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Model of voluntary ethanol intake in zebrafish: Effect on behavior and hypothalamic 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 of time 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.

Keywords

zebrafish; ethanol; orexin; galanin

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

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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 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, New York, 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, .25ppt and 500–700uS). Adult male and female fish between 6 and 12 months of age were used in this study, maintained on a 12:12 hr 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 3L 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 4 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) determine the effect of ethanol consumptions on BEC at different time point, 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 hr in a sealed cup at 4°C and then kept on ice. These ethanol-gelatin meals were made fresh daily and were given 1 hr into the light cycle. For the first 3 days of training, the fish were housed in groups of 4 in 3L 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 3L 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-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-D19, was caught in a soft white net (PetSmart, Phoenix, AZ) and held out of their 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 with gelatin containing 0%, 10% or 20% ethanol, as described above, and their intake was measured. Fifteen min after completion of this meal, the fish were individually netted into the test tank filled with system water at 25.5°C, and their behavior was video recorded twice for 60 s: first, 30 s after having been placed in the tank, indicating their response to novelty (1st min), and second, 10 min later, indicating their response in a habituated state (10th min).

The Novel Test tank was used first to assess the effect of ethanol-gelatin ingestion on locomotor activity and anxiety, using methods established in other laboratories [7, 42]. Briefly, the tank (2 L, 2 L, rectangular, Colombia University) was divided into 4 equal vertical sections and 3 horizontal sections by drawing lines on the outside of the tank, with each resulting grid being 5 × 5 cm, double the average adult body length. The following behaviors were scored during the 1st and 10th min of the test: 1) locomotor activity (calculated as the total number of horizontal + vertical crossings), and 2) exploratory behavior, an indicator of anxiety (calculated as the time spent in the top, middle and bottom sections and latency to reach the top). Behaviors were scored from videotapes, with the camera facing the longer, gridded wall of the tank.

The Mirror test was then used to assess in individual fish the effect of ethanol-gelatin intake on aggressive behavior, using methods established in other laboratories [6, 7, 43, 44] that scored fish responses to their own mirror image. A mirror was cut to fit into the tank and was placed at a 22.5° angle behind a partition, in the back or front of the experimental 1.5 L trapezoidal tank (Aquatic Habitats) on alternate days. The mirror was placed inside rather than outside the tank, in order to focus the fish on their reflection in the mirror instead of on the wall of the tank. The tank was divided into 4 equal sections drawn with a black marker on the bottom of the tank. In addition, a line was drawn 0.5 cm from the mirror, representing the “contact zone”, and another line was drawn 2.5 cm from the first line (based on the

average adult body length), representing the “approach zone”. With the camera placed above the tanks, the following behaviors were scored: 1) percent time in S1 (contact zone + approach zone); 2) latency to 1st approach and 1st contact; 3) number of entries into approach zone and contact zone; and 4) percent time in contact zone.

2.2.3 Quantification of behavior—All videos were recorded with a Panasonic Lumix DMC TS25 in 720p HD and viewed in MPEG-4 Movie format. All behaviors were manually scored using a stop watch by a trained observer blind to the treatment of the groups. Locomotion was scored by counting entries into each of the 4 horizontal sections [6, 7] and 3 vertical sections [42, 45]. Time in each layer was recorded and calculated as percent time spend in each section relative to the total time of 60s [6, 7]. Latency to reach the top layer of the tank was scored as the time it took for two-thirds of the body length to cross the line that separated the middle from the top layer of the tank [46]. The Mirror test was scored by measuring the time it took for fish to approach and contract the mirror and the number of entries into the approach zone and contact zone, and then calculating the percent time spent in the S1 zone (“contact zone” plus “approach zone”) and the contact zones [42, 46].

2.3. Digoxigenin-labeled in situ hybridization (ISH) histochemistry to measure peptide neuronal density

In situ hybridization with digoxigenin-labeled probes was used to measure the density of neurons expressing the orexigenic peptide genes. Fish were fed for 5 min with gelatin containing 0%, 10% or 20% ethanol, and at 30 min after completion of the meal, they were anesthetized in an ice water bath for 5–10 min until they lost equilibrium and ocular movement. Immediately after, blood was collected, and fish were euthanized by decapitation. For the brain dissection, the fish head was placed ventral side up on moistened filter paper which was placed on the top of a petri dish filled with ice. As previously described [47], forceps were used to remove the gills, jaw, eyes and surrounding tissue. The skull was cracked open, and the base of the skull was gently removed. The whole brain was pulled out of the skull by the spinal cord. The brain was immediately placed in 4% paraformaldehyde in phosphate buffer solution (0.1 M, pH 7.2), fixed overnight at 4°C, and then cryoprotected in 25% sucrose for 24 to 48 hrs at 4°C. Afterwards, the brain was frozen at −80°C for 1–2 hrs, then cut with a cryostat in 40 μm serial sagittal sections, allowing alternate free-floating sections to be used for measurement of GAL and OX mRNA using ISH. Specifically, ISH was performed by processing the sections consecutively as follows: 10 min each in 0.001% proteinase K, 4% paraformaldehyde, and acetylation solution, between each step, 2 × 5 min wash in PB (0.1 M pH 7.2), followed by hybridization for 18 hrs at 65°C and then a 20-min wash in 5 × SSC and 30-min wash in 50% formamide, with both at 65°C, and 30 min in RNase A (1 μg/ml) at 37°C. After 2–4 hrs in a 1.5% blocking solution, the sections were then incubated in AP-conjugated sheep anti-digoxigenin Fab fragments (1:500, Roche, Indianapolis, IN) for 18 hrs and developed in freshly prepared color developer (50 μl of 4-nitroblue tetrazolium chloride solution, 37.5 μl of 5-bromo-4-chloro-3-indolyl-phosphate solution in 10 ml Tris buffer, pH 9.5; Roche). Finally, the sections were fixed for 10 min in 4% paraformaldehyde, then mounted, air-dried, dehydrated, cleared and coverslipped. The sense probe control was performed in the same

tissue, and no signal was detected. All procedures were conducted at room temperature, unless otherwise indicated. Zebrafish GAL and OX cDNA plasmids were generously provided by Dr. Pertti Panula (University of Helsinki, Finland), and the digoxigenin (DIG)-labelled riboprobes (sense and antisense) were made as described [34, 48].

2.4. Semiquantification of digoxigenin-labeled ISH

The GAL- and OX-expressing cells in the hypothalamus were viewed using a Leitz microscope with a 20X illumination objective, and the images were captured with a Nikon DXM 1200 digital camera (Nikon, Tokyo, Japan). The density of the GAL- and OX-expressing neurons was measured using Image-Pro Plus software (Version 4.5, Media Cybernetics Inc., Silver Spring, MD), as described in detail [49, 50], and the measure of cell density is expressed as number of cells/μm^2. In all analyses, only those cells containing a nucleus ($\geq 20\ \mu m^2$) on one plane in each section were counted. The average cell density for the 0% (control), 10% and 20% ethanol groups was then compared and analyzed statistically, with the GAL cells evident in the ventral and caudal zones of the periventricular hypothalamus and the OX cells evident in the anterior hypothalamus.

2.5. Ethanol exposure in tank water

In addition to the effect of ethanol consumption on hypothalamic peptides, we also examined peptide responses to ethanol in the tank water by holding the fish in an ethanol-system water solution (0%, 0.25% or 0.5% ethanol) for 1 hr in a 3L home tank with a lid [7, 51]. Blood collection was carried out as described below (Section 2.6) and assayed for levels of BEC. To determine the effect of ethanol treatment on orexigenic peptides, an additional group of fish was treated with 0% or 0.25% ethanol, and as described below, their blood was collected for measurements of BEC and their brain was collected for analysis of the peptides.

2.6. Blood ethanol concentration

As previously described [52, 53], immediately after the fish were anesthetized in ice water bath, each one was wrapped in a paper towel with its tail end exposed to air and was then placed on a second paper towel on top of a Petri dish filled with ice, with its tail hanging off. The tail was cut above the anal fin to sever the dorsal aorta. Holding the fish between the fingers at a 60–90° angle, blood was collected in a heparinized capillary tube by gently squeezing the fish from head to tail. The entire procedure took less than 1.5 min per fish. The capillary tubes were capped with clay on the bottom, covered with parafilm, and centrifuged at 3.1 RPM for 8 min at 4°C, with each fish yielding 5–10 ul of blood and 2–7 ul of serum. Serum was removed from the tube and stored in −80°C until it was assayed for BEC using an Analox GM8 Alcohol Analyzer (Lunenberg, MA). When the serum volume was not sufficient for standard BEC measurements, it was diluted with PBS (pH 7.4), and BEC levels were calculated accordingly.

2.7 Specific Experiments

2.7.1. Experiment 1: Voluntary ingestion of ethanol-gelatin and effect on BEC in individual zebrafish—The goal of this experiment in zebrafish was to establish a

model of voluntary ethanol ingestion and to relate this ingestion to BEC. After the initial 3-day training to consume gelatin, the zebrafish (N = 48) on the fourth day of training (D4) were randomly assigned to one of three groups (n = 16/group), and for the next 11 days (D4-D14), were given a daily meal of gelatin containing 0% (control), 10% or 20% ethanol and the amount ingested was measured. To determine the effect of ingesting the ethanol-gelatin mixture on BEC, a different set of fish (N = 42) was similarly trained to consume 0%, 10% or 20% ethanol-gelatin and their individual intake was recorded daily from D4-D14. They were then sacrificed on D14 at 5, 15 or 30 min after the 5-min meal, and their blood was collected for measurements of BEC. With our evidence showing that the zebrafish ingest more of the 10% ethanol-gelatin compared to gelatin alone, we examined an additional set of fish (n = 6/group) to determine if this could be due to a change in appetite induced by the ethanol. We found that fish that had consumed a 10% ethanol-gelatin meal tended to consume less of a subsequent meal of 0% ethanol-gelatin provided 3 hrs later (49 ± 6 mg/g body weight) than did fish that consumed 0% ethanol-gelatin at both meals (85 ± 10 mg/g bodyweight, *ns*), suggesting that the increased intake of the ethanol-gelatin mixture was not due specifically to ethanol-induced changes in appetite.

2.7.2. Experiment 2: Voluntary ingestion of ethanol-gelatin and effects on behavior—

This experiment examined the effects of voluntary intake of ethanol-gelatin vs. gelatin alone on different behaviors, using the same set of zebrafish examined in Experiment 1 after completing the intake measurements from D4-D14. After these fish were habituated to handling from D15-D19, their behaviors were first examined on D20 in a Novel Test tank and then on D22 in a Mirror Test, 15 min after completion of the 0%, 10% or 20% ethanol-gelatin meal. To determine the effect of ethanol-gelatin intake on locomotion and anxiety, the Novel Test tank was used to measure locomotor activity, latency to reach the top zone of the tank, and location of swimming during the 1^{st} and 10^{th} min of the test. To determine the impact of ethanol-gelatin intake on aggressive behavior, the Mirror test was used to examine their responses to their own mirror image, by measuring the percent time spent in the S1 zone, including both the approach and contact zones of the mirror.

2.7.3. Experiment 3: Voluntary intake of ethanol-gelatin and effects on expression of orexigenic peptides GAL and OX—

This experiment examined the effect of voluntary ethanol-gelatin ingestion on mRNA expression of the orexigenic peptides, GAL and OX, in the hypothalamus. Zebrafish (N = 18) were trained to consume 0%, 10% or 20% ethanol-gelatin as described above (n = 8/group), and then on the last day of training (D14), they were sacrificed 30 min after meal completion and their blood was collected for measurements of BEC and their brains were dissected for peptide analyses using *in situ* hybridization. To compare these results to those produced by ethanol in the tank water, we measured in an additional set of zebrafish (N = 8) gene expression of the two peptides along with BEC after 1 hr of soaking in 0.25% ethanol (v/v) compared to water (n = 4/group).

2.8. Statistical Analysis

Correlations between gelatin intake at each day, ethanol intake with BEC, and BEC with peptide expression, were analyzed using the Pearson product-moment correlation

coefficient. Differences between the effects of ethanol on behaviors or gene expression at single time-points were tested with a one-way ANOVA, followed up by Tukey's post-hoc test or by unpaired, two-tailed *t*-tests as appropriate. Differences in the effects of multiple doses of ethanol at multiple time-points were tested with a repeated-measures ANOVA (when time was a within-subject factor) followed up by pairwise comparisons using Tukey's HSD, or with a one-way ANOVA followed up by Tukey's post-hoc test as appropriate. Differences in the effects of multiple doses of ethanol in multiple brain regions (in the same fish) were tested with a repeated-measures ANOVA (with brain area as the within-subject factor), with a significant interaction effect followed up by a one-way ANOVA and then pairwise comparisons using Tukey's HSD. Data were determined to be distributed normally using the Shapiro-Wilk test. Significance was determined at $p < 0.05$. Data are reported as mean ± standard error of the mean (S.E.M.).

3. Results

3.1. Experiment 1: Voluntary ingestion of ethanol-gelatin and effects on BEC in individual zebrafish

The goal of this experiment was to establish a model of voluntary ethanol ingestion and to characterize the ingestion pattern of gelatin with or without ethanol (0%, 10%, 20%, $n = 16$/group) and determine the relationship between the amount of ethanol-gelatin consumed and measurements of BEC. As shown in Fig. 1A, daily ingestion (mg/g body weight) started to stabilize around D9, with day-to-day intake from D11 through D14 significantly, positively correlated ($r = +0.69$, $p < 0.01$). Gelatin intake from D9 to D14 averaged 43 ± 4.7 mg with 0% ethanol (control), 73 ± 7 mg with 10% ethanol, and 75 ± 4 mg with 20% ethanol, yielding an average ethanol intake by each fish of 7.3 ± 0.7 mg at 10% ethanol and 15.0 ± 0.7 mg at 20% ethanol. These data from D4 to D14 yielded a significant main effect of ethanol-gelatin intake [$F(2,27) = 4.42$, $p < 0.01$] and time [$F(10,270) = 4.70$, $p < 0.01$] but no ethanol-gelatin x time interaction [$F(20, 270) = 1.30$, *ns*], with post-hoc comparisons showing the main effect on intake to be due to a significant increase in intake of 10% ethanol-gelatin (+50%, $p < 0.01$) with no change in intake of 20% ethanol-gelatin (*ns*) compared to 0% ethanol-gelatin control. To measure the impact of ethanol-gelatin ingestion on BEC over time, a separate set of zebrafish ($N = 42$) was trained to consume 0%, 10% or 20% ethanol-gelatin ($n = 10$–12/group/time point) and sacrificed on D14 at either 5, 15 or 30 min after 5 min of consumption, at which time their trunk blood was collected. Analysis with a one-way ANOVA revealed a significant main effect of ethanol-gelatin intake on BEC at 5 min [$F(2,31) = 21.14$, $p < 0.01$], 15 min [$F(2,29) = 40.01$, $p < 0.01$] and 30 min [$F(2,29) = 28.34$, $p < 0.01$] (Fig. 1B), with post-hoc comparisons showing this effect to be due to a significant increase in BEC from 10% and 20% ethanol-gelatin at all three time points compared to 0% ethanol-gelatin control ($p < 0.01$) and higher after intake of 20% ethanol-gelatin compared to 10% ethanol-gelatin at all time points ($p < 0.01$). Further, the amount of ethanol-gelatin consumed (mg gelatin/g body weight) was strongly, positively correlated with BEC when measured at 5 min ($r = +0.65$, $p < 0.05$), 15 min ($r = +0.77$, $p < 0.01$) and 30 min ($r = +0.73$, $p < 0.05$) after intake of 10% ethanol-gelatin and also at 5 min ($r = +0.61$, $p < 0.05$), 15 min ($r = +0.88$, $p < 0.01$) and 30 min ($r = +0.92$, $p < 0.01$) after intake of 20% ethanol-gelatin. To compare these BEC values to those measured in fish that were soaked in

ethanol, we examined an additional set of fish (n = 8/group) and found that soaking for 1 hr in 0.25% or 0.5% ethanol, a paradigm commonly used [6, 7, 43, 54], has a significant effect on BEC [$F(2,23) = 141.56$, $p < 0.01$], with a significant increase at both the 0.25% (129 ± 9 mg/dl, $p < 0.05$) and 0.5% (222 ± 15 mg/dl, $p < 0.05$) ethanol concentrations. Together, these results indicate that zebrafish voluntarily ingest stable amounts of ethanol in gelatin and that this consumption increases BEC to levels that are pharmacologically relevant.

3.2. Experiment 2: Voluntary ingestion of ethanol-gelatin and effects on different behaviors in zebrafish

This experiment examined the effects of voluntary ingestion of ethanol-gelatin on different behaviors in zebrafish, first in a Novel Tank test and then in a Mirror test. Table 1 displays the results for all the ANOVAs used to analyze the behavioral tests.

Novel Tank Test—To determine the impact of ethanol-gelatin intake on locomotor behavior and anxiety in a novel tank, we recorded different behavioral measures in zebrafish (N = 47) during the 1st and 10th min of the test after the 0%, 10% and 20% ethanol-gelatin meal. We observed a main effect of ethanol-gelatin intake on locomotor activity, calculated as the total number of horizontal and vertical crossings, with a significant change in activity over time (Fig. 2A) but no ethanol x time interaction, with a significant effect of 20% ($p < 0.001$) but not 10% (*ns*) ethanol-gelatin intake compared to 0% ethanol-gelatin control. We also observed a significant main effect of ethanol-gelatin intake on the latency to reach the top zone, with post-hoc tests showing 20% ethanol-gelatin compared to control to significantly decrease the time taken for zebrafish to reach the top zone ($p < 0.01$) and 10% ethanol-gelatin to produce a small, statistically insignificant decrease in time (*ns*) (Fig. 2B).

Examination of the location of swimming during the 1st and 10th min of the test revealed changes in the measures of percent time spent in the top, middle and bottom sections of the tank (Table 2). Analysis of time spent in the top section revealed a significant main effect of ethanol-gelatin intake and time with an interaction between ethanol-gelatin and time, and post-hoc tests showing a significant increase in percent time spent on top after consumption of 20% ($p < 0.01$) but not 10% (*ns*) ethanol-gelatin compared to control and no difference between the two ethanol groups (*ns*). Pairwise comparisons showed the percent time spent in the top section to increase with time ($p < 0.01$). Analysis of the time spent in the middle section also revealed a significant main effect of ethanol-gelatin and time but no interaction between ethanol-gelatin and time, with post-hoc tests showing a significant increase after consumption of 20% ($p < 0.05$) but not 10% (*ns*) ethanol-gelatin and no difference between the two ethanol groups (*ns*) and with pairwise comparisons showing the percent time spent in the middle section overall to increase over time ($p < 0.05$). The time spent in the bottom section was also significantly affected by ethanol-gelatin and time as was the interaction between ethanol-gelatin and time, with post-hoc tests showing a significant decrease after consumption of both 20% ($p < 0.05$) and 10% ($p < 0.05$) ethanol-gelatin and with pairwise comparisons showing the percent time spent at the bottom of the tank to decrease after consumption of 10% ($p < 0.01$) but not 20% (*ns*) ethanol-gelatin compared to control. Together, these data demonstrate that voluntary consumption of ethanol-gelatin significantly increases anxiety in zebrafish.

Mirror Test—To determine the impact of voluntary ethanol-gelatin consumption on aggressive behavior in zebrafish (N = 48), individual fish were tested for their responses to their own mirror image. Analysis of the percent time spent in the S1 zone, including both the approach and contact zones of the mirror, revealed a significant main effect of ethanol-gelatin intake, with no main effect of time and no interaction between time and treatment (Fig. 2C) and post-hoc tests showed this main effect to reflect a significant increase in time spent in the S1 zone after ingestion of both the 10% and 20% ethanol-gelatin compared to control ($p < 0.05$) and no difference between the two ethanol-gelatin groups (*ns*). Analysis of the percent time spent specifically in the contact zone of the mirror revealed a significant main effect of ethanol-gelatin as well as time and a significant interaction between ethanol-gelatin and time, with post-hoc analysis showing this effect to be due to intake of both the 10% (+80%, $p < 0.01$) and 20% (+77%, $p < 0.01$) ethanol-gelatin with no difference between the two ethanol groups (*ns*). A significant main effect of ethanol-gelatin was also obtained for the latency to approach and contact the mirror, with a significant decrease in both measures observed after consumption of 10% ($p < 0.05$) and 20% ($p < 0.05$) ethanol-gelatin but no difference between the two ethanol groups (*ns*) (Table 2). While there was no main effect of ethanol-gelatin intake on the number of entries into the approach or contact zones, there was a significant main effect of time on these measures, with pairwise comparisons showing that entries into these zones increased with time ($p < 0.05$). There were no time x treatment interaction effects for the number of entries into approach and contact zones. Together, these behavioral tests demonstrate that voluntary ethanol consumption has significant, stimulatory effects on both exploration and aggressive behavior.

3.3. Experiment 3: Voluntary intake of ethanol-gelatin and effects on expression of orexigenic peptides, GAL and OX

With no studies to date examining the impact of ethanol on orexigenic peptides in the brain, this experiment examined the effect of voluntary ethanol-gelatin intake (0%, 10% or 20% ethanol-gelatin) on mRNA expression of the orexigenic peptides, GAL and OX, in the hypothalamus (n = 8/group). As in Experiment 1, measurements of BEC revealed a significant main effect of ethanol-gelatin intake [$F(2,21) = 28.0$, $p < 0.01$], with both the 10% and 20% ethanol-gelatin producing a significant increase in BEC ($p < 0.01$), with the 20% ethanol-gelatin producing higher BEC (63 ± 8 mg/dl) than 10% ethanol-gelatin (31 ± 5 mg/dl, $p < 0.001$), and with BEC being strongly, positively correlated with the amount of 10% ($r = +0.82$, $p < 0.01$) and 20% ($r = +0.91$, $p < 0.01$) ethanol-gelatin consumed. Analysis of the peptides revealed a main effect of ethanol-gelatin on mRNA expression of GAL [$F(2, 21) = 140.19$, $p < 0.01$] and OX [$F(2, 23) = 11.06$, $p < 0.01$] in the hypothalamus (Fig. 3). The effect on GAL was anatomically localized [$F(2,21) = 162.17$, $p < 0.01$], occurring specifically in the ventral (Hv) [$F(2,21) = 23.98$, $p < 0.01$] and caudal (Hc) [$F(2,21) = 149.35$, $p < 0.01$] zones of the periventricular hypothalamus, with no significant interaction between ethanol-gelatin intake and brain area [$F(2,21) = 60.65$, $p < 0.01$] (Fig 4). In both groups (10% and 20%), pairwise comparisons showed ethanol-gelatin consumption to significantly increase the expression of GAL mRNA in both the Hv (+22%, $p < 0.01$) and Hc (+239%, $p < 0.01$), with a significantly larger effect observed in the Hc zone ($\eta_p^2 = 0.93$ vs. $\eta_p^2 = 0.69$) (Fig 4). Ethanol-gelatin intake also increased OX mRNA expression in the

anterior hypothalamic region (+60%, $p < 0.01$) (Fig 4), again with no difference observed between the two ethanol groups (*ns*). Exposure to ethanol by soaking in 0.25% ethanol (v/v) compared to water ($n = 4$/group), which increased BEC to 114 ± 9 mg/dl, also significantly increased the density of GAL mRNA in both the Hv (5.0 vs 3.8 objects/μm^2 $x10^{-5}$, $p < 0.05$) and Hc (5.2 vs 1.3 objects/μm^2 $x10^{-5}$, $p < 0.05$) zones of the periventricular hypothalamus and of OX mRNA in the anterior region of the hypothalamus (2.4 vs 1.4 objects/μm^2 $x10^{-5}$, $p < 0.05$). Together, these two experiments demonstrate that voluntary ethanol-gelatin intake has a stimulatory effect on hypothalamic expression of both GAL and OX and that these effects are similar to those observed with exposure to ethanol in tank water.

4. Discussion

This study describes a method for inducing fish to voluntary ingest ethanol by mixing it with gelatin, which allows one to measure ethanol-gelatin intake in individual fish that can be related to their BEC. This novel model produces pharmacologically relevant BEC, which leads to profound changes in behavior that in rodent and human studies are associated with alcohol abuse. These behavioral changes induced by ethanol-gelatin ingestion are accompanied by a strong stimulatory effect on orexigenic peptides known in other species to have a role in mediating these behaviors.

4.1. Model of voluntary intake of ethanol-gelatin in zebrafish

In this study, we established a model that is easy to set up and allows one to reliably measure voluntary ingestion of ethanol mixed with gelatin and the resulting BEC in individual zebrafish. There is only one publication that has measured BEC in zebrafish, and this study pooled samples from 12 fish and showed that soaking for 10 min in 0.125–1% ethanol solution increased BEC to 50–170 mg/dl [55]. Our present results demonstrate that ingestion of the 10% or 20% ethanol-gelatin mixture causes an increase in BEC, to 86 mg/dl and 152 mg/dl, respectively, during the first 15 min after completion of this 5-min meal. These levels are comparable to those produced by soaking zebrafish for 1 hr in 0.25% ethanol (129 mg/dl), allowing us to compare behavioral changes induced by our model of ethanol ingestion to those induced by the commonly-used procedure of ethanol soak (0.025–1%) [7]. These BEC levels produced by ethanol-gelatin intake were also comparable to those observed in rats (up to 134 mg/dl) consuming a pharmacologically relevant amount of ethanol [18] and higher than the minimum value in humans (80 mg/dl) that qualifies as binge drinking [56]. Measurements of ethanol concentration in the zebrafish brain have shown that soaking in 1% ethanol in the water increases brain levels to 4.2 ug/mg in 20 min and 6.3 ug/mg in 1 hr [45], with 6 hr required for equilibration of the brain concentration with tank concentration [9]. Of particular note is our additional finding that the amount ethanol-gelatin consumed by each zebrafish is strongly, positively correlated with BEC ($r = +0.76$). It is interesting that the fish consumed significantly more of the gelatin when it contained 10% ethanol vs. 0% ethanol. As fish that consumed this 10% ethanol-gelatin did not consume more of a subsequent 0% ethanol-gelatin meal than did fish that consumed 0% ethanol-gelatin at both meals, the increased intake of ethanol-gelatin may not due to an ethanol-induced change in appetite. Instead, zebrafish may have a preference for the pharmacological effects or the taste of ethanol-gelatin, at least at the 10% concentration,

which in humans and rodents is perceived as containing both sweet and bitter taste components [57, 58]. This preference is similar to what we and others have observed in outbred rodents when presented with 10% or 20% ethanol using an intermittent-access model [18, 40, 41]. These findings suggest that our new model of voluntary ingestion of ethanol-gelatin, which yields stable measures of intake within just 14 days and increases BEC in individual fish to pharmacologically-relevant levels, will provide a valuable tool for investigating the effects of ethanol intake on behavior and brain neurochemical systems, as well as the effect of genetic and pharmacological manipulations on ethanol consumption.

4.2. Effect of voluntary intake of ethanol-gelatin on behavior in zebrafish

The results indicate that our model of voluntary ethanol-gelatin intake produces pharmacologically relevant BEC levels that significantly affect behavior in zebrafish. Overall, the behavioral changes induced by ingestion are similar to those produced by soaking in ethanol, validating this new model of voluntary consumption. The ethanol-gelatin caused a significant increase in locomotion and a decrease in anxiety, as indicated by increased exploration in zebrafish that ingested the 20% ethanol-gelatin. Measurements of locomotor activity (number of vertical and horizontal transitions) in a novel environment revealed a significant increase after ingestion of the 20% ethanol-gelatin, during the 1st min as well as the 10th min of the test after habituation. The long-lasting nature of this hyperactivity suggests that that the ethanol-exposed fish failed to habituate to this novel environment [7], although it cannot be ruled out that it does not reflect increased sensitivity over the course of the test to the pharmacokinetic-induced changes in ethanol levels. This increased activity is similar to that observed in wildtype zebrafish exposed for 1 hr to a low or intermediate concentration (0.25% and 0.5%) of ethanol in the water, in contrast to the reduced locomotor activity induced by a higher concentration (1%) of ethanol [7]. We additionally found the latency to reach the top of the tank to be significantly reduced by ingestion of the 20% ethanol-gelatin, indicating increased exploratory behavior that is associated with reduced anxiety [51]. This behavioral change caused by ethanol-gelatin intake in AB zebrafish is similar to that observed in wildtype zebrafish exposed for 5 min to 0.3% ethanol in the water [5]. The location of swimming was also affected by 20% ethanol-gelatin, with an increase in the percent time spent in the top of the tank during both the 1st and 10th min of the test again reflecting an increase in exploratory activity and possibly reduced anxiety with time. These results are consistent with findings in wildtype zebrafish exposed repeatedly for 1 hr to 0.25% and 0.5% ethanol in the water [7, 51] and also in AB zebrafish exposed for 20 min to 1% and 1.5% ethanol [10, 43]. Further measurements revealed significant effects of ethanol-gelatin intake on aggressive behavior, with the both 10% and 20% ethanol-gelatin significantly increasing the time spent in close proximity to the mirror and decreasing the latency to approach and contact the mirror, suggesting an increase in aggression. Again, these effects induced by ethanol-gelatin intake are similar to those observed in wildtype zebrafish with 0.25% and 0.50% ethanol in the water [6, 7, 43], although they are opposite to findings in AB zebrafish [10, 43]. In addition to these similarities between the effects of ethanol-gelatin intake and ethanol in the tank water, it is interesting that these behavioral effects in zebrafish are similar to those observed in rodents and humans, which in response to ethanol at low-to-moderate doses exhibit an increase in locomotor activity and aggressive behavior along with a decrease in anxiety [12, 18, 22, 36,

59]. The finding that 10% ethanol-gelatin significantly increases aggressive behavior while having no impact on exploratory behavior suggests that these specific behaviors are differentially sensitive to BEC [36, 60]. Together, these findings validate our new model of voluntary ingestion of ethanol-gelatin in zebrafish, which increases BEC to pharmacological levels that are strongly, positively correlated with the amount consumed and produces behavioral changes similar to those induced by ethanol in the water, as well as by ethanol consumption in rodents.

4.3. Effect of voluntary ingestion of ethanol-gelatin intake on hypothalamic orexigenic peptides in zebrafish

Our results additionally demonstrate that voluntary intake of ethanol-gelatin stimulates the expression of the orexigenic peptides, GAL and OX, in the hypothalamus of zebrafish. This effect was found to be anatomically specific, occurring for GAL in the ventral (+22%) and caudal (+239%) zones of the periventricular hypothalamus, with a significantly greater effect in the caudal compared to ventral zone, and for OX (+60%) in the anterior periventricular hypothalamus. These findings with ethanol-gelatin intake in zebrafish are similar to evidence obtained in rats, showing ethanol consumption [18–20] as well as ethanol administration [20, 21] to increase mRNA levels of these two peptides. The lack of a clear dose response relationship for the two ethanol concentrations, as also shown in rats [20, 21], suggests that mRNA expression may not be sensitive to differential BEC levels within the first 30 min following ethanol ingestion. Although we cannot rule out the possibility that intake of gelatin itself affected the orexigenic peptides, the findings that 20% ethanol-gelatin stimulated the peptides without gelatin intake beyond that of 10%, together with our result that ethanol soak also stimulated GAL and OX mRNA, supports the idea that ethanol intake under our new model of voluntary ethanol-gelatin intake is sufficient to significantly alter neurochemical processes in the brain.

Whereas these peptides have yet to be examined in zebrafish in relation to their effects on ethanol intake, there is evidence that both GAL and OX mRNA expression in the hypothalamus of fish is stimulated by fasting [61–63] and that intracerebroventricular injection of GAL or OX increases food intake in fish between 1 hr and 8 hrs post-injection [27–29, 61, 64], pointing to their involvement in feeding regulation in fish. While yet to be tested, the possibility that they are involved in promoting ethanol ingestion in fish is supported by abundant evidence in the rodent literature, showing central injection of GAL or OX to stimulate ethanol drinking as well as food intake and genetic manipulation of these peptides to affect behaviors known to predict or be associated with ethanol overconsumption, including novelty seeking, locomotor activity, anxiety and aggression [3, 21, 26, 36, 65]. With this study focusing on the measures of exploration in a novel environment, which is an indicator of anxiety, and aggressive behavior, there is some evidence in the fish literature pointing to the possibility that orexigenic peptides mediate the effects of ethanol on behavior. While little is known about the relationship of these peptides to aggressive behavior and of GAL to ethanol intake, intracerebroventricular injection of OX in goldfish does stimulate locomotor activity [61, 64], a behavior closely associated with the overconsumption of ethanol in the rodent [11, 35], and studies in rats have linked endogenous GAL expression to increased locomotor activity in a novel environment [11]

and show central injection of GAL to reduce anxiety [66, 67]. Collectively, our findings demonstrate for the first time a direct relationship between ethanol and the orexigenic peptides in zebrafish, demonstrating that voluntary ingestion of ethanol dramatically changes peptide expression. Future studies are needed to determine whether these peptides, in turn, promote novelty seeking, anxiety and aggression, behaviors which increase the probability of ethanol drinking.

5. Conclusion

This novel model of voluntary ethanol consumption in zebrafish, closely resembling the pattern of ethanol consumption observed in humans and rodents, results in pharmacologically relevant BEC that leads to significant changes in behavior and orexigenic peptides which parallel changes previously observed in other species. These results support the ability of this model to be used for forward genetic studies that may allow one to characterize the effect of a single gene mutation on ethanol consumption and vulnerability to ethanol abuse in zebrafish.

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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

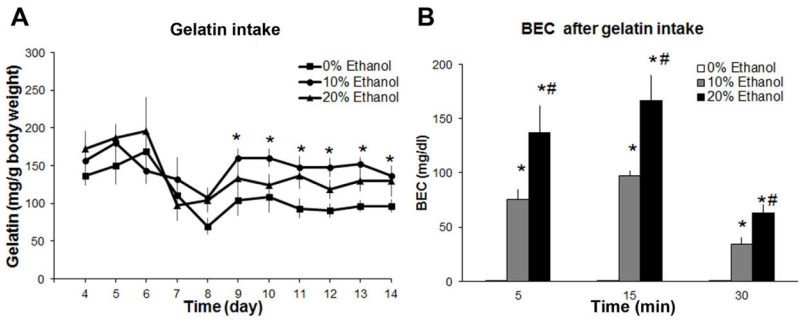

Fig. 1.

Daily feeding with gelatin containing 0%, 10% or 20% ethanol produced stable intake in zebrafish. (**A**) Intake of gelatin containing 10% ethanol resulted in greater consumption than that of gelatin with 0% ethanol (starting on D9) (n = 16/group). (**B**) Intake of 10% or 20% ethanol-gelatin led to greater blood ethanol concentration (BEC) levels than consumption of 0% ethanol-gelatin, while consumption of 20% ethanol-gelain led to higher BEC levels than consumption of 10% ethanol-gelatin (n = 10–12/group). *p < 0.05 vs. 0% ethanol-gelatin, $^{\#}p$ < 0.05 vs. 10% ethanol-gelatin.

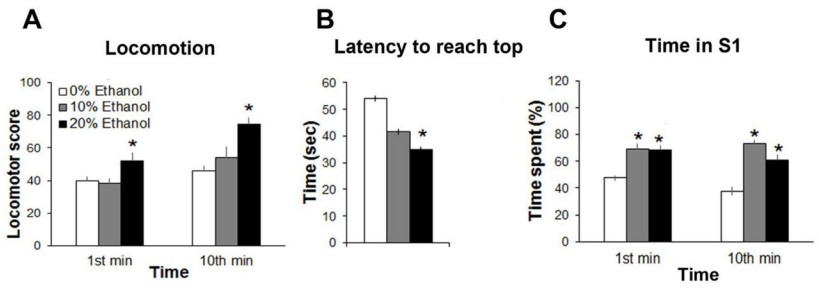

Fig. 2.

Intake of ethanol-gelatin significantly altered zebrafish behavior. (**A**) Zebrafish in the novel tank test became hyperactive following intake of 20% ethanol-gelatin compared to 10% or 0% ethanol-gelatin. (**B**) Latency to reach the top of the tank was decreased after intake of 20% but not 10% ethanol-gelatin compared to 0% ethanol-gelatin in the novel tank test. (**C**) Intake of 10% or 20% ethanol-gelatin increased the time spent in the S1 zone, which represents “contact zone” plus “approach zone” in the mirror stimulation test. *$p < 0.05$ vs. 0% or 10% ethanol-gelatin, $n = 16$/group.

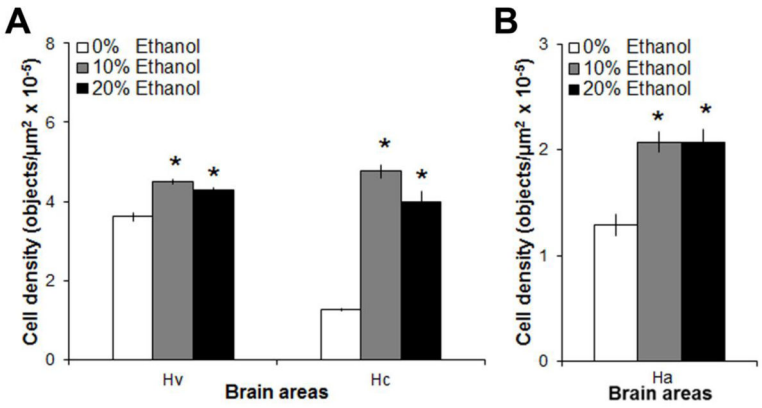

Fig. 3.

Intake of 10% or 20% ethanol-gelatin increased the density of (**A**) galanin mRNA-expressing neurons in the ventral (Hv) and caudal (Hc) region of the periventricular nucleus and of (**B**) orexin mRNA-expressing neurons in the anterior hypothalamus (Ha), as measured by digoxigenin-labeled *in situ* hybridization. *$p < 0.05$ vs. 0% ethanol-gelatin, n = 8/group.

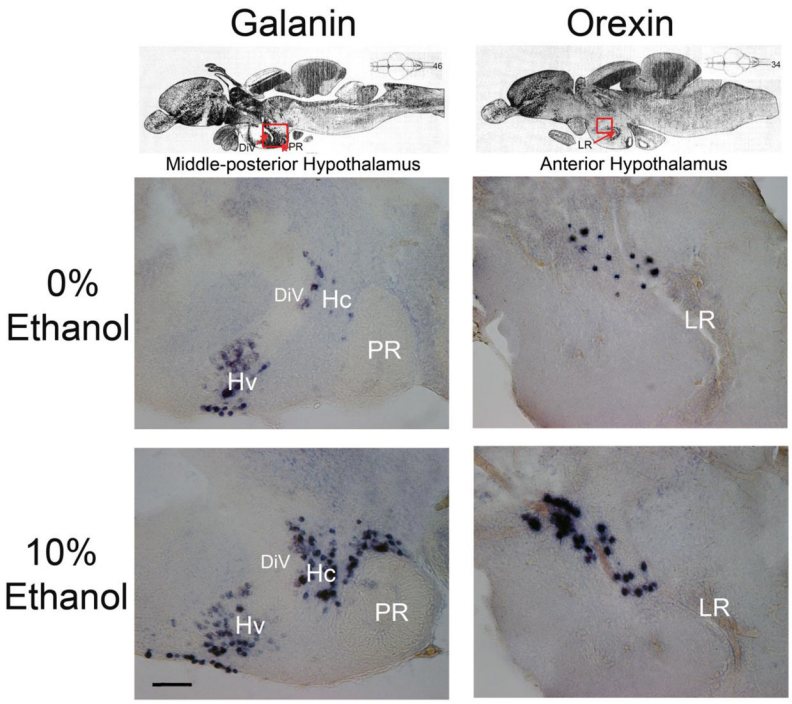

Fig. 4.

Photomicrographs illustrate changes in the density of galanin (column A) and orexin (column B) mRNA-expressing neurons in the hypothalamus in response to consumption of 20% ethanol-gelatin compared to 0% ethanol-gelatin, as revealed in sagittal sections with digoxigenin-labeled *in situ* hybridization. (**A**) Representative images from sagittal sections at the 46th section plane of the zebrafish brain, demonstrating a stimulatory effect of ethanol on galanin expression in the mid-posterior hypothalamus, outlined in red in the top panel. (**B**) Representative images from sagittal sections at the 34th section plane of the zebrafish brain, demonstrating a stimulatory effect of ethanol on orexin expression in the anterior hypothalamus, outlined in red in the top panel. DiV, diencephalic ventricle; Hc, caudal zone of periventricular hypothalamus; Hv, ventral zone of periventricular hypothalamus; LR, lateral recess of diencephalic ventricle; PR, posterior recess of diencephalic ventricle. Scale bar = 100μm. Top panels adapted from Wullimann, Rupp, and Reichert, Neuroanatomy of the Zebrafish Brain (1996), with permission from Birkhäuser Verlag [64].

Table 1

Statistical results for the effect of ethanol-gelatin ingestion on behavior in the Novel Tank and Mirror Tests. Groups were 0%, 10%, and 20% ethanol-gelatin. Time-points were 1st and 10th minute.

	df	F	p
Novel Tank Test			
Locomotor activity			
Ethanol-gelatin	1,42	69.61	0.01
Time	1,42	22.10	0.01
Ethanol-gelatin x Time	2,42	2.16	*ns*
Latency to reach top			
Ethanol-gelatin	2,45	4.37	0.01
Location of swimming			
% time on top			
Ethanol-gelatin	1,44	3.28	0.05
Time	1,44	19.10	0.02
Ethanol-gelatin x time	2,44	5.55	0.05
% time in the middle			
Ethanol-gelatin	1,44	4.53	0.01
Time	1,44	13.2	0.01
Ethanol-gelatin x time	3,44	2.83	*ns*
% time in the bottom			
Ethanol-gelatin	1,44	33.01	0.01
Time	2,44	5.43	0.01
Ethanol-gelatin x time	2,44	5.55	0.01
Mirror Test			
% time in S1			
Ethanol-gelatin	2,46	14.01	0.01
Time	1,46	1.31	*ns*
Ethanol-gelatin x time	2,46	1.81	ns
% time in contact zone			
Ethanol-gelatin	2,46	6.12	0.05
Time	1,46	10.39	0.01
Ethanol-gelatin x time	2,46	5.01	0.05
Latency to approach			
Ethanol-gelatin	2,44	7.92	0.05
Latency to contact			
Ethanol-gelatin	2,44	1.25	*ns*
Entries into approach zone			
Ethanol-gelatin	2,46	2.21	*ns*
Time	1,46	6.20	0.05

	df	F	p
Ethanol-gelatin x time	2,46	0.11	*ns*
Entries into contact zone			
Ethanol-gelatin	2,46	1.25	*ns*
Time	1,46	10.01	0.05
Ethanol-gelatin x time	2,46	0.76	*ns*

Table 2

Consumption of ethanol increases: 1) exploration in a novel environment as demonstrated by a decrease in the time that fish spent in the bottom of the tank and an increase in the time spent in the top of the tank; and 2) aggressive behavior in a mirror test as demonstrated by a decrease in the latency to approach and contact the mirror and an increase in the time spent in close proximity to the mirror. $p < 0.05$ vs. 0% ethanol, $n = 16$/group.

	1st min			10th min		
	0%	**10%**	**20%**	**0%**	**10%**	**20%**
Novel Tank Test						
% Time on top	2 ± 0.9	5 ± 2.7	14 ± 5.1	16 ± 4.4	32 ± 5.9*	29 ± 2.1*
% Time in middle	8 ± 2.9	14 ± 1.1	32 ± 2.9*	27 ± 4.6	36 ± 4.1	33 ± 3.9*
% Time on bottom	91 ± 3.5	81 ± 7.1	55 ± 9.2*	57 ± 8.0	33 ± 5.4*	48 ± 5.6
Mirror Test						
Latency to approach	12.2 ± 3.2	2.8 ± 0.6*	4.1 ± 1.5*	n/a	n/a	n/a
Latency to contact	12.8 ± 3.3	3.7 ± 0.9*	4.4 ± 1.5*	n/a	n/a	n/a
Entries into contact zone	5.8 ± 0.6	4.6 ± 0.5	5.9 ± 1.0	6.8 ± 0.8	7.4 ± 0.9	8.3 ± 0.8
Entries into approach zone	6.1 ± 0.9	5.1 ± 0.8	5.9 ± 1.6	7.0 ± 0.7	7.6 ± 1.0	9.3 ± 0.7
% Time in contact zone	34.4 ± 2.6	57.6 ± 6.9*	65.4 ± 4.9*	32.0 ± 5.1	61.6 ± 5.5*	52.3 ± 7.4*
% Time in S1	48.0 ± 3.0	65.8 ± 4.7*	68.4 ± 5.1*	37.7 ± 5.6	73.0 ± 6.3*	61.4 ± 6.6*