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# Paternal preconception ethanol exposure blunts hypothalamic-pituitary-adrenal axis responsivity and stress-induced excessive fluid intake in male mice

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ABSTRACT

A growing number of environmental insults have been shown to induce epigenetic effects that persist across generations. For instance, paternal preconception exposures to ethanol or stress have independently been shown to exert such intergenerational effects. Since ethanol exposure is a physiological stressor that activates the hypothalamic-pituitary-adrenal (HPA) axis, we hypothesized that paternal ethanol exposure would impact stress responsivity of offspring. Adult male mice were exposed to chronic intermittent vapor ethanol or control conditions for 5 weeks before being mated with ethanol-naïve females to produce ethanol (E)- and control (C)-sired offspring. Adult male and female offspring were tested for plasma corticosterone (CORT) levels following acute restraint stress and the male offspring were further examined for stress-evoked 2-bottle choice ethanol-drinking. Paternal ethanol exposure blunted plasma CORT levels following acute restraint stress selectively in male offspring; females were unaffected. In a stress-evoked ethanol-drinking assay, there was no effect of stress on ethanol consumption. However, C-sired males exhibited increased total fluid intake (polydipsia) in response to stress while E-sired males were resistant to this stress-induced phenotype. Taken together, these data suggest that paternal ethanol exposure imparts stress hyporesponsivity to male offspring.



## Introduction

Epigenetic inheritance has been gaining acceptance as a plausible explanation for transmission of complex behavioral traits across generations (Bohacek & Mansuy, 2013; Vassoler & Sadri-Vakili, 2014). Several studies have shown that paternal preconception exposures to stress (Dietz et al., 2011; Gapp et al., 2014; Rodgers, Morgan, Bronson, Revello, & Bale, 2013) or addictive substances (Byrnes, Johnson, Schenk, & Byrnes, 2012; Vassoler, Johnson, & Byrnes, 2013; Vassoler, White, Schmidt, Sadri-Vakili, & Pierce, 2013) can impart adaptive behavioral phenotypes to offspring. Similarly, various chronic paternal ethanol exposures induce intergenerational phenotypes (see Finegersh, Rompala, Martin, & Homanics, 2015 for review). Recently, we reported that exposing adult male mice to vapor ethanol over 5 weeks prior to mating with ethanol-naïve females conferred attenuated 2-bottle choice ethanol-drinking behavior and increased sensitivity to an anxiolytic dose of ethanol selectively in male offspring (Finegersh & Homanics, 2014). The neurobiological mechanisms underlying these effects of paternal ethanol on intergenerational ethanol-modulated behaviors are unknown.

One neurobiological system that is overactivated by ethanol abuse is the hypothalamic-pituitary-adrenal (HPA) axis endocrine stress response pathway. Ethanol acutely engages the HPA-axis (Rivier, 2014) and the transition to ethanol dependence are characterized by sustained HPA-axis tolerance to ethanol and other stressors (Stephens & Wand, 2012). Interestingly, non-ethanol dependent individuals with a family history of alcoholism also show aberrant HPA-axis responsivity to stress or ethanol exposure (Dai, Thavundayil, & Gianoulakis, 2002; Evans, Greaves-Lord, Euser, Franken, & Huizink, 2012; Schuckit, 1988; Sorocco & Ferrell, 2006). While there is evidence that maternal ethanol exposure during gestation impacts HPA-axis responsivity in offspring (Govorko, Bekdash, Zhang, & Sarkar, 2012), it is not known whether preconception ethanol dependence causally impacts stress responsivity in

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the next generation. Notably, paternal chronic stress exposures have been found to blunt HPA-axis responsivity in offspring (Pisu et al., 2013; Rodgers et al., 2013). Therefore, we hypothesized that paternal ethanol similarly impacts intergenerational stress responsivity. As stress is a major risk factor for excessive and problematic ethanol-drinking (Becker, Lopez, & Doremus-Fitzwater, 2011; Koob et al., 2014), this has significant implications for intergenerational ethanol-drinking behavior.

In the current study, we test the hypothesis that paternal ethanol exposure blunts HPA-axis responsivity to acute stress and alters stress-induced ethanol-drinking behaviors. Our findings suggest that paternal ethanol exposure prior to conception may have an underappreciated impact on stress responsivity in the next generation.

## Materials and methods

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh and were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Eight-week-old, ethanol-naïve, C57BL/6J (B6) and Strain 129S1/SvImJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and CD-1 mice were purchased from Charles River Laboratories (Burlington, MA). Unless otherwise specified, specific pathogen-free mice were group-housed in individually ventilated micro-isolater cages under 12 h light/dark cycles and had *ad libitum* access to food and water.

### *Paternal ethanol exposure*

The paternal ethanol exposure model was modified slightly from previously published methods (Finegersh & Homanics, 2014). Briefly, group-housed 8 week-old, B6 male mice were exposed to vapor ethanol (E) or room air control conditions (C) for 8 h/day (0900–1700), 5 days/week (M–F) for 5 weeks. Notably, this method has been optimized to produce stable blood ethanol concentrations (BECs) without the use of an alcohol dehydrogenase inhibitor. Immediately after the final ethanol exposures of week 5, each E- and C-exposed male was mated in the home cage of two 8 week-old Strain 129S1/SvIMJ ethanol-naïve female mice for 48 h. Breeding was limited to 48 h to minimize the influence of paternal ethanol exposure on maternal care. Strain 129S1/SvIMJ mice were chosen for mothers in accordance with our published paternal preconception ethanol exposure breeding scheme (Finegersh & Homanics, 2014). Moreover, this breeding scheme was used in a relevant study which found that paternal stress experience impacts HPA-axis reactivity in offspring (Rodgers et al., 2013). Two different paternal ethanol cohorts were used to produce all offspring used in the current study. Cohort 1 contributed mice to the acute restraint and HPA-axis responsivity assay while cohort 2 contributed offspring for the stress-evoked ethanol-drinking experiments. Sires were weighed weekly and BECs were measured following the final exposure of each week. Ethanol in plasma was measured with an Analox Ethanol Analyzer (AM1, Analox Instruments, London, UK).

### *Acute restraint stress and measurement of plasma corticosterone (CORT)*

Twelve-week-old male and female E- and C-sired offspring were subjected to a 15 min restraint stress exposure. All animals were tested between 3 and 5 h after lights-on (10:00 AM to 12:00 noon). 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 returned to a single novel cage until the 90 min time point. Only one mouse was tested per group-housed cage to avoid pre-stressing any test animals. Tail blood ($<$10 μL) was collected with heparin-coated capillary tubes (Drummond, Broomall, PA) at time points 0, 15, 30, and 90 min from the onset of restraint. Blood samples were centrifuged for 10 min at 5000 rpm to separate plasma for measurement of CORT with an enzyme immunoassay (Enzo Life Sciences, Farmingdale, NY). Statistical analysis was performed by repeated-measures ANOVA and Bonferroni *post hoc* tests where appropriate. For both males and females in Experiment 1, mice were derived from 6 E-sired and 6 C-sired litters with no more than two mice selected per litter.

### *Chronic Variable Stress (CVS) with 2-bottle free-choice ethanol-drinking*

Mice were first acclimated to 2-bottle free-choice ethanol-drinking. E- and C-sired male offspring were single-housed and habituated to two sipper tubes filled with water. After one week, one tube was filled with escalating ethanol concentrations of 2 and 4% for 4 days at each concentration, followed by 8% ethanol for the remainder of testing. Baseline 2-bottle free-choice ethanol-drinking continued at 8% for 3 weeks before the onset of stress. Baseline drinking measures used in the study were obtained over the final 8 days preceding stress. Tube position was changed daily to control for side preference.

### *CVS*

Following acclimation to 2-bottle free-choice drinking and the baseline 2-bottle choice ethanol-drinking period, mice were exposed to CVS and drinking behavior was measured. Over the 4 week CVS period, each week began with 3 consecutive days of the same unique stress exposure (described below). Each stress exposure occurred between 1400 and 1700 h during the light cycle. Two-bottle free-choice ethanol-drinking was ongoing daily throughout and between each stress exposure period. Statistical analysis of 2-bottle choice drinking behavior was performed using two-way repeated-measures ANOVA and Bonferroni *post hoc* tests where appropriate. One outlier (defined here as mean $\pm$ 2 SD over at least 2 weeks of testing) was removed from the E-sired group. Male mice used in this test were derived from 6 E-sired litters and 6 C-sired litters with no more than two mice selected per litter.

### *CVS week 1: social-defeat stress*

Test mice were introduced to the home cage of a 10-month-old outbred CD-1 male aggressor mouse. All aggressors were retired breeders and screened for reliable attack behavior prior to use in the CVS experiment using published methods (Golden, Covington, Berton, & Russo, 2011). Body weights for aggressors were at least 25% greater than those for each test mouse. After the aggressor mouse completed one 3–5 s attack, the test mouse was isolated in a wire cup within the aggressor cage for another 30 min before being returned to the home cage, where 24 h 2-bottle choice drinking was continued. The social-defeat procedure was repeated for two additional days, each time with new pairings of aggressor and test mice.

### *CVS week 2: forced-swim stress*

The forced-swim stressor was completed in a 12 cm diameter glass cylinder filled with 23 °C water. Each mouse was placed in the cylinder for a 5 min period. Following the test, mice were briefly dried and placed under a heating lamp for 3 min before being returned to their home cages.