Coincubation of sperm with epididymal extracellular vesicle preparations from chronic intermittent ethanol-treated mice is sufficient to impart anxiety-like and ethanol-induced behaviors to adult progeny

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A B S T R A C T

We previously reported that paternal preconception chronic ethanol exposure in mice imparts adult male offspring with reduced ethanol drinking preference and consumption, increased ethanol sensitivity, and attenuated stress responsivity. That same chronic ethanol exposure paradigm was later revealed to affect the sperm epigenome by altering the abundance of several small noncoding RNAs, a mechanism that mediates the intergenerational effects of numerous paternal environmental exposures. Although recent studies have revealed that the unique RNA signature of sperm is shaped during maturation in the epididymis via extracellular vesicles (EVs), formal demonstration that EVs mediate the effects of paternal preconception perturbations is lacking. Therefore, in the current study we tested the hypothesis that epididymal EV preparations are sufficient to induce intergenerational effects of paternal preconception ethanol exposure on offspring. To test this hypothesis, sperm from ethanol-naive donors were incubated with epididymal EV preparations from chronic ethanol (Ethanol EV-donor) or control-treated (Control EV-donor) mice prior to in vitro fertilization (IVF) and embryo transfer. Progeny were examined for ethanol- and stress-related behaviors in adulthood. Ethanol EV-donors imparted reduced body weight at weaning and imparted modestly increased limited access ethanol intake to male offspring. Ethanol-EV donors also imparted increased basal anxiety-like behavior and reduced sensitivity to ethanol-induced anxiolysis to female offspring. Although Ethanol EV-donor treatment did not recapitulate the ethanol- or stress-related intergenerational effects of paternal ethanol following natural mating, these results demonstrate that coincubation of sperm with epididymal EV preparations is sufficient to impart intergenerational effects of ethanol through the male germline. This mechanism may generalize to the intergenerational effects of a wide variety of paternal preconception perturbations.

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Introduction

While the high heritability associated with alcohol use disorder (Prescott & Kendler, 1999; Young-Wolff, Enoch, & Prescott, 2011; Ystrom, Reichborn-Kjennerud, Aggen, & Kendler, 2011) has long been exclusively attributed to genetic factors, over the past several years, many studies now suggest that familial risk may be significantly mediated by germline epigenetic mechanisms (see Rompala & Homanics, 2019 for review). For instance, previous work from our lab revealed that paternal chronic ethanol exposure reduced ethanol drinking preference and consumption, increased ethanol sensitivity, and attenuated stress responsivity in male mice on both hybrid and pure genetic backgrounds (Beeler, Nobile, & Homanics, 2019; Finegersh & Homanics, 2014; Rompala, Finegersh, & Homanics, 2016; Rompala, Finegersh, Slater, & Homanics, 2017). In addition, a plethora of studies have demonstrated intergenerational effects of paternal ethanol exposure on wide ranging
phenotypes including offspring weight, learning and activity, anxiety-related behaviors, and various molecular and physiologic effects (see Finegersh, Rompala, Martin, & Homanics, 2015 for review).

While it is clear that paternal preconception ethanol exposure induces persistent effects that impact the next generation, the molecular mechanism(s) responsible for these intergenerational effects are largely unknown. Over the last five years, evidence from studies in other fields has established a causal relationship between environmentally responsive sperm noncoding RNAs and diverse intergenerational phenotypes (Benito et al., 2018; Chen, Yan, Cao, et al., 2016a; Gapp et al., 2018, 2014; Rodgers, Morgan, Leu, & Bale, 2015; Sharma et al., 2016). For example, the intergenerational effects of paternal stress were partially recapitulated in mice derived from embryos injected with sperm noncoding RNAs from stressed fathers (Gapp et al., 2018, 2014; Rodgers et al., 2015). Consistent with this mechanism of epigenetic inheritance, we found that chronic intermittent ethanol exposure altered several small noncoding RNAs in sperm (Rompala et al., 2018). Such findings have prompted intense interest in understanding the biogenesis of the sperm RNA milieu and how sperm RNAs are altered in response to environmental exposures.

Following spermatogenesis in the testis, sperm are transcriptionally quiescent (Martins & Krawetz, 2007). Despite this, the composition of small noncoding RNA (ncRNA) in sperm shifts dramatically as sperm mature during migration through the epididymis, suggesting the involvement of the extracellular luminal environment (Nixon et al., 2015). Throughout the epididymis, small (50–150 nm) membrane-bound vesicles are secreted from the epithelium into the sperm-enriched lumen (Trigg, Eamens, & Nixon, 2019). These extracellular vesicles (EVs) are critical for trafficking key proteins to sperm in support of motility and capacity for fertilization (see Sullivan, 2016 for review). More recently, deep sequencing efforts have elucidated that the RNA cargo of epididymal EVs closely reflects that of mature sperm (Sharma et al., 2016). Furthermore, epididymal EVs are capable of delivering small ncRNAs to sperm in vitro (Reilly et al., 2016; Sharma et al., 2016, 2018). Intriguingly, given this ability to shape the sperm small ncRNA profile, epididymal EVs may play a critical role in RNA-mediated intergenerational inheritance through the male germline (Morgan, Chan, & Bale, 2019); nevertheless, this remains to be directly tested. Therefore, in the present study, we examined the hypothesis that epididymal EV preparations are sufficient to recapitulate the intergenerational effects of paternal chronic ethanol exposure.

Materials and methods

Animals

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh and conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Specific pathogen-free C57BL/6J (B6) and CD-1 mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, United States). Mice were habituated to the University of Pittsburgh animal facility for at least 1 week prior to initiation of experiments. Mice were housed in ventilated caging (Allentown, Inc., Allentown, New Jersey, United States) under 12-h light/dark cycles (lights on at 7:00 AM and lights off at 7:00 PM), and had ad libitum access to food (irradiated SP76 ProLab IsoPro RMH 3000 [LabDiet; St. Louis, Missouri, United States]) and water.

Chronic intermittent ethanol vapor inhalation

Chronic intermittent ethanol vapor exposure was performed as previously described (Finegersh & Homanics, 2014; Rompala et al., 2016, 2017). Briefly, 8-week-old male B6 mice were randomly assigned to one of two treatments: half of the mice were exposed to ethanol inhalation chambers in the home cage with water and food for 5 weeks from 09:00 AM to 5:00 PM over five consecutive-day blocks, with 2 days in between blocks. The other half of the mice were assigned to the room-air control group in identical chamber conditions without ethanol vapor. All animals were group-housed throughout the experiment, and cages, food, and water were all changed routinely after the final exposure of each week. Blood ethanol concentration was measured after the final ethanol exposure of each week by extracting tail vein blood (<10 μL) using heparin-coated capillary tubes (Drummond; Broomall, Pennsylvania, United States) and running plasma samples (extracted from blood by centrifugation at 2300 × g for 10 min) on an Analox Ethanol analyzer (AM1, Analox Instruments; London, United Kingdom). Tail blood was drawn from all groups to control for stress. Ethanol content in the ethanol inhalation chambers was monitored using a custom sensor generously provided by Brian McCool, PhD (Wake Forest University), and flow rates in the chambers were adjusted weekly based on blood ethanol concentration measurements made during the preceding week. Importantly, animals do not lose significant body weight during this experimental protocol (defined as >10%). In addition, the effects of ethanol vapor on lungs, heart, and liver were comparable to those associated with other chronic ethanol exposure models (Mouton et al., 2016).

Isolation of extracellular vesicles from the epididymis

EV samples were isolated from adult male mice sacrificed –16–19 h following the final ethanol or room air exposure during the light cycle (8:00 AM to 11:00 AM). Briefly, after euthanasia by CO2 asphyxiation, left and right epididymides were dissected into 1.5 mL of EmbryoMax Human Tubal Fluid (HTF) (Sigma–Aldrich; St. Louis, Missouri) at 37 °C. Several small cuts were made in each epididymide to release the epididymal lumen contents, including sperm and EVs, into solution. The sperm-EV medium was then transferred to a 1.5-mL Eppendorf tube and incubated for 20 min at 37 °C. The top 1.2 mL of supernatant was carefully collected for further processing, leaving behind large tissue pieces. Next, the recovered supernatant was centrifuged at 2000 × g for 5 min to pellet the sperm. EVs were then isolated from the supernatant by filtration and ultracentrifugation. First, the EV-containing medium was centrifuged at 10 000 × g for 30 min at 4 °C before being passed through a 0.2-μm nylon syringe filter. Finally, EVs were pelleted on a tabletop ultracentrifuge at 120,000 × g for 2 h at 4 °C, washed once with ice-cold 1.5-mL PBS to remove excess protein aggregates, centrifuged again at 120 000 × g for 2 h at 4 °C, and then snap-frozen on dry ice. EV concentration was quantified using the BCA protein assay.

In vitro fertilization

All media were equilibrated with mineral oil (Sigma–Aldrich) and kept at 37 °C in a 5% CO2 incubator. For 6-week-old B6 oocyte donor females (habituated to the animal colony for at least 1 week) were superovulated by intraperitoneal (i.p.) injection with 5 IU pregnant mare serum gonadotropin (Sigma–Aldrich) and 5 IU human chorionic gonadotropin (hCG) (Sigma–Aldrich) 48 h later. The following day, 10.5 h after the hCG injection, one 10-week-old B6 donor male (habituated to the mouse colony for 2 weeks) was sacrificed for rapid collection of cauda epididymis into HTF. The left
and right cauda epididymis were split into separate 500-μL HTF preparations and assigned to either Control-EV or Ethanol-EV donor treatment at random. Small cuts were made to release sperm into solution, and sperm were incubated for 2 min. Next, the epididymal tissue was removed and the sperm suspension was centrifuged at 300 × g for 1 min. The supernatant was discarded, and the remaining sperm pellet was resuspended in 500-μL HTF. Sperm concentration was quantified with a hemocytometer, and 6 × 10⁵ sperm (in 30 μL) were coincubated with 190 μg (in 10 μL) of the epididymal EV preparation (pooled from four mice from the same group-housed home cage) in HTF supplemented with 1 mM ZnCl₂, adjusted to pH 6.5 at a final volume of 40 μL, and incubated at 37 °C in 5% CO₂ for 3 h. For each IVF culture, three oocyte donor females were sacrificed for rapid collection of oviducts into HTF media supplemented with 1 mM glutathione (GSH) (Sigma-Aldrich) to increase zona pellucida permeability. For each oocyte donor, oviducts were torn at the ampulla to release oocyte masses into solution and moved to a 300-μL HTF+GSH drop on the IVF culture dish. Finally, 20 μL of the EV-mixed sperm was added to each IVF dish and incubated for 6 h. Following IVF, oocytes were washed in three different 100-μL HTF drops to remove debris and excess sperm. Presumptive zygotes were then cultured overnight. The next morning, 2-cell embryos were counted and separated from unfertilized or degenerating oocytes and cultured in KSOM media (Sigma-Aldrich) for 1–3 h prior to transfer to pseudopregnant CD-1 foster mothers.

Embryo transfer

CD-1 females (Charles River Labs) at 8–12 weeks old were naturally mated to CD-1 vasectomized males (Charles River Labs; Fig. 1. Examining the effects of Ethanol-EVs on IVF-derived mice (A) Electron micrograph demonstrating enrichment of extracellular vesicles (EV) isolated from adult mouse epididymis. Arrows indicate EVs and scale bar = 100 nm (B) Experimental design for examining the effect of EVs on intergenerational ethanol- and stress-related behaviors. After adult males were exposed to chronic ethanol or control treatment, they were sacrificed to isolate epididymal EVs. For each Ethanol EV- and Control EV-donor pool, EVs were pooled from four mice and incubated with sperm during capacitation immediately preceding in vitro fertilization. Fertilized oocytes were implanted into foster dams and adult progeny were phenotyped for ethanol- and stress-related behaviors (C) Average blood ethanol concentrations (BECs) for all Ethanol EV-donors (D) No effect of chronic ethanol exposure on body weight of Ethanol EV-donors (E) No effect of Ethanol-EVs on IVF success rate (F) No effect of Ethanol-EVs on litter size. Data are presented as μ ± SEM. Error bars are obscured by data points in panel D. Numbers in bars denote group sizes.

Fig. 2. Ethanol-EVs reduce body weight selectively in male progeny (A) Ethanol-EVs conferred reduced body weight to postnatal day (PND) 21 males vs. Control EV-donors (Ctrl-EVs) (B) There was no effect of Ethanol-EVs on body weights in females. *** = p < 0.001. Data are presented as μ with SEM bars obscured by data points.
−6 months of age). The following morning, females were checked for vaginal plugs; plug-positive (pseudo-pregnant) females were segregated to be used as recipients. 2-Cell embryos (15–30 embryos per recipient) were surgically transferred to both oviducts of anesthetized recipients. Pregnant dams were maintained in single housing and were housed with pups until weaning at 3 weeks postnatal.

**Behavioral testing**

For all behavioral testing, no more than two mice of the same sex were examined per litter. For each treatment group, 8-week-old offspring were split into one of two behavioral batteries with at least 2 weeks between each behavioral assay. Behavioral battery 1 was carried out in the following order: 1) elevated plus maze (following saline injection), 2) two-bottle free choice ethanol drinking, 3) light/dark test. Behavioral battery 2 was carried out in the following order: 1) elevated plus maze (following ethanol injection), 2) HPA axis responsivity to acute restraint, 3) drinking in the dark.

**Elevated plus maze**

Adult mice were single-housed and habituated to the test room for 1 h in the home cage prior to the test trial. The elevated plus maze apparatus was fitted with two closed and open arms, and both the floors and walls were made of opaque white plexiglass. Light intensity directly over the apparatus was set to 35 lux. Ten minutes prior to the test trial, mice received IP injections of 5% (w/vol) ethanol (1.0 g/kg) or saline (0.9% NaCl) and were returned to the home cage. After 10 min, mice were placed in the center of the elevated plus maze, always positioned with the snout-end facing...
Mal es

Four paws needed to be visible in the light region of the box. Camera and scored manually. To be scored as in the light region, all time spent in the light region were recorded with an overhead into the dark region of the apparatus, and latencies to enter and room. At the beginning of the 5-min trial, test mice were placed preceding the trial, single-housed mice were habituated to the test

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Flap aperture in the dividing wall. The dark region features black plex-

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The light/dark box features adjacent light and dark compartments through which the test mouse can move freely through an aperture in the dividing wall. The dark region features black plexiglass flooring and walls with a removable cover to place the animal inside (light intensity of 2 lux). The light region has transparent flooring and walls with no roof (light intensity of 390 lux). One hour preceding the trial, single-housed mice were habituated to the test room. At the beginning of the 5-min trial, test mice were placed into the dark region of the apparatus, and latencies to enter and time spent in the light region were recorded with an overhead camera and scored manually. To be scored as in the light region, all four paws needed to be visible in the light region of the box.

Light/dark box

The drinking in the dark assay was performed based on published methods (Thiele, Crabbe, & Boehm, 2014). For 4 nights, mice were habituated to a 10-mL sipper tube filled with water that replaced their regular water bottle 2 h into the animal’s dark cycle. Sipper tubes were designed by fitting ball-bearing sippers into modified 10-mL serological pipets (Corning Incorporated; Durham, North Carolina, United States) and sawed off at the tip, with the fit secured with heat-shrink and Parafilm®. After the final habituation trial, the 10-mL sipper tube was then filled with 20% (w/vol) ethanol, and consumption was measured for 2 h over three consecutive nights, and finally was measured for 4 h on the fourth and final night. Tail blood was collected immediately following the 4-h trial to measure BECs as described above. As a control measure, 1 week after the 4-h trial, saccharine consumption was examined 2 h into the dark cycle in 4-h trials over two consecutive days.

Acute HPA axis responsivity

Sixteen-week-old male and female mice were subjected to a 15-min restraint stress exposure. All animals were tested between 10:00 AM and 1:00 PM of the light cycle. Briefly, mice were removed from group housing and restrained in conical plastic tubes with several air hole perforations near the animal’s head and an opening for the tail. After the 15-min restraint, each mouse was housed in a single novel cage in a fume hood for another 15 min. Only one mouse was tested per group-housed cage to avoid pre-stressing any test animals. Tail blood was collected at time points 0 and 30 min from the onset of restraint stress. Blood samples were centrifuged for 10 min at 2300×g to separate plasma for measurement of corticosterone with an enzyme immunoassay (Enzo Life Sciences; Farmingdale, New York, United States).

Transmission electron microscopy

Exosome microscopy was performed with a JEOL JEM-1011 transmission electron microscope using negative staining procedures at the Center for Biological Imaging at the University of Pittsburgh (Pittsburgh, Pennsylvania).

Statistical analysis

As we have previously observed, a significant interaction between paternal ethanol exposure and offspring sex (Finegersh & Homanics, 2014; Rompala et al., 2016, 2017), males and females were examined separately for all tests. For IVF experiments, the unpaired Student’s t test was used to compare Control EV-donor and Ethanol EV-donor group means for IVF efficiency, litter size, light/dark box measures, open field measures, and BECs. A two-way ANOVA was used for elevated plus maze measures (factors of ethanol injection and EV-donor), and a two-way repeated-measures ANOVA was used for two-bottle choice ethanol drinking, drinking in the dark, HPA axis responsivity (factors of EV-donor and

A. Males

100

Latency to enter light

Males

6

7

100

150

Time in light region

Males

6

7

C. Females

100

Latency to enter light

Females

12

11

D. Females

100

Time in light region

Females

12

11

Fig. 4. Ethanol-EVs confer increased anxiety-like behavior to IVF-derived females in the light/dark box transition test. No effect of Ethanol-EVs on (A) latency to enter light or (B) total time spent in the light region in male progeny (C) Ethanol-EVs increased latency to enter the light region in female progeny (D) No effect of Ethanol-EVs on time in light region for female progeny. ** = p < 0.01. Data are presented as ± SEM. Numbers in bars represent group sizes.

Two-bottle free choice ethanol drinking test

Mice were single-housed for 1 week while habituating to two 25-mL sipper tubes filled with autoclaved water. After the 1-week habituation, ethanol-drinking behavior was assessed by filling one tube with ethanol. Consumption of ethanol and water was measured daily, and the positions of the ethanol and water tubes were rotated each day. Ethanol concentration started at 3% (w/vol) and was increased every 4 days to 6, 9, 12, and 15%, successively. Cages were changed and animals were weighed every 4 days.

Drinking in the dark assay

The drinking in the dark assay was performed based on published methods (Thiele, Crabbe, & Boehm, 2014). For 4 nights, mice were habituated to a 10-mL sipper tube filled with water that replaced their regular water bottle 2 h into the animal’s dark cycle. Sipper tubes were designed by fitting ball-bearing sippers into modified 10-mL serological pipets (Corning Incorporated; Durham, North Carolina, United States) and sawed off at the tip, with the fit secured with heat-shrink and Parafilm®. After the final habituation trial, the 10-mL sipper tube was then filled with 20% (w/vol) ethanol, and consumption was measured for 2 h over three consecutive nights, and finally was measured for 4 h on the fourth and final night. Tail blood was collected immediately following the 4-h trial to measure BECs as described above. As a control measure, 1 week after the 4-h trial, saccharine consumption was examined 2 h into the dark cycle in 4-h trials over two consecutive days.

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Ethanol EV-donors confer reduced body weight selectively to male progeny

Analysis of IVF-derived males at weaning (postnatal day (PND) 21) and adulthood (PND 56) revealed significant effects of postnatal age (F(1,144) = 1989, p < 0.001), EV-donor (F(1,144) = 21.03, p < 0.001) and EV-donor × age (F(1,144) = 5.66, p < 0.05) (Fig. 2A). Post hoc analysis revealed a significant reduction of body weight by Ethanol EV-donor treatment vs. Controls, specifically at PND 21 (p < 0.001). There was no effect of EV-donor treatment, but there was a significant effect of postnatal age (F(1,57) = 835.0, p < 0.001) and EV-donor × age (F(1,57) = 6.85, p < 0.05) on body weights in females (Fig. 2B). Post hoc test revealed no significant difference for female body weights between Control and Ethanol EV-donor groups at PND 21 or 56.

Ethanol EV-donors confer reduced anxiety-like behavior to female progeny

IVF-derived adult males and females were examined for basal anxiety-like behavior and ethanol-induced anxiolysis on the elevated plus maze. Basal anxiety-like behavior was assessed in mice injected with saline prior to testing. Mice injected with 1 g/kg ethanol were assessed for ethanol-induced anxiolysis. For males, there was a trending effect of ethanol injection on open arm time (F(1,46) = 3.75, p < 0.06; Fig. 3A), a significant effect of ethanol injection on open arm entries (F(1,47) = 31.27, p < 0.001; Fig. 3B), and no effect on total arm entries (Fig. 3C). There was no effect of EV-donor or EV-donor × ethanol injection on any measure in males.

For females, there was a significant effect of ethanol injection on open arm time (F(1,40) = 28.56, p < 0.001; Fig. 3D), open arm entries (F(1,40) = 15.43, p < 0.001; Fig. 3E), and total arm entries (F(1,40) = 10.54, p < 0.01; Fig. 3F). In addition, there was a significant effect of EV-donor on open arm time (F(1,40) = 10.65, p < 0.01; Fig. 3D) and open arm entries (F(1,40) = 11.44, p < 0.01; Fig. 3E), but...
not total arm entries (Fig. 3F). There was no interaction of EV-donor with ethanol injection on any measure for females. Post hoc analysis revealed a significant reduction of open arm time after ethanol treatment in Ethanol EV-donor females (p < 0.01) and a significant reduction in open arm entries for Ethanol EV-donor females in both saline- (p < 0.05) and ethanol- (p < 0.05) treatment groups.

To further examine basal anxiety-like behavior, males and females were tested in the light/dark transition test. Here, there was no effect of EV-donor on latency to enter the light or time spent in the light region in males (Fig. 4A and B). For females, a significant increase in latency to enter the light region for Ethanol EV-donor vs. Control EV-donor females (t(22) = 2.99, p < 0.01; Fig. 4C) was observed. There was no effect of EVs for time spent in the light region (Fig. 4D).

No effect of Ethanol EV-donor on two-bottle choice ethanol drinking

In the two-bottle choice test, there was a significant effect of ethanol concentration on ethanol consumption for both male (F(4,100) = 95.96, p < 0.001) and female (F(4,80) = 115.3, p < 0.001) mice with no effect on ethanol preference or total fluid intake. There was no effect of EV-donor or EV-donor × ethanol concentration on ethanol drinking preference, ethanol consumption, or total fluid intake in IVF-derived male (Fig. 5A–C) or female (Fig. 5D–F) mice.

**Ethanol EV-donors increase binge-like ethanol consumption in male progeny**

In the limited access drinking in the dark assay, for IVF-derived males, there was no effect of EV-donor, but there was a significant effect of EV-donor × trial on ethanol consumption (F(3,75) = 3.68, p < 0.05; Fig. 6A). Post hoc analysis revealed significantly (p < 0.05) increased ethanol consumption in Ethanol EV-donor males compared to Control EV-donor males during the 4-h test. For females, there was no effect of EV-donor or EV-donor × trial (Fig. 6B). In males, there was a trending effect (p < 0.09) of Ethanol EV-donor on BECs measured following the 4-h drinking in the dark test (Fig. 6C). The BECs of Ethanol EV-donor males and both groups of females exceeded binge levels (>80 mg/dL), whereas Control EV-
donor males did not. In addition, there was no effect of EV-donor or EV-donor × stress interaction on corticosterone levels in males ($F_{(1,15)} = 278.6, p < 0.001$) and females ($F_{(1,17)} = 73.30, p < 0.001$). However, there was no effect of EV-donor or EV-donor × stress interaction on corticosterone levels in males (Fig. 7A) or females (Fig. 7B).

**Discussion**

In the present study, we utilized an established paternal preconception ethanol exposure model to test the hypothesis that epididymal EVs play a causal role in intergenerational ethanol-related phenotypes. Remarkably, incubating epididymal EV preparations from ethanol-treated males with sperm impacts body weight and modestly alters binge ethanol drinking in IVF-derived adult males and basal anxiety-like behavior and sensitivity to an anxiolytic dose of ethanol in IVF-derived adult females. Importantly, these results provide the first evidence establishing sufficiency of epididymal EV preparations in mediating paternally driven intergenerational phenotypes.

The chronic ethanol exposure in the current study was previously found to affect small noncoding RNA in sperm (Rompala et al., 2018) and to have intergenerational effects on ethanol- and stress-related behaviors (Beeler et al., 2019; Finegersh & Homanics, 2014; Rompala et al., 2016, 2017). In addition, a chronic binge ethanol-drinking exposure has also been shown to affect sperm small noncoding RNAs (Bedi, Chang, Gibbs, Clement, & Golding, 2019) and to impact metabolic and growth measures in offspring (Chang, Wang, Bedi, & Golding, 2019). Therefore, given the capacity for epididymal EVs to traffic noncoding RNAs to sperm (Reilly et al., 2016; Sharma et al., 2016, 2018), the findings from the current study have broad implications for further investigations into heritable epigenetic mechanisms across paternal ethanol exposure studies.

While the exact mechanism underlying the intergenerational effects of ethanol-EV coincubation with sperm prior to IVF remains to be explored, several recent studies suggest that small noncoding RNAs may be responsible. For example, different species of sperm small noncoding RNA, such as microRNAs and tRNA-derived small RNAs, are sufficient to drive cross-generational effects of paternal environmental exposures (Chen, Yan, & Duan, 2016b; Rodgers et al., 2015). Furthermore, in vitro coinoculation of epididymal EVs alters the RNA profile of sperm (Reilly et al., 2016; Sharma et al., 2018).

Indeed, we have previously shown that some tRNA-derived small RNAs are similarly affected in both sperm and epididymal EVs by chronic ethanol exposure (Rompala et al., 2018). Alternatively, it is possible that ethanol-exposed EVs uniquely affected the internal and surface protein content of sperm (Martin-DeLeon, 2015). Epididymal EV-derived proteins influence immunoprotection, capacitation, and acrosomal exocytosis, all of which may conceivably affect embryonic development (Martin-DeLeon, 2015). In addition, ultracentrifugation of EV preparations may have included contaminants such as protein aggregates, ribonucleoprotein complexes, and DNA fragments that cannot be ruled out as causal factors in the current study (Li, Kaslan, Lee, Yao, & Gao, 2017; Shurtleff et al., 2017). Thus, additional mechanistic studies are needed to determine whether the cross-generational effects of Ethanol-EV preparations were specific to EV trafficking of RNA cargo to sperm.

It is critical to note that, with the exception of reduced body weight in males (Fig. 2A), the observed effects of Ethanol-EV preparations on the resulting progeny were inconsistent with the effects of paternal ethanol exposure on offspring following natural mating, i.e., increased ethanol-induced anxiolysis, decreased ethanol drinking, and blunted HPA axis responsivity selectively in male offspring (Finegersh & Homanics, 2014; Rompala et al., 2016, 2017). One likely explanation for this discrepancy is that in vitro coinoculation of EV preparations with sperm poorly models the in vivo spatial and temporal dynamics of EV/sperm interactions. Indeed, while our coinoculation occurred over 3 h, rodent sperm spend as long as 1 month between migration through and storage in the epididymis (Jones, 1999). Moreover, the epididymal EV preparations in the current study were pooled from the whole epididymis, while under biological conditions, sperm would be exposed to caput-, corpus-, and cauda-derived EVs sequentially, which may be especially important given that the RNA cargo varies dramatically between EVs derived from each region (Reilly et al.,
It is also important to note that we utilized mature sperm that have already migrated into the epididymis and therefore have already undergone significant epididymal EV exposure endogenously. Future studies should utilize alternative methods such as intracytoplasmic injection of immature sperm from testes that were not exposed to epididymal EVs in vivo. Finally, it is notable that the EV preparations lack natural breeding-specific factors such as seminal plasma, which may contribute to epigenetic mechanisms of inheritance (Watkins et al., 2018).

In summary, we report that epididymal EV preparations from ethanol-exposed mice are capable of transmitting unique intergenerational ethanol drinking and anxiety-like phenotypes to offspring. Future studies are imperative to determine whether the heritable effects of epididymal EV preparations were driven by trafficking of their EV RNA cargo to sperm. Overall, the evidence strongly implicates a soma-to-germline epigenetic mechanism underlying the intergenerational effects of paternal chronic ethanol exposure. Importantly, this novel mechanism may generalize to the intergenerational effects of a wide variety of paternal preconception perturbations.

Author statement

Contribution of individual authors: Dr. Rompala: conceptualization, methodology, investigation, data analysis, drafted manuscript; Ms. Ferguson: methodology, investigation, review and editing of manuscript; Dr. Homannis: conceptualization, methodology, supervision, review and editing of manuscript, funding acquisition, project administration.

Note added in proof

While this manuscript was under review, Chan et al. (2020) reported that epididymal epithelial cell-derived EVs were sufficient to alter neurodevelopment and offspring stress responsivity.

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