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Effects of embryonic ethanol exposure at low doses on neuronal development, voluntary ethanol consumption and related behaviors in larval and adult zebrafish: Role of hypothalamic orexigenic peptides

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Abstract

Embryonic exposure to ethanol is known to affect neurochemical systems in rodents and increase alcohol drinking and related behaviors in humans and rodents. With zebrafish emerging as a powerful tool for uncovering neural mechanisms of numerous diseases and exhibiting similarities to rodents, the present report building on our rat studies examined in zebrafish the effects of embryonic ethanol exposure on hypothalamic neurogenesis, expression of orexigenic neuropeptides, and voluntary ethanol consumption and locomotor behaviors in larval and adult zebrafish, and also effects of central neuropeptide injections on these behaviors affected by ethanol. At 24 h post-fertilization, zebrafish embryos were exposed for 2 h to ethanol, at low concentrations of 0.25% and 0.5%, in the tank water. Embryonic ethanol compared to control dose-dependently increased hypothalamic neurogenesis and the proliferation and expression of the orexigenic peptides, galanin (GAL) and orexin (OX), in the anterior hypothalamus. These changes in hypothalamic peptide neurons were accompanied by an increase in voluntary consumption of 10% ethanol-gelatin and in novelty-induced locomotor and exploratory behavior in adult zebrafish and locomotor activity in larvae. After intracerebroventricular injection, these peptides compared to vehicle had specific effects on these behaviors altered by ethanol, with GAL stimulating consumption of 10% ethanol-gelatin more than plain gelatin food and OX stimulating novelty-induced locomotor behavior while increasing intake of food and ethanol equally. These results, similar to those obtained in rats, suggest that the ethanol-induced increase in genesis and expression of these hypothalamic peptide neurons contribute to the behavioral changes induced by embryonic exposure to ethanol.

Keywords

zebrafish; ethanol; orexin; galanin; consumption; locomotion

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1. Introduction

Preference for and overconsumption of alcohol, in addition to being genetically determined, are affected by early environmental factors, including exposure to ethanol early in development that produces profound disturbances in growth and development, with long-term behavioral consequences. In humans, maternal drinking during pregnancy increases the risk for development of alcohol use and abuse and early onset of alcohol-related disorders [1–3]. Rodents exposed during gestation to ethanol also exhibit an increase in ethanol preference and consumption [4–6] and other behavioral disturbances, including hyperactivity and impulsive behaviors, which are associated with drug abuse [7–9]. In recent years, the zebrafish has emerged as an important vertebrate species in behavioral neuroscience showing a great translational relevance in studies of alcohol-related disorders [10, 11]. There is evidence in this species that exposure to ethanol early in development can affect certain behaviors associated with drug abuse [12–14], causing an increase in preference for ethanol as measured in a conditioned place preference test [15], an impairment of social behavior as indicated by an increase in distance among members of the shoal [16], and an increase in locomotor activity in a novel tank [17]. While studies have yet to determine whether these behavioral effects induced by embryonic ethanol exposure are accompanied by an increase in voluntary consumption of ethanol in zebrafish, this phenomenon would be expected in light of evidence consistently showing prenatal ethanol exposure to increase the drinking of alcohol in rodents and humans [1–6].

Rodent studies suggest several neurochemical and molecular mechanisms that may mediate the effects of early ethanol exposure on the consumption of ethanol in the offspring. These include the neurotransmitters, dopamine, serotonin, and gamma-aminobutyric acid, which are known to have a role in mediating ethanol acquisition, consumption, reward, reinforcement and tolerance [18–21] and also to be stimulated by ethanol in adult animals [22–25] and affected by prenatal exposure to ethanol at moderate to high doses [4]. In addition, there are considerable clinical and preclinical studies supporting a role for the orexigenic neuropeptides in alcohol abuse [26–31], including galanin (GAL) and orexin (OX) expressed in the hypothalamus. In adult rats, consumption or injection of ethanol stimulates endogenous expression and synthesis of GAL and OX [29, 30], and central injection of these peptides, in turn, increases the drinking of ethanol, while their receptor antagonists induce the opposite effect [32–35]. Moreover, prenatal exposure to ethanol in rodents has been shown to stimulate the genesis and long-term expression of these orexigenic peptides in the hypothalamus [36]. Together, these reports suggest that these orexigenic peptides may have a role in mediating the increased ethanol consumption and related behaviors affected by prenatal ethanol exposure.

Zebrafish (*Danio rerio*) have gained great popularity as an excellent model for studying the effects of harmful substances including ethanol and the mechanisms that lead to different physical, behavioral and neurochemical phenotypes. While there are no studies in this species investigating the effect of early ethanol exposure on these orexigenic peptides, there is evidence that embryonic ethanol impairs a number of neurotransmitter systems in zebrafish, causing a reduction in dopamine and serotonin levels in adult fish [16] and

altering the expression of nicotinic acetylcholine receptors and μ -opioid receptor encoding gene [15]. In our recent study describing a model of voluntary ethanol consumption in adult zebrafish [37], we demonstrated that intake of ethanol increases in the expression of the orexigenic peptides, GAL and OX, in the hypothalamus, suggesting the existence of a positive relationship between ethanol and these neuropeptides in zebrafish, similar to that described in rodents [38]. Whereas there are no reports in zebrafish examining the direct effects of these peptides on ethanol consumption, there is evidence in teleosts that central injection of GAL or OX can significantly increase the intake of food [39–41]. Also, central injection of OX in zebrafish [40] and the overexpression of OX in mutant fish [42, 43] are shown to increase locomotor behavior, while the overexpression of GAL in mutant mice is associated with a decrease in locomotor activity [43]. These studies hint at the possibility that these orexigenic peptides have a role in mediating ethanol consumption and related behaviors in zebrafish, similar to their functions in rodents, suggesting that this vertebrate species may be a good model for investigating using genetic techniques the precise mechanisms underlying these behavioral phenomena.

Building on this evidence, we investigated in the present study the involvement of hypothalamic GAL and OX in the behavioral changes induced by embryonic exposure to ethanol in zebrafish. This report examined in both larval and adult zebrafish the effects of: 1) embryonic ethanol exposure, at low concentrations, on GAL and OX expression in the hypothalamus and on the proliferation in the embryo of neurons and specifically peptide-expressing neurons; 2) embryonic exposure to ethanol on consummatory, locomotor, exploratory and aggressive behaviors; and 3) central injection of GAL and OX on the specific behaviors affected by embryonic ethanol exposure. The results obtained suggest a role for these neuropeptides in the stimulatory effects of embryonic ethanol exposure on subsequent behaviors in larval and adult zebrafish, similar to results obtained in rodents.

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 [44], from breeding pairs purchased from ZIRC (Eugene, Oregon) as described in our recent report [37]. 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. 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 μ S). The breeders were 5–8 mos old, maintained on a 12:12 h light-dark cycle (9 am lights on and 9 pm lights off) in 28.5°C water, and they were fed twice daily at 10 am and 4 pm with Zeigler Adult Diet (Aquatic Habitats). Embryos were collected 30 min after spawning and grown in a 3 L tank with 300 mL Embryo Medium in an incubator at 28.5°C, with the same light/dark cycle as the adults. Both the brain and behaviors were examined in larval and adult zebrafish raised and housed in this system, with 13 different sets of fish used in this study as listed in Table 1.

2.2 Embryonic ethanol treatment

The dosing regimen utilized in this study has been described previously by other studies [16] and is summarized briefly here. At 24 h post fertilization (hpf), the embryos were removed from the incubator, placed in a fresh solution of either 0.0, 0.25, or 0.5% ethanol (vol/vol%, pH 7.5), and then immediately returned to the incubator for 2 h, with lids covering their containers to prevent ethanol evaporation. After this 2 h-period, the embryos were once again removed from the incubator, washed for 30 min (3×10 min) in Embryo Medium, and again immediately returned to the incubator. For analysis of the larval brain, the embryos were then treated at 27 hpf with a 0.03% PTU solution.

2.3 Whole-mount immunofluorescence histochemistry (WIFH)

Whole-mount immunofluorescence histochemistry (WIFH) was used to determine in two sets of larval zebrafish (5 dpf) the effect of embryonic ethanol exposure (0.25% or 0.5%) compared to 0.0% ethanol control group ($n = 5/\text{group/set}$) on cell proliferation and neurogenesis in the anterior and posterior hypothalamus. First, a cell proliferation marker 5-Bromo-2'-deoxyuridine (BrdU) was incorporated into 48 hpf zebrafish embryos by incubating them in 10 mM BrdU solution containing 15% DMSO in Embryo Medium for 30 min at room temperature, with further incubation in Embryo Medium at 28.5° C continuing until 5 dpf. The larvae were then sacrificed and fixed in 4% paraformaldehyde (PF) overnight at 4°C. Their brains were dissected and processed for WIFH to examine hypothalamic cells that single-labeled BrdU or a neuronal differentiation marker, HuC/D (Set 1) and also cells that double-labeled BrdU and HuC/D (Set 2) to indicate the proliferation of neurons. The brains were processed as follows: 1) permeabilization in pre-cold acetone at -20° C for 10 min after 30 min wash in PBS-T (T: 0.25% TritonX-100); 2) After 30 min wash in PBS, further permeabilization in proteinase K (PK, 10 ug/ml) for 30 min followed by 20 min post-fixation in 4% PF, 15 min wash in PBS-T, and 1 h incubation in 2.0 N HCL, then 30 min wash in PBS-T, 2–4 h blocking in 0.5% TritonX-100, 5% normal donkey serum (NDS), and 2% DMSO in PBS; and 3) incubation for 48–60 h in a primary antibodies mixture containing rat anti-BrdU (1:200, Abcam MA) and mouse anti-HuC/D (1:500, Molecular Probes, Eugene, OR) at 4° C, followed by 4×10 min wash in PBS-T and further 2 h incubation in secondary antibodies mixture of Cy3-Donkey anti-mouse at 1:100 and FITC-Donkey anti-rat at 1:50 (JacksonImmunoResearch Lab. Inc. PA). Finally, the brains were mounted and coverslipped with Vectashield mounting medium (Vector, CA). They were viewed, and fluorescence images were captured using a Zeiss fluorescence microscope with MetaVue software. The density of BrdU⁺ and HuC/D⁺ immunofluorescent cells in the anterior and posterior hypothalamus was quantified with Image-Pro Plus software (Version 4.5; Media Cybernetics) as described [45], and this measure is reported as number of cells/ μm^2 . Average cell density of the ethanol (0.25% and 0.5%) and control groups was compared and analyzed statistically. For analysis of cells that double-labeled BrdU with HuC/D, the images were captured with a 20x objective, and the double-labeled cells were confirmed with a 40x objective and further validated by confocal Z-sectioning with a 40x water-immersion lens on a Zeiss LSM 510 META confocal microscope. The double-labeled cells were counted and are expressed as percent of total single-labeled cells.

2.4 Whole-mount digoxigenin-labeled in situ hybridization histochemistry (WISH)

Whole-mount in situ hybridization (WISH) was used to measure in two sets of larval zebrafish (4 dpf) the effect of embryonic ethanol exposure on the density of GAL-expressing neurons (Set 3) and density of OX-expressing neurons (Set 4) in the anterior and posterior hypothalamus. As described above, the embryos at 24 hpf were placed for 2 h in a 0.0, 0.25, or 0.5% ethanol solution ($n = 6/\text{group/set}$) and then kept until 4 dpf, when the larvae were sacrificed and fixed immediately in 4% PF at 4° C for 22–24 h. After a 10-min wash (2×5 min) in PBS, the larvae were dehydrated with a series of phosphate-buffer saline solution (0.01 M, pH 7.4) that contained 0.1% Tween 20 (PBST)-methanol (3:1, 1:1, 1:3), with 10 min in each, and then stored in 100% methanol overnight at –20° C. For the probes, zebrafish GAL and OX cDNA plasmids, generously provided by Dr. Pertti Panula (University of Helsinki, Finland), and digoxigenin-labeled riboprobes (sense and antisense) were made as described [46, 47]. To perform WISH, the larvae were rehydrated in a series of PBST-methanol solutions (1:3, 1:1, 3:1), with 10 min in each, followed by a wash in PBST (4×5 min), PB (2×5 min), and DEPC water (1×5 min). They were then processed for WISH using the same procedures as previously described [37], with the temperature of the hybridization and post-hybridization washes increased to 70° C. After color developing, the larvae were fixed for 30 min in 4% PF, and their yolk was removed. They were then immersed in 50% glycerol in PBS for 1 h, then transferred into 100% glycerol overnight at 4° C in dark, afterwards, mounted with 100% glycerol. The sense probe control was performed in additional fish receiving the same treatment, and no signal was detected. Unless otherwise indicated, all procedures were conducted at room temperature. The GAL- and OX-expressing neurons 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 [45, 48], and is expressed as number of cells/ μm^2 . The average cell density for the different groups was then compared and analyzed statistically.

2.5 Digoxigenin-labeled ISH of peptides with BrdU immunofluorescence histochemistry to measure genesis of neuropeptide neurons

In addition to using double-labeling immunofluorescence to identify BrdU cells that label HuC/D in the hypothalamus, digoxigenin-labeled ISH of peptides was combined with BrdU immunofluorescence histochemistry to determine in two sets of larval zebrafish whether the BrdU-labeled cells express the orexigenic neuropeptides, GAL (Set 6) or OX (Set 7). For this procedure, the embryos were exposed at 24 hpf to 0.0 or 0.5% ethanol for 2 h ($n = 7/\text{group/set}$), with BrdU incorporated at 48 hpf as described above, and the larvae were allowed to grow and were sacrificed at 50 dpf when the density of their peptide neurons was sufficiently high to reveal double labeling. The brains were dissected, fixed in 4% PF immediately at 4° C overnight, then cryoprotected in 25% sucrose at 4° C for 24–48 h, and then frozen at –80° C. For processing, 40 μm serial sagittal sections were cut using a cryostat, and alternate free-floating sections were prepared for digoxigenin-labeled ISH of GAL or OX, which was performed using the same procedures described in our recent publication [37], except for a lower probe concentration (2.5 $\mu\text{l/ml}$) that revealed enough but

not too strong a signal to allow double labeling with BrdU immunofluorescence. After the ISH signal was visualized in NBT/BCIP, the sections were then further processed for BrdU immunofluorescence using the same procedures as described [45, 48], BrdU immunoreactivity was revealed by rat anti-BrdU (1:100) and Cy3-conjugated donkey anti-rat secondary antibody (1:100). Double labeling was examined with a Zeiss fluorescence microscope using a 20x objective, the peptide-expressing neurons with dark blue digoxigenin-labeled signal were viewed and captured with the differential interference contrast (DIC) filter first, and then the red rhodamine/Cy3 fluorescence filter was applied to reveal the BrdU⁺ signal in the same field. The images were merged, and the double-labeled cells were confirmed with a 40x objective, counted and reported as a percentage of total single-labeled cells.

2.6 Voluntary consumption in adult zebrafish

One set of adult zebrafish individual housed at 6 mos of age (Set 8), which were embryonically exposed to 0.0% or 0.5% ethanol solution ($n = 12/\text{group}$), were trained to voluntarily consume ethanol in the form of gelatin mixed with either 10% ethanol or 0% ethanol (plain gelatin), as described in our recent publication [37]. These ethanol-gelatin meals were made fresh daily and were given 5 h into the light cycle (2 pm). The system temperature was maintained between 26.0–26.9° C and was cooled down before feeding to 24.4° C, a temperature that was optimal for keeping gelatin pieces from dissolving while not altering the normal behavior of the zebrafish. To reduce disintegration of the food, the water flow to the tanks was also stopped before the gelatin feeding and then resumed immediately after the gelatin had been removed. The fish were first trained to consume 0% ethanol-gelatin for 5 days until their baseline intake stabilized [37]. Approximately one 500 mg cube of the gelatin was given to each fish, which were allowed 10 min to consume it. The remaining gelatin was then scooped out with a fine brine shrimp net (PetCo, NYC) that had been tied taught at the head to form a scoop, blotted on a paper towel, and returned to its container. On the 6th day, the fish were then given a 500 mg cube of 10% ethanol-gelatin for 10 min, again over a period of 5 days that allowed the intake baseline to stabilize [37].

2.7 Behavioral tests in adult zebrafish

In addition to gelatin intake, other behaviors were examined in two sets of adult fish (6 mos of age), in a Novel Tank test (Set 9) and a Mirror test (Set 10), that were exposed as embryos to 0.0 or 0.5% ethanol ($n = 12\text{--}16/\text{group/set}$). These behaviors were recorded twice for 60 s, with the first recording occurring immediately after the fish were placed in the tank and reflecting their response to novelty (1st min) and the second recording occurring 10 min later and indicating their response in a habituated state (10th min). The Novel Tank test was used to assess the effect of ethanol exposure on locomotor activity and exploratory behavior, using methods established in other laboratories [10, 49] and described in our recent report [37]. Briefly, the tank (2 L, rectangular, Columbia 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. With the tests starting at 5 h after light onset, 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 latency to

reach the top and the time spent in the top, middle and bottom sections of the tank. These behaviors were scored from recordings made by a camera facing the longer, gridded wall of the tank. The Mirror test, as previously described [37] using methods established in other laboratories [10, 11, 50], was employed to assess the effect of embryonic ethanol exposure on the responses of the fish to their own mirror image, reflecting aggressive behavior. Briefly, the mirror was placed inside the 1.5 L tank (Aquatic Habitats), and 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 a camera stationed above the tanks, the following behaviors were scored: 1) percent time spent in the S1 zone (contact zone + approach zone); 2) latency to 1st approach and 1st contact; and 3) number of entries into the contact and approach zones. These behaviors were recorded by a camera placed to have a bird’s eye view of the tank.

2.8 Behavioral tests in larval zebrafish

A Novel Environment test was used to assess the locomotor activity of larval zebrafish at 6 dpf (Set 5), after embryonic exposure to 0.5% compared to 0% ethanol control group ($n = 10/\text{group}$), as described in other studies [51]. A mini square weigh boat (4.2×4.2 cm and 0.8 cm deep) was marked with a fine Sharpie on the outside with a grid of 25 squares (0.7×0.7 cm). Embryo Medium at 28.5°C was poured in to fill the weigh boat to 0.6 cm deep. The larvae were fed at 9 am and tested 3 h after feeding. An individual fish was caught with a transfer pipette and released into the novel environment. Recording commenced 30 s after the release of the larva from an overhead camera, and the number of grids entered by the larva were counted and scored as total locomotion. After 4 min of recording, the larva was returned to its home tank. Also, a thigmotaxis test, as described in other studies [51, 52], was then carried out in a standard 6 well plate, with a 4 mm circle drawn from the walls to mark the outer and inner zones. The wells were filled with Embryo Medium at 28.5°C . Larval fish were gently pipetted into the center of the well and given 2 min to habituate before a 4-min recording period started, and the percent time spent in each zone was calculated.

2.9 Quantification of different behaviors

All videos were recorded with a Panasonic Lumix DMC TS25 in 720 p HD and viewed in MPEG-4 movie format. All behaviors were manually scored using a stopwatch by a trained observer blind to the treatment of the groups. Locomotion was scored by counting entries into each of the 4 horizontal sections [10, 50] and 3 vertical sections [49, 53]. 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 [54]. Time in each layer was recorded and calculated as percent time spent in each section relative to the total time of 60 s [10, 50]. The Mirror test was scored by measuring the percent time spent in the S1 zone (contact + approach zone), the time it took for fish to first approach and contact the mirror, and the number of entries into the approach and contact zones, as previously described [49, 55].

2.10 Neuropeptides

Synthetic porcine galanin (GAL) and human orexin A (OX-A) and were purchased from American Peptide Company (Vista, CA, USA) and were dissolved in teleost saline (0.6% NaCl and 0.02% Na₂CO₃) at a dose of 0.05 ug per 0.5 uL for GAL and of 0.3 pmol per 0.5 uL for OX. These doses were chosen based on other studies showing them be to be effective in stimulating feeding behavior in fish [39, 41].

2.11 Intracerebroventricular injection of peptides in adult zebrafish

Intracerebroventricular (icv) injections were made in adult fish (6 mos of age), weighing 0.29–0.75g, using procedures described in other studies [41, 56]. To perform the injection, a piece of Styrofoam was cut to form a fish sized notch in which the fish can be placed and secured. A double-folded piece of wetted paper towel was placed under and over the fish to help secure and keep them wet. Also, clean Reverse Osmosis water, which had Instant Ocean added to 500uS and a pH of 7.4, was pipetted over the animal to help it breathe as well as remain wet. Then, fish were anesthetized in MS-222 (0.21 mg/mL) at 17.8°C for 3 min until they were no longer responding to ventral prodding with forceps. Once they were secured, a 26.5 gauge needle (Becton Dickinson, NJ, USA) was used to puncture part of the frontal parietal bone and held at a slight, 20° angle to minimize damage to the brain. The parietal bone was peeled back using fine forceps (DuMont No. 5), and a glass capillary needle was connected via a polyethylene tube to a Hamilton syringe (#80134, Hamilton Co., NA) to perform the injection into the telencephalic ventricle. This apparatus was used to inject the peptide solution, containing a marking dye (0.2 uL of 1g/1L Methylene Blue), with the injection needle at a 25° angle that placed it into the posterior region of the telencephalic ventricle close to the diencephalic ventricle and 2.0 mm below the surface of the cortex. The fish were injected with either GAL (0.05 ug) or OX-A (0.3 pmol) dissolved in 0.5 uL teleost saline, in counterbalanced order with 0.5 uL teleost saline vehicle for comparison. After the injection, the bone gap was sealed with a surgical sealing agent (Vet Bond), and the fish were placed in a clean recovery tank for about 2 min until they were swimming upright and then returned to their home tank in the system. There was a 100% survival rate, with all fish appearing normal during the following hours, as well as days and month after injection. At 2 h after icv injection, the different measures of consummatory, locomotor and exploratory behaviors were recorded in 3 sets of fish, using the test procedures described above. For the voluntary consumption tests ($n = 8-11$ /group/set), the adult fish injected with GAL or saline (Set 11) or with OX-A or saline (Set 12) were first trained to consume 0% ethanol-gelatin over a 5-day period, followed by the injection test on the 6th day, and then after a month for recovery, they were trained to consume 10% ethanol-gelatin for 5 days, followed by the injection test on the 6th day. In Set 13, the fish ($n = 8$ /group) were given icv injection of GAL, OX-A or saline and were examined 2 h later in a Novel Tank test for measurement of locomotor activity and exploratory behaviors.

2.12 Histological analysis of icv injection site

The accuracy of the injection site was confirmed by histological slides of the brain. Immediately after injection, the fish were sacrificed, and the brains were dissected and fixed in 4% PF for 2 h. After wash in PBS and briefly drying on paper towel, the fish brains were

frozen, and 40 μm serial coronal sections were cut with a cryostat. Without any staining, the sections were directly viewed with a Leitz microscope, according to the zebrafish atlas [57].

2.13 Statistical analyses

Differences between the effects of ethanol on behaviors were tested with unpaired, two-tailed t -tests. 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 by pairwise comparisons using Tukey's HSD, or with a one-way ANOVA followed 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 by a one-way ANOVA and then pairwise comparisons using Tukey's HSD. Differences in the effects of icv peptide injections on intake of gelatin with or without ethanol were tested using Univariate Analysis of Variance, followed by unpaired, two-tailed t -tests as appropriate. Data were determined to be distributed normally using the Shapiro-Wilk test, and significance was determined at $p < 0.05$. Data are reported as mean $\pm$ standard error of the mean (S.E.M.).

3. Results

3.1 Experiment 1: Effect of embryonic ethanol exposure on hypothalamic neurogenesis in larval zebrafish

This experiment examined the effect of embryonic exposure to ethanol on neurogenesis in the anterior and posterior areas of the hypothalamus, as revealed in two sets of larval zebrafish (5 dpf) using WIFH. Ethanol treatment at 0.25% and 0.5% compared to water control group ($n = 5/\text{group/set}$) induced in Set 1 a significant, overall main effect in these two areas on the density of newly generated cells single-labeled with BrdU ($F(2,30) = 28.00$, $p < 0.01$) and density of neurons single-labeled with HuC/D ($F(2,12) = 41.06$, $p < 0.01$), with no effect of hypothalamic area on either measure of BrdU ($F(1,12) = 0.26$, ns) or HuC/D ($F(1,12) = 0.18$, ns). Separate analyses revealed a significant main effect in both hypothalamic areas, of ethanol on BrdU⁺ cells in the anterior [$F(2,14) = 27.39$, $p < 0.01$] and posterior [$F(2,14) = 13.28$, $p < 0.01$] hypothalamus and on HuC/D⁺ neurons in the anterior [$F(2,14) = 24.18$, $p < 0.01$] and posterior [$F(2,14) = 10.49$, $p < 0.01$] hypothalamus, with direct comparisons showing ethanol to significantly increase ($p < 0.01$) at both concentrations the density of BrdU⁺ and HuC/D⁺ cells in these two hypothalamic areas (Table 2). In Set 2, a similar analysis of double-labeled BrdU⁺/HuC/D⁺ cells revealed a significant increase in neurogenesis (Figs. 1 and 2). The statistical analysis yielded a significant main effect of ethanol on the density of these double-labeled neurons relative to BrdU⁺ cells in the anterior ($F(2,14) = 22.61$, $p < 0.01$) and posterior ($F(2,14) = 26.06$, $p < 0.01$) hypothalamus and relative to HuC/D⁺ in the anterior ($F(2,14) = 15.09$, $p < 0.01$) and posterior ($F(2,14) = 12.78$, $p < 0.01$) hypothalamus. These main effects reflected a significant increase ($p < 0.01$) at both ethanol concentrations in the density of BrdU⁺/HuC/D⁺ cells relative to single-labeled BrdU⁺ cells (Fig. 1, left) or single-labeled HuC/D⁺ cells (Fig. 1, right), as illustrated in the photomicrographs (Fig. 2). These results provide the first

evidence that embryonic exposure to ethanol, at relatively low concentrations, can stimulate neurogenesis in the zebrafish hypothalamus.

3.2 Experiment 2: Effect of embryonic ethanol exposure on GAL and OX mRNA and behavior in larval zebrafish

This experiment examined in two sets of fish whether embryonic exposure to ethanol at 0.25% and 0.5% also affects gene expression of the orexigenic neuropeptides, GAL (Set 3) and OX (Set 4), in the anterior and posterior hypothalamus, as revealed in larval zebrafish (at 4 dpf) using WISH. Ethanol treatment compared to water control group ($n = 6/\text{group/set}$) had a significant, overall main effect on GAL mRNA expression ($F(2,14) = 16.65, p < 0.01$), with a significant main effect of brain area ($F(1,14) = 43.34, p < 0.01$) and a significant interaction between treatment and brain area ($F(2,14) = 19.84, p < 0.01$). A separate analysis of GAL mRNA expression in the two brain areas yielded a significant, main effect of ethanol in the anterior hypothalamus ($F(2,16) = 21.78, p < 0.05$) but not the posterior hypothalamus ($F(2,16) = 1.48, ns$) (Fig. 3A), as illustrated in the photomicrographs (Fig. 4), with post-hoc analyses in the anterior region showing a significant increase (+125%) in GAL mRNA expression ($p < 0.01$) after 0.5% ethanol but not after 0.25% concentration (ns) compared to control. Analysis of OX mRNA expression in the anterior hypothalamus where its neurons are primarily located similarly revealed a significant, overall main effect of ethanol ($F(2,17) = 56.24, p < 0.01$), which as with GAL reflected a significant increase (+154%) in OX mRNA after 0.5% ethanol ($p < 0.01$) compared to control but not after the lower 0.25% concentration (ns) (Fig. 3B), as illustrated in the photomicrographs (Fig. 4). To determine if these stimulatory effects of embryonic ethanol on orexigenic peptide neurons in larvae are accompanied by changes in behavior, another set of larval zebrafish (Set 5) at 6 dpf ($n = 10/\text{group}$) was examined in a novel tank with measures of locomotor behavior and thigmotaxis. These behavioral tests in fish embryonically exposed to 0.5% ethanol compared to water control revealed a significant increase in locomotor activity, as indicated by the greater number of grids crossed (97.7 ± 9.1 vs $65.4 \pm 6.1, t(18) = -3.20, p < 0.05$), but no effect of ethanol on thigmotaxis, as indicated by no change in percent time spent in the center (22.94 ± 9.9 vs $17.17 \pm 8.2, t(18) = -0.10, ns$) or at the edge (89.72 ± 4.1 vs $92.85 \pm 4.1, t(18) = 0.23, ns$) of the tank. These results show in larval zebrafish that the dose-dependent increase in expression of GAL and OX in the anterior hypothalamus induced by embryonic ethanol exposure can be linked to a specific behavioral change, namely, increased locomotor activity in a novel tank.

3.3 Experiment 3: Effect of embryonic ethanol exposure on the proliferation of hypothalamic peptide-expressing neurons

To determine if the newly generated neurons stimulated by embryonic ethanol as shown in Experiment 1 express the orexigenic peptides examined in Experiment 2, we used in this third experiment a combination of IF to label BrdU⁺ cells and ISH to label GAL-expressing neurons (Set 6) or OX-expressing neurons (Set 7). As described in the Methods section, we found that adult zebrafish (50 dpf) with a denser population of peptide neurons and a low concentration of the peptide probes were needed to reveal the co-labeling of BrdU with a peptide. Using these procedures to examine fish exposed at 24 hpf to 0.5% ethanol compared to water control group ($n = 7/\text{group/set}$), we were able to observe a small number

of GAL- and OX-expressing neurons specifically in the anterior hypothalamus. These adult fish treated as embryos to 0.5% ethanol had an average of six GAL-expressing neurons in the ventral region and two OX-expressing neurons in the dorsal region, with only one GAL neuron or no OX neurons detected in the water control group. When stained for BrdU, we observed in these ethanol-treated fish compared to control a significant increase in the density of BrdU⁺ cells ($\times 10^{-4}$) in the anterior hypothalamus (3.75 ± 0.012 vs 2.25 ± 6.1 , $t(12) = -12.44$, $p < 0.05$), confirming the results of Experiment 1. In addition, we found that two of the six GAL-expressing neurons (33%) co-labeled BrdU, as illustrated by the double-labeled BrdU⁺/GAL⁺ neuron in the photomicrograph (Fig. 5), while the OX-expressing neurons were too few to exhibit BrdU co-labeling. These results provide the first evidence that embryonic exposure to ethanol at 0.5% for 2 h is effective in stimulating neurogenesis in the zebrafish hypothalamus, specifically of neurons that express an orexigenic peptide in the anterior hypothalamus, and that these newly generated neurons are still evident in adult zebrafish.

3.4 Experiment 4: Effect of embryonic ethanol exposure on ethanol consumption in adult zebrafish

With these results showing embryonic ethanol exposure to stimulate the proliferation and expression of anterior hypothalamic peptide neurons known to promote consummatory behavior in rodents, we next tested whether embryonic ethanol affects voluntary consumption of ethanol in adult zebrafish (Set 8), similar to its effects described in rodents and humans (see Introduction). Using our recently published model of voluntary ethanol-gelatin consumption [37], we examined zebrafish at 6 mos of age that were exposed as embryos to 0.5% ethanol as compared to water control group ($n = 12$ /group) and measured changes in the consumption of plain 0% ethanol-gelatin and of 10% ethanol-gelatin during a 10-min feeding test. Measurements of intake during this 10-min period showed that embryonic exposure to 0.5% ethanol compared to control, while having no effect on the consumption of plain 0% ethanol-gelatin ($t(20) = -0.47$, ns), significantly increased by 25% the consumption of 10% ethanol-gelatin ($t(20) = -2.65$, $p < 0.05$) (Fig. 6). These data demonstrate that embryonic ethanol exposure at 24 hpf increases in adult zebrafish the consumption specifically of ethanol, while having no impact on the intake of plain food.

3.5 Experiment 5: Effect of embryonic ethanol exposure on other behaviors in adult zebrafish

This experiment examined in two sets adult zebrafish the effects of embryonic exposure to 0.5% ethanol compared to water control group on locomotor behavior and exploration in a Novel Tank test (Set 9, $n = 15$ – 16 /group) and aggressive behavior in a Mirror test (Set 10, $n = 12$ /group). To measure locomotor behavior and exploration, we recorded different behavioral measures, during the 1st min of the test when the tank was novel and then during the 10th min when the tank was familiar. In the 1st min (Fig. 7), embryonic exposure to ethanol compared to control significantly increased locomotor activity, as indicated by the total number of horizontal and vertical grid crossings ($t(29) = -3.47$, $p < 0.05$), significantly decreased latency to reach the top zone of the tank ($t(29) = 3.203$, $p < 0.05$), and significantly increased percent time spent on top ($t(29) = -2.40$, $p < 0.05$) and in the middle ($t(29) = -3.028$, $p < 0.05$) of the tank while decreasing the percent time spent on the bottom

($t(29) = 2.77, p < 0.05$). This is in contrast to these measures during the 10th min after acclimation to the environment (Table 3), when no significant changes were observed in locomotor behavior ($t(29) = 0.24, ns$) and location of the swimming whether on the top ($t(29) = -0.16, ns$), middle ($t(29) = 0.18, ns$) or bottom ($t(29) = -0.21, ns$) of the tank. To measure aggressive behavior in the zebrafish, we examined their response to their own mirror image during the 1st and 10th min of the Mirror test. The results revealed no significant differences (ns) between the ethanol-exposed and control fish during the 1st and 10th min of the test (Table 4), in the measures of percent time spent in the S1 zone ($t(22) = 0.15$ and $t(22) = 0.24$, respectively), latency to approach ($t(22) = -1.05, ns$ and $t(22) = -0.23$) and contact ($t(22) = -0.20$ and $t(22) = 0.17$) the mirror, and number of entries into the contact ($t(22) = -1.20$ and $t(22) = 0.94$) and approach zones ($t(22) = -0.20$ and $t(22) = -0.99$). Together, these behavioral tests in adult zebrafish reveal a clear stimulatory effect of embryonic exposure to 0.5% ethanol on both locomotor activity and exploratory behavior specifically in a novel environment, but no effect on aggressive behavior.

3.6 Experiment 6: Effect of central injection of GAL and OX-A on ethanol intake in adult zebrafish

With embryonic ethanol exposure stimulating the proliferation and expression of GAL and OX neurons in the anterior hypothalamus, we next tested with direct icv injections in adult zebrafish (6 mos of age) whether these peptides in the brain can themselves induce the same behaviors found to be stimulated by ethanol. The accuracy of the icv injection site was examined histologically, with methylene blue presented into the ventricle (see Methods section). In addition to light methylene blue evident within the ventricle anterior to and at the level of the hypothalamus, moderately stained cells could also be seen outside the ventricle, as illustrated in a representative image (Fig. 8) in a section between the levels 136–149 according to the atlas [57]. These dye-stained cells (purple) were concentrated in the midline along the ventricle and can be seen in the ventral region of the anterior hypothalamus.

In this experiment, we tested in two sets of adult zebrafish the effects of icv injection (Set 11) of GAL (0.05 ug) vs saline ($n = 11$ /group) and icv injection (Set 12) of OX-A (0.3 pmol, $n = 11$) vs saline ($n = 8$) on voluntary consumption of plain 0% ethanol-gelatin or 10% ethanol-gelatin in a 10-min test given 2 h after injection. Analysis of the feeding response to GAL revealed a significant, main effect of peptide vs saline injection on consumption ($F(1,40) = 71.19, p < 0.01$) and of ethanol-gelatin content ($F(1,40) = 14.30, p < 0.01$), along with a significant interaction between peptide injection and ethanol-gelatin content ($F(1,40) = 7.45, p < 0.01$). Separate analyses revealed a significant, stimulatory effect of GAL (Fig. 9A) on intake of both 0% ethanol-gelatin ($t(20) = -6.42, p < 0.01$) and 10% ethanol-gelatin ($t(20) = -6.05, p < 0.01$), with the increase in intake of 10% ethanol-gelatin (+150%) significantly greater than the increase in intake of plain 0% ethanol-gelatin (+83%). A similar analysis of the effects of OX-A also revealed a significant, main effect of this peptide vs saline injection on consumption ($F(1,34) = 20.64, p < 0.01$) and of ethanol-gelatin content ($F(1,34) = 5.73, p < 0.05$); however, in contrast to GAL, it showed no significant interaction between peptide injection and ethanol-gelatin content ($F(1,34) = 1.77, ns$), indicating that the stimulatory effect of OX-A on intake of plain 0% plain ethanol-gelatin

(+82%) and 10% ethanol-gelatin (+80%) were similar in magnitude (Fig. 9B). These results in adult zebrafish, in addition to revealing clear, stimulatory effects of icv GAL or OX injection on the consumption of ethanol-gelatin and plain gelatin, show that GAL has a considerably stronger effect on the consumption of ethanol than it does on intake of food, while OX has a general stimulatory effect that is similar for ethanol and food.

3.7 Experiment 7: Effect of central injection of GAL and OX-A on other behaviors in adult zebrafish

This experiment tested in one set of fish (Set 13) whether icv injection of GAL (0.05 ug), OX-A (0.3 pmol) or saline ($n = 8/\text{group}$) in adult zebrafish (6 mos of age) affects the additional behaviors, novelty-induced locomotor activity and exploratory behavior, that were also significantly stimulated by embryonic exposure to ethanol. Analysis of these behaviors during the 1st min of the test in a novel tank revealed a significant, main effect of peptide vs saline injection on locomotor activity ($F(2,23) = 12.41, p < 0.01$), reflecting a significant increase in the total number of horizontal and vertical crossings after injection of OX-A (+76%, $p < 0.05$) but not of GAL (ns) (Fig. 10). The measures of exploratory behavior during the 1st min of the 10-min test, in contrast, revealed no main effect of either peptide injection compared to saline (Table 5), as indicated by no change in the latency to reach the surface ($F(2,23) = 0.32, ns$) or in the location of swimming, whether percent time spent at the top ($F(2,23) = 1.02, ns$), middle ($F(2,23) = 0.94, ns$) or bottom ($F(2,23) = 0.94, ns$) of the tank. Together, these results indicate that, while neither of the peptides affected exploratory behavior, icv injection of OX-A caused a significant increase in locomotor activity in the novel environment, while GAL injection failed to alter this behavior.

4. Discussion

Results described in this report reveal a stimulatory effect of embryonic exposure to ethanol, at relatively low concentrations, on the proliferation of hypothalamic neurons and the genesis and expression of orexigenic peptide neurons, GAL and OX, in the anterior hypothalamus. It also demonstrates a stimulatory effect on different behaviors, locomotion in larvae and consummatory, locomotor and exploratory behaviors in adult zebrafish. The possibility that the endogenous GAL and OX systems are involved in mediating these behavioral changes induced by embryonic ethanol exposure is supported by two additional findings in adult zebrafish, showing that central injection of GAL preferentially stimulates the consumption of 10% ethanol-gelatin and central injection of OX stimulates novelty-induced locomotor behavior, similar to the behavioral effects induced by embryonic exposure to ethanol.

4.1 Embryonic ethanol exposure and neurogenesis in the hypothalamus of larval zebrafish

The results in this study are the first to describe a stimulatory effect of embryonic ethanol exposure on the generation of new cells, specifically neurons, in the zebrafish hypothalamus. This is revealed by a significant increase in the number of single-labeled BrdU⁺ cells and of HuC/D⁺ neurons and also in the density of BrdU⁺ cells that co-label HuC/D, indicating an increase specifically in neurogenesis. These effects, evident in the anterior and posterior regions of the hypothalamus, are induced by both concentrations of ethanol, 0.25% and

0.5%. While neurogenesis in the brain of zebrafish has yet to be examined in fish exposed as embryos to low concentrations of ethanol, moderate to high ethanol concentrations have been tested and found to have very different effects in fish, causing an increase in cell death and morphological malformations in the brain and also a decrease in neuronal differentiation in the spinal cord [58, 59]. Thus, the concentration of ethanol during embryonic development is critical for the outcome, with low concentrations stimulating neurogenesis in the hypothalamus as demonstrated here in larval zebrafish.

4.2 Embryonic ethanol and expression of hypothalamic neuropeptide systems in anterior hypothalamus of larval zebrafish

To understand whether specific neurochemical mechanisms are affected by embryonic ethanol exposure and thus have a possible role in mediating the behavioral changes, studies to date in zebrafish have focused primarily on the neurotransmitters, dopamine, serotonin, and gamma-aminobutyric acid, which are known to influence ethanol drinking, locomotor activity and exploratory behavior in rodents and humans [20, 21, 60]. In most reports, these investigations in zebrafish have shown embryonic ethanol exposure, at moderate to high concentrations, to suppress the functioning of these neurotransmitter systems in the brain [61–63]. The results of the present study reveal a very different response, with a lower concentration of ethanol (0.5%) significantly increasing in larval zebrafish the expression of the neuropeptides, GAL and OX, which are known to affect these behaviors in rodents [38]. This stimulatory effect of ethanol on peptide expression was observed specifically in the anterior hypothalamus, and it was dose dependent, seen at the 0.5% but not 0.25% concentration of ethanol. This effect is similar to that recently described in adult zebrafish when given the 10% ethanol-gelatin diet to consume [37]. Notably, it is also consistent with the stimulatory effect of prenatal ethanol exposure on the expression of these neuropeptides in adolescent rats [64]. Thus, this evidence confirms in zebrafish the responsiveness of hypothalamic GAL and OX neurons to embryonic exposure to ethanol and the persistent nature of this effect possibly reflecting an increase in neurogenesis in the embryo.

4.3 Embryonic ethanol and proliferation of peptide-expressing neurons in the anterior hypothalamus of larval zebrafish

These findings in Experiments 1 and 2, showing embryonic ethanol at low concentrations to increase neurogenesis and stimulate the expression of peptide neurons in the hypothalamus, led us to examine the possibility that the newly generated neurons labeled by BrdU co-express the orexigenic peptides. While allowing us to identify only a small number of peptide-expressing neurons, the specific procedures used enabled us to demonstrate, in zebrafish exposed as embryos to 0.5% ethanol, the existence of neurons specifically in the anterior hypothalamus that co-label GAL with BrdU. This stimulatory effect on the proliferation of GAL neurons is similar to that observed in rats exposed *in utero* to low doses of ethanol [64]. Thus, this low concentration of ethanol during embryonic development at 24 hpf is effective in stimulating the proliferation of specific hypothalamic neurons that later express an orexigenic peptide in adult fish.

4.4 Embryonic ethanol and consummatory behavior in adult zebrafish

There are no studies to date of the effects of embryonic ethanol exposure on voluntary consummatory behavior in zebrafish. Our results demonstrate for the first time that embryonic exposure to ethanol in the tank water at a relatively low concentration produces significant changes in ethanol intake of adult offspring. Specifically, 0.5% ethanol at 24 hpf significantly increases voluntary consumption of 10% ethanol-gelatin in the zebrafish at 6 months of age, showing the persistence of this behavioral change after only a brief period of exposing the embryo to ethanol. This result is consistent with the finding that embryonic ethanol increases the time spent in the presence of ethanol-conditioned cues in a conditioned place preference test, suggesting an increase in ethanol preference and the propensity for habit formation [15], a key component of drug addiction in humans [65]. It also agrees with our recent study, showing that exposure of adult zebrafish to ethanol in the tank water increases their voluntary consumption of the ethanol-gelatin food that elevates blood ethanol to > 150 mg/dL [37]. Our additional finding here, that embryonic ethanol exposure has no effect on the intake of plain 0% ethanol-gelatin, demonstrates that this change in consummatory behavior is specific to the ingestion of ethanol. This behavioral effect, induced by soaking zebrafish embryos for only 2 h in a low concentration of ethanol, substantiates the results obtained in rodents and humans (see Introduction) which consistently demonstrate a similar increase in ethanol drinking in offspring exposed *in utero* to ethanol.

4.5 Embryonic ethanol and other behaviors in larval and adult zebrafish

In addition to this increase in ethanol consumption, there are other behavioral changes in the offspring induced by embryonic exposure to ethanol at 0.5%. Our results show that novelty-induced locomotor behavior is increased in adult zebrafish, as indicated by a significantly greater number of vertical and horizontal crossings during the 1st min in a novel tank. This effect is consistent with that observed in adolescent zebrafish embryonically exposed to 1% ethanol [17] and the behavioral response of zebrafish exposed as adults to 0.25% or 0.5% ethanol [10]. The importance of a novel environment in revealing this behavioral change is indicated by our additional finding that embryonic ethanol has no effect on these measures during the 10th min of the test, after the fish had become habituated to the environment. This is in agreement with other studies, showing embryonic ethanol exposure to produce little change in the total distance traveled in a tank during a 10- or 30-min test and the total number of entries into all zones of a plus maze during a 20-min test [15, 66, 67]. The stimulatory effect of embryonic ethanol on novelty-induced locomotor activity revealed here in adult fish was similarly evident in larval zebrafish exposed as an embryo to ethanol consistent with a prior study [68], showing this behavioral change to occur early and to persist from the larval stage into adulthood. In addition to novelty-induced locomotor behavior, our results show embryonic ethanol exposure to increase exploratory behavior, as indicated by a reduction in latency to reach the top of the tank, suggesting a reduction in anxiety [54]. This same behavioral effect has been described in zebrafish exposed as adults to ethanol [10, 37, 69, 70]. This increase in exploratory behavior is consistent with our additional finding, that embryonic ethanol affects the location of swimming during the 1st min of the test, causing an increase in percent time spent in the top of the tank and a

decrease in percent time spent at the bottom of the tank. Contrary to these changes in locomotor and exploratory behavior, our tests failed to reveal in the mirror test any effect of embryonic ethanol exposure on aggressive behavior, as indicated by no change in the time spent in close proximity to the mirror or in the latency to approach and contact the mirror. Although not previously examined in fish after embryonic ethanol exposure, these measures of aggressive behavior are found to be increased in zebrafish exposed as adults to ethanol in the water [10, 50, 55] and also in zebrafish given 20% ethanol-gelatin to consume [37]. Thus, the evidence reported here clearly demonstrates that exposure of the embryo to 0.5% ethanol has a clear, stimulatory effect specifically on novelty-induced locomotor behavior in larval and adult zebrafish as well as on exploratory behavior in adult fish.

4.6 Central injection of neuropeptides and different behaviors in adult zebrafish

This increase in peptide neurogenesis induced by embryonic ethanol exposure suggests a possible involvement of these peptides in the behavioral changes induced by ethanol. While yet to be tested in zebrafish, there is ample evidence in the rodent literature linking GAL and OX to the consumption of ethanol, with studies in rats showing ethanol to stimulate peptide gene expression [28, 29] and central injection of GAL or OX, in turn, to stimulate ethanol drinking, in addition to food intake [32–35]. These peptides centrally injected in zebrafish have also been found to increase food intake [39–41, 71], consistent with the results of the present study with icv injection, and this effect has been shown to be blocked by their specific receptor antagonists [39, 41]. Our new finding, that icv injection of GAL or OX also stimulates consumption of ethanol-gelatin, supports a possible relationship between these peptides and ethanol intake. Of particular note is our additional finding that, while the effect of OX on the consumption of plain 0% ethanol-gelatin is similar in magnitude to its effect on 10% ethanol-gelatin suggesting a general effect on consummatory behavior, the stimulatory effect of GAL on intake of ethanol-gelatin is significantly stronger than its effect on intake of plain gelatin, suggesting that its actions are more specific to the intake of ethanol. A further difference between these peptides was revealed by our analyses of novelty-induced locomotor behavior, a behavior that is closely associated with the overconsumption of ethanol in rodents [72] and found here to be stimulated by injection of OX but not GAL. These findings are consistent with a study in goldfish, showing icv injection of OX to stimulate locomotor activity [71, 73, 74], and also with studies in rodents, showing central administration of OX to stimulate locomotor activity and arousal [75, 76] while GAL has no effect on these behaviors [77]. The finding here, that injection of neither GAL nor OX affect exploratory activity indicating no change in anxiety, contrasts with results in rats showing GAL to cause a reduction in anxiety [77, 78] while OX stimulates it [79]. Thus, the evidence described here suggests that GAL and OX have distinct functions in zebrafish, with GAL acting more specifically to stimulate ethanol intake and OX stimulating locomotor activity while acting more generally to increase consummatory behavior.

4.7 Possible underlying mechanisms

The results of this study lead us to postulate that the stimulatory effects of ethanol on the genesis of GAL and OX neurons are causally related to the increase in ethanol intake and locomotor behavior in the offspring, although the specific mechanisms mediating ethanol's stimulatory effect on neurogenesis is largely unknown. There is some evidence that the

maternal CCL2/CCR2 chemokine system may contribute to this effect of ethanol on the development of peptide neurons. This is indicated in recent studies from this lab [80, 81] and other reports [82–84] showing that prenatal ethanol exposure increases maternal CCL2, a chemokine that stimulates the proliferation, differentiation and migration of neurons in the offspring hypothalamus, including those that co-express its receptor, CCR2, and an orexigenic peptide. There is also evidence from *in vitro* studies showing that low doses of ethanol affects the proliferation and differentiation of neurospheres and the migration of neurons [7, 85].

5. Summary and Conclusion

The results of this study demonstrate that embryonic exposure to ethanol, at a relatively low level and for only 2 h at 24 hpf, has strong effects on neural and behavioral responses in larval and adult zebrafish. These results are remarkably similar to those obtained in both rodents and humans, indicating that the zebrafish is a useful model for investigating these phenomena. Our novel findings reveal that ethanol at low doses which do not cause apoptosis actually stimulates the expression and genesis of orexigenic peptide neurons and produces behavioral changes possibly mediated by these neurons. Furthermore, they demonstrate that embryonic exposure to ethanol stimulates voluntary ethanol consumption and novelty-induced locomotor behavior, which are also shown here in adult zebrafish to be stimulated by central administration of GAL and OX, respectively. This evidence reveals for the first time that early ethanol exposure can effect consummatory behavior in zebrafish similar to that observed in rodents and humans, indicating the translational relevance of this research in zebrafish. It is particularly important in setting a strong foundation for experiments using genetic techniques designed to investigate in depth these phenomena and their underlying mechanisms and permitting one to focus on specific genes that are relevant to the human condition of Fetal Alcohol Syndrome Disorder.

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Highlights

- Embryonic ethanol exposure stimulates neurogenesis in zebrafish hypothalamus
- Embryonic ethanol exposure stimulates expression of galanin and orexin neurons
- Embryonic ethanol exposure stimulates proliferation of galanin neurons
- Central injection of GAL preferentially stimulates ethanol intake in zebrafish
- Central injection of OX stimulates novelty-induced locomotor activity in zebrafish

Fig. 1.

Embryonic exposure to ethanol (0.25% or 0.50%) compared to control ($n = 5/\text{group}$) significantly increased the density of BrdU⁺/HuC/D⁺ neurons in the anterior (aHYP) and posterior (pHYP) hypothalamus of zebrafish larvae (5 dpf), as measured using WIFH (Experiment 1). Ethanol exposure increased the percentage of BrdU⁺/HuC/D⁺ double-labeled neurons relative to the total number of BrdU⁺ cells (left) or HuC/D⁺ cells (right) in the hypothalamus. Data are mean $\pm$ S.E.M., * $p < 0.05$ vs control.

Fig. 2.

Photomicrographs illustrate the stimulatory effect of ethanol (0.5%) compared to water control in the anterior hypothalamus on double-labeled BrdU⁺/HuC/D⁺ neurons (yellow), identified by arrowheads (Experiment 1). Images on the bottom are higher magnifications of the six neurons in the white square of an ethanol-exposed zebrafish, showing the single-labeled BrdU⁺ (green) and HuC/D⁺ (red) cells and the double-labeled BrdU⁺/HuC/D⁺ neurons (yellow). Scale bar = 100 μ m.

Fig. 3.

Embryonic exposure to ethanol compared to control ($n = 6/\text{group/peptide}$) significantly increased expression of GAL and OX peptides neurons in the hypothalamus of larval zebrafish (4 dpf), as measured by WISH and indicated by cell density (Experiment 2). A) Ethanol exposure at the 0.50% but not 0.25% concentration significantly increased GAL mRNA expression in the anterior hypothalamus (aHYP) but had no effect in the posterior hypothalamus (pHYP). B) Ethanol exposure at 0.50% but not 0.25% concentration significantly increased OX mRNA expression in the anterior hypothalamus (aHYP). Data are mean $\pm$ S.E.M., * $p < 0.05$ vs control.

Fig. 4.

Photomicrographs illustrate the significant, stimulatory effect of ethanol (0.50%) compared to control on neurons expressing GAL (top panel) or OX (bottom panel) in the anterior hypothalamus (outlined by black oval), with no significant effect on GAL-expressing neurons in the posterior hypothalamus below the oval outline, as indicated by data in Fig. 3 (Experiment 2). Scale bar = 100 μ m.

Fig. 5.

Photomicrograph illustrates the $\text{BrdU}^+/\text{GAL}^+$ double-labeled neuron (identified by the arrowhead) in the anterior hypothalamus of adult zebrafish (50 dpf) exposed as embryos to 0.50% ethanol (Experiment 3). Black: GAL^+ cells; Red: BrdU^+ cells; Double-labeled $\text{BrdU}^+/\text{GAL}^+$ cell with a red BrdU nucleus and black GAL-expressing cell perikaryon. Right column is a higher magnification of the boxed area in left image, showing from top to bottom a GAL^+ cell, BrdU^+ cell, and double-labeled $\text{BrdU}^+/\text{GAL}^+$ cell indicated by the arrowhead. Scale bar = 100 μm .

Fig. 6.

Embryonic exposure to ethanol (0.50%) compared to control ($n = 12/\text{group}$), while having no effect on intake of plain 0% ethanol-gelatin, significantly increased the intake of 10% ethanol-gelatin in adult zebrafish (Experiment 4). Data are mean $\pm$ S.E.M., * $p < 0.05$ vs control with no ethanol.

Fig. 7.

Embryonic exposure to ethanol (0.50%) compared to control ($n = 12/\text{group}$) significantly altered various behaviors in adult zebrafish when measured during the first min of a 10-min test (Experiment 5). Exposure to ethanol: A) significantly increased locomotor activity in a novel tank; B) significantly decreased latency to reach the top of the tank; and C) significantly increased the percent time spent in the top and middle zones while reducing the percent time spent in the bottom zone of the tank. Data are mean $\pm$ S.E.M., * $p < 0.05$ vs no ethanol control.

Fig 8.

Photomicrographs showing representative image of the injection site, in a section between levels 136–149 according to the atlas [57] (Experiment 6). Top image shows methylene blue-stained cells concentrated in the midline along the ventricle (V). Bottom image is a higher magnification of the boxed in area of the top image, illustrating the methylene blue-stained cells in the ventral area of the anterior hypothalamus. Scale bar = 100 μ m.

Fig. 9. Central intracerebroventricular (icv) injection of the neuropeptides compared to saline vehicle ($n = 11/\text{group}$) increased voluntary consumption of ethanol-gelatin in adult zebrafish (Experiment 6). A) GAL (0.05 μg) stimulated intake of 10% ethanol-gelatin to a significantly greater extent than intake of plain 0% ethanol-gelatin. B) OX (0.3 pmol) had a similar stimulatory effect on the consumption of 0% and 10% ethanol-gelatin. Data are mean $\pm$ S.E.M., $*p < 0.05$ vs saline vehicle.

Fig. 10.

Central injection (icv) of orexin (0.3 pmol) compared to saline ($n = 8/\text{group}$) significantly increased locomotor activity (number of grids crossed) during the first min in a novel tank, while icv injection of galanin (0.05 ug) had no effect on this behavior (Experiment 7). Data are mean $\pm$ S.E.M., $*p < 0.05$ vs saline vehicle.

Table 1

Seven experiments were performed in 13 sets of zebrafish at different ages, with some treated as embryos to ethanol in the water or given as adults intracerebroventricular injections of galanin (GAL) or orexin (OX-A) and with different procedures used for brain or behavioral measurements as described in the text.

Experiments	Sets	Age	Groups	n/group	Procedures	Measures
1	1	5 dpf	0.0, 0.25, 0.50%	5	WIFH	BrdU, HuC/D single-label
	2	5 dpf	0.0, 0.25, 0.50%	5	WIFH	BrdU/HuC/D double-label
2	3	4 dpf	0.0, 0.25, 0.50%	6	WISH	GAL mRNA cell density
	4	4 dpf	0.0, 0.25, 0.50%	6	WISH	OX mRNA cell density
	5	6 dpf	0.0, 0.50%	10	Novel Environment	Locomotion, Thigmotaxis
3	6	50 dpf	0.0, 0.50%	7	IF + ISH	BrdU/GAL mRNA
	7	50 dpf	0.0, 0.50%	7	IF + ISH	BrdU/OX mRNA
4	8	6 mos	0.0, 0.50%	12	Voluntary consumption	Ethanol-gelatin intake
5	9	6 mos	0.0, 0.50%	15, 16	Novel Tank test	Locomotion, Exploration
	10	6 mos	0.0, 0.50%	12	Mirror test	Aggressive behavior
6	11	6 mos	Saline, GAL (icv)	11	Voluntary consumption	Ethanol-gelatin intake (0%, 10%)
	12	6 mos	Saline, OX-A (icv)	8, 11	Voluntary consumption	Ethanol-gelatin intake (0%, 10%)
7	13	6 mos	Saline, GAL, OX-A	8	Novel Tank test	Locomotion, Exploration

Table 2

Embryonic exposure to ethanol (0.25% and 0.50%) compared to control ($n=5/\text{group}$) had a significant stimulatory effect on the density of BrdU⁺ and HuC/D⁺ cells in the anterior (aHYP) and posterior (pHYP) hypothalamus of larval zebrafish (5 dpf) as measured using WIFH. Data are mean $\pm$ S.E.M.

Group	BrdU ⁺ cell density (cells/ $\mu\text{m}^2 \times 10^{-4}$)		HuC/D ⁺ cell density (cells/ $\mu\text{m}^2 \times 10^{-4}$)	
	aHYP	pHYP	aHYP	pHYP
Control	1.40 $\pm$ 0.011	0.99 $\pm$ 0.01	2.45 $\pm$ 0.096	1.78 $\pm$ 0.32
0.25% ethanol	1.88 $\pm$ 0.036 [*]	2.28 $\pm$ 0.21 [*]	3.27 $\pm$ 0.081 [*]	3.46 $\pm$ 0.43 [*]
0.50% ethanol	2.22 $\pm$ 0.033 [*]	2.38 $\pm$ 0.26 [*]	3.55 $\pm$ 0.079 [*]	3.55 $\pm$ 0.16 [*]

^{*}
 $p < 0.05$ vs control.

Table 3

Embryonic exposure to ethanol (0.50%) compared to water control ($n = 15\text{--}16/\text{group}$) had no effect in adult zebrafish during the last min of the 10-min tank test, on the measures of locomotor activity and location of swimming in the familiar tank. Data are mean $\pm$ S.E.M.

Group	Locomotor behavior (Total # grids crossed)	Location of swimming (% time)		
		Top	Middle	Bottom
Control	69 $\pm$ 4.8	7 $\pm$ 1.2	17 $\pm$ 2.1	34 $\pm$ 3.8
0.50% ethanol	59 $\pm$ 7.1	8 $\pm$ 1.0	13 $\pm$ 2.5	38 $\pm$ 4.1

Table 4

Embryonic exposure to ethanol (0.50%) compared to control ($n = 12/\text{group}$) had no effect in adult zebrafish during the 1st and 10th min of the 10-min Mirror test, on the measures of percent time spent in the S1 zone, latency to approach and contact the mirror, and number of entries into the contact and approach zones. Data are mean $\pm$ S.E.M.

Time period	Group	% Time spent in S1 zone	Latency to approach	Latency to contact	# entries into contact zone	# entries into approach zone
1 st min	Control	30.5 $\pm$ 4.5	17.6 $\pm$ 2.6	29.6 $\pm$ 3.5	7.3 $\pm$ 1.5	6.5 $\pm$ 1.2
	0.50% ethanol	24.0 $\pm$ 2.4	27.5 $\pm$ 4.7	28.9 $\pm$ 3.9	6.8 $\pm$ 2.1	6.5 $\pm$ 1.4
10 th min	Control	38.4 $\pm$ 5.1	n/a	n/a	5.4 $\pm$ 2.4	4.1 $\pm$ 1.5
	0.50% ethanol	39.5 $\pm$ 4.1	n/a	n/a	8.3 $\pm$ 3.1	7.5 $\pm$ 2.1

Table 5

Central injection (icv) of orexin (0.3 pmol) or galanin (0.05 ug) compared to saline vehicle ($n = 8/\text{group}$) in a Novel Tank test had no effect on exploratory behavior, as indicated by no change in the latency to reach the top of the tank or location of the swimming.

Group	Latency to reach top of tank	Location of swimming (% time)		
		Top	Middle	Bottom
Saline	43.10 $\pm$ 8.0	29.2 $\pm$ 9.6	26.3 $\pm$ 8.5	44.5 $\pm$ 11.2
Orexin	31.63 $\pm$ 8.9	27.1 $\pm$ 8.2	30.0 $\pm$ 7.1	42.9 $\pm$ 13.5
Galanin	38.60 $\pm$ 8.5	15.3 $\pm$ 7.9	19.2 $\pm$ 5.1	65.5 $\pm$ 6.4

Data are mean $\pm$ S.E.M.