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Drinking beyond a lifetime: New and emerging insights into paternal alcohol exposure on subsequent generations

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Abstract

Alcohol-use disorder (AUD) is prevalent and associated with substantial socioeconomic costs. While heritability estimates of AUD are ~50%, identifying specific gene variants associated with risk for AUD has proven challenging despite considerable investment. Emerging research into heritability of complex diseases has implicated transmission of epigenetic variants in the development of behavioral phenotypes, including drug preference and drug-induced behavior. Several recent rodent studies have specifically focused on paternal transmission of epigenetic variants, which is especially relevant because sires are not present for offspring rearing and changes to offspring phenotype are assumed to result from modifications to the sperm epigenome. While considerable interest in paternal transmission of epigenetic variants has emerged recently, paternal alcohol exposures have been studied for 30+ years with interesting behavioral and physiologic effects noted on offspring. However, only recently, with improvements in technology to identify epigenetic modifications in germ cells, has it been possible to identify mechanisms by which paternal ethanol exposure alters offspring behavior. This review presents an overview of epigenetic inheritance in the context of paternal ethanol exposure and suggests future studies to identify specific effects of paternal ethanol exposure on offspring behavior and response to ethanol.

Keywords

alcoholism; alcohol; ethanol; transgenerational inheritance; epigenetics; DNA methylation; histones; ncRNAs

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Introduction

A large number of recent studies have challenged traditional views of Mendelian inheritance by showing that offspring phenotype can be modified by parental exposure to nutritional changes, stress, drugs of abuse, and other factors. These studies have implicated heritability of germ-line encoded epigenetic variants in mediating these effects. Converging evidence has also shown that ethanol is an epi-mutagen in several tissue types, including germ cells. Based on known heritability of alcohol-use disorder (AUD) and difficulty in identifying specific gene variants associated with AUD, studying heritability of ethanol-induced epigenetic modifications and their impact on ethanol-related behaviors in subsequent generations has the potential to advance our understanding of the etiology of AUD and to elucidate new biomarkers for AUD.

Heritability of alcohol-use disorder

The lifetime prevalence of AUD is 30% in the United States (Hasin, Stinson, Ogburn, & Grant, 2007), highlighting the likelihood of significant genetic and environmental differences among such a large cohort. This diversity is further complicated by the wide spectrum of alcohol (ethanol) consumption among humans, contributing to varied thresholds for tolerance and dependence among those who meet DSM-V criteria for AUD (American Psychiatric Association, 2013). Heterogeneity has made drug development for AUD challenging, since factors promoting pathological alcohol consumption are likely to be complex and varied between subjects. Despite this diversity, twin and adoption studies consistently find that AUD has a heritability of ~50% (Prescott & Kendler, 1999; Young-Wolff, Enoch, & Prescott, 2011; Ystrom, Reichborn-Kjennerud, Aggen, & Kendler, 2011), indicating transmission of risk alleles from parents to offspring independent of environment.

Based on the known heritability of AUD, studies have extended to heritable physiological markers that may account for increased risk for AUD, including level of response to ethanol and ethanol metabolism. An early meta-analysis of several studies found that sons of alcoholics had decreased subjective response to a low dose of ethanol (Pollock, 1992). Utilizing subjects from the Australian Alcohol Challenge Twin Study, a more recent study quantified heritability of subjective response to a low dose (0.75 g/kg) of ethanol as ~60% in both men and women (Heath et al., 1999). These studies indicate that decreased level of response to ethanol, which is associated with increased risk for AUD (Schuckit, 1994), may be a component of AUD heritability. Observer-measured studies of sensitivity to ethanol have focused on heritability of static ataxia (body sway) after ethanol consumption and have generally noted lower increases in body sway after ethanol in children of alcoholics (Lex, Lukas, Greenwald, & Mendelson, 1988; Schuckit, 1985). Physiological measures of response to ethanol also show evidence for heritability. One study noted that sons of alcoholics had lower plasma cortisol levels after receiving a 1.1 mL/kg dose of ethanol (Schuckit, Gold, & Risch, 1987). Notably, several groups have studied the p300 event-related potential, an electroencephalographic finding associated with attending to a stimulus (Polich, 2012), in people with a family history of alcoholism. These studies have found decreased p300 amplitude in children of alcoholics and have inversely correlated p300

amplitude with future risk for AUD (Begleiter, Porjesz, Bihari, & Kissin, 1984; Costa et al., 2000; Hesselbrock, Begleiter, Porjesz, O'Connor, & Bauer, 2001).

While complex ethanol-related behaviors are difficult to associate with heritable genetic variants, changes in ethanol metabolism have been linked to mutations in single genes. In particular, heritable variants of alcohol (ADH) and acetaldehyde (ALDH) dehydrogenase enzymes are known to be modifiers of alcohol consumption and risk for AUD. Polymorphisms inactivating the ALDH2 gene have been found almost exclusively in Asian populations and are associated with decreased risk for developing AUD (Higuchi, Matsushita, Murayama, Takagi, & Hayashida, 1995; Li, Zhao, & Gelernter, 2012; Thomasson et al., 1991). ADH1 and ADH7 single-nucleotide polymorphisms (SNP) have been associated with alcohol metabolism and consumption in European and African populations (Bierut et al., 2012; Birley et al., 2008). ADH1 polymorphisms also modulate vulnerability to fetal alcohol syndrome disorders (FASD) in fetuses exposed to ethanol (Warren & Li, 2005).

Increasing evidence implicating heritability of ethanol response and metabolism in modifying risk for AUD has led to a broader search for genetic variants associated with AUD. These studies have utilized population-level genetics to measure this association in large numbers of subjects across millions of SNPs. However, while such efforts have identified several potential loci that modify AUD risk, they have also left many questions unanswered regarding how alcohol-related behaviors are inherited. In particular, one recent genome-wide association study (GWAS) did not identify any SNPs significantly associated with alcoholism risk and estimated that all of the SNPs studied accounted for only 0.1% of the genetic risk for developing alcoholism (Heath et al., 2011). Other groups have found SNPs significantly associated with AUD using GWAS but later failed to replicate their results (Bierut et al., 2010; Treutlein & Rietschel, 2011). More recent meta-analyses and expanded studies have identified novel SNPs significantly associated with AUD (Gelernter et al., 2013; Wang et al., 2013), though differences in the SNPs discovered between studies suggest they may not be meaningful across the entire population. "Missing heritability" is a recent concept that refers to the inability of GWAS to uncover risk alleles that explain a substantial portion of the heritability of complex diseases. While technical issues and the contribution of rare genetic variants may be masking these alleles (Manolio et al., 2009), it is also possible that heritable variants outside the DNA sequence, known as "epi-alleles", may be contributing to complex phenotypes such as those mediating susceptibility to AUD.

Epigenetic modifications

Epigenetics deals with a broad group of processes that drive stable states of gene expression without changing nucleotide sequence. These processes are mediated by a diverse set of epigenetic modifications of DNA, including histone modifications and the proteins that create them, covalent modifications to DNA, large and small non-coding RNAs, and proteins that interact with DNA and its epigenetic modifications (Watanabe et al., 2011). The complement of epigenetic modifications associated with a genome is termed the "epigenome". Epigenetic mechanisms are the primary drivers of transcriptional regulation, and allow a single genome to give rise to the hundreds of stable cell lineages within an

organism; thus, the genome of an organism is associated with many epigenomes in distinct cell types, and these epigenomes control the expression of genes that determine the cell's phenotype. The environment can dramatically impact epigenetic modifications (Feil & Fraga, 2012), including those within the germ line. Since some epigenetic modifications are mitotically and meiotically heritable (Reik, 2007), germ-line encoded epigenetic modifications may function as "epi-alleles" if they alter development and/or phenotype in the next generation.

The basic unit of the eukaryotic epigenome is the nucleosome, which consists of ~147 base pairs of DNA wrapped around a core of 8 histone proteins. All histone proteins are rich in basic amino acids that carry a net positive charge, imparting a strong affinity for the negatively charged DNA phosphodiester backbone. The affinity between histones and DNA is critical for regulation of gene expression and is altered by covalent modifications to histone N-terminal tails (Smith & Shilatifard, 2010). For example, acetylation of lysine residues neutralizes the positive charge on lysine's ammonium group, reducing its affinity for DNA; weaker histone-DNA interactions increase accessibility of DNA to transcription factors, which recruit RNA polymerase to initiate transcription (Lee, Hayes, Pruss, & Wolffe, 1993). Histone modifications are catalyzed by a diverse group of histone-modifying enzymes and are rapidly reversible, so that they are a key mechanism of cellular adaptation to the environment (Smith & Shilatifard, 2010). However, their role in gene regulation is complex, as recent studies have identified over 100 post-translational modifications to histones and the function of most of these is unknown (Tan et al., 2011).

The primary covalent modification to DNA in vertebrates is the methylation of cytosine preceding guanine (the CpG dinucleotide). CpGs occur much less frequently throughout the genome than would be expected by chance, most likely due to deamination of methylcytosine to thymine (Schorderet & Gartler, 1992). However, near transcriptional start sites of most mammalian genes, the density of unmethylated CpG dinucleotides is increased at regions known as "CpG islands" (Bird, Taggart, Frommer, Miller, & Macleod, 1985; Gardiner-Garden & Frommer, 1987). CpG islands show tissue-specific patterns of methylation; only ~8% are hypermethylated in most cell types and methylation is associated with transcriptional silencing of associated genes (Illingworth et al., 2008). Importantly, DNA methylation at CpG islands may indicate a relatively stable mechanism of transcriptional repression. Several studies have now shown that nucleosome repression, through histone methylation and induction of the polycomb repressive complex, precedes DNA methylation and that DNA methylation may "lock" gene promoters into a repressive state (Gal-Yam et al., 2008; Jones, 2012; Kass, Landsberger, & Wolffe, 1997). However, though DNA methylation is a critical component of gene regulation and suppression of retroviral elements (Jones, 2012), mechanisms for induction of DNA methyltransferases (DNMT) and pathways for demethylation are still poorly understood (Kohli & Zhang, 2013).

Noncoding RNAs function in both transcriptional and post-transcriptional control of gene expression. ncRNAs comprise all genome-encoded RNAs that are not translated into proteins, but instead carry out diverse functions in the regulation of RNA transcription and translation. The best-studied ncRNA species are the microRNAs (miRNA), which are

important mediators of post-transcriptional gene regulation (Siomi & Siomi, 2009). miRNAs repress gene expression both by destabilizing mRNAs and by repressing their translation (Fabian, Sonenberg, & Filipowicz, 2010). Each miRNA-guided complex can recognize and downregulate hundreds of RNA targets that contain a complementary “seed” sequence, and thus altered expression of a single miRNA can impact expression of hundreds of genes (Huntzinger & Izaurralde, 2011). Small ncRNAs associated with Piwi proteins (piRNAs) are involved in transcriptional silencing of repeat elements in germ cells, although the mechanisms by which silencing occurs is incompletely understood (Brennecke et al., 2008). Short interfering RNAs (siRNAs) are small noncoding RNAs that form RNA-induced silencing complexes (RISC) with dicer and Argonaute proteins to bind and degrade complementary RNA target sequences. siRNAs are distinguished from miRNAs by their exogenous origin, specificity for a single mRNA target, and degradation of the targeted mRNA (Carthew & Sontheimer, 2009). Other ncRNAs such as the long noncoding RNAs (>200 nucleotides) and tRNA-derived fragments add further complexity to the range of epigenetic functions completed by ncRNA species through targeted chromatin regulation (Mercer & Mattick, 2013), competition with other RNAi signaling processes (Haussecker et al., 2010), and probably other means.

Intergenerational inheritance of epigenetic variants

Epigenetic states were once thought to be a somatic phenomenon, reset in developing germ cells so that each generation started with a “clean slate” and recapitulated the laying down of epigenetic modifications that underlie cell differentiation and development. Although plants have been known to exhibit inheritance of epigenetic states at least since Brink’s and Coe’s demonstrations of paramutation in maize in the 1950s (Brink, 1958, 1973; Coe, 1959), it was long thought that the segregation of germ lines in animal species prevented the transmission of acquired epigenetic states. This view has been challenged in the past 20 years by multiple examples of trans-generational inheritance of epigenetic states in animals (briefly reviewed in Suter, Boffelli, & Martin, 2013). These examples indicate that it is possible for purely epigenetic states (i.e., alternative epigenetic states that are not driven by genetically encoded differences) to be maintained in the germ line for one or more generations and survive the phases of epigenetic resetting. In several of these examples, parental exposure to an environmental factor induces a phenotypic change in offspring, and this change is sometimes passed on for one or more generations.

Rodent models allow for identification of specific epigenetic variants that mediate an effect on phenotype as well as the study of multiple generations in a relatively short time frame. Studying the transmission of these variants across multiple generations has led to a distinction between intergenerational and transgenerational effects in the literature. Intergenerational inheritance refers to passage of epigenetic variants from parents to offspring (F1 generation). Because developing germ cells in a fetus can also be affected by *in utero* exposures, intergenerational exposure may extend into the F2 generation. Transgenerational inheritance refers to heritability of an environmentally acquired phenotype into the F2 generation for parental exposures or F3 generation for *in utero* exposures. Therefore, transgenerational inheritance requires persistence of epigenetic variants or phenotypic effects through epigenetic reprogramming during both primordial

germ cell development and the epigenetic resetting events in early embryogenesis. Distinctions are also made between maternal and paternal exposures. Notably, paternal exposures may provide a more direct way of studying molecular mechanisms of epigenetic inheritance. Because the influence of changes to maternal physiology is avoided, sires can be removed during offspring rearing, and *in vitro* fertilization can eliminate any contribution of the sire apart from its germ cell. Paternal studies have built on known effects of diet on sperm (Barazani, Katz, Nagler, & Stember, 2014; Palmer, Bakos, Owens, Setchell, & Lane, 2012). These early studies noted that manipulation of paternal nutrition, including low protein diet (Carone et al., 2010), high fat diet (Ng et al., 2010), fasting (Anderson et al., 2006), and folate deficiency (Kim, Kim, Choi, & Chang, 2013), lead to changes in offspring metabolism and epigenetic modifications in several tissue types.

Recent studies have extended beyond nutritional exposures to reveal that paternal stressors can alter the sperm epigenome and offspring development. Unpredictable chronic stress in sires altered their sperm miRNA content and led to blunting of the hypothalamic-pituitary-adrenal (HPA) axis in their offspring (Rodgers, Morgan, Bronson, Revello, & Bale, 2013). Sires exposed to chronic social defeat stress had offspring that displayed increased anxiety-like behaviors (Dietz et al., 2011). Paternal olfactory fear conditioning to acetophenone enhanced the fear response to acetophenone but not other odors in offspring, and decreased the DNA methylation of an olfactory receptor responsible for detecting acetophenone (Dias & Ressler, 2014).

Studies are also identifying mechanisms of paternal exposures on offspring phenotypes. In one recent study, injection of sperm ncRNAs from postnatally stressed males into fertilized embryos was sufficient to recapitulate the effects of paternal early life stress on depression-like behaviors in offspring (Gapp et al., 2014). Early life paternal stress alters behavior as well as brain miRNA expression of several transcripts for three subsequent generations (Gapp et al., 2014). A recent study of *in utero* ethanol exposure demonstrated decreased DNA methylation of the pro-opiomelanocortin (POMC) gene promoter and altered LPS-induced corticosterone levels through the F3 generation and transmitted via the male germ line (Govorko, Bekdash, Zhang, & Sarkar, 2012). While transgenerational effects are less likely due to epigenetic reprogramming during primordial germ cell development, at least two studies have observed maintenance of epigenetically encoded phenotypes despite loss of transmission of altered ncRNAs to offspring (Gapp et al., 2014; Radford et al., 2014). These findings demonstrate that changes to germline ncRNAs that modify offspring phenotype may become encoded and transmitted in subsequent generations without regeneration of altered ncRNAs in gametes. Mechanisms explaining this idea are still unknown but may involve induction of other epigenetic modifications, such as DNA methylation or histone modifications that stably modify gene expression through cell divisions.

Investigations of other drugs of abuse have yielded similarly compelling evidence for intergenerational epigenetic inheritance of drug-induced behaviors and drug consumption in offspring. Adult offspring derived from dams exposed to preconception morphine had enhanced behavioral sensitivity to morphine and other behavioral alterations (Byrnes, 2005). Male offspring of cocaine-exposed sires surprisingly displayed a cocaine-resistance phenotype and increased expression of BDNF in the prefrontal cortex (Vassoler, White,

Schmidt, Sadri-Vakili, & Pierce, 2013). Chronic cocaine was also found to enhance histone acetylation in the testes, and increased histone acetylation was discovered at the BDNF promoter in sperm (Vassoler et al., 2013), suggesting altered histone modifications may have been inherited through development. Studies have now extended to cross-drug interactions. Parental methamphetamine exposure enhanced cocaine-induced locomotion and reward as well as altered DNA methylation in offspring brains (Itzhak, Ergui, & Young, 2014). Parental tetrahydrocannabinol (THC) exposure was associated with increased heroin-seeking and altered heroin withdrawal in offspring (Szutorisz et al., 2014). It remains to be seen whether these drugs are acting on common pathways to modulate drug-induced signaling in offspring or if their effects are more specific to particular psychoactive agents. Considering the complex pathways that regulate drug intake, including roles for memory, learning, stress, and reward pathways, it may prove difficult to identify a single mechanism driving altered drug preference in offspring following a parental exposure.

Despite compelling evidence for paternally transmitted intergenerational and transgenerational inheritance in mammals, discovering mechanisms that underlie these effects likely requires more rigorous investigation and control of under-recognized variables. Notably, perturbations to the paternal environment can influence maternal investment in offspring care, a concept termed maternal provisioning. For example, offspring of sires exposed to social enrichment were licked and nursed by dams more frequently than those of sires raised in isolation (Mashoodh, Franks, Curley, & Champagne, 2012). Along these lines, some paternal effects on offspring phenotypes disappear after *in vitro* fertilization (Dietz et al., 2011). Therefore, cross-fostering may represent an important control to eliminate effects of maternal provisioning. Additionally, there has been recent concern about inflation of statistical significance in high-profile studies of epigenetic inheritance (Churchill, 2014; Francis, 2014). While we cannot speak to individual studies, confirmation of results and follow-up studies are as essential to this relatively recent field as any in science.

Effects of paternal ethanol exposure on offspring

While molecular evidence for epigenetic inheritance is only recently emerging, paternal ethanol has long been hypothesized to modulate offspring development. These studies have historically been undertaken from a FASD perspective, which posits that like maternal alcohol use during pregnancy, paternal preconception alcohol use also induces a spectrum of morphological and cognitive deficits in offspring.

In humans, several groups have now shown that children of fathers with AUD have higher risk for psychosocial abnormalities, including increased risk for psychiatric disorders (Ozkaragoz, Satz, & Noble, 1997; Pihl, Peterson, & Finn, 1990), decreased performance on measures of intelligence (Ervin, Little, Streissguth, & Beck, 1984), personality changes (Christensen & Bilenberg, 2000; Ervin et al., 1984), and increased incidence of attention-deficit hyperactivity disorder (ADHD) (Knopik et al., 2005). Some of these effects are specific to fathers who had active AUD compared to those who were in remission (Ozkaragoz et al., 1997). Physiological deficits in offspring of alcoholic fathers have also been noted, including electroencephalographic changes (Ramsey & Finn, 1997),

neuroimaging findings (Cservenka, Fair, & Nagel, 2014), a potential increase in the rate of childhood cancers (Infante-Rivard & El-Zein, 2007), and decreased intracranial volumes (Gilman, Bjork, & Hommer, 2007). While these results are confounded by social and environmental factors associated with being raised by a father with AUD, they also raise the alternative possibility that acquired changes to male gametes are being transmitted to offspring.

Rodent studies provide clearer evidence for transmission of acquired effects of ethanol, since sires do not contribute to offspring rearing and isogenic strains minimize potential genetic effects. A comprehensive list of the ethanol exposures used and primary findings of these studies is shown in Table 1. Several groups have found that paternal ethanol induces physiologic abnormalities in offspring in the absence of maternal ethanol exposure, including low birth weight (Bielawski, Zaher, Svinarich, & Abel, 2002; Ledig et al., 1998), increased number of runts (Bielawski & Abel, 1997; Bielawski et al., 2002), altered organ weights (Abel, 1993b; Ledig et al., 1998; Lee et al., 2013), thickening of layers of the cerebral cortex (Jamerson, Wulser, & Kimler, 2004), and low testosterone levels (Abel, 1989b). Several behavioral abnormalities have also been noted, including decreased spatiotemporal learning (Wozniak, Cicero, Kettinger, & Meyer, 1991), decreased novelty-seeking behavior (Ledig et al., 1998), increased immobility on the forced-swim test (Abel & Bilitzke, 1990; Liang et al., 2014), and decreased grooming (Abel, 1991a). Studies which are more recent have found increased anxiety- and impulsivity-like behaviors in offspring of ethanol-exposed sires (Kim et al., 2014; Liang et al., 2014). One of these groups found altered expression of DNMT1 and MeCP2 in brains of paternal ethanol-sired offspring (Kim et al., 2014), suggesting potentially widespread epigenetic abnormalities in these animals. Changes in hypothalamic gene expression of offspring from sires exposed to ethanol through adolescence has also been recently noted (Przybycien-Szymanska, Rao, Prins, & Pak, 2014). While these effects are varied, it is important to note that animals show deficits in some but not all behaviors, suggesting that paternal ethanol affects discrete neurobiological pathways. Moreover, recurrent changes in behavior across multiple animals indicate that the changes are not caused by random mutations in the sperm genome, which would be expected to produce effects with low penetrance.

Despite evidence from several groups indicating that paternal ethanol exposure alters offspring development, until recently, no studies had examined its intergenerational effects on ethanol-drinking behavior and/or behavioral sensitivity to ethanol. This was surprising, considering the high heritability of AUD and difficulty in uncovering risk alleles associated with AUD, though the dearth of studies may also reflect the general neglect until recently of epigenetic inheritance as a factor in the transmission of phenotypes. Our group recently demonstrated that male offspring of ethanol-exposed sires have decreased ethanol drinking and preference on the two-bottle free-choice drinking assay and increased sensitivity to ethanol (Finegersh & Homanics, 2014b). We also identified changes in expression of *Bdnf* in the ventral tegmental area (VTA), and maintenance of ethanol-induced DNA hypomethylation of the *Bdnf* promoter in sires' sperm and offsprings' VTA. Decreased ethanol preference and increased sensitivity to ethanol were not present in females despite maintaining DNA hypomethylation of the *Bdnf* promoter in the VTA, suggesting a sexually

dimorphic pattern of inheritance. Moreover, while decreased ethanol consumption in ethanol-sired male mice is opposite to findings reported in humans, these results provide an invaluable model to probe heritable ethanol-induced factors that influence ethanol-related behaviors, some of which may be relevant to humans. Furthermore, elucidation of the mechanisms responsible for the apparent protective effect of paternal preconception ethanol exposure may yield insights that can be harnessed to decrease problem drinking.

While rodent studies do not fully recapitulate models of human drinking, there are similarities between human and animal models. Notably, evidence for an effect of paternal alcohol on fetal and birth weights in humans has been mixed in the literature (Little & Sing, 1987; Savitz, Zhang, Schwingl, & John, 1992; Windham, Fenster, Hopkins, & Swan, 1995), and this is reflected in the diversity of effects reported in rodent studies (Table 1). Several rodent studies have also noted increased activity and decreased performance on learning paradigms in offspring of ethanol-exposed sires (Table 1), and these are supported by human studies showing increased ADHD (Knopik et al., 2005) and decreased intelligence (Ervin et al., 1984) in children of male alcoholics. Altered patterns of DNA methylation in sperm have also been noted in humans (Ouko et al., 2009) and mice (Finegersh & Homanics, 2014b) exposed to ethanol. However, most human studies have relied on self-reported ethanol consumption, which makes it challenging to predict ethanol use around the time of conception and generalize results to rodent studies.

How does ethanol modify heritable variants in germ cells?

Based on numerous reports of a wide variety of behavioral changes in offspring of ethanol-exposed sires (Table 1), ethanol likely affects several components of spermatogenesis. In particular, epigenetic modifications during spermatogenesis are highly plastic and are regulated by coordinated induction of DNA methylation, histone modifications at developmentally important loci, RNAs, and probably epigenetic mechanisms that have not yet been discovered. These systems interact to produce a cell with a compact and transcriptionally silent nucleus, minimal RNA content, and a paternal imprinting pattern. While sperm have historically been viewed as passive carriers of genetic information, recent studies have demonstrated the importance of these sperm-encoded epigenetic modifications for offspring development.

Condensation of sperm chromatin in mammals involves replacement of nearly all (95–99%) histones with highly basic protamines, which facilitate nuclear condensation by tightly binding DNA (Oliva, 2006). Retained histones in mature sperm were largely ignored until recently, when it was discovered that histone subunit H3 methylated at either lysine 4 and/or 27 was associated with several loci important for early embryogenesis (Hammoud et al., 2009). Moreover, perturbing protamine incorporation into the sperm nucleosome by blocking poly-ADP ribosylation caused aberrant histone retention and altered the expression of hundreds of genes in two-cell embryos (Ihara et al., 2014). While the effects of ethanol on sperm histones have not been directly studied, acute ethanol does increase histone acetylation in rat testes, of which a major component is likely to be in developing germ cells (Kim & Shukla, 2006). Ethanol is also a modulator of histone-modifying enzymes in the brain (Finegersh & Homanics, 2014a; Pandey, Ugale, Zhang, Tang, & Prakash, 2008) and

liver (You, Liang, Ajmo, & Ness, 2008). Notably, altered histone acetylation in the testes after chronic cocaine intake was associated with heritability of decreased cocaine preference in male offspring (Vassoler et al., 2013), so it is possible that altering the distribution of retained histones in sperm can affect drug-seeking behavior. Whether and how ethanol alters histone modifications and histone-modifying enzymes during spermatogenesis, and where in the genome these changes may occur, are critical questions that remain to be answered.

Maintenance and patterning of DNA methylation during spermatogenesis is emerging as a mediator of offspring development. In rodent sires, systemic inhibition of DNA methylation results in decreased sperm count, infertility, and a ~30-fold increase in abnormal preimplantation embryos (Doerksen & Trasler, 1996). Hypomethylated regions of the sperm genome are enriched in transcription-factor binding sites and mark promoters of genes expressed in early embryogenesis (Hammoud et al., 2009; Molaro et al., 2011). Retrotransposable elements are heavily methylated in sperm and resistant to DNA demethylation during embryogenesis (Smith et al., 2012), suggesting that altered programming of DNA methylation in these regions could affect offspring development (Whitelaw & Martin, 2001). While a causal role of sperm DNA methylation in development has yet to be established, these studies support its role in patterning offspring phenotypes. Ethanol has emerged as a modifier of DNA methylation patterns in sperm through multiple mechanisms. Two weeks of ethanol injections decreased sperm counts and plasma testosterone as well as increased oxidative stress in mice (Jana, Jana, De, & Guha, 2010). Increased oxidative stress has been associated with global DNA hypomethylation in human sperm (Tunc & Tremellen, 2009), suggesting a role for oxidative stress in ethanol's effects on sperm DNA methylation. Ethanol may have a particularly robust effect on DNA methylation during spermatogenesis due to aberrant one-carbon metabolism after chronic alcohol use. In particular, alcohol negatively regulates s-adenosylmethionine (SAM) (Hamid, Wani, & Kaur, 2009; Kruman & Fowler, 2014), which is the primary methyl donor for DNMTs.

Several studies now provide evidence for a specific effect of ethanol on DNA hypomethylation in sperm. Ethanol gavage 3 times per week for 9 weeks in rats was associated with decreased expression of the maintenance methyltransferase DNMT1 in sperm (Bielawski et al., 2002). Locus-specific studies have focused on the effect of ethanol consumption on DNA methylation at heavily methylated, imprinted regions in sperm that are resistant to genome-wide demethylation. One study did not find changes in sperm 1 week after ethanol exposure but noted small but statistically significant decreases in DNA methylation at paternally imprinted regions in the offspring of ethanol-exposed sires in mice (Knezovich & Ramsay, 2012). Another study found similarly small but significant decreases in DNA methylation at one of these paternally imprinted regions in sperm after 4 weeks of ethanol treatment using mice (Liang et al., 2014). Consistent with these studies, our group found a more marked decrease in DNA methylation at a paternally imprinted region following 5 weeks of vapor ethanol (Finegersh & Homanics, 2014b). Extending these studies to humans, men with moderate alcohol consumption exhibited a trend for decreased DNA methylation of paternally imprinted genes compared to those who did not drink alcohol (Ouko et al., 2009). Global studies of DNA methylation using promoter microarrays or sequencing are needed to identify ethanol-induced epigenetic modifications that underlie

altered behaviors in ethanol-sired offspring. These studies may be especially interesting because large regions of the genome around intracisternal-A particles (IAP) and imprinted genes were recently discovered to retain DNA methylation during early embryogenesis and primordial germ cell development, providing a potential mechanism for transgenerational inheritance (Lane et al., 2003; Reik, 2007; Seisenberger et al., 2012).

Sperm RNAs have gained significant attention for their role in gametogenesis and, more recently, offspring development. Both male and female gametes are enriched with small ncRNA species (Krawetz, 2005; Ostermeier, Dix, Miller, Khatri, & Krawetz, 2002). Early evidence supports the idea that ncRNAs from paternal sperm and maternal oocytes are important for coordinating normal zygotic and early embryonic gene expression (Liu et al., 2012; Tang et al., 2007). Studies have also recently identified tens of thousands of distinct small ncRNAs in spermatozoa, comprising miRNAs, piRNAs, tRNA fragments, and other sequences (Krawetz et al., 2011; Peng et al., 2012). Sperm miRNAs are also emerging as a modulator of offspring development that may underlie epigenetic inheritance. While an early study found only a minimal contribution of sperm miRNAs to zygotes (Amanai, Brahmajosyula, & Perry, 2006), others have found a role for specific miRNAs in embryo cleavage (Liu et al., 2012) and offspring phenotypes (Rassoulzadegan et al., 2006). Recent studies have found a more general role of altered sperm miRNA populations in heritability of paternally transmitted behavioral phenotypes (Gapp et al., 2014; Rodgers et al., 2013). For instance, postnatal stress altered sperm miRNAs in adult sires, and several of these miRNAs maintained a change in expression in the brains of offspring through the F3 generation (Gapp et al., 2014). Interestingly, methylation of ncRNAs via DNMT2 may be required for RNA-mediated intergenerational inheritance (Kiani et al., 2013). While no studies have directly tested the effects of ethanol on small ncRNAs in sperm, ethanol consumption is known to alter miRNA expression in the brain (Gorini, Nunez, & Mayfield, 2013) and gut (Tang et al., 2008), and it remains to be seen whether these effects extend to gametes. Sperm-derived small ncRNAs represent an exciting new avenue of study that may elucidate molecular mechanisms of intergenerational ethanol-drinking behavior.

While ethanol-induced changes to DNA methylation, retained histones, and ncRNAs in sperm likely each contribute to the effects of paternal ethanol, it will be challenging to identify specific contributions without a better understanding of mechanisms of epigenetic inheritance. Several approaches are emerging for mechanistic studies of epigenetic inheritance. New technology for altering locus-specific epigenetic modifications in the brain (Heller et al., 2014; Konermann et al., 2013) could be used to alter epigenetic modifications in sperm at developmentally relevant promoters. Injection of specific ncRNAs derived from sperm into zygotes is also promising for elucidating mechanisms that underlie behavior (Gapp et al., 2014). These strategies will be possible after a better characterization of ethanol-induced epigenetic modifications emerges.

Toward a specific phenotype for ethanol-sired offspring

The range of behavioral findings across multiple ethanol exposures and research groups makes it challenging to ascribe a particular phenotype to ethanol-sired offspring (Table 1). For example, our study found increased weight gain after weaning in male ethanol-sired

mice (Finegersh & Homanics, 2014b), while other groups have found decreased weight in ethanol-sired mice (Knezovich & Ramsay, 2012). Behavioral findings have also varied among (Finegersh & Homanics, 2014b; Kim et al., 2014; Liang et al., 2014) and between (Abel & Bilitzke, 1990) rodent strains used to model paternal ethanol exposure. These studies suggest complex factors at play, including strain-specific effects, mode and duration of ethanol exposure, and duration of time spent with dams following ethanol exposure. Notably, mouse strain does influence epigenetic inheritance at the *axin/fu* allele (Rakyan et al., 2003). Variable inheritance of epi-alleles is also supported by human studies of colorectal cancer, which indicate some but not all children inherit altered DNA methylation at a promoter region for a mismatch repair enzyme that confers risk for colorectal cancer (Hitchins et al., 2007). Therefore, while ethanol is known to alter epigenetic modifications in sperm, the way in which these modifications are inherited and maintained during development likely varies across strains and species. Characterizing the effects of paternal ethanol on multiple strains using the same ethanol exposure or more global analyses of epigenetic modifications in offspring will elucidate to what extent these mechanisms are reproducible across a heterogeneous population.

Characterizing mechanisms that underlie paternal ethanol's role in ethanol-induced behaviors also requires consideration of the complex mechanisms that regulate these behaviors. In particular, our group noted decreased ethanol preference and increased sensitivity to the anxiolytic effect of ethanol in male ethanol-sired offspring (Finegersh & Homanics, 2014b). Changes to any one of several behavioral pathways could regulate the ethanol-drinking phenotype in mice, including taste perception of ethanol, operant learning, stress associated with social isolation, or sensitivity to the effects of ethanol. Importantly, earlier studies found an effect of paternal ethanol on learning (Wozniak et al., 1991) and behaviors that are affected by stress, such as grooming (Abel, 1991a) and the forced-swim test (Abel, 1991b). While we did not see changes in basal behaviors in mice, other measures of stress, like measurement of corticosterone levels during a stressful paradigm, may uncover effects on these measures in our model. Studying other models of ethanol preference or drinking, such as the conditioned place-preference test (Bozarth, 1990), may disentangle complex effects of paternal ethanol on neurodevelopment from a specific effect on ethanol preference. Stress studies in ethanol-sired offspring may be especially important because of the previously identified effects of paternal stress on male offspring, which showed blunting of the hypothalamic-pituitary axis and changes in several behavioral measures (Gapp et al., 2014; Rodgers et al., 2013). Additionally, considering the fact that ethanol exposure potentiates the hypothalamic-pituitary-adrenal axis and is a potent stressor (Koob, 2003), sires exposed to ethanol may also experience chronic stress prior to mating.

While much work remains to uncover which behavioral phenotypes are reliably caused by paternal ethanol exposure, studies consistently find effects on metabolism or weight gain (Finegersh & Homanics, 2014b; Knezovich & Ramsay, 2012; Ledig et al., 1998) and stress or anxiety-related behaviors (Abel, 1991b; Finegersh & Homanics, 2014b; Kim et al., 2014; Liang et al., 2014) in offspring. The latest findings from our lab have now extended the reported effects of paternal ethanol to regulation of ethanol drinking and ethanol-related behaviors in offspring (Finegersh & Homanics, 2014b). A broader characterization of these

behaviors using standardized ethanol exposures and strains will likely identify common pathways that underlie effects of paternal ethanol and may make results in rodent models more translatable to AUD. Additionally, better characterizing this intergenerational phenotype will facilitate additional research to test for transgenerational effects of paternal ethanol extended out to the F2 and F3 generations.

Conclusion

Epigenetic inheritance is emerging as an adjunct to Mendelian inheritance and may account for some portion of the missing heritability of complex human diseases. Rodent studies have now shown that various perturbations to the paternal environment can alter a male's offspring (and in some cases his offspring's offspring), and these include drug-seeking behaviors. Importantly, paternal preconception ethanol exposure has long been known to alter offspring physiology and behavior, but its role in regulating ethanol drinking in offspring is only now emerging. Similar to human studies that dissect physiological and behavioral correlates of AUD, a detailed analysis of ethanol-sired offspring is necessary to determine how changes in ethanol preference are inherited. Additionally, expanding studies on ethanol's epigenetic effects in germ cells will identify molecular markers that underlie these behavioral effects. While much work needs to be done to definitively establish mechanisms by which paternal ethanol affects offspring, the potential to study a new mode of heritability of AUD represents an exciting new avenue for ethanol research.

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HIGHLIGHTS

- Epigenetic variants can be transmitted between generations
- Numerous studies demonstrate paternal ethanol exposure impacts subsequent generations
- Additional studies of the epigenetic effects of ethanol are desperately needed

Table 1

Rodent models of intergenerational effects of paternal ethanol exposure

Reference	Species	Route	Duration	Primary findings in offspring
<i>Offspring weight and development</i>				
Anderson, Furby, Oswald, & Zaneveld, 1981	SW Mice	LD	4 weeks	↓ birth weight
Mankes et al., 1982	LE Rats	DW	8.5 weeks	↑ malformations, ↓ litter weight
Randall, Burling, Lochry, & Sutker, 1982	C3H Mice	LD	4 weeks	No change in fetal weight
Leichter, 1986	Rats	LD	6 weeks	No change in fetal weight
Abel & Moore, 1987	SW Mice	LD	6 weeks	No change in fetal weight, mortality
Abel & Tan, 1988	SD Rats	LD	7.5 weeks	No change in birth or adult weight
Abel, 1989a	SW Mice	LD	7 weeks	No change in birth or adult weight
Abel, 1989c	LE Rats	LD	9 weeks	No change in fetal weight
Abel, 1993b	SD Rats	Gavage	9 weeks	↑ runts; no change in birth weight
Abel, 1995	SD Rats	Gavage	9 weeks	↑ fetal weight; no change in birth weight
Bielawski & Abel, 1997	SD Rats	Gavage	16 hours	↑ runts and malformations
Ledig et al., 1998	IW Rats	DW	13 weeks	↓ birth and adult weight in males
Bielawski et al., 2002	SD Rats	Gavage	9 weeks	↑ runts, ↓ fetal weight
Knezovich & Ramsay, 2012	C57 Mice	Gavage	5 weeks	↓ postnatal growth at day 35
Lee et al., 2013	CD1 Mice	Gavage	7 weeks	↑ fetal malformations
Kim et al., 2014	CD1 Mice	Gavage	7 weeks	No change in body weight
Finegersh & Homanics, 2014b	C57 Mice	Vapor	5 weeks	↑ weight after weaning in males
<i>Learning and activity</i>				
Abel & Tan, 1988	SD Rats	LD	7.5 weeks	↓ activity, ↓ learning in females
Abel, 1989a	SW Mice	LD	7 weeks	↓ activity prior to weaning
Abel, 1989b	LE/SD Rats	LD	4 weeks	Strain-dependent ↓ activity
Abel, 1989c	LE Rats	LD	9 weeks	↓ activity
Wozniak et al., 1991	SD Rats	LD	5.5 weeks	↓ learning in males
Abel, 1993a	SD Rats	Gavage	13 weeks	↑ amphetamine-induced activity
Ledig et al., 1998	IW Rats	DW	13 weeks	↑ activity and novelty seeking
Kim et al., 2014	CD1 Mice	Gavage	7 weeks	↑ activity and impulsivity
Finegersh & Homanics, 2014b	129xB6 Mice	Vapor	5 weeks	No change in open field activity

Reference	Species	Route	Duration	Primary findings in offspring
<i>Anxiety-related behaviors</i>				
Abel & Bilitzke, 1990	Mice/Rats	LD	14 weeks	Species-dependent FST immobility
Abel, 1991a	SD Rats	LD	5.5 weeks	↓ grooming
Abel, 1991b	SD Rats	LD	30 weeks	↓ immobility on FST
Ledig et al., 1998	IW Rats	DW	13 weeks	↑ light-dark transitions
Meek, Myren, Sturm, & Bureau, 2007	SW Mice	IP inj.	12 hours	↑ aggression and ↓ fear
Liang et al., 2014	KM Mice	Gavage	4 weeks	↑ anxiety-like behaviors
Finegersh & Homanics, 2014b	129xB6 Mice	Vapor	5 weeks	No changes on basal anxiety tests
Rompala, Finegersh, & Homanics, 2015	B6 mice	Vapor	6 weeks	No changes on basal anxiety tests
<i>Molecular and physiologic effects</i>				
Abel & Lee, 1988	SW Mice	LD	7.5 weeks	↓ serum testosterone
Nelson, Brightwell, MacKenzie-Taylor, Burg, & Massari, 1988	SD rats	Vapor	6 weeks	Altered neurotransmitter levels
Berk, Montgomery, Hazlett, & Abel, 1989	SW Mice	LD	7 weeks	↑ ocular infections
Hazlett, Barrett, Berk, & Abel, 1989	SD Rats	LD	3 weeks	↑ severity of ocular infections
Cicero et al., 1990	SD Rats	LD	5.5 weeks	↓ sexual maturation in males
Abel, 1993b	SD Rats	Gavage	9 weeks	↑ adrenal and ↓ spleen weights
Ledig et al., 1998	IW Rats	DW	13 weeks	↓ glial enolase, SOD, GS
Jamerson et al., 2004	SD Rats	DW	7 weeks	↑ CCx thickness
Knezovich & Ramsay, 2012	C57 Mice	Gavage	5 weeks	↓ DNA methylation imprinting
Liang et al., 2014	KM Mice	Gavage	4 weeks	Altered imprinted gene expression
Kim et al., 2014	CD1 Mice	Gavage	7 weeks	↓ DAT, DNMT1, MeCP2 expression
Finegersh & Homanics, 2014b	129xB6 Mice	Vapor	5 weeks	↑ BDNF, ↓ methylation in males
Rompala et al., 2015	129xB6 Mice	Vapor	5 weeks	Blunting of HPA axis in males
<i>Drinking and alcohol-induced behaviors</i>				
Finegersh & Homanics, 2014b	129xB6 Mice	Vapor	5 weeks	↓ ethanol preference on 2BC, ↑ ethanol-induced anxiolysis on EPM in males
Rompala et al., 2015	B6 Mice	Vapor	6 weeks	↓ ethanol preference on 2BC, ↑ ethanol-induced anxiolysis on EPM in males
Rompala et al., 2015	129xB6 Mice	Vapor	5 weeks	Resistance to stress-induced polydipsia, No change in DID

Strains: LE = Long Evans; SW = Swiss Webster; IW = Italian Webster; KM = Kunming. Route of ethanol administration: LD = Liquid Diet; DW = Drinking Water; IP inj. = Intraperitoneal Injection. Other Abbreviations: FST = Forced Swim Test; EPM = Elevated Plus Maze; CCx = Cerebral Cortex; DAT = Dopamine Transporter; SOD = Superoxide Dismutase; GS = Glutamine Synthetase; DAT= Dopamine transporter; HPA = Hypothalamic-pituitary-adrenal; 2BC = 2-bottle choice; DID = Drinking in the dark.