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Maternal ethanol consumption before paternal fertilization: Stimulation of hypocretin neurogenesis and ethanol intake in zebrafish offspring

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Abstract

There are numerous clinical and pre-clinical studies showing that exposure of the embryo to ethanol markedly affects neuronal development and stimulates alcohol drinking and related behaviors. In rodents and zebrafish, our studies show that embryonic exposure to low-dose ethanol, in addition increasing voluntary ethanol intake during adolescence, increases the density of hypothalamic hypocretin (hcr) neurons, a neuropeptide known to regulate reward-related behaviors. The question addressed here in zebrafish is whether maternal ethanol intake *before* conception also affects neuronal and behavioral development, phenomena suggested by clinical reports but seldom investigated. To determine if *preconception* maternal ethanol consumption also affects these hcr neurons and behavior in the offspring, we first standardized a method of measuring voluntary ethanol consumption in AB strain adult and larval zebrafish given gelatin meals containing 10% or 0.1% ethanol, respectively. We found the number of bites of gelatin to be an accurate measure of intake in adults and a strong predictor of blood ethanol levels, and also to be a reliable indicator of intake in larval zebrafish. We then used this feeding paradigm and live imaging to examine the effects of preconception maternal intake of 10% ethanol-gelatin compared to plain-gelatin for 14 days on neuronal development in the offspring. Whereas ethanol consumption by adult female *HuC:GFP* transgenic zebrafish had no impact on the number of differentiated HuC⁺ neurons at 28 hours post-fertilization (hpf), ethanol consumption by adult female *hcr:EGFP* zebrafish significantly increased the number of hcr neurons in the offspring, an effect observed at 28 hpf and confirmed at 6 and 12 days post-fertilization (dpf). This increase in hcr neurons was primarily present on the left side of the brain, indicating asymmetry in ethanol's actions, and it was accompanied by behavioral changes in the offspring, including a significant increase in novelty-induced locomotor activity but not thigmotaxis measured at 6 dpf and also in voluntary consumption of 0.1% ethanol-gelatin at 12 dpf. Notably, these measures of ethanol intake and locomotor activity stimulated by preconception ethanol were strongly, positively

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Declaration of interest

The authors declare no conflict of interest.

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correlated with the number of hcrt neurons. These findings demonstrate that preconception maternal ethanol consumption affects the brain and behavior of the offspring, producing effects similar to those caused by embryonic ethanol exposure, and they provide further evidence that the ethanol-induced increase in hcrt neurogenesis contributes to the behavioral disturbances caused by ethanol.

Keywords

hypocretin; orexin; ethanol; preconception alcohol; zebrafish

1. Introduction

Alcohol use disorder (AUD) is a major health problem impacting over 15 million adults in the USA and 76 million worldwide at great personal and societal cost (World Health Organization, 2014; Bose et al., 2016). While there is evidence that AUD is heritable (Bierut et al., 2002; Kendler et al., 2015; Verhulst et al., 2015; Reilly et al., 2017), exposure to ethanol early in life is also an important factor that increases the risk for developing AUD (Baer et al., 2003; Alati et al., 2006; Malone et al., 2010). In rodent models, maternal consumption of ethanol during pregnancy is found to increase alcohol intake and preference in the offspring (Chotro et al., 2007; Chang et al., 2015), an effect that can persist for multiple generations (Nizhnikov et al., 2016), and it also causes a range of other behavioral disturbances that further promote alcohol intake, including hyperactivity, anxiety, impulsivity and cognitive dysfunction (Carneiro et al., 2005; Stettner et al., 2011; Cullen et al., 2013; Drew & Kane, 2014; Geil et al., 2014; Hoyne et al., 2016). Whereas this evidence is clear in revealing marked neuronal and behavioral effects produced by ethanol exposure of the embryo during pregnancy, little is known about the effects on the brain and behavior caused by maternal ethanol consumption *before* conception. While the risk of developing AUD has yet to be examined, clinical studies of maternal drinking before pregnancy have revealed negative outcomes in the offspring, including a decrease in birth weight (Nykjaer et al., 2014), increase in behavioral problems (Knudsen et al., 2014), and abnormal neuropsychological functioning (Kesmodel et al., 2015), and reports in animals indicate that paternal ethanol consumption increases ethanol drinking in the offspring across multiple generations (Nizhnikov et al., 2016; Hollander et al., 2019). Also, rodent and primate models demonstrate that preconception maternal ethanol exposure increases in offspring different anxiety behaviors and corticosterone levels induced by an immune challenge, and it affects the expression and methylation profile of hypothalamic stress regulatory genes (Jabbar et al., 2016) in addition to the oocyte quality and cumulus cell gene expression (VandeVoort et al., 2015; Xu et al., 2017). These reports provide some evidence that maternal ethanol consumption before conception may have effects on both the brain and behavior of the offspring.

To test this possibility, we focused here on a specific population of neurons expressing the orexigenic neuropeptide, hypocretin/orexin (hcrt). These neurons exist in the hypothalamus while having projections throughout the brain, and they are known to be involved in promoting substance abuse and related behaviors, including ethanol consumption as well as

arousal, anxiety, motivation and stress(DiLeone et al., 2003; Li et al., 2014). This hcrt system is related to AUD, as shown by evidence in humans that blood hcrt levels are elevated in alcohol-dependent individuals during the early stages of withdrawal(Bayerlein et al., 2011; Ziółkowski et al., 2016) and are positively correlated with depression-like symptoms of alcohol withdrawal(von der Goltz et al., 2011). Hcrt expression is also shown to be stimulated by ethanol in adult rats(Moorman et al., 2016; Barson & Leibowitz, 2017) and by prenatal exposure to ethanol(Chang et al., 2012; Chang et al., 2015). Further, its injection directly into the hypothalamus increases ethanol intake and behaviors such as locomotor activity (Barson et al., 2011), suggesting a positive feedback loop.

To characterize these neurons and their relation to behaviors that may be affected by preconception maternal ethanol consumption, we utilized zebrafish, an especially useful model for *in vivo* developmental and neurobehavioral analyses. The advantages of zebrafish are considerable, due in part to their optical accessibility during early development, their genetic tractability, small size and low cost, as well as their external development. In addition, zebrafish have high physiological and genetic homology to humans, a similar CNS with neuropeptide systems that develop early and rapidly(Faraco et al., 2006; Berman et al., 2009; Podlasz et al., 2012; Gerlai, 2014), and a relatively sophisticated behavioral repertoire, including consummatory and emotional behaviors related to substance abuse(Gerlai, 2010; Kalueff et al., 2016). Of particular note is that the behavioral effects in zebrafish produced by embryonic exposure to ethanol in the water(Mathur & Guo, 2011) are very similar to those produced by intraoral administration of ethanol in pregnant rodents(Valdez et al., 2002) as well as by alcohol consumption in humans(Caldwell et al., 2002). This conservation across species is confirmed by studies in our lab and others, revealing a stimulatory effect of embryonic ethanol at different concentrations on locomotor activity, anxiety, and reward-related behaviors(Gerlai et al., 2000; Collier & Echevarria, 2013; Fontana et al., 2016). The hcrt system is well conserved in zebrafish and organized in a similar manner as in mammals, consisting of bilateral hypothalamic cell clusters that are heterogeneous(Yelin-Bekerman et al., 2015), with widespread projections to noradrenergic, dopaminergic, serotonergic, cholinergic and histaminergic nuclei(Kaslin et al., 2004; Elbaz et al., 2017). The hcrt neurons are glutamatergic in zebrafish(Appelbaum et al., 2009), as they are in mammals(Rosin et al., 2003), and they control behaviors involving feeding, reward, sleep and wakefulness(Zhdanova, 2011; Elbaz et al., 2017). It has been shown that optogenetic activation(Singh et al., 2015), chemogenetic activation(Chen et al., 2016), and overexpression(Prober et al., 2006) of hcrt neurons in zebrafish stimulate locomotor activity and decrease sleep, and chemogenetic ablation(Chen et al., 2016) as well as ablation using nitroreductase(Elbaz et al., 2012) have the opposite effect of reducing locomotor activity and increasing sleep.

The goal of this study was to examine in zebrafish the effects of voluntary maternal ethanol consumption before conception on both the brain and behavior of the offspring. To achieve this goal, we needed a reliable paradigm for feeding the adult female zebrafish some kind of food that contains ethanol. In a recent study, we developed a behavioral model of voluntary ethanol consumption in adult zebrafish with 10% or 20% ethanol mixed with gelatin, a binding agent used for nutrient or chemical administration to aquarium fishes(Royes & Chapman, 2003). In that study, we found that consumption of this ethanol-gelatin by adult

zebrafish raises their blood ethanol concentration (BEC) to pharmacologically-relevant levels and that these measures of BEC are strongly, positively correlated with the amount consumed. We also observed that consumption of this ethanol-gelatin stimulates hypothalamic expression of *hcrt* mRNA and causes an increase in locomotor activity (Sterling et al., 2015).

Using this model with some modifications to reliably measure voluntary ethanol consumption in adult as well as larval zebrafish, we investigated how preconception maternal ethanol consumption affects in the offspring: (1) early development of the *hcrt* system and neurogenesis *in-vivo*; (2) voluntary ethanol intake and related behaviors; and (3) the relationship of these changes in the *hcrt* system to the behavioral disturbances.

2. Methods and Materials

2.1. Animals and housing

AB strain zebrafish and transgenic *hcrt:EGFP* (Appelbaum et al., 2009) and *HuC:GFP* (Park et al., 2000) zebrafish (*Danio rerio*) of an unknown strain origin were used for this study. Adult zebrafish were initially group housed in 3 L tanks (Aquatic Habitat, Apopka, FL), with recirculating water flow at a temperature between 28–29°C and a pH between 6.9–7.4. Water was first filtered by reverse osmosis followed by the addition of CrystalSea Marinemix (Baltimore, MD) Bioassay Laboratory Formula salts, which primarily contains chloride, sodium, sulfate, magnesium, calcium, potassium and bicarbonate, in addition to other minor and trace elements. Conductivity of zebrafish housing water was maintained at 0.5–0.6 mS/cm. For the gelatin feeding tests, adult zebrafish were individually housed in 1.5 L tanks within the same housing system. All animals were maintained on a 12:12 hour light-dark cycle (8 AM lights on and 8 PM lights off) within an AAALAC accredited facility. Embryos were produced by natural spawning and raised in embryo media (E2) (29.2g NaCl, 1.27g KCl, 4.86 g CaCl₂, 8.14g MgSO₄ dissolved in 1 L of deionized water with 250 µl of Methylene Blue). Embryos were maintained in 250 mL beakers with lids in an incubator at 28–29°C and on a 12:12 light cycle. At 24 hpf, embryos were transferred to E2 + 0.003% phenothiourea (PTU) to inhibit melanin pigmentation and retain optical transparency for live-imaging (Kamei & Weinstein, 2005; Pan et al., 2011; Cunningham & Monk, 2018). All protocols were approved by the Rockefeller University Institutional Animal Care and Use Committee and followed the NIH Guide for the Care and Use of Laboratory Animals.

The outline of experimental procedures is presented in Figure 1.

2.2. Voluntary ethanol consumption in adult zebrafish

AB adult females of approximately 6 months of age were randomly assigned to one of two groups (n=8/group), a control group fed plain gelatin or an ethanol group fed gelatin mixed with ethanol, referred to here as “control-gelatin” and “ethanol-gelatin” groups, respectively. All adult zebrafish groups were first trained for 7 days to consume the plain gelatin (Knox Gelatin, Kraft Foods, Northfield, IL), which contained brine shrimp nauplii (Brine Shrimp Direct, Ogden, UT) and was prepared as we have previously described (Sterling et al., 2015;

Sterling et al., 2016). Every morning the gelatin was freshly prepared within a small condiment cup using 2.5ml of a 2.6% gelatin stock mixed with 2.5ml of either DI methylene blue water for the control-gelatin group or 20% ethanol that yielded a 10% ethanol-gelatin concentration for the ethanol-gelatin group. The gelatin mixture was then cooled at room temperature until it reached a honey like consistency, at which time fresh brine shrimp blotted to remove excess water were mixed in and suspended evenly throughout the gelatin. This final mixture was then placed in the freezer at -20°C for 3 minutes (min), transferred to the refrigerator at 4°C for 30 min to finish setting, and then kept on ice to keep the temperature at approximately 9°C until it was placed in the tank. To minimize possible dissolving of the gelatin cubes, each individual tank was removed from the housing system and gradually cooled to approximately 25°C prior to the feeding test. Every morning at approximately 10 AM, four gelatin cubes each weighing 250 mg were placed in each tank and then after the 10-min test were gently removed, blotted once with paper towels to remove excess water, and then re-weighed, with intake measured by calculating the change in weight divided by body weight (gelatin weight/body weight) as previously described (Sterling et al., 2015; Sterling et al., 2016). Animals were fed Zeigler Adult Zebrafish Diet (Zeigler, Gardners, PA) each afternoon at approximately 3:30 PM. With further observations leading us to consider another measure of intake, we also examined in these zebrafish the number of bites taken of the gelatin cubes. Thus, in addition to the change in gelatin weight/body weight, the number of bites was manually recorded in real-time by at least 2 observers blind to experimental conditions during each 10-min feeding session, with the final scores averaged across observers and the number of bites generally ranging from 20 to 50. After the initial training period with plain gelatin for 7 days, the zebrafish were fed for their daily morning meal over the next 14 days either the plain or 10% ethanol-gelatin for 10 min, and the two measures of change in gelatin weight/body weight and number of bites were recorded for the control-gelatin and ethanol-gelatin groups.

2.3. Blood ethanol concentration

The same two groups of adult female AB zebrafish ($n=8/\text{group}$) following 14 days of control-gelatin or ethanol-gelatin consumption were anesthetized in an ice water bath, and their blood was collected via tail incision into heparinized capillary tubes. Blood samples were centrifuged for 10 min at 4°C and 0.5 g, and blood ethanol concentration (BEC) was measured using an Analox GM7 Alcohol Analyzer (Lunenburg, MA).

2.4. Preconception maternal ethanol consumption in transgenic zebrafish

To form the two groups involving preconception ethanol exposure compared to control, *HuC:GFP* and *hcrt:EGFP* adult females of approximately 6 months of age were trained to consume control-gelatin and 10% ethanol-gelatin as described above. Following 14 days of daily consumption for their morning meal, these animals were then bred with either ethanol-naïve *HuC:GFP* or ethanol-naïve *hcrt:EGFP* males, respectively, and their offspring were grouped together and grown to 28 hpf, 6 dpf and 12 dpf for the subsequent behavioral and live-imaging experiments.

2.5. Novelty-induced locomotor activity and thigmotaxis behavior in larval zebrafish

Two groups of *hcr:EGFP* offspring at 6 dpf (n=10/group) born from either control-gelatin or ethanol-gelatin consuming adult females were individually removed from their home tank, gently placed into a circular, 36 mm diameter, open-field well of a 6-well culture plate containing 3.5 mL of embryo media, and allowed to explore for 10 min. An infrared light was mounted below the plate to enhance contrast, and due to the small size of the larval zebrafish, videos were recorded from directly overhead using a Raspberry Pi NoIr Camera V2 connected to a Raspberry Pi 3 model B+ (Raspberry Pi Foundation, Cambridge, United Kingdom). To measure novelty-induced locomotor activity, the bottom of the well was marked with a 7.9×7.9 mm square grid using a fine sharpie, and the number of grid crossings was used as the measure of novelty-induced locomotor activity. For measurements of thigmotaxis, we delineated the circular open field environment into an outer zone (1/3 of total diameter) and an inner zone (2/3 of total diameter), and thigmotaxis was measured as the percent time spent in the outer zone (Lockwood et al., 2004; Schnörr et al., 2012). All behaviors were manually scored independently by multiple investigators.

2.6. Voluntary ethanol consumption in larval zebrafish

Using the same procedures as described above for the adult zebrafish model, we measured voluntary ethanol consumption in larval zebrafish at 12 dpf, the youngest age that yielded reliable patterns of feeding. For the 10-min feeding test, 0.02 g of zebrafish AP 100 Diet <50 microns (Zeigler Bros, Gardners, PA, USA) was mixed with dissolved 2.6% gelatin stock (300 μ L) and 0.001% methylene blue DI water (300 μ L) within a small condiment cup and was pipetted until homogeneous. This control-gelatin mixture was then left at room temperature until becoming viscous and placed at -20° C for 3 min and then at 4° C for 30 min to set. The 0.1% ethanol-gelatin was prepared in the same manner as control-gelatin, except with 300 μ L of 0.2% ethanol used in place of methylene blue DI water for a final concentration of 0.1% ethanol-gelatin. Prior to feeding, the tank water of each zebrafish was cooled to approximately 25° C to minimize gelatin dissolving during the test. Each zebrafish received four small 30 mg cubes of plain gelatin for 10 min as their morning meal within a single well of a 6-well plate, with gelatin-cubes being gently placed into the water using a small spatula. For the actual experiment, *hcr:EGFP* offspring at 6 dpf, which were trained on control-gelatin until 11 dpf, were randomly separated into four groups (n=8/group), with two groups born from adults that consumed control-gelatin and two from adults that consumed ethanol-gelatin. At 12 dpf, for their morning meal, one group of control-gelatin offspring and one of ethanol-gelatin offspring continued to receive the control-gelatin, while the two remaining groups received gelatin containing 0.1% ethanol. As described above, ethanol consumption was quantified by manually counting the number of bites from the recorded videos simultaneously by multiple observers, with the final scores averaged across observers.

2.7. Live imaging and analysis of the brain

The *hcr:EGFP* and *HuC:GFP* zebrafish offspring (n=13/group) born from control-gelatin and ethanol-gelatin consuming females were mounted ventral side at 28 hpf down within 1% low-melting-point agarose containing 15 mM Tricaine Methanesulfonate (Pentair, Cary, NC)

within a metal slide with a 15 mm diameter well drilled out from the center and a $24 \times 50 \times 1$ mm glass coverslip adhered to the bottom via high vacuum grease. Following behavioral testing, the same *hcr:EGFP* zebrafish at 6 dpf ($n=10/\text{group}$) and 12 dpf ($n=16/\text{group}$) were mounted dorsal side down, and all z-stack images were acquired with an inverted Leica SP5 laser scanning confocal microscope with a 20 x or 40x water immersion lens and 1 μm step size. Deconvolution of confocal images was performed using AutoQuant X3 software (Media Cybernetics), and images were analyzed using Imaris software (Bitplane, Zurich, Switzerland). HuC^+ and hcr^+ neurons were automatically identified by the software using a 6 μm threshold for cell size, and neurons not accounted for by the software were manually added.

2.8. Statistical analyses

Comparisons between BEC levels, total HuC^+ neuron number, total hcr^+ neuron number, and behaviors were performed using two-tailed Student's t-tests. Multiple regression analysis was conducted to determine predictors of BEC using the enter method, and adjusted R-squared values are reported to account for the number of predictors in the model. Pearson's correlation was used to determine the relationship between BEC and the measures of bites of gelatin and change in gelatin weight/body weight, as well as between hcr^+ neuron number and behavior. Two-way ANOVA was used to test the within-subject main effect of brain side, the between-subject main effect of ethanol, and the interactions between the two, with significant interactions followed by post-hoc Holm-Sidak's multiple comparisons test. Significance was determined at $p < 0.05$ with Bonferroni adjusted alpha levels used when appropriate. All data are reported as mean $\pm$ SEM. Statistical analysis was performed using GraphPad Prism (GraphPad, La Jolla, CA).

3. Results

3.1. Measurements ethanol-gelatin intake are positively correlated with and predictive of BEC

Building on our prior studies (Sterling et al., 2015; Sterling et al., 2016), our first goal was to further test and substantiate the model of voluntary ethanol consumption in zebrafish that will enable us to investigate the effects of preconception maternal ethanol consumption on neuronal development in the brain of the offspring and also on their behavior, including their own intake of ethanol-gelatin. As previously reported (Sterling et al., 2015), we found during the 10-min feeding session that the intake of gelatin measured by the change in gelatin weight/body weight stabilized after about 7 days, with the intake of ethanol-gelatin over the next 14 days somewhat greater than control-gelatin intake ($t_{(14)} = 2.2$, $p < 0.05$). A similar result was obtained with the new measure of number of bites, which again was greater with the ethanol-gelatin than the control-gelatin ($t_{(14)} = 3.9$, $p < 0.01$). Consumption of 10% ethanol-gelatin during the 10-min feeding session resulted in a significant increase in BEC ($t_{(14)} = 7.18$, $p < 0.0001$), with a mean BEC of 76 ± 7 mg/dl (Fig. 2A). In the control-gelatin group, there was no correlation between BEC and the number of bites ($r = -0.08$, ns) or change in gelatin weight/body weight ($r = -0.19$, ns). Also, a multiple regression analysis calculated to predict BEC based on these two measures failed to reveal a significant model ($F_{(2,5)} = 0.8$, ns) with an adjusted R^2 of -0.05 , indicating that both measures in zebrafish

consuming the control-gelatin are not predictive of BEC, as expected. In the 10% ethanol-gelatin group, however, BEC was significantly, positively correlated with the number of ethanol-gelatin bites ($r = +0.93$, $p < 0.001$) (Fig. 2B), more strongly than that exhibited for the change in gelatin weight/body weight measure ($r = +0.72$, $p < 0.05$). Further, a multiple regression analysis revealed a significant model ($F_{2,5} = 16.6$, $p < 0.01$), with an adjusted R^2 of 0.8 and with the number of ethanol-gelatin bites being the only significant predictor of BEC ($\beta = 0.69$, $p < 0.01$). These results, revealing a close relationship between the number of bites of ethanol-gelatin and BEC, led us to use this measure of intake to examine the effects of preconception maternal consumption of 10% ethanol-gelatin compared to control-gelatin for 14 days on the brain and behavior of the offspring early in development.

Based on these findings in adult zebrafish, we then adapted this model of voluntary ethanol consumption for use in larval zebrafish, also using the number of bites to measure their voluntary consumption of 0.1% ethanol-gelatin and control-gelatin (as described in Methods and Materials). In our pilot tests with larval zebrafish of AB strain, we found that from 6 to 11 dpf they readily took bites out of the gelatin cubes, consuming progressively more gelatin with each feeding session, and their intake stabilized by 12 dpf. During the 10-min feeding session, these larval zebrafish at 12 dpf exhibited a range of 5–15 bites of the control-gelatin-cubes, and when fed 0.1% ethanol-gelatin, their number of bites was found to be comparable ($t_{14} = 2.08$, ns).

3.2. Preconception maternal ethanol consumption stimulates neurogenesis specifically of hcrt neurons

Based on previous studies showing postconception ethanol exposure to stimulate the neurogenesis of hcrt neurons (Chang et al., 2012; Sterling et al., 2016) while sometimes reducing overall neurogenesis (Gil-Mohapel et al., 2010; Buckley et al., 2019), we examined using live imaging in 28 hpf offspring of adult female zebrafish whether maternal consumption for 14 days of 10% ethanol-gelatin before conception alters the total number of differentiated neurons in *HuC:GFP* transgenic offspring and the number of hcrt neurons in transgenic *hcrt:EGFP* offspring. Compared to 545 ± 7.3 HuC^+ neurons detected in control animals at 28 hpf, we found that the offspring from the 10% ethanol-gelatin group had a similar number, an average of 542 ± 13.4 HuC^+ neurons, showing no significant difference ($t_{18} = 0.2$, ns) (Fig. 3A) as illustrated in the photomicrographs (Figs. 3B). Also, examination of the HuC^+ neurons separately on both sides of the brain revealed no main effect of ethanol-gelatin ($F_{1,36} = 0.008$, ns) or brain side ($F_{1,36} = 3.9$, ns). Very different results, however, were obtained with analyses of the hcrt neurons in *hcrt:EGFP* offspring at 28 hpf after preconception maternal intake of ethanol. Compared to 9.6 ± 0.7 hcrt neurons in control-gelatin offspring, the 10% ethanol-gelatin offspring had an average of 18.4 ± 1 hcrt neurons, almost a 2-fold increase over control ($t_{24} = 7.2$, $p < 0.0001$) (Fig. 3C). There was a significant ethanol-gelatin x brain side interaction ($F_{1,48} = 5.1$, $p < 0.05$), and the post-hoc Holm-Sidak analysis, while showing hcrt neuron number in the ethanol-gelatin compared to control-gelatin offspring to be increased on both the left ($p < 0.0001$) and right ($p < 0.001$) sides of the brain, revealed in the ethanol-gelatin offspring a significantly greater number of hcrt neurons on the left compared to the right ($p < 0.05$), an effect not present in control-gelatin offspring (ns), as illustrated in the photomicrographs (Figs. 3D). These

findings demonstrate that preconception 10% ethanol consumption by adult females, while having no effect on total number of differentiated neurons, has a stimulatory effect specifically on hcrt neurogenesis in the embryonic offspring, an asymmetric effect that occurs only on the left side of the brain.

3.3. Preconception maternal ethanol consumption stimulates hcrt neurogenesis at 6 dpf and 12 dpf

With preconception maternal consumption of 10% ethanol-gelatin found to produce a marked increase in the neurogenesis of hcrt neurons in *hcrt:EGFP* offspring at 28 hpf, we next tested whether these effects are persistent and still evident at 6 and 12 dpf. In line with our finding of increased hcrt neuron number at 28 hpf, *hcrt:EGFP* offspring of the 10% ethanol-gelatin group had significantly more hcrt neurons than control-gelatin offspring at 6 dpf, showing that the early stimulation of ethanol on their number is persistent. The 10% ethanol-gelatin offspring had an average of 22.5 ± 0.9 hcrt neurons, an 18% increase over the 18.5 ± 0.7 hcrt neurons in control-gelatin offspring ($t_{(18)} = 3.4$, $p < 0.01$) (Fig. 4A). With a separate analysis of the two sides of the brain, we found a significant main effect of ethanol-gelatin ($F_{(1,36)} = 10.7$, $p < 0.01$) but no effect of brain side ($F_{(1,36)} = 0.007$, *ns*), indicating that the ethanol-gelatin offspring at 6 dpf showed no difference in the number of hcrt neurons between the left and right sides of the brain, as shown in the photomicrographs (Figs. 4B). In 12 dpf offspring, a similar stimulatory effect was observed, with the ethanol-gelatin offspring having significantly more hcrt neurons than control-gelatin zebrafish, an average of 23.4 ± 0.7 hcrt neurons compared to 18.7 ± 0.8 neurons in control ($t_{(30)} = 4.3$, $p < 0.001$) (Fig. 4C). Interestingly, separate analyses of hcrt neurons on the two sides of the brain revealed a significant main effect of ethanol-gelatin ($F_{(1,60)} = 23.3$, $p < 0.0001$) and also a significant main effect of brain side ($F_{(1,60)} = 7.7$, $p < 0.01$). The post-hoc Holm-Sidak analysis showed in ethanol-gelatin compared to control-gelatin offspring a marked increase in neurons on the left side of the brain ($p < 0.0001$) with no effect on the right (*ns*), and in ethanol-gelatin offspring significantly more hcrt neurons on the left compared to the right side of the brain ($p < 0.01$), a difference not evident in control-gelatin offspring (*ns*), as illustrated in the photomicrographs (Figs. 4D). These results demonstrate that the stimulatory effect of preconception maternal ethanol consumption on hcrt neurogenesis observed at 28 hpf persists at least until 12 dpf and that this effect is asymmetric in these older offspring, with a greater number of hcrt neurons on the left side of the brain.

3.4. Preconception maternal ethanol consumption increases ethanol intake and locomotor activity in the offspring in close relation to the increase in hcrt neurons

We next sought to determine if preconception maternal consumption of 10% ethanol-gelatin alters voluntary ethanol consumption and also novelty-induced locomotor activity and thigmotaxis in the offspring and if these ethanol-induced changes in behavior are related to the increase in hcrt neuronal development. In 6 dpf offspring, we observed in the ethanol-gelatin compared to control-gelatin group a significant increase in locomotor activity measured in a novel open-field environment ($t_{(18)} = 2.65$, $p < 0.05$) (Fig. 5A). This measure of locomotor activity was positively correlated with the total number of hcrt neurons ($r = +0.65$, $p < 0.05$) (Fig. 5B), but not with the number of hcrt neurons on the left ($r = +0.57$, *ns*) or right ($r = +0.27$, *ns*) side of the brain, indicating the absence of an asymmetric

relationship with locomotor behavior. While preconception maternal intake of 10% ethanol-gelatin had no effect on thigmotaxic behavior in the offspring at 6 dpf as indicated by no difference in the percentage of time spent in the perimeter in control-gelatin ($t_{(18)} = 1.2$, *ns*), it markedly affected the consumption of 0.1% ethanol-gelatin in the offspring at 12 dpf. In this experiment, the offspring of the maternal control-gelatin or 10% ethanol-gelatin consumption groups were each separated into two groups that were then trained to eat either the control-gelatin or 0.1% ethanol-gelatin, with the number of bites used as the measure of intake (see Methods and Materials). In the offspring of control-gelatin females, there was no difference in their number of bites of 0.1% ethanol-gelatin compared to control-gelatin ($t_{(14)} = 2.08$, *ns*) (Fig. 5C) and no significant correlations (ranging from $r = -0.29$ to $r = 0.35$) between total, left or right hcrt neuron number and the number of bites of the control-gelatin or 0.1% ethanol-gelatin. The offspring of 10% ethanol-gelatin females, however, exhibited at 12 dpf a marked increase in their consumption of 0.1% ethanol-gelatin compared to the control-gelatin ($t_{(14)} = 4.5$, $p < 0.001$) (Fig. 5C). In addition, their total number of hcrt neurons was strongly, positively correlated with their number of bites of ethanol-gelatin ($r = +0.87$, $p < 0.01$) (Fig. 5D) but not with the number of bites of control-gelatin ($r = +0.26$, *ns*). Further, whereas the number of hcrt neurons on the right side of the brain showed no significant correlation with the measure of bites of control-gelatin ($r = +0.05$, *ns*) or ethanol-gelatin ($r = +0.51$, *ns*), the number of hcrt neurons on the left side were strongly, positively correlated with the number of bites of ethanol-gelatin ($r = +0.83$, $p < 0.05$) but not with the bites of control-gelatin ($r = +0.32$, *ns*). These results demonstrate that preconception maternal ethanol intake stimulates the consumption of ethanol in the offspring at an early age and that this increase has a strong relationship in the offspring with the total number of hcrt neurons, specifically on the left but not right side of the brain.

4. Discussion

This study demonstrates that voluntary consumption of 10% ethanol-gelatin as well as control-gelatin can be precisely and reliably measured in adult and larval zebrafish, by quantifying the number of bites taken in a 10 min feeding session, and this measure in adults is strongly, positively correlated with and predictive of BEC measures. It also shows that preconception voluntary consumption of ethanol by female adult zebrafish, while having no effect on the total number of differentiated neurons in the embryonic brain, significantly stimulates the neurogenesis specifically of hcrt neurons in *hcrt:EGFP* offspring, an effect observed at 28 hpf and confirmed at 6 and 12 dpf. Moreover, this stimulatory effect of ethanol on hcrt neurogenesis is found to be asymmetric, significantly stronger on the left than right side of the brain, and it is accompanied by and strongly positively correlated with changes in the offspring's behavior, an increase in locomotor activity measured at 6 dpf and in voluntary consumption of ethanol-gelatin at 12 dpf. These results, indicating a close relationship between these neuronal and behavioral changes, provides the first evidence that preconception maternal consumption of ethanol-gelatin disturbs the development of hypothalamic hcrt-expressing neurons while also affecting the offspring's behavior and, interestingly, has effects similar to those produced by ethanol exposure after conception (Chotro et al., 2007; Chang et al., 2015; Collier et al., 2019).

Previous work from our laboratory has demonstrated that adult female zebrafish voluntarily consume 10% and 20% ethanol-gelatin, as measured by the change in gelatin weight/body weight during the feeding session, and that they consume somewhat more of the ethanol-gelatin than the control-gelatin (Sterling et al., 2015). In the present study, we confirmed this finding in adult female zebrafish, with the greater intake of the ethanol-gelatin consistent with other evidence showing a preference for ethanol in adult zebrafish (Mathur et al., 2011; Parmar, 2011; Collier et al., 2014) and rodents (Avena et al., 2004; Barson et al., 2015). We also showed the number of bites of gelatin during the feeding session to be a more accurate, reliable and quantifiable measure than that of change in gelatin weight/body weight, perhaps due to some gelatin dissolving in the water, and the bites to be more strongly, positively correlated with BEC and the only significant predictor of BEC. After consumption of 10% ethanol-gelatin, we found BEC to be significantly elevated to 76 mg/dl, nearly reaching the minimum BEC to be qualified as binge drinking in humans (National Institute on Alcohol Abuse and Alcoholism, 2004). These findings indicate that this model of preconception maternal ethanol consumption results in pharmacologically relevant BEC levels, with the number of gelatin bites the most reliable measure of voluntary ethanol-gelatin consumption in zebrafish.

Our prior studies in rats show that postconception embryonic exposure to low-dose ethanol stimulates the expression of orexigenic neuropeptides, including hcrt neurons in the hypothalamus (Chang et al., 2012; Chang et al., 2015). It also increases at birth and in postnatal offspring the number of peptide neurons that co-express the proliferation marker BrdU, indicating that prenatal ethanol stimulates their neurogenesis (Chang et al., 2012). We have confirmed in zebrafish these effects of embryonic exposure to ethanol (0.25% or 0.50% v/v from 22–24 hpf) in the water, revealing in the hypothalamus at 4–5 dpf an increased expression and density of hcrt as well as galanin-expressing neurons and of HuC/D⁺ neurons that co-express BrdU (Sterling et al., 2016). In the current study, we obtained comparable results with preconception maternal consumption of ethanol, which significantly stimulated hcrt neurogenesis in the offspring at different ages, 28 hpf as well as 6 dpf and 12 dpf. These similar results obtained with pre- and postconception ethanol demonstrate that fertilization is not necessary for these neuronal changes to occur, indicating that they may result from epigenetic remodeling (Haycock, 2009; Ramsay, 2010). This is evidenced by a report in mice, showing both preconception and postconception maternal ethanol exposure to be similar in producing hypermethylation of the epigenetically sensitive Agouti viable yellow allele (Kaminen-Ahola et al., 2010).

To our knowledge, there have been no studies of the effects of preconception ethanol exposure on neurogenesis in the brain. Studies with postconception ethanol generally reveal an inhibitory effect on neuronal development. This is shown in zebrafish by reduced number of HuC⁺ neurons in spinal cord (Joya et al., 2014) and of cells expressing the pan-neuronal marker *elavl3:gfp* (Buckley et al., 2019) and reduced expression of the neural progenitor marker *sox2* as well as HuC⁺ and mature glial marker myelin-associated glycoprotein (Yin et al., 2014), results consistent with reports in children with fetal alcohol spectrum disorders (Riikonen et al., 2005) and rodents (Livy et al., 2003; Gil-Mohapel et al., 2010). Our observation here of preconception maternal ethanol consumption, showing no change in total number of differentiated neurons in *HuC:GFP* offspring along with an increase in hcrt

neurogenesis, suggests that ethanol exposure before conception may affect specific, but not all, neuronal subpopulations during early development. This idea is supported by evidence in zebrafish, showing that embryonic ethanol exposure disturbs facial motor neuron migration while having little to no effect on *olig2* and *ptf1a* neurons in the cerebellum and spinal cord (Buckley et al., 2019), and by reports in rats, showing prenatal ethanol to stimulate hypothalamic expression and levels of enkephalin (Druse et al., 1999; Lugo et al., 2006) and *hcrt* (Chang et al., 2012) while having little effect on expression of the orexigenic peptide, neuropeptide Y (Dembele et al., 2006). Thus, the differential effects of ethanol on specific neuronal systems, increasing *hcrt* neurogenesis but not total neuronal differentiation, may be a factor in determining the particular behavioral effects produced by preconception as well as postconception ethanol exposure.

In addition to the increase in *hcrt* neurons, we found preconception maternal ethanol consumption to stimulate novelty-induced locomotor activity in offspring at 6 dpf, a behavior closely related to and predictive of ethanol consumption (Chen et al., 2011; Barson et al., 2013). This finding is consistent with reports of low-dose postconception ethanol exposure in zebrafish from our lab (Sterling et al., 2016) and others (Tsang et al., 2019) and also in rodents (Choi et al., 2012). Further, there is evidence in zebrafish that optogenetic activation (Singh et al., 2015) and overexpression (Prober et al., 2006) of *hcrt* neurons, as well as intracerebroventricular injection of *hcrt* (Sterling et al., 2016), stimulate arousal and novelty-induced locomotor activity. Our additional finding of a strong positive correlation between total *hcrt* neuron number and locomotor activity in the offspring lends further support for a direct relationship between the stimulation of *hcrt* neurogenesis and this behavior after preconception ethanol exposure. Our evidence that ethanol has no effect on thigmotaxis behavior in 6 dpf offspring suggests a lack of anxiety, consistent with our previous study of embryonic ethanol exposure in zebrafish (Sterling et al., 2016). While this negative result is surprising given a strong relationship between *hcrt* and anxiety states (Johnson et al., 2012) and the stimulatory effect on anxiety-like behavior of optogenetic *hcrt* activation (Bonnavion et al., 2015) and central *hcrt* injection (Suzuki et al., 2005) in rodents, there is evidence that embryonic ethanol exposure may affect anxiety behavior at later ages, as we (Sterling et al., 2016) and others (Baggio et al., 2018) have previously shown in adult zebrafish.

With clinical studies showing parental drinking to be a strong predictor of the initiation and development of ethanol consumption in the offspring (Vermeulen-Smit et al., 2012), we examined the effect of maternal ethanol consumption before conception on ethanol intake in the zebrafish offspring and observed a significant increase in this behavior as measured by the number of bites during the 10-min feeding session. To our knowledge, this is the first description in any species of this behavioral change produced by preconception ethanol exposure, an effect which is similar to that produced by postconception ethanol in the rat (Chang et al., 2012; Chang et al., 2015) as well as zebrafish (Sterling et al., 2016). While there is little information in humans or animal models on the effects of maternal ethanol consumption before conception, there is evidence in rodents that preconception paternal ethanol exposure can alter ethanol intake and preference in the offspring, although the effects described vary with the paradigm tested. One study of paternal intermittent ethanol exposure via ethanol vapor inhalation reported a decrease in ethanol intake and preference in

male mice(Finegersh & Homanics, 2014; Rompala et al., 2017), while another showed paternal binge ethanol via intragastric administration to increase oral infusion of ethanol in 14-day-old rat offspring(Hollander et al., 2019), similar to our findings here in larval zebrafish.

Interestingly, the stimulatory effect of preconception maternal ethanol consumption on hcrt neuron number was found to be asymmetric, with significant increases in hcrt neurogenesis on the left but not right side of the brain, similar to the findings in our recent report of embryonic ethanol exposure in zebrafish after fertilization has occurred(Collier et al., 2019). Asymmetries in the brain are known to be important factors in the development of AUD. They are associated with an increased risk for this disorder in adolescents with asymmetrical brain morphology(Hill et al., 2001; Sharma & Hill, 2017), and there is evidence that postconception ethanol exposure produces asymmetries across brain regions in humans(Gordon, 2016, 2017; Zhou et al., 2018), enlarges the hippocampus on the left side in mice(Marjonen et al., 2015), and asymmetrically alters the migration of neural crest cells (Boric et al., 2013) as well as hcrt neurons(Collier et al., 2019) in zebrafish embryos. Thus, the asymmetric nature of the increased hcrt neurogenesis produced here by preconception maternal ethanol consumption may be a factor contributing to behavioral disturbances observed in the offspring. Notably, we found the maternal ethanol-induced increase in ethanol-gelatin intake of the offspring at 12 dpf to be strongly positively correlated with the total number of hcrt neurons and specifically with the number on the left but not right side of the brain. Evidence in zebrafish shows that hcrt neurons exhibit heterogeneous gene expression(Yelin-Bekerman et al., 2015), and rodent studies demonstrate that hcrt neurons, in addition to being neurochemically heterogeneous(Mickelsen et al., 2017), exhibit elevated Fos expression in a topographically specific manner that is positively correlated with the degree of seeking and preference for ethanol(Moorman et al., 2016) and other rewarding substances(Harris et al., 2005; Aston-Jones et al., 2009). Thus, the hcrt neurons on the left side, increased by preconception maternal ethanol consumption, may be functionally distinct in promoting ethanol intake in the offspring.

Conclusion

In conclusion, this study provides new evidence that preconception maternal ethanol intake produces marked changes in hcrt neurons and behavior of the offspring early in development, and that these preconception effects parallel those produced by postconception ethanol exposure, suggesting that fertilization may not be necessary for them to occur. With the hcrt system regulating a range of behaviors including the consumption of rewarding substances, our observation in ethanol-exposed offspring that hcrt neurogenesis is increased and strongly positively correlated with both voluntary ethanol consumption and novelty-induced locomotor activity, suggests that there may be a direct relationship between these neuronal and behavioral changes. Our additional finding of a greater increase in hcrt neurogenesis on the left side of the brain and its strong relationship with ethanol consumption in the offspring indicates that this neuronal asymmetry caused by preconception maternal ethanol exposure may have particular importance in producing alcohol-related behaviors.

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Abbreviations

Hcr	hypocretin
hpf	hours post-fertilization
dpf	days post-fertilization
AUD	alcohol use disorder
BEC	blood ethanol concentration
DI	deionized
E2	embryo media
PTU	phenylthiourea
ns	non-significant
BrdU	Bromodeoxyuridine

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Highlights

- Adult female zebrafish voluntarily consume ethanol-gelatin that increases BEC
- Preconception ethanol exposure stimulates hypocretin neurogenesis in offspring
- This increase in hypocretin is asymmetric with greater effect on the left side
- Offspring show increased locomotion and voluntary ethanol consumption
- These behavioral changes are positively correlated with hypocretin neuron number

Figure 1.

Outline of experimental procedures. AB adult female zebrafish were fed control or 10% ethanolgelatin for 14 days followed by BEC analysis. Adult *HuC:GFP* female zebrafish were also fed controlgelatin or 10% ethanol-gelatin; they were then bred with ethanol-naïve males on day 14, and the embryo offspring were live-imaged at 28 hpf. Adult *hcrt:EGFP* zebrafish were fed and bred in this same manner, with offspring live-imaged at 28 hpf, 6 dpf, or 12 dpf and their locomotor activity and thigmotaxis behavior assessed at 6 dpf and voluntary ethanol-consumption measured at 12 dpf in these same zebrafish.

Figure 2.

Effects of maternal consumption of 10% ethanol-gelatin on BEC levels and relationship to two measures of consumption. (A) Consumption of 10% ethanol-gelatin for 10 min significantly increased BEC in adult female zebrafish. **** $p < 0.0001$. (B) The measure of bites of gelatin was positively correlated with BEC. *** $p < 0.001$.

Figure 3.

Effects of maternal 10% ethanol consumption on HuC^+ and $hcrt^+$ neuron number in 28 hpf offspring. (A) There was no change in the total number of HuC^+ neurons in 28 hpf offspring of the ethanol-gelatin compared to control-gelatin consumption groups, or in the number on the left and right sides of the brain. (B) Frontal views of 28 hpf $HuC:GFP$ offspring born from the control-gelatin (top) or 10% ethanol-gelatin (bottom) consumption group. Scale bar = 30 μm . (C) 28 hpf offspring of the 10% ethanol-gelatin group had significantly more total $hcrt$ neurons and more $hcrt$ neurons on the left and right sides of the brain, with the ethanol-gelatin offspring having more $hcrt$ neurons on the left compared to the right side. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. (D) Frontal views of 28 hpf $hcrt:EGFP$ offspring born from the control-gelatin (top) or 10% ethanol-gelatin (bottom) consumption group. Scale bar = 10 μm

Figure 4.

Effects of maternal 10% ethanol consumption on *hcr*⁺ neuron number in 6 and 12 dpf offspring. (A) 6 dpf offspring of the 10% ethanol-gelatin group had significantly more total *hcr* neurons. ** $p < 0.01$ (B) Dorsal view of 6 dpf *hcr:EGFP* offspring born from control-gelatin (top) or 10% ethanol-gelatin (bottom) consumption groups. Scale bar = 10 μ m (C) 12 dpf offspring of the 10% ethanol-gelatin group had more total *hcr* neurons and more *hcr* neurons on the left side of the brain compared to control-gelatin offspring. In ethanol-gelatin offspring there were more *hcr* neurons on the left compared to the right side. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (D) Dorsal view of 12 dpf *hcr:EGFP* offspring born from control-gelatin (top) and 10% ethanol-gelatin (bottom) consumption groups. Scale bar = 10 μ m

Figure 5.

Effects of maternal 10% ethanol consumption on behavior in 6 and 12 dpf offspring and the relation to hcrt neuron number. (A) 6 dpf offspring of the 10% ethanol-gelatin group had increased novelty-induced locomotor activity. $*p < 0.05$ (B) Novelty-induced locomotor activity in 6 dpf ethanol-gelatin offspring was significantly positively correlated with total hcrt neuron number. $*p < 0.05$. (C) 12 dpf offspring of the ethanol-gelatin group consumed significantly more 0.1% ethanol-gelatin compared to control gelatin, an effect not seen in control-gelatin offspring. $***p < 0.001$. (D) The number of bites of 0.1% ethanol-gelatin was strongly positively correlated with the total number of hcrt neurons in 12 dpf offspring, a relationship similarly seen with hcrt neuron number on the left but not right side of the brain. $**p < 0.01$.