



HHS Public Access

Author manuscript

Alcohol Clin Exp Res. Author manuscript; available in PMC 2020 June 01.

Published in final edited form as:

Alcohol Clin Exp Res. 2019 June ; 43(6): 1032–1045. doi:10.1111/acer.14029.

Intergenerational effects of alcohol: a review of paternal preconception ethanol exposure studies and epigenetic mechanisms in the male germline

Gregory R. Rompala, Ph.D.^{a,e} and Gregg E. Homanics, Ph.D.^{a,b,c,d,*}

^aCenter for Neuroscience, University of Pittsburgh School of Medicine, Biomedical Science Tower 3, 3501 Fifth Avenue, Pittsburgh, PA USA 15261

^bDepartment of Pharmacology & Chemical Biology, University of Pittsburgh School of Medicine, Biomedical Science Tower 3, 3501 Fifth Avenue, Pittsburgh, PA USA 15261

^cDepartment of Anesthesiology and Perioperative Medicine, University of Pittsburgh School of Medicine, Biomedical Science Tower 3, 3501 Fifth Avenue, Pittsburgh, PA USA 15261

^dDepartment of Neurobiology, University of Pittsburgh School of Medicine, Biomedical Science Tower 3, 3501 Fifth Avenue, Pittsburgh, PA USA 15261

^eCurrent Address: Department of Psychiatry, Icahn School of Medicine at Mount Sinai, 1470 Madison Ave, New York, NY USA 10029

Abstract

While alcohol use disorder (AUD) is a highly heritable psychiatric disease, efforts to elucidate that heritability by examining genetic variation (e.g., single nucleotide polymorphisms) have been insufficient to fully account for familial AUD risk. Perhaps not coincidentally, there has been a burgeoning interest in novel non-genomic mechanisms of inheritance (i.e., epigenetics) that are shaped in the male or female germ cells by significant lifetime experiences such as exposure to chronic stress, malnutrition, or drugs of abuse. While many epidemiological and preclinical studies have long pointed to a role for the parental preconception environment in offspring behavior, over the last decade many studies have implicated a causal relationship between the environmentally-sensitive sperm epigenome and intergenerational phenotypes. This critical review will detail the heritable effects of alcohol and the potential role for epigenetics.

Keywords

alcohol; stress; epigenetic inheritance; germline

*Corresponding Author: Gregg E. Homanics, PhD, University of Pittsburgh, Biomedical Science Tower-3, Rm 6060, 3501 Fifth Avenue, Pittsburgh, PA 15261, Telephone: 412-648-8172, FAX: 412-383-5267.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

INTRODUCTION

Alcohol use disorder (AUD) is a mental health disorder with an astounding lifetime prevalence of 29.1 % (Grant et al., 2016), exerting massive societal costs that account for ~1% of the gross national product in high- to middle-income countries (Rehm et al., 2009). As risk for AUD is increased several fold by a family history of alcoholism (Cotton, 1979, Kendler et al., 2015, Morean et al., 2009, Worobec et al., 1990), much of the research to date has focused on elucidating the genetic underpinnings of AUD pathology. However, there is a growing appreciation that epigenetic mechanisms, which govern gene expression without a change in the underlying DNA sequence, are critical at fertilization to drive gene expression patterns and development at the earliest stages of embryonic growth. Unlike the inflexible genome, epigenetic mechanisms are sensitive to environmental perturbations. Indeed, many recent studies have found that alcohol has diverse epigenetic effects in somatic and germinal tissue (Chastain and Sarkar, 2017) which play a causal role in the developmental and adult features of subsequent generations (Finegersh et al., 2015).

Given the long-held belief that only genetic information is passed through the germline, the potential for preconception alcohol exposure to affect offspring health and development has been understudied. Still, there is evidence dating as far back as one hundred years (Stockard and Papanicolaou, 1918) directly implicating parental preconception alcohol exposure as a driver of offspring development. Particularly with the discovery that environmentally-sensitive epigenetic mechanisms in the germline are causally related to cross-generational phenotypes, such studies have now attracted major scientific consideration and public enthusiasm (Yehuda et al., 2018). This intrigue is warranted as the potential for parental preconception alcohol exposure to affect offspring behavior such as alcohol drinking has radical implications for how we appreciate the mechanistic role of family history in AUD risk.

The goal of this critical review is to summarize work examining the effects of preconception alcohol exposure on offspring health and behavior. While gestational alcohol exposure has wide-ranging effects on child development and behavior, this review will primarily focus on such studies where preconception alcohol exposure impacts subsequent generations (i.e., the effects are transmitted through the germline) (Govorko et al., 2012, Nizhnikov et al., 2016). Notably, as preconception maternal exposures may have lasting effects on *in utero* conditions and the quality of maternal care (requiring embryo transfer and/or cross-fostering to elucidate the germline vs somatic origin of offspring phenotypes), the majority of studies examining cross-generational effects of preconception alcohol exposure have utilized male subjects. Therefore, this review will focus primarily on findings following paternal exposure, although maternal preconception studies are considered. Overall, this review will emphasize the causal role of alcohol in the transmission of complex behavioral phenotypes across generations, such as alcohol drinking, and how these effects are likely attributable to reprogramming of epigenetic mechanisms in the germline.

THE MISSING HERITABILITY OF ALCOHOL USE DISORDER

Individual risk of developing most psychiatric disorders, including AUD, is widely conceptualized as the product of gene \times environment interactions (Meaney, 2017). That is, both heredity (i.e., familial factors presumed to be primarily genetic) and experiences throughout the lifetime (i.e., environmental factors) are considered to best define at risk populations for disease. For the clinical pathologist, the genetic component of the risk equation has been especially intriguing, given the implications for biomarker discovery and targeted disease treatment and prevention. Collectively, twin and adoption studies estimate that AUD is ~50% heritable (Prescott and Kendler, 1999, Young-Wolff et al., 2011, Ystrom et al., 2011). Thus, to examine the genetic component of AUD, investigators throughout the world have widely employed genome wide association studies (GWAS) to survey diverse populations for genetic marks -- commonly single nucleotide polymorphisms (SNPs) -- that predict alcohol-related phenotypic variation (e.g., AUD diagnosis).

The most well-recognized studies linking genetic variants and AUD risk, implicate genes directly involved in alcohol metabolism -- alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) enzymes (Bierut et al., 2012, Birley et al., 2008, Higuchi et al., 1995, Li et al., 2012). Identified SNPs on ADH1B and ALDH2 are associated with impaired clearance of acetaldehyde, the toxic byproduct of alcohol metabolism, and reduced AUD risk (Cederbaum, 2012), suggesting a function for alcohol metabolism-related genetic variants in AUD heritability. Nevertheless, although these risk variants are common in Asian populations, they are rarely identified in populations of central European origin (Bach et al., 2017). In addition, such reproducible and robust findings have been the few exceptions in an otherwise conflicted field of study. The results of many GWAS efforts often fail to replicate across studies (Treutlein and Rietschel, 2011, Bierut et al., 2010). Moreover, none of the SNPs identified are estimated to account for more than 0.1% of AUD heritability (Heath et al., 2011). Such outcomes are not unique to AUD. For example, Crohn's disease is estimated to have 80% heritability, but the additive effect of all SNPs associated with the disease account for only ~20% of that estimate (Park et al., 2010). This common discrepancy between estimated heritability of disease predicted by familial vs genetic variation, is referred to as the "missing" heritability problem.

There are numerous factors that may help explain missing AUD heritability. For example, the innumerable variables that comprise the environmental component of the G \times E interaction (e.g., cultural diversity, drug availability in addiction, early-life stress, environmental toxins, microbiota), as well as gender and age all severely compound the challenge of replication across heterogeneous sample populations (Ober and Vercelli, 2011). Moreover, psychiatric disorders feature complex symptomology comprised of multiple "intermediate" phenotypes (e.g., alcohol-induced body sway) that further challenge efforts to discover a unifying genetic signature for AUD (Blanco-Gomez et al., 2016). Finally, in addition to all of these plausible explanations for missing heritability, there is growing evidence to suggest that a portion of the heritability of complex phenotypes may result from parental preconception experience.

HERITABILITY OF THE PRECONCEPTION ENVIRONMENT

The genetic theory of evolution has been the unifying standard for modern biology. Harmonious with Charles Darwin's theory of natural selection, genetic theory posits that, for a given environment, success or fitness of the species will be determined by selective pressure for phenotypic traits that are genomic in origin (Orr, 2005). By this principle, the ancestral or parental environment has no targeted mechanism to influence the phenotype in subsequent generations. However, even preceding Darwin's *On the Origin of Species*, the French biologist Jean-Baptiste Lamarck described a more flexible theory of heredity, positing that the ancestral environment is the primary driver of phenotypic variation in posterity (Lamarck, 1802). While long-rejected in favor of a singular genetic-basis for heredity, over the past twenty years, a surfeit of clinical and preclinical evidence has reignited interest in Lamarckian theory and a potential complimentary role for the parental environment in heredity (Skinner, 2015).

Nongenomic inheritance of a given phenotype is described as intergenerational if it is imparted from father (i.e., the F0 generation) to offspring (i.e., the F1 generation). If the given phenotype is observed in the F2 or subsequent generations, it is described as transgenerational. In the context of germline inheritance, the transgenerational terminology indicates that the given phenotype was transferred through germ cells never harbored by the exposed F0 generation. Thus, for females, as the F0 female will harbor both the F1 fetus and the primordial germ cells from which the F2 generation will spawn, transgenerational effects are reserved for the F3 generation and beyond. This review will focus largely on mechanisms of intergenerational germline inheritance, though transgenerational effects will also be discussed.

Studies examining effects of ancestral environmental exposures require careful monitoring of subjects across multiple generations and are therefore difficult to conduct on human populations. Nonetheless, some of the earliest human studies reporting multigenerational effects stem from the 1944–1945 Dutch Famine Cohort. These studies revealed that men exposed to famine during prenatal development were more likely to have offspring with increased body weight and more adiposity in adulthood (Veenendaal et al., 2013, Painter et al., 2008). Other early study focused on the remote Överkalix population of northern Sweden which maintained cross-generational historical records of harvest and food supply throughout the 20th century. The Överkalix study found that the food supply of paternal grandparents was inversely related to male and female longevity (Bygren et al., 2001, Pembrey et al., 2006). Additionally, fathers that smoked prior to puberty were more likely to have sons with increased body-mass-index (Pembrey et al., 2006). More recent findings from Rachel Yehuda's group demonstrate intergenerational effects of parental stress. Both paternal and maternal post-traumatic stress disorder (PTSD) --from traumatic episodes preceding conception-- were associated with reduced basal cortisol levels and greater dexamethasone-suppression of cortisol in adult offspring (Lehrner et al., 2014, Yehuda et al., 2007). In another study, Yehuda et al. found that offspring of women that survived the Holocaust prior to conception were at increased risk for the development of PTSD, depression, and anxiety disorders (Yehuda et al., 2008). Furthermore, a new study found that male descendants of civil war prisoners of war exhibited increased mortality (Costa et al.,

2018). Finally, a family history of alcoholism is associated with altered inheritance of a unique epigenetic signature at two putative oncogenes (Hill et al., 2017).

Despite these intriguing studies demonstrating effects of parental lifetime exposures on offspring health and raising the possibility of epigenetic inheritance manifesting in the human population, they cannot rule out a genetic contribution based on potential pre-existing genetic variation in the sample population. Additionally, maternal *in utero* effects and parental investment in child development top a long list of potential confounding variables, making a germline-specific mechanism extremely difficult to discern. Thus, to test the hypotheses inspired by cross-generational epidemiological data, preclinical investigators have utilized isogenic rodent strains under controlled laboratory conditions to directly test the effects of various paternal preconception exposures to a vast range of environmental insults on an equally expansive number of biological and behavioral measures in offspring. Most prominently featuring studies pertaining to dietary change, chronic stress and various drugs of abuse, many paternal preconception exposures with rodents have now been found to directly impart complex physiological and behavioral phenotypes to offspring (for excellent reviews, see: Rando and Simmons, 2015, Chan et al., 2017b, Goldberg and Gould, 2018). For instance, obese fathers confer deficits in glucose metabolism to offspring (Chen et al., 2016a, Cropley et al., 2016, de Castro Barbosa et al., 2016, Fullston et al., 2013). Furthermore, numerous paternal chronic stress paradigms reshape physiological and behavioral stress vulnerability across generations (Rodgers et al., 2013, Dietz et al., 2011, Gapp et al., 2014, Short et al., 2016) and paternal cocaine exposure alters cocaine preference and hippocampal-dependent memory in male offspring (Vassoler et al., 2013, Le et al., 2017, Wimmer et al., 2017). Other paternal conditions with cross-generational effects in rodents include exposures to environmental toxicants such as bisphenol A (Fan et al., 2018) as well as exposures to beneficial conditions such as enriched housing (Yeshurun et al., 2017) or free access to a running wheel (Short et al., 2017). As rodent sires are not involved in offspring gestation or rearing in these studies, the contribution of the father to offspring development is likely restricted to the germline. While factors such as sire “fitness” can influence maternal care for offspring through social interaction during breeding (Mashoodh et al., 2012), several paternal exposure studies have partially or fully validated the germline origin of intergenerational effects using *in vitro* fertilization experiments (Dias and Ressler, 2014, Chen et al., 2016a, Sharma et al., 2016, Dietz et al., 2011, Huypens et al., 2016).

HERITABILITY OF PRECONCEPTION ALCOHOL EXPOSURE

To date, there have been over forty published paternal preconception alcohol exposure studies in rodents (for review, see: Finegersh et al., 2015), most varying in species, route of administration, and duration. Despite these differences, some intergenerational (F1) effects have been consistent such as low fetal and birth weight (Bielawski et al., 2002, Ledig et al., 1998, Chang et al., 2017, Chang et al., 2019), altered organ weights (Abel, 1993b, Ledig et al., 1998, Lee et al., 2013, Chang et al., 2017), and increased number of runts (Bielawski et al., 2002, Bielawski and Abel, 1997). Additional phenotypes reported include increased cortical thickness (Jamerson et al., 2004), reduced testosterone (Abel and Lee, 1988), and altered neurotransmitter levels (Nelson et al., 1988). Furthermore, several intergenerational behavioral alterations have been reported including reduced spatiotemporal learning

(Wozniak et al., 1991), increased anxiety- and impulsivity-like phenotypes (Kim et al., 2014, Liang et al., 2014), and increased sensitivity to amphetamine-induced hyperlocomotion (Abel, 1993a). Overall, these preclinical studies affirm that paternal alcohol exposure can confer fetal alcohol syndrome-like defects, molecular and physiological alterations, and behavioral phenotypes to offspring.

Most of the above referenced studies were conducted from the perspective of the paternal contribution to fetal alcohol syndrome-like phenotypes. Surprisingly, until recently, no published studies had examined the effect of paternal alcohol exposure on offspring alcohol drinking or alcohol sensitivity-related phenotypes. In 2014, Finegersh and Homanics started to fill this gap in the literature, by examining a battery of alcohol-related behaviors in the offspring of sires that were exposed to chronic alcohol exposure. They discovered that C57BL/6J (B6) adult male mice exposed intermittently to alcohol vapor over five weeks (average blood alcohol concentration ~160 mg/dL following each 8 hour exposure) sired hybrid B6 × Strain 129 adult male offspring with increased sensitivity to the anxiolytic effects of a low dose alcohol injection and decreased alcohol drinking preference and consumption (Finegersh and Homanics, 2014). Those key findings were later replicated with mice on a pure B6 background (Rompala et al., 2017). Using a chronic alcohol liquid diet exposure, another study reported a bidirectional effect of paternal alcohol exposure on conditioned place preference (CPP) for alcohol in male offspring depending on the paternal alcohol exposure dosage (i.e., increased CPP at a low dose (0.5 g/kg), decreased CPP at a high dose (1.5 mg/kg)) (Ceccanti et al., 2016). Another study examining cross-generational alcohol drinking behaviors found that paternal binge-alcohol exposure over eight days increased alcohol intake by intraoral infusion in postnatal offspring (Hollander et al., 2018). Finally, female mice exposed to alcohol during postnatal development exhibited reduced sensitivity to alcohol-induced loss of righting reflex and passed this phenotype to male and female offspring (Popoola et al., 2017). Intriguingly, in three of these studies, the cross-generational alcohol-related phenotypes coincided with altered BDNF signaling in reward-related brain regions (Ceccanti et al., 2016, Finegersh and Homanics, 2014, Rompala et al., 2017). In addition, a recent study found that like alcohol, paternal cocaine reduced cocaine seeking behavior in male offspring and the phenotype was reversible with a BDNF antagonist (Vassoler et al., 2013). This suggests that the BDNF locus may be an attractive target for examining mechanistic effects of paternal alcohol exposure on offspring neurobiology across reward-related brain regions. Taken together, these novel studies suggest that paternal preconception alcohol exposure is a heritable factor capable of driving alcohol-related phenotypes in the next generation.

Given the complexity of factors driving alcohol drinking in humans relative to rodents, more work needs to be done with rodent models to more fully characterize how paternal preconception alcohol affects the offspring alcohol drinking phenotype. Illustrating this point, while it was initially reported that chronic paternal cocaine self-administration reduced cocaine preference in male offspring, another study found that sires selected for high cocaine motivation imparted increased cocaine preference to offspring (Le et al., 2017). Moreover, paternal cocaine exposure had divergent effects on offspring cocaine preference depending on whether the cocaine exposure was voluntarily (self-administered) or forced (Le et al., 2017). Thus, the results from the handful of studies to date are insufficient and

more cross-generational alcohol exposure paradigms need to be evaluated to better understand their translational potential. Likewise, as no studies to date have employed next generation sequencing techniques (e.g., RNA-seq, ATAC-seq) to examine the effects of paternal alcohol on offspring brain, such experiments are needed for an unbiased characterization of transcriptomic and epigenetic mechanisms potentially underlying intergenerational alcohol-related behaviors.

CROSS-GENERATIONAL RELATIONSHIP BETWEEN ALCOHOL AND STRESS

Many studies have characterized the positive feedback loop propelling the relationship between stress and alcohol (i.e., stress promotes chronic alcohol abuse and chronic alcohol abuse enhances vulnerability to subsequent stressors) (see Koob et al., 2014 and Becker et al., 2011 for excellent reviews). Now, several studies indicate a unique relationship between alcohol and stress stretching across generations. For instance, alcohol exposure paradigms such as parental adolescent injections (Przybycien-Szymanska et al., 2014), maternal preconception gavage (Jabbar et al., 2016) and paternal exposure during prenatal development (Govorko et al., 2012) were found to alter hypothalamic-pituitary-adrenal (HPA) axis function in offspring. These studies are notable in light of the extensive literature showing that a family history of alcoholism is associated with impaired HPA axis function (Stephens and Wand, 2012).

One recent investigation carried out by our lab found that male offspring of sires exposed to preconception alcohol exhibit blunted stress responsivity (Rompala et al., 2016). Interestingly, this blunted HPA axis phenotype in the F1 generation has also been observed in response to paternal preconception chronic stress exposure (Rodgers et al., 2013). Similarly, we have found that both paternal preconception alcohol and paternal preconception stress reduce alcohol drinking selectively in males (Rompala et al., 2018b, Finegersh and Homanics, 2014), thus suggesting that paternal alcohol and stress act similarly to influence the alcohol drinking and stress phenotypes of the next generation. Indeed, alcohol acts directly on the HPA axis, acutely increasing corticosterone/cortisol levels in rodents (Rivier, 2014) and humans (Valimaki et al., 1984, Mendelson and Stein, 1966), indicating a likely shared mechanism of the paternal alcohol and stress exposures. Notably, glucocorticoid receptors are expressed throughout the male reproductive tract (Silva et al., 2010, Schultz et al., 1993) and may be a shared somatic mechanism to influence epigenetic factors in the germline (Chan et al., 2018).

GERMLINE EPIGENETIC MECHANISMS

As many of the studies demonstrating an intergenerational effect of alcohol exposure were carried out with males which do not contribute to offspring fetal and postnatal development, the effects are likely transmitted through the germline. This section will review the heritable mechanisms implicated in non-genomic inheritance of complex behavior phenotypes such as alcohol drinking behavior.

Epigenetic processes are broadly defined as the molecular factors that drive gene expression while not altering the primary DNA nucleotide sequence. Among the most well-understood epigenetic mechanisms are cytosine methylation, chromatin modifications, and noncoding RNAs. Methylation of DNA at a cytosine nucleotide that is followed by a guanine nucleotide (CpG) within gene promoters is associated with transcriptional silencing by local recruitment of heterochromatic proteins that impede transcription factor binding (Illingworth et al., 2008). Post-translational chromatin modifications regulate the affinity of positively-charged histone proteins for the phosphate-rich DNA, in turn influencing gene expression by determining whether the chromatin is “open” or “closed” to transcription (Smith and Shilatifard, 2010). Finally, noncoding RNAs function by regulating diverse transcriptional and translational processes, often targeting messenger RNAs with sequence homology (Cech and Steitz, 2014). Collectively, epigenetic mechanisms are the primary regulators of transcription and underlie the cellular diversity able to emerge from a single genome. This section will focus primarily on the unique epigenomic state of the male germline (see Figure 1 for overview of sperm epigenome).

Given the specialized function of the male germline in fertilization, sperm feature a unique nuclear structure. Most histones are replaced in the early stages of spermatogenesis with highly basic protamines that robustly neutralize the phosphate bond-rich DNA. The nucleus is further compressed by inter- and intra-molecular disulfide bonds, contributing to a condensed state at approximately 1/13 the volume of the oocyte nucleus (Martins and Krawetz, 2007). In this heavily neutralized state, and lacking major ribosomal machinery, mature sperm cells exhibit minimal transcriptional activity (Gur and Breitbart, 2006). Upon fertilization, most of the sperm genome is stripped of epigenetic marks to facilitate pluripotency in early embryo development (Feng et al., 2010). Thus, until recently, the male gametes were presumed to deliver little to no epigenetic memory to the fertilized oocyte. However, with the advancement of next generation sequencing technology allowing for enhanced resolution in methylation, chromatin modification, and noncoding RNA analysis, studies are beginning to characterize a unique, environmentally-responsive, and functional epigenetic landscape in sperm.

Indeed, not all genomic loci are stripped of DNA methylation at fertilization. For instance, large regions around intracisternal A particle (IAP) retrotransposons (Popp et al., 2010) and imprinting regions (Feng et al., 2010) are protected from global epigenetic reprogramming during embryogenesis. Additionally, only a small percentage of histones are retained in sperm (~1% in mouse, ~10% in humans) as the majority are exchanged for protamines during spermatogenesis to facilitate DNA condensation and stabilization (Bogliotti and Ross, 2012). Retained histones are concentrated at promoter regions of key genes for embryo development, thereby well-positioned to influence initial zygotic gene expression (Brykczynska et al., 2010, Hammoud et al., 2009). Moreover, adult exposure to the environmental toxin DDT or gestational exposure to vinclozolin lead to ectopic histone retention sites in sperm, showing that environmental exposures can influence germline histone displacement mechanisms (Skinner et al., 2018, Ben Maamar et al., 2018).

In addition to chromatin, sperm contain RNA, though only ~ 1% of that carried by somatic cells (Zhang et al., 2017). Due to the largely arrested transcriptional state of fully mature

sperm, it was long assumed that this limited amount of RNA in sperm was purely comprised of degraded transcripts from the earlier transcriptionally-active stages of spermatogenesis in the testis. However, the discovery that sperm RNAs are delivered to the oocyte at fertilization raised the possibility that they could be functional epigenetic molecules in the early embryo (Ostermeier et al., 2004). Supporting this notion, subsequent deep sequencing studies have helped characterize a diverse and unique population of intact, functional noncoding RNA species in sperm. The majority of RNA species identified in sperm are “small” noncoding RNA (18–40 nucleotides), mainly microRNA (miRNA), transfer RNA-derived small RNAs (tDR), and piwi-interacting RNAs (piRNA) (Chen et al., 2016b). The miRNAs are the most well-studied small noncoding RNA with a putative function in mRNA silencing via binding to homologous “seed” regions of the 3'-UTR of target transcripts (Cai et al., 2009). The function of tDR is not well-understood in sperm though they have been implicated in various biological functions including transcriptional regulation (Chen et al., 2016a), miRNA-like mRNA silencing (Haussecker et al., 2010), and translational inhibition (Yamasaki et al., 2009). The piRNAs regulate expression of transposable elements during spermatogenesis and are the dominant noncoding RNA species in immature testis spermatozoa (Ernst et al., 2017). Finally, sperm also contain long noncoding RNAs (>200 nucleotides) involved in diverse transcriptional regulatory processes and additional novel RNA species will likely continue to be identified in the coming years. Overall, while the precise mechanism of these noncoding RNAs either in mature sperm, or upon delivery to the oocyte, remains to be determined, multiple studies have report an essential role for sperm RNA in early embryonic development (Yuan et al., 2016, Liu et al., 2012, Conine et al., 2018).

EFFECTS OF ENVIRONMENTAL INSULTS ON EPIGENETIC MECHANISMS IN SPERM

The earliest evidence of intergenerational/transgenerational phenotype associated with environmentally-induced epigenetic mechanisms in sperm showed that transient exposure of pregnant rats to endocrine disruptors (e.g., vinclozolin) imparts reduced fertility to males across multiple generations through the male germline (Anway et al., 2005, Nilsson and Skinner, 2015). This phenomenon was associated with altered DNA methylation patterns in sperm (Anway et al., 2005, Nilsson and Skinner, 2015) and those effects have more recently been found to expand to histone retention and altered small RNA content (Ben Maamar et al., 2018, Schuster et al., 2016).

Additional studies have found that the male germline is sensitive to environmentally-induced changes in DNA methylation well beyond prenatal development. For instance, adult-onset prediabetic conditions (Wei et al., 2014), obesity (Fullston et al., 2013), and cocaine-seeking motivation (Le et al., 2017) all modify the DNA methylome in sperm. One recent study reported similar changes in DNA methylation in sperm between tetrahydrocannabinol (THC)-treated rats and cannabis-smoking adults (Murphy et al., 2018). Other studies have reported effects of cocaine and stress on histone modifications in developing or mature sperm at specific-gene loci that coincided with altered expression of the same gene in adult offspring brain, suggesting cross-generational epigenetic memory (Vassoler et al., 2013,

Dias and Ressler, 2014). Finally, various paternal preconception environmental exposures directly affect the noncoding RNA milieu in sperm (Rodgers et al., 2013, Fullston et al., 2013, Gapp et al., 2014, Chen et al., 2016a, Sharma et al., 2016, Short et al., 2017). In humans, alterations in sperm small noncoding RNAs have been associated with obesity (Donkin et al., 2016), early life stress (Dickson et al., 2018), and smoking history (Marczylo et al., 2012). Aside from sperm RNA abundance, there is increasing interest in post-transcriptional RNA modifications (i.e., the epitranscriptome) as some of these modifications on sperm RNA were found to be sensitive to paternal diet (Chen et al., 2016a) and deletion of the RNA methyltransferase gene DNMT2 is necessary for an established model of RNA-mediated inheritance of glucose intolerance from paternal high fat diet (Zhang et al., 2018). Interestingly, dissimilar to DNA methylation and histone modifications in sperm, which are hypothesized to be vulnerable to environmental exposures in the testis preceding or during spermatogenesis, the sperm RNA profile is dynamically shaped in the epididymis during final maturation (Nixon et al., 2015). Whether the sperm RNA profile is especially sensitive to environmental exposures during epididymal transit remains to be tested.

While experimental methods for loci-specific manipulation of DNA methylation or histone modifications in the germline have yet to be developed (although the relevant technology is progressing rapidly (Kungulovski and Jeltsch, 2016)), techniques have been established for directly testing the role of sperm RNA in established animal models of environmentally-induced intergenerational inheritance. Using 1-cell embryos, isolated sperm RNA can be directly injected into the fertilized oocyte to examine resultant progeny and determine if sperm RNA is sufficient to recapitulate the cross-generational phenotypes in question. To date, there have already been at least six studies using this method to draw a causal link between environmentally-responsive sperm RNAs and heritable phenotypes (see Table 1) (Rodgers et al., 2015, Gapp et al., 2014, Benito et al., 2018, Grandjean et al., 2015, Gapp et al., 2018, Chen et al., 2016a). Beginning in 2014, Isabella Mansuy and colleagues found that the intergenerational effects of maternal separation stress could be partially recapitulated in mice derived from one cell embryos from control, unstressed parents that were injected with total sperm RNA from stressed males (Gapp et al., 2014). Subsequently, Tracey Bale and colleagues discovered that the intergenerational effect of paternal chronic stress on stress responsivity in offspring could be recapitulated in mice derived from normal embryos injected with synthetic oligos for nine stress-enriched sperm miRNAs (Rodgers et al., 2015). Another study examining the intergenerational effects of paternal high fat diet found that sperm tDR could reproduce the diet-induced intergenerational effects on glucose tolerance in the next generation (Chen et al., 2016a). Finally, a similarly important role for sperm long noncoding RNAs was discovered in a model of postnatal trauma (Gapp and Bohacek, 2018). Collectively, this evidence supports a functional role for sperm-derived RNA in the early embryo and epigenetic inheritance. However, in each of these studies, the specific causal embryonic mechanism of the altered sperm RNA remains unclear. In Rodgers et al., stress-increased sperm miRNAs were found to reduce expression of canonical mRNA targets (Rodgers et al., 2015). In addition, one paternal diet-sensitive tRNA fragment (tRNA-Glu-CTC) was discovered to be essential for suppression of retrotransposon-dependent zygotic gene expression (Sharma et al., 2016). Given the pluripotent state of the early embryo, it is

conceivable that transient sperm RNA-induced alterations in zygotic transcription may offset other mechanisms such as *de novo* DNA methylation to ultimately program a stable epigenetic state in the developing offspring (Greenberg et al., 2017).

It does not appear that the effects of a direct environmental exposure on sperm RNA are maintained in the unexposed sperm of male offspring. Both paternal stress and high fat diet studies have reported inconsistent sperm RNA profiles between F0 and F1 generations (Gapp et al., 2014, Fullston et al., 2016). Conversely, alcohol exposure during gestation and adult cocaine addiction have been found to induce similar effects on gene promoter cytosine methylation in F0 and F1 sperm (Govorko et al., 2012, Le et al., 2017). How environmentally-induced changes in sperm methylation may escape reprogramming or undergo reestablishment in the germline after global demethylation remains unclear and an intriguing area for future study.

EFFECTS OF ALCOHOL ON EPIGENETIC MECHANISMS IN SPERM

Alcohol reduces sperm count (Rahimipour et al., 2013), circulating testosterone levels (Widenius et al., 1989, Kucheria et al., 1985), and overall fertility (Anderson et al., 1983) in rodents and similar effects on reproductive health have been reported in alcoholic men (reviewed in La Vignera et al., 2013). In addition to these deleterious effects of alcohol on reproductive health, numerous studies support a significant effect of alcohol on the epigenetic milieu in sperm.

DNA Methylation

As alcohol acts directly on DNA methylation machinery, reducing levels of the cytosine methyltransferase DNMT1 (Ponomarev et al., 2012) and S-Adenosyl-methionine (SAM) in somatic cells (Lu and Mato, 2005), many studies have investigated whether alcohol similarly affects DNA methylation in the male germline. Indeed, DNMT1 is reduced in sperm of animals undergoing intragastric chronic alcohol exposure (Bielawski et al., 2002). Moreover, several studies have found that DNA methylation at imprinted gene loci is reduced in sperm of chronic alcohol-treated mice (Knezovich and Ramsay, 2012, Finegersh and Homanics, 2014, Liang et al., 2014) as well as men with alcohol use disorder (Ouko et al., 2009). Recently, Finegersh and Homanics found that paternal preconception alcohol exposure reduced methylation of the BDNF promoter in sperm which coincided with increased BDNF in adult VTA of male offspring, thus implicating the BDNF locus in the cross-generational effects of alcohol (Finegersh and Homanics, 2014). In addition to these effects demonstrating vulnerability of the adult sperm methylome to alcohol, animals exposed to prenatal alcohol exhibited increased CpG methylation at the pro-opiomelanocortin (POMC) gene promoter in sperm that is associated with reduced POMC expression and suppression of its HPA-axis mediating function in offspring (Govorko et al., 2012). Not all results support methylation changes in response to ethanol, as one recent study found that paternal voluntary alcohol consumption, which altered cholesterol trafficking and hepatic fibrosis markers in offspring, had no effect on DNA methylation in sperm (Chang et al., 2017). In addition, despite a number of studies demonstrating effects of environmental perturbations on methylation in sperm, the effect sizes of significant changes in methylation at individual

cytosines are often small (e.g., ~10–20 %) and thereby the significance for cross-generational inheritance is hard to discern, given the high penetrance observed in many preclinical paternal epigenetic inheritance models (Shea et al., 2015). Overall, emerging techniques that allow for loci-specific manipulation of epigenetic marks such as methylation will be critical for further examining the role of methylation in epigenetic inheritance.

Chromatin Modifications

While several studies have found effects of alcohol on histone post-transcriptional modifications in somatic tissue such as the liver or brain (see Berkel and Pandey, 2017 for an excellent review), only one study has directly examined the effect of alcohol on chromatin modifications in sperm. Chronic exposure to liquid alcohol (10% v/v) was found to impair chromatin condensation in mice (Rahimipour et al., 2013). Few studies to date have examined the effect of environmental insults on the chromatin profile with sequencing resolution in sperm, likely due to the minimal histone content retained following spermatogenesis. One recent study used novel next generation sequencing techniques to examine chromatin accessibility and conformation (e.g., ATAC-seq, Hi-C) in sperm, revealed a unique euchromatic landscape that was reflected in the developing embryo (Jung et al., 2017). However, another study found that studies such as Jung et al. using swim-up based sperm purification methods are likely to capture a significant fraction of infertile sperm characterized by incomplete histone to protamine transition (Yoshida et al., 2018). Nonetheless, as the unique sperm chromatin profile continues to be detailed with enhanced resolution, it will be essential to examine the effects of alcohol on these emerging epigenetic mechanisms.

Noncoding RNAs

To date, there has been one study examining the effects of alcohol exposure on small noncoding RNA in sperm. Chronic intermittent alcohol exposure, the same exposure found to reduce alcohol drinking and blunt stress responsivity in male offspring (Finegersh and Homanics, 2014, Rompala et al., 2016), was found to alter several miRNA and tDR species in sperm (Rompala et al., 2018a). Shared predicted mRNA targets of alcohol-responsive miRNAs were enriched for transcriptional regulators including some with defined roles in embryonic development. Prominently among the small RNAs increased in sperm by alcohol was miR10a, one of the few microRNAs found to be significantly enriched in the sperm relative to the oocyte, (Yang et al., 2016) and a putative post-transcriptional regulator of the alcohol-related gene BDNF (Jiajie et al., 2017). Among alcohol-responsive sperm tDR, tRNA-Glu-CTC was prominent as, along with tRNA-Gly-GCC, it is one of two extremely enriched small RNAs in mammalian sperm, together comprising more than half of the small RNA content (Peng et al., 2012). Supporting the importance of these sperm tDR, tRNA-Gly-GCC is necessary for controlling retrotransposon-regulated gene networks in the fertilized oocyte (Sharma et al., 2016). Moreover, *in silico* analysis revealed that tRNA-Glu-CTC is highly enriched for overall mRNA targets relative to other tDR species (Rompala et al., 2018a). Interestingly, both miR10a and tDR-Glu-CTC were prominently altered in F1 sons of obese fathers that impart glucose intolerance to the F2 male descendants (Cropley et al., 2016). Thus, interrogating the casual role of these and other alcohol-responsive small RNAs

in intergenerational inheritance has great potential to unveil novel heritable mechanisms driving alcohol-related behavior.

Notably, the sperm RNA profile is shaped during epididymal transit via extracellular vesicles (i.e., epididymosomes) (Reilly et al., 2016, Sharma et al., 2016, Sharma et al., 2018). Indeed, the effects of alcohol on mature sperm were reflected in epididymosomes for some tDR species (Rompala et al., 2018a), suggesting a potential effect of alcohol on soma-to-germline RNA trafficking. Given the evidence implicating epididymosomes as the source of major RNA species in mature sperm, there is a fast-growing interest in characterizing gonadal and neuroendocrine regulation of epididymosome function. For instance, testosterone levels mediate epididymosomal protein trafficking to the sperm membrane (Suryawanshi et al., 2012) and emerging studies support such a role for glucocorticoids in RNA trafficking to sperm (Chan et al., 2018). Future studies should further investigate how adaptive changes in epididymal gene expression affect epididymosome RNA cargo sorting and delivery to sperm. One powerful tool for such experiments was recently introduced allowing for epididymal-synthesized RNAs to be tagged *in vivo* and then detected in sperm RNA using next generation sequencing (Sharma et al., 2018). This methodology can be used to examine the effects of various environmental perturbations directly on soma-to-germline RNA trafficking.

In addition to small RNA abundance, two post-transcriptional RNA modifications were altered in sperm by chronic alcohol exposure (Rompala et al., 2018a). These modifications were specific to mitochondrial tRNAs and could affect expression of novel small mitochondrial RNA species (Ro et al., 2013) as well as sperm mitochondrial function. Notably, while Rompala et al. examined twenty-two RNA modifications, there have been over one hundred and fifty identified to date (Boccaletto et al., 2018). Moreover, Rompala et al. examined only the 30–40 nucleotide fraction of small RNAs, excluding all miRNA species (~21–23 nucleotides). Given that RNA modifications underlie small RNA stability (Chen et al., 2016a) and are essential to models of RNA-mediated inheritance (Kiani et al., 2013), future studies will need to fully characterize the effects of alcohol on the epitranscriptome.

Moving forward: linking alcohol-responsive germline epigenetics with alcohol drinking in offspring

Future studies will need to examine the causal role of alcohol-induced changes to the sperm epigenome on paternal alcohol-imparted offspring behavior. As novel genome editing tools are developed for applying locus-specific CpG methylation and histone modifications in the germline, the many cited effects of paternal environmental exposures and alcohol on the sperm chromatin will need to be tested directly. Conversely, there has been significant progress in examining the causal role of sperm RNA, as several studies examining the effects of paternal diet and paternal stress have directly tested whether injecting 1-cell embryos with altered paternal sperm RNA is sufficient to recapitulate intergenerational phenotypes in the resulting progeny. While this may not be a physiological manipulation (and does not establish necessity of the affected RNAs), it would be a critical step in establishing a role for sperm RNAs in driving the paternal effects. Studies targeting specific

RNAs with mimics and/or inhibitors could also be useful, although RNA mimics were found to be sensitive to rapid degradation and lack potentially crucial post-transcriptional modifications (Chen et al., 2016a). Alternatively, given that extracellular vesicles are emerging as the likely mode of RNA biogenesis in epididymal sperm and that epididymosome RNAs can be trafficked to developing sperm *in vitro* (Sharma et al., 2018), the causal role of epididymosomal RNA in cross-generational inheritance remains to be studied. Finally, as novel candidate epigenetic marks emerge, clinical studies examining relevant population of subjects with alcohol abuse and AUD will be needed to define the translational relevance of current and forthcoming preclinical findings.

SUMMARY

Overall, the last decade has generated a wealth of evidence supporting inheritance driven by preconception environmental perturbations such as chronic alcohol exposure. While there has long been evidence for preconception alcohol exposures inducing fetal alcohol syndrome-like growth abnormalities, more recent studies have extended these findings to include intergenerational alcohol-related behaviors and the ability of alcohol to exert cross-generational effects similar to chronic stress. Finally, studies have begun to delineate genomic loci and RNAs in sperm that are sensitive to alcohol and associated with cross-generational effects (see Figure 2). All in all, these studies highlight the need to continue investigating the role of epigenetics to fully appreciate the heritability of AUD. Given the prevalence and societal impact of alcoholism, the effects of heavy alcohol exposure (or other stressors) on cross-generational alcohol drinking behavior have major implications for predicting familial risk for alcohol use disorder and development of novel disease prevention strategies.

Acknowledgments

Funding support: NIH/NIAAA AA010422, AA020889 and AA024670

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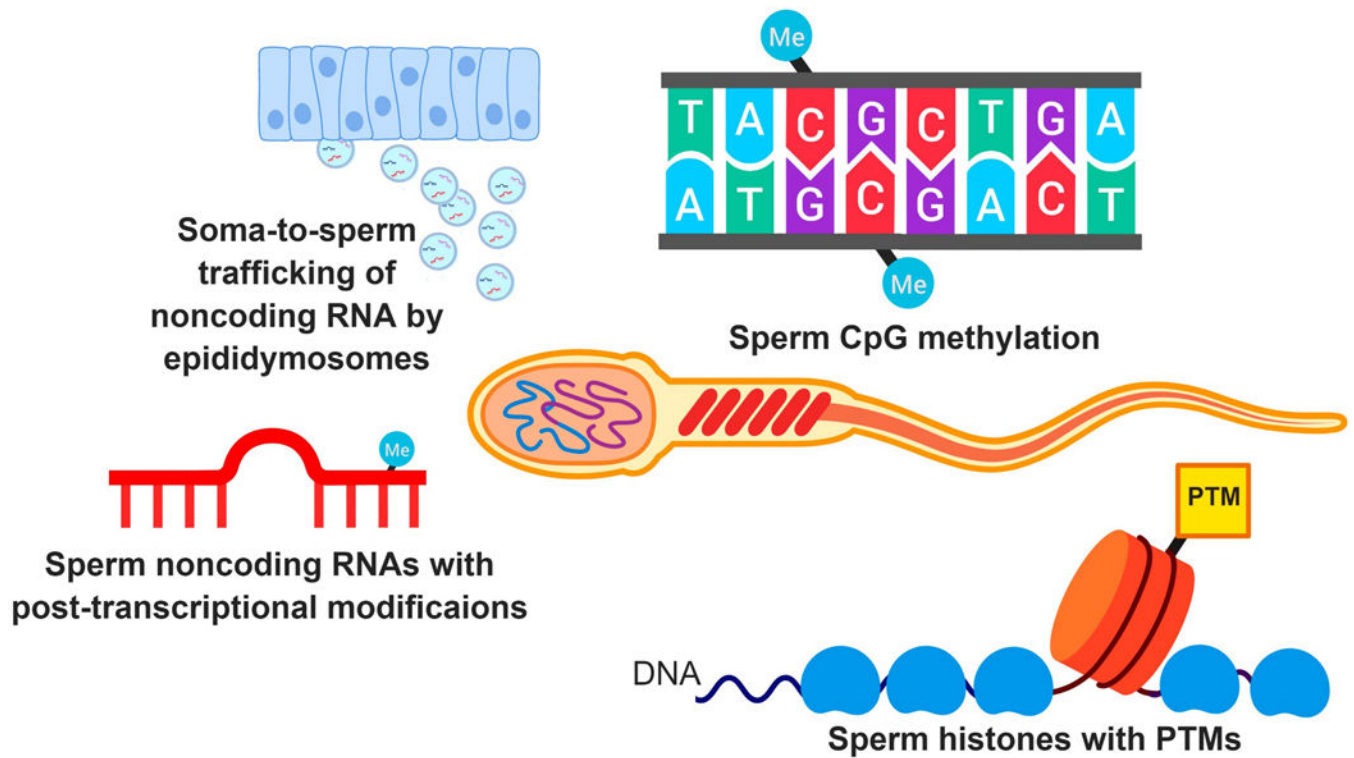


Figure 1. Mechanisms of the sperm epigenome.

Sperm feature various epigenetic mechanisms, most notably DNA methylation, histone post-translational modifications (PTMs) and noncoding RNAs. The high levels of DNA methylation in sperm are primarily localized to cytosine phosphate guanine (CpG) dinucleotides. While many of the histones in sperm are exchanged for protamines, a small percentage of histones are maintained and contain PTMs. Finally, sperm contain diverse noncoding RNA types such as miRNAs and tDRs with accompanying post-transcriptional modifications. Many of the sperm noncoding RNAs are derived from extracellular vesicles secreted by epididymal somatic cells (i.e., epididymosomes).

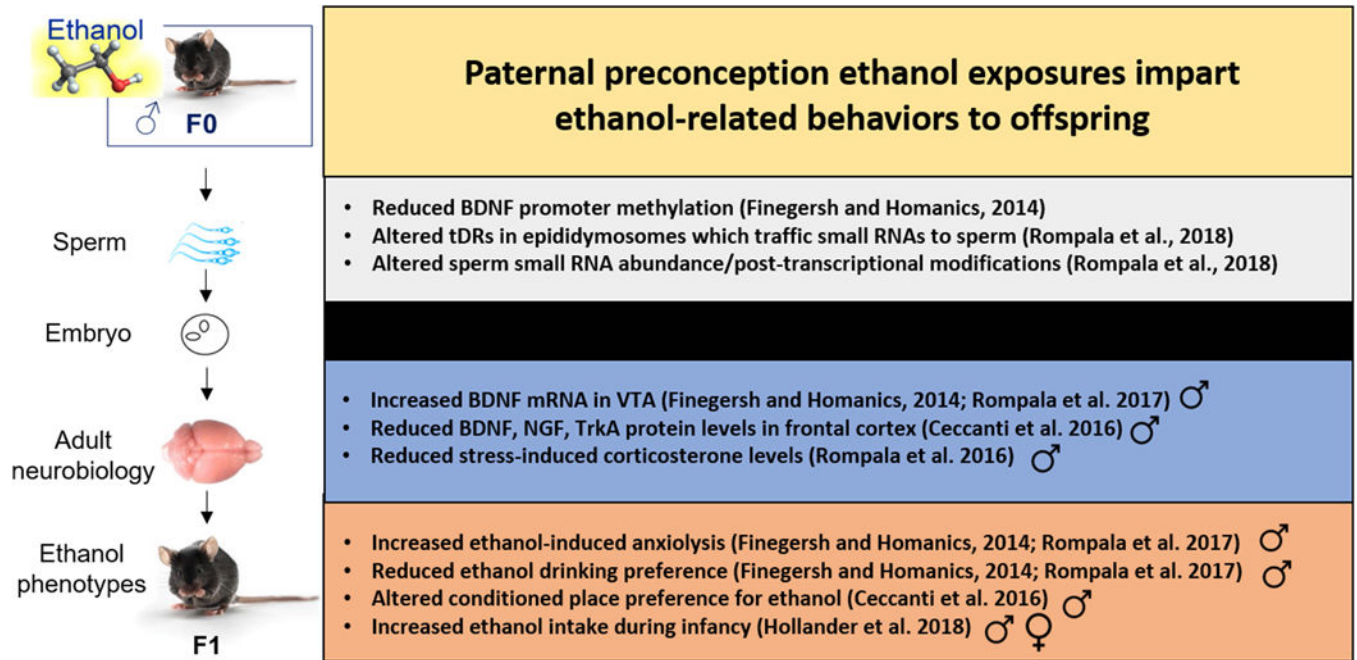


Figure 2. Summary of evidence for paternal preconception alcohol exposure imparting alcohol-related behaviors to offspring.

Studies investigating the intergenerational effects of paternal preconception alcohol have found effects of alcohol on the germline epigenome associated with alterations to neurobiology and alcohol-related behavior in adult male offspring.

Table 1:

Studies where paternal exposure-induced alterations in sperm RNA coincide with intergenerational offspring phenotypes

	Paternal Exposure	Offspring Phenotype	Altered sperm noncoding RNA types and/or species	Citation
DIET	low protein diet	impaired hepatic cholesterol biosynthesis	↓ let7c, ↑ tRNA-Gly-GCC	Sharma et al. 2016
	offspring of obese fathers	glucose intolerance	↑miR10a, ↓ tRNA-Glu-CTC	Cropley et al. 2016
	high fat diet	glucose intolerance *, ↑ weight *	altered tDR, altered RNA modifications	Chen et al. 2016
	obesity	↑obesity, ↑insulin resistance	11 altered miRNA	Fullston et al. 2013
	western diet	↑obesity *	↑ miR19b	Grandjean et al. 2015
	high fat diet	↑weight, ↓glucose metabolism	↑ let7c across both F0, F1 generations	de Castro Barbosa et al. 2016
	obesity	↑obesity, ↓fertility	altered long noncoding RNAs, mRNA	An et al. 2017
STRESS	chronic variable stress	↓HPA responsivity *	9 increased miRNAs	Rodgers et al. 2013
	maternal separation stress	↓anxiety-like *, ↑depression-like behaviors *	several altered miRNA, piRNA, long noncoding RNA	Gapp et al. 2014, Gapp et al. 2018
	chronic glucocorticoid treatment	↑ anxiety-like behavior, ↓fear extinction	altered miRNAs	Short et al. 2016
	chronic social instability	↑anxiety-like behavior, ↓socialization	↓ miR34c, ↓ miR449	Saavenra et al. 2015, Dickson et al. 2018
ENRICHMENT	chronic voluntary exercise	↓ anxiety-like behavior, ↓ fear reinstatement	altered miR19b, miR455, miR133a, tRNA-Gly-GCC	Short et al. 2016
	environmental enrichment	↑ hippocampal-dependent learning *	↑ miRNA 212/132	Benito et al. 2018
TOXICANTS	ancestral vinclozolin exposure	↓ fertility	piRNAs most affected	Ben Maamar et al. 2018, Schuster et al. 2016
	DTT exposure	obesity	piRNA, tRNA most affected	Skinner et al. 2015, Skinner et al. 2018
ALCOHOL	chronic intermittent alcohol	↓alcohol drinking, ↑ alcohol sensitivity, ↓HPA responsivity	↑miR10a, ↑ tRNA-Glu-CTC, two altered RNA modifications	Finegersh et al. 2014, Rompala

	Paternal Exposure	Offspring Phenotype	Altered sperm noncoding RNA types and/or species	Citation
				et al. 2016, Rompala et al. 2018

Summary of rodent studies demonstrating heritable effects of paternal preconception exposures associated with epigenetic reprogramming of sperm noncoding RNA. Legend: mt-tRNA = mitochondrial tRNA.

* = offspring phenotype can be recapitulated by zygotic injection of altered sperm RNA.

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