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Research report

Model of voluntary ethanol intake in zebrafish: Effect on behavior and hypothalamic orexigenic peptides

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HIGHLIGHTS

- Zebrafish consume stable and pharmacologically relevant levels of ethanol–gelatin.
- Ethanol–gelatin intake correlates with blood ethanol concentration.
- Ethanol–gelatin intake leads to similar blood ethanol levels to ethanol soak.
- Ethanol–gelatin intake significantly changes behavior and orexigenic peptides.

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ABSTRACT

Recent studies in zebrafish have shown that exposure to ethanol in tank water affects various behaviors, including locomotion, anxiety and aggression, and produces changes in brain neurotransmitters, such as serotonin and dopamine. Building on these investigations, the present study had two goals: first, to develop a method for inducing voluntary ethanol intake in individual zebrafish, which can be used as a model in future studies to examine how this behavior is affected by various manipulations, and second, to characterize the effects of this ethanol intake on different behaviors and the expression of hypothalamic orexigenic peptides, galanin (GAL) and orexin (OX), which are known in rodents to stimulate consumption of ethanol and alter behaviors associated with alcohol abuse. Thus, we first developed a new model of voluntary intake of ethanol in fish by presenting this ethanol mixed with gelatin, which they readily consume. Using this model, we found that individual zebrafish can be trained in a short period to consume stable levels of 10% or 20% ethanol (v/v) mixed with gelatin and that their intake of this ethanol–gelatin mixture leads to pharmacologically relevant blood ethanol concentrations which are strongly, positively correlated with the amount ingested. Intake of this ethanol–gelatin mixture increased locomotion, reduced anxiety, and stimulated aggressive behavior, while increasing expression of GAL and OX in specific hypothalamic areas. These findings, confirming results in rats, provide a method in zebrafish for investigating with forward genetics and pharmacological techniques the role of different brain mechanisms in controlling ethanol intake.

1. Introduction

Alcohol is one of the most abused drugs in the world. Studies of neurobiological mechanisms underlying alcohol abuse have demonstrated the importance of different neurochemical systems that are responsive to ethanol exposure and, in turn, promote intake [1,2]. While these studies have been performed mostly in humans and rodents [3,4], zebrafish are being increasingly utilized as an animal model for studying the effects of ethanol on behavior and brain neurotransmitters known to be involved in alcoholism. Zebrafish are highly prolific, resilient, and one of the lower order vertebrate species in which complex brain function and behavior may be studied in the laboratory. A large number of zebrafish mutants have already been described (www.zfin.org), providing a valuable tool for researchers interested in ethanol-gene interactions. Studies in zebrafish show that acute exposure to ethanol in the tank water, at low to moderate concentrations, reduces anxiety and increases locomotion, aggression, conditioned place preference and shoaling [5–8], whereas chronic exposure to ethanol in the water leads to the development of tolerance and withdrawal which then increases anxiety and decreases shoaling [9,10]. These ethanol-induced behavioral changes in zebrafish, which are

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shown to enhance consummatory behavior in humans and rodents [11–13], are associated with marked changes in neurotransmitters in the zebrafish brain. Acute ethanol exposure increases whole brain levels of dopamine, serotonin and their metabolites and suppresses levels of glutamate and gamma-aminobutyric acid (GABA) [14–16], while chronic ethanol exposure has little impact on these neurotransmitters [15]. Ethanol consumption in rodents has similar effects on these neurotransmitters, which are sometimes stimulated by acute consumption of low amounts of ethanol while unaffected or suppressed by chronic consumption of high amounts of ethanol [17]. This evidence supports the use of zebrafish in investigating the relationship of ethanol with these neurochemicals.

In addition to these neurotransmitters, there are a number of orexigenic peptides, which in rodents are found to stimulate the consumption of ethanol and to be strongly affected by ethanol [18–21] and in humans are believed to have a role in alcoholism [22,23]. These include galanin (GAL) and orexin/hypocretin (OX), which show increased expression in rats administered or trained to drink ethanol [19,20,24] and higher expression in inbred and outbred rodent strains that spontaneously overconsume ethanol [25,26]. Whereas central injections of GAL and OX have been shown to stimulate food intake in zebrafish [27,28], these peptides have yet to be examined in zebrafish exposed to ethanol in the water and in relation to various behaviors known to promote intake. To date, studies of OX in zebrafish have focused on arousal-related behaviors and shown this peptide to regulate the sleep–wake cycle, as in rodents [25,29]. The evidence demonstrates that OX overexpression increases locomotor activity, decreases rest, and consolidates wakefulness [30,31], while the ablation of OX neurons increases sleep and alters behavioral responses to external stimuli [32] and OX receptor mutants exhibit short and fragmented sleep in the dark [33]. There are few studies of GAL in zebrafish, with one report showing overexpression of GAL to increase rest and decrease locomotor activity [31] and another study showing the inhibition of GAL translation to have little impact on other orexigenic peptides [34]. With no studies examining the effect of ethanol exposure on orexigenic peptides in zebrafish, the possibility that the peptides are stimulated by ethanol in this species, similar to rodents, would provide support for their role in mediating the ingestion of ethanol and also other behaviors known to predict or be associated with alcohol abuse [3,11,35,36].

Building on the reports in zebrafish examining the effects of ethanol exposure in the tank water, our goals for this investigation were to: (1) establish a model for inducing voluntary ethanol ingestion in zebrafish, which could reliably elevate blood ethanol concentration (BEC) to pharmacological levels; (2) characterize the effect of voluntary ethanol intake on different behaviors that have an established role in promoting ethanol consumption in rodents and humans; and (3) determine whether exposure to ethanol through voluntary ingestion has effects on orexigenic peptides known to stimulate ethanol intake in rodents and believed to have a role in alcoholism in humans. The model presented in this study could be valuable in performing more targeted genetic, epigenetic, molecular and pharmacological studies of mechanisms controlling ethanol consumption and abuse.

2. Methods

2.1. Animals and housing

Adult zebrafish (*Danio rerio*) of the AB strain were bred in our facility (Rockefeller University, NY) following standard procedures [37], from the breeding pairs purchased from ZIRC (Eurgene, Oregon). Fish were housed in 3 L tanks with constant water flow (Aquatic Habitats, Apopka, FL), which consisted of reverse osmosis water with salts (Instant Ocean, 0.25 ppt and 500–700 μS). Adult male and female fish between 6 and 12 months of age were used in this study, maintained on a 12:12 h light–dark cycle (9 am lights on and 9 pm lights off) in 24.5–25 °C water. All fish were housed in groups of 10 in 3 L tanks (Aquatic Habitats). Fish were fed twice daily, at 10 am with gelatin containing shrimp as described below and at 4 pm with Zeigler Adult Diet (Aquatic Habitats). The facility was fully accredited by AAALAC. Protocols were approved by the Rockefeller University Animal Care and Use Committee and followed the NIH Guide for the Care and Use of Laboratory Animals. We used 5 different sets of zebrafish in this study in the following manner: Set 1 ($N = 48$) to establish the model of voluntary ethanol consumption and measure brain peptides and BEC, Set 2 ($N = 48$) to measure the effect of ethanol consumption on behavior, Set 3 ($N = 42$) to determine the effect of ethanol consumptions on BEC at different time points, Set 4 ($N = 16$) to measure the effect of soaking in ethanol on BEC and Set 5 ($N = 8$) to measure the effect of soaking in ethanol on brain peptides and BEC.

2.2. Experimental design and procedures

2.2.1. Model of voluntary ethanol consumption

With gelatin-based foods used for administering special nutrients and antibiotics to aquarium fish [38,39], we chose to use this gelatin diet to train the zebrafish to consume ethanol. Numerous studies conducted in our laboratory and others, indicating that rats not specially bred to drink ethanol will readily consume a 10% or 20% ethanol solution that in turn leads to pharmacologically relevant BEC levels when presented in a binge model [18,40,41], led us to formulate three types of gelatin that contained either 0%, 10% or 20% ethanol (v/v). We first prepared the gelatin (Knox Gelatin, Kraft Foods, Northfield, IL) by combining one packet (1.8 g) of gelatin with 120 mL of hot water and stirring until dissolved. Then, we poured 2.5 mL of melted gelatin into a condiment cup and combined it with 100 mg (wet weight, with excess water removed) of 2-day-old brine shrimp nauplii (Brine Shrimp Direct, Ogden, UT). We then added into each cup 2.5 mL of liquid consisting of water or 20% or 40% ethanol solution, for a final gelatin mixture containing 0%, 10%, or 20% ethanol (v/v) in 5 mL of gelatin, respectively. The mixture of gelatin with shrimp and different ethanol concentrations was allowed to set for 1 h in a sealed cup at 4 °C and then kept on ice. These ethanol–gelatin meals were made fresh daily and were given 1 h into the light cycle. For the first 3 days of training, the fish were housed in groups of 4 in 3 L tanks and fed with plain gelatin (500 mg/per feeding/tank), with water flow turned off during feeding and resumed after gelatin removal. Starting on the 4th day (first day of ethanol feeding, D1), fish in the 3 L tanks were group fed for 5 min with pre-weighed 0%, 10% or 20% ethanol–gelatin mixture, and then the gelatin was removed with a fine brine shrimp net, blotted and re-weighed. After 3 days of group training, the fish on D4 were individually housed in 1.5 L tanks and fed daily for 5 min from D4 to D14 with 500 mg of gelatin containing 0%, 10% or 20% ethanol, and then the gelatin was re-weighed to determine the amount consumed. Blood was collected from individual fish in the manner described below, to determine the effect of ethanol–gelatin intake on BEC.

2.2.2. Behavioral testing

In addition to gelatin intake, other behaviors of the fish were also examined in a Novel test tank on D20 and then in a Mirror test on D22. To acclimate them to handling, each animal, once daily from D15 to D19, was caught in a soft white net (PetSmart, Phoenix, AZ) and held out of its tank for 10 s before being gently released back into the tank. On test days, fish were fed for 5 min in their home cage