Behavioral And Epigenetic Consequences of Paternal Alcohol Exposure

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ABSTRACT

Familial transmission of alcohol use disorders reflects genetic and environmental factors. For decades, studies in rodents demonstrated that paternal alcohol exposure produces cognitive and physiological abnormalities in offspring. The mechanisms of these effects may reflect epigenetic modifications transmitted through the male germ line. While mouse studies show that paternal alcohol exposure alters sensitivity to alcohol in offspring, no studies have examined whether paternal alcohol exposure impacts sensitivity to unconditioned and reinforcing effects of alcohol using genetically diverse rat strains. We exposed male Wistar rats to a chronic intermittent ethanol procedure (CIE) in alcohol vapor chambers (16 h/day; 5 days/week; 6 weeks) or to air. Eight weeks later, rats were mated with alcohol-naive females and separate groups of adult offspring (F1) were assessed on a range of alcohol-induced behaviors and operant alcohol self-administration. In Experiment 1, separate groups of alcohol- and control-sired offspring were intragastrically administered alcohol (1.5 g/kg) or water 30 min prior to testing for general locomotor activity (open field), anxiety-like behaviors (elevated plus maze [EPM]), and motor coordination (rotarod). We found that alcohol reduced locomotor activity in alcohol-sired male offspring but not alcohol-sired female or control-sired offspring. Alcohol-sired males showed less anxiety-like behavior on the EPM regardless of treatment. Alcohol-sired males were resistant but alcohol-sired females were more sensitive to alcohol-induced impairments in motor coordination relative to their respective controls. In Experiment 2, alcohol- and control-sired offspring were

trained to lever press for increasing alcohol concentrations (2.5%, 5%, & 10%, v/v). Tests were conducted under a progressive ratio (PR) schedule of reinforcement at 5% and 10% alcohol. Extinction training was followed by reinstatement tests and reinitiation procedures. During acquisition training sessions, alcohol-sired offspring self-administered less alcohol (5% & 10%) relative to control-sired offspring. Under progressive ratio tests, alcohol-sired offspring self-administered less alcohol (5% & 10%) relative to control-sired offspring. Alcohol-sired offspring displayed lower responding during extinction training and blunted relapse-like behavior during reinstatement. During reinitiation, alcohol-sired offspring self-administered less alcohol relative to control-sired offspring. In Experiment 3, global and *brain-derived neurotrophic factor* (*Bdnf*) DNA methylation levels were measured in sperm, the medial prefrontal cortex, and the nucleus accumbens of sires and adult offspring. Global methylation levels varied by tissue in alcohol sires compared to controls, but no changes were seen in offspring. Alcohol sires had lower *Bdnf* DNA methylation levels in the nucleus accumbens but higher methylation levels in the medial prefrontal cortex relative to control sires. Alcohol-sired offspring also had aberrant *Bdnf* DNA methylation levels in the nucleus accumbens that varied as a function of sex and CpG site. Overall, results indicate that paternal alcohol exposure prior to conception induces long-lasting behavioral and epigenetic effects that reflect an alcohol resistant phenotype in offspring.

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CHAPTER ONE- INTRODUCTION

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Substance use disorders

Substance use disorders (SUDs) occur when the recurrent use of alcohol/drugs leads to clinical and functional impairments that are detrimental to a person's health, or the welfare of others (APA, 2013). According to the 2015 National Survey on Drug Use and Health, SUDs are highly prevalent in the United States with ~20 million adults meeting clinical criteria for a substance or alcohol use disorder. In addition, the economic costs associated with SUDs are greater than 740 billion/year (NIDA, 2017), largely due to costs associated with crime, health care, and lost work productivity. Chronic drug and alcohol use increases the risk of many negative health consequences, including cardiovascular and neurological problems and cancer (NIDA, 2017). Given the substantial economic and individual costs, it is essential to identify risk factors that predispose individuals to developing a SUD.

Genetics play a prominent role in the development of SUDs independent of the environment. Family, twin, and adoption studies find that SUD heritability ranges from 40% for hallucinogens to 72% for cocaine (Ducci and Goldman, 2012). Parental drug use has long-lasting ramifications on child outcomes. The consequences of maternal drug use during pregnancy are a well-studied area. In animal and human studies, maternal drug use associates with several developmental, cognitive, and emotional impairments in offspring (Bandstra et al., 2010; Minnes et al., 2011; O'Connor and Paley, 2009; Schempf, 2007). In contrast, the consequences of

paternal drug use, especially in periods prior to conception, have received relatively little attention. This is unfortunate given that drugs and alcohol can modify sperm in humans and animals (Finegersh and Homanics, 2014; Li et al., 1997; Misra et al., 1977; Ouko et al., 2009; Vassoler et al., 2013); studies in the latter show that these changes can be passed to future generations. Thus, drug or alcohol exposure can have long-lasting implications for subsequent generations.

Given this significant genetic influence, candidate gene and genome wide association studies have aimed to identify genetic variants that contribute to SUDs. These investigations have been challenging given the polygenic nature of SUDs. Considering these challenges, hypothesis-driven candidate gene studies have identified several genes involved in drug metabolism and the monoamine and serotonin systems (Ducci and Goldman, 2012). Genome-wide association studies have identified novel variants that associate with smoking behaviors. However, genome wide association studies have been less successful in identifying loci associated with other substances, particularly alcohol (Ducci and Goldman, 2012). Thus, there may be other factors that contribute to this missing heritability. For instance, rare variants of strong effect remain unidentified (Manolio et al., 2009). Another possibility is the growing attention to molecular epigenetic factors in human diseases, including addiction (Maze and Nestler, 2011; Nestler, 2014; Nielsen et al., 2012). The primary goal of this review is to determine the current state of the preclinical literature on the inter- and trans-generational consequences of paternal drug exposure, as well as to highlight areas for further study that may improve prevention and treatment approaches for SUDs.

Epigenetics

Epigenetics refers to a range of mitotically and meiotically heritable molecular modifications that alter gene expression without changing the underlying DNA sequence (Maze and Nestler, 2011). Several related epigenetic mechanisms regulate gene expression: chromatin remodeling, DNA methylation, and non-coding RNAs. These mechanisms are essential to normal cell function allowing diverse cell types to emerge from a single genome. Additionally, some epigenetic alterations can have an acute onset (1 hr) and offset (24 hr), while others have a more stable profile reflecting events from prior decades (Heijmans et al., 2008). The epigenetic mechanisms described below work collectively to regulate gene expression and a wide array of biological functions.

Chromatin remodeling

Chromatin consist of a complex of DNA and histone proteins. DNA is tightly wrapped around eight core histone proteins, two copies each of H2A, H2B, H3, and H4, within a nucleosome. Histone tails that project from the histone core are the sites for post-translational modifications. Covalent modifications, such as acetylation, methylation, and phosphorylation, at histone tails modify the chromatin structure leading to open (active) or closed (repressive transcriptional state), or a somewhere in between these two states (Kornberg and Lorch, 1999; Kouzarides, 2007). An open chromatin state, or euchromatin, enhances gene expression and occurs when acetyl groups attach to lysine residues located on histone tails (Gardner et al., 2011; Jenuwein and Allis, 2001). Acetylation loosens the electrostatic bond between histones and DNA, providing transcription factors access to promoter regions.

Histone acetyltransferases increase acetylation and histone deacetylases maintain it (Jenuwein and Allis, 2001). Relative to acetylation, histone methylation is a more complex histone modification system that, depending on the site and number of methyl groups bonded, can facilitate or repress gene transcription. Histone methylation is controlled by both histone methyltransferases and histone demethylases. Some methyl marks are found in inactive chromatin (i.e., H3K27me3), while others are found in transcriptionally active chromatin (i.e., H3K4me3) (Barski et al., 2007). In addition, phosphorylated histones are found in both active and inactive chromatin (Ito, 2007). It is important to note that these and other histone modifications, such as SUMOylation, ubiquitination, citrullination, and ADP-ribosylation form a “histone code” to govern gene expression (Jenuwein and Allis, 2001).

DNA methylation

DNA methylation is the most well studied epigenetic modification and is involved in regulating gene expression by marking genes for silencing or activation. Specifically, DNA methylation occurs when methyl groups attach to the 5’ pyrimidine ring via DNA methyltransferases (DNMT) and methyl CpG-binding protein 2 (Mecp2) enzymes (Bestor, 2000). DNMT3a and DNMT3b are involved in *de novo* DNA methylation, while DNMT1 maintains DNA methylation after DNA replication. DNA methylation occurs often at cytosine:guanine dinucleotides (CpG) to form 5’-methylcytosine guanine dinucleotides (mCG). (Bestor, 2000; Bird and Macleod, 2004; Fazzari and Greally, 2004; Lande-Diner et al., 2004; Robertson and Wolffe, 2000b); however, DNA methylation can also occur at other dinucleotide pairings

(Varley et al., 2013; Xie et al., 2012). Promoter regions of genes contain a high density of CpG dinucleotides called “CpG islands” (Larsen et al., 1992). A substantial percentage (~70%) of CpG islands are methylated while a smaller percentage (~2%) are unmethylated (Ziller et al., 2013). Typically, DNA methylation near transcription start sites represses gene transcription while methylation within the gene body activates gene transcription (Bird and Macleod, 2004; Campanero et al., 2000; Heller et al., 2008; Hwang et al., 2007; Iguchi-Ariga and Schaffner, 1989; Jaenisch and Bird, 2003; Lande-Diner et al., 2004; Robertson and Wolffe, 2000b; Tong et al., 2010). DNA demethylation (e.g. hydroxymethylation) is facilitated by ten eleven translocation (TET) proteins and typically activates transcription (Ito et al., 2010; Shen and Zhang, 2012). Normal developmental processes, such as genomic imprinting and X chromosome inactivation, rely on DNA methylation.

DNA methylation can also interact with histone modifying enzymes to affect chromatin. MeCP2 binds DNA at methylated cytosines to inhibit transcription (Boyes and Bird, 1991; Cross et al., 1997; Gabel et al., 2015; Hendrich and Bird, 1998; Prokhortchouk et al., 2001). Additionally, MeCP2 may recruit histone deacetylases to deacetylate proximal histones, thereby attenuating gene expression (Jones et al., 1998; Nan et al., 1998; Razin, 1998). Conversely, MeCP2 may be involved in recruiting transcription factors, such as CREB in active promoters (Chahrour et al., 2008).

Non-coding RNA’s

Non-coding RNAs can also alter gene expression. MicroRNA’s (miRNA’s) are short (~20 nucleotides) non-coding RNA’s that are involved in post-transcriptional

silencing. miRNA's are transcribed from genomic DNA and a single strand can suppress protein translation of dozens of genes (Mercer et al., 2009). The literature examining the role of miRNA's in the intergenerational effects of drugs is limited. However, non-coding RNAs are hypothesized to be passed down to future generations via the male germ line (Murashov et al., 2016).

Epigenetic reprogramming in male gametes

It is becoming clearer that male germ cells do more than passively carry genetic information. Sperm can alter the epigenetic profile and regulate the expression of hundreds of genes in embryos (Ihara et al., 2014). Mammalian germ cells undergo two rounds of epigenetic reprogramming throughout the lifecycle, 1) during preimplantation development and 2) during germ cell development (Abe et al., 2011; Feng et al., 2010; Monk et al., 1987; Reik et al., 2001; Seisenberger et al., 2013). Reprogramming during the former is important for naïve pluripotency in the zygote epigenome while the latter erases parental and somatic epigenetic marks and enables gametogenesis (Messerschmidt et al., 2014; Saitou et al., 2012). During the early embryonic period, the primordial germ cells that give rise to spermatogenic cells in males demethylate from around 70% to 4% as they migrate and colonize the gonadal region (Kobayashi et al., 2013; Seisenberger et al., 2012). At this point, even imprinted loci are hypomethylated. Chromatin modifications maintain genomic integrity during this period of demethylation. For example, repressive chromatin modifications suppress retrotransposon activity (Tang et al., 2016). Eventually, methylation is reestablished in a sex-dependent manner,

~embryonic day 13.5 for males and after birth for females (Messerschmidt et al., 2014).

Some genomic loci can escape global demethylation. Most of these loci are associated with retrotransposons (Guibert et al., 2012; Hackett and Surani, 2013; Seisenberger et al., 2012) while others are found in pericentromeric satellite repeats (Tang et al., 2015) and in subtelomeric regions (Guibert et al., 2012). In addition, single-copy sequences and genes expressed in the brain and ubiquitously can also escape global demethylation (Guibert et al., 2012; Hackett and Surani, 2013; Seisenberger et al., 2012; Tang et al., 2015). It is important to note that preserved methylation at these sites is not necessarily maladaptive and may be important for maintaining chromosome stability and chromosome alignment and segregation during mitosis (Tang et al., 2016).

Inter- and trans-generational consequences of paternal drug exposure

Paternal exposure to environmental stimuli can result in several intergenerational consequences. At the preclinical level, paternal diet manipulations alter glucose metabolism and brain development in offspring (Anderson et al., 2006; Kim et al., 2013; Ng et al., 2010). Sires exposed to stress paradigms have offspring with blunted stress responses and greater depression- and anxiety-like behaviors (Dietz et al., 2011; Gapp et al., 2014; Rodgers et al., 2013). Conditioned fear to odors is also enhanced in offspring of sires exposed to olfactory fear conditioning (Dias and Ressler, 2014). Some of the behavioral and physiological effects seen in offspring are accompanied by changes in DNA methylation levels (Carone et al., 2010; Dias and Ressler, 2014; Kim et al., 2013). In each section below, we will

review the behavioral and epigenetic consequences of paternal drug exposure (Table 1.1).

Alcohol

Developmental findings

Paternal alcohol exposure induces several developmental aberrations. It reduces litter sizes (Abel, 1989b; Cicero et al., 1994a; Cicero et al., 1994b; Emanuele et al., 2001b; Mankes et al., 1982; Tanaka et al., 1982) and increases the number of runts (Abel, 1993; Bielawski and Abel, 1997; Bielawski et al., 2002; Chang et al., 2017; Meek et al., 2007), malformations (Bielawski and Abel, 1997; Mankes et al., 1982), and pup mortality (Cicero et al., 1994a; Cicero et al., 1994b; Meek et al., 2007) in rats and mice. Litters from alcohol-exposed sires also exhibit increased (Abel, 1995; Emanuele et al., 2001a) or decreased male-to-female ratios (Abel, 1993). Yet, several groups find that these litter parameters are unaltered in rats (Abel, 1989c; Abel and Tan, 1988; Bielawski and Abel, 1997; Bielawski et al., 2002; Cake and Lenzer, 1985; Leichter, 1986) and mice (Abel and Lee, 1988; Ceccanti et al., 2016; Finegersh and Homanics, 2014; Randall et al., 1982). Alcohol-sired offspring also display increased (Emanuele et al., 2001b; Finegersh and Homanics, 2014; Rompala et al., 2016), decreased (Bielawski et al., 2002; Ceccanti et al., 2016; Ledig et al., 1998; Mankes et al., 1982; Meek et al., 2007; Rompala et al., 2017; Tanaka et al., 1982), and no change (Abel, 1989b, c, 1993; Abel and Lee, 1988; Abel and Tan, 1988; Bielawski and Abel, 1997; Leichter, 1986; Livy et al., 2004; Randall et al., 1982) in body weights at birth, weaning, or adulthood. At times, changes in body weights occur in a sex-dependent manner. Overall, paternal alcohol

exposure alters several developmental parameters across strains in rats and mice, but results are inconsistent across studies.

Learning and locomotor activity findings

Paternal alcohol exposure alters learning and memory and locomotor activity in offspring. Alcohol-sired offspring exhibit greater impairments in inhibitory (Abel, 1994) and active avoidance (Abel and Tan, 1988), and working memory (Abel and Lee, 1988; Wozniak et al., 1991). Across several studies using rats and mice, alcohol-sired offspring exhibit hyperactivity (Abel, 1989b, 1994; Ledig et al., 1998), hypoactivity (Abel, 1989a, b, c; Abel and Lee, 1988; Abel and Tan, 1988), and unaltered (Finegersh and Homanics, 2014; Rompala et al., 2017) activity levels when measured in pre-adolescence or adulthood. Alcohol-sired offspring also show greater amphetamine-induced hyperactivity (Abel, 1993). Alcohol-sired offspring display normal motor coordination on the rotarod (Nelson et al., 1988), but male offspring are less sensitive to alcohol-induced impairment in motor coordination (Finegersh and Homanics, 2014).

Affective findings

Paternal alcohol exposure alters baseline and alcohol-induced affective behaviors, sometimes in a species-dependent manner. Swiss Webster alcohol-sired males exhibit greater aggression and less fear behaviors (Meek et al., 2007). Alcohol-sired males show less anxiety-like behavior at baseline (Abel, 1991; Ledig et al., 1998) and after alcohol administration (Finegersh and Homanics, 2014; Rompala et al., 2017). C57/BL6J alcohol-sired offspring display greater depression-like

behavior; however, Long Evans alcohol-sired offspring display less depression-like behavior (Abel and Bilitzke, 1990).

Reward-related findings

Paternal alcohol alters sensitivity to the rewarding effects of alcohol in a sex-dependent manner. In two-bottle choice procedures, C57/BL6J alcohol-sired males consume less alcohol (Finegersh and Homanics, 2014; Rompala et al., 2017). CD1 alcohol-sired males also exhibit greater place preference at a lower alcohol dose, while place aversion is seen at a higher dose that induced a preference in control-sired offspring (Ceccanti et al., 2016). Thus, paternal alcohol exposure may confer a phenotype that is protective against alcohol-motivated behaviors in male offspring or lead to a leftward shift in the alcohol dose response function. Studies using rats and operant self-administration procedures are lacking.

Molecular and physiological findings

Paternal alcohol exposure results in several molecular and physiological abnormalities in offspring, such as alterations in organ weights, gonadal hormones, neurotransmitter and stress systems, and neurotrophic factors. Alcohol-sired offspring display greater brain (Cake and Lenzer, 1985), thymus (Abel and Lee, 1988), and adrenal weights (Abel, 1993); while spleen weights are lower (Abel, 1993). Alcohol-sired male offspring have lower testosterone levels (Abel, 1989b; Abel and Lee, 1988). Preadolescent alcohol-sired offspring have greater leptin levels. The glutamate, serotonin, norepinephrine, and opioid systems are also altered in alcohol-sired offspring (Ledig et al., 1998; Nelson et al., 1988). Alcohol-sired male offspring have greater *Brain-derived neurotrophic factor (Bdnf)* mRNA

expression in the ventral tegmental area (Finegersh and Homanics, 2014; Rompala et al., 2017), while protein levels are lower in the kidneys, frontal cortex, and olfactory lobes at baseline and after alcohol (Ceccanti et al., 2016). Nerve growth factor protein levels are also lower at baseline and after alcohol exposure in alcohol-sired males (Ceccanti et al., 2016). In response to acute restraint stress, alcohol-sired males show lower (Rompala et al., 2016) or unaltered corticosterone levels (Rompala et al., 2017). Paternal alcohol exposure also increases deafness (Liang et al., 2015) and susceptibility to ocular infections in offspring (Berk et al., 1989).

Epigenetic findings

Paternal alcohol exposure alters DNA methylation levels of paternally imprinted and neurotrophic factor genes. Alcohol exposure increases *Paternally expressed gene 3 (Peg3)* (Liang et al., 2014) and decreases *Bdnf* (Finegersh and Homanics, 2014) methylation levels in the sperm of sires. These changes are maintained in the cerebral cortices (*Peg3*) and ventral tegmental area (VTA; *Bdnf*) in the brains of offspring (Finegersh and Homanics, 2014; Liang et al., 2014). *Bdnf* methylation and mRNA changes in VTA associate with lower sensitivity to alcohol-induced anxiolysis and lower alcohol consumption in male offspring (Finegersh and Homanics, 2014). However, a recent study in mice found no changes in sperm-inherited DNA methylation in sires after voluntary alcohol consumption (Chang et al., 2017). Thus, in some instances, alcohol-induced changes to the sperm epigenome can have long-term functional consequences in male offspring.

Cocaine

Developmental findings

There has been little research examining for developmental consequences of paternal cocaine exposure. Studies in Sprague-Dawley and Long Evans rats that have passively received cocaine or self-administered cocaine from 2-2.5 months show no changes in several developmental outcomes including litter size, sex ratio, and weights at birth and weaning (Abel, 1989c; Wimmer et al., 2017). Lower birth weights are found after more than 3 months of paternal cocaine exposure in Sprague-Dawley rats (George et al., 1996). Thus, longer durations of paternal cocaine exposure may impact developmental outcomes.

Learning and locomotor activity findings

Cocaine-sired offspring also show deficits in learning and memory tests and greater hyperactivity. In Sprague-Dawley rats, cocaine-sired male offspring display impaired long-term object memory and decreased hippocampal long-term potentiation (Fischer et al., 2017). In CD 1 mice, cocaine-sired offspring of both sexes show impaired sustained visuospatial attention and spatial working memory (He et al., 2006). In addition, C57/BL6J and Long Evans cocaine-sired offspring display greater hyperactivity at baseline and after psychostimulant exposure (Abel, 1989c; Fischer et al., 2017). No changes have been seen in C57/BL6J cocaine-sired offspring on spatial and working memory, novel object discrimination, and social behavior (Fischer et al., 2017; Killinger et al., 2012). Overall, paternal cocaine exposure induces learning and memory deficits and increased baseline and

psychostimulant-induced activity in offspring. Impairments in learning and memory appear to be strain-specific in mice.

Affective findings

Cocaine-sired offspring also show altered affective behaviors. Paternal cocaine exposure increases anxiety-like behavior in Sprague-Dawley and C57/BL6J male offspring on the elevated plus maze, novelty-induced hypophagia, and marble burying tests (Fischer et al., 2017; White et al., 2015). It should be noted that findings in mice have been inconsistent as anxiety-like behavior is unchanged on open field and elevated plus maze in cocaine-sired offspring (Killinger et al., 2012). Additionally, C57/BL6J cocaine-sired offspring show greater depression-like behaviors on the tail suspension test (Killinger et al., 2012); however, no change in depression-like behavior on the forced swim test has been observed in C57/BL6J and Sprague-Dawley cocaine-sired offspring (Fischer et al., 2017; White et al., 2015). Taken together, paternal cocaine treatments result in an anxiogenic phenotype in male offspring across rodent species, but findings on depression-like behavior are inconsistent.

Reward-related findings

Mice and rat studies show that paternal cocaine exposure alters sensitivity to cocaine in offspring. Male Sprague-Dawley cocaine-sired offspring show reduced cocaine sensitization (Wimmer et al., 2018). C57/BL6J cocaine-sired females display lower cocaine place preference (Fischer et al., 2017). Sprague-Dawley cocaine-sired offspring exhibit delayed acquisition and motivation during cocaine self-administration (Le et al., 2017; Vassoler et al., 2013) but unaltered nicotine self-

administration (Wimmer et al., 2018). It is possible that the intergenerational effects of paternal cocaine exposure on reward measures are cocaine-specific. While a recent study shows that cocaine-sired grand offspring (F2 generation) exhibit normal cocaine self-administration (Wimmer et al., 2018), another finds that sires that both self-administer high amounts of cocaine and display greater levels of cocaine motivation have male offspring and grand offspring that exhibit addiction-like behaviors (Le et al., 2017). Thus, high cocaine intake alone, but not a high motivation + high intake combination, confers a protective effect against the rewarding and reinforcing effects of cocaine in offspring.

Molecular and physiological findings

Cocaine-sired offspring show altered neurotransmitter levels and expression of genes related to amino acid degradation and the stress axis. Sprague-Dawley cocaine-sired male offspring show lower levels of hippocampal D-serine glutamine, glutamate, *D-amino oxidase 1* mRNA, and *Corticotropin releasing hormone receptor 2* mRNA and protein levels (White et al., 2015; Wimmer et al., 2017). In the medial prefrontal cortex, cocaine-sired males have greater *Bdnf* exon IV mRNA and protein levels (Le et al., 2017; Vassoler et al., 2013). Increased levels of BDNF protein in the mPFC correlate with cocaine intake in sires and not cocaine motivation (Le et al., 2017). In summary, paternal cocaine treatments alter gene expression and protein levels in the hippocampus and medial prefrontal cortex in male, but not female, offspring.

Epigenetic findings

Paternal cocaine exposure induces several histone modifications in the brains of offspring. Sprague-Dawley cocaine-sired males show greater global histone 3 acetylation downstream of *D-amino oxidase 1*, H3k4me1 (histone 3 lysine 4 methylation), H3K9ac (histone 3 lysine 9 acetylation), H3K18me1, H3K23me1, H3K27me1, and H4K16ac in the hippocampus (Wimmer et al., 2017). These epigenetic changes associate with deficits in a hippocampal memory task and synaptic plasticity. Interestingly, these deficits were reversed by hippocampal administration of the NMDA receptor co-agonist D-serine; however, it is unclear whether D-serine reversed the epigenetic marks on the histone proteins. In the medial prefrontal cortex, Sprague-Dawley cocaine-sired males also display greater histone 3 acetylation and *Bdnf* exon IV associations, in addition to lower cocaine self-administration (Vassoler et al., 2013). Importantly, these functional and epigenetic changes are not a result of altered maternal behavior (Vassoler et al., 2013). The blunted cocaine sensitization in Sprague-Dawley cocaine-sired male offspring was accompanied by lower abundance of H3K4me2, H3K20me2, H3K27me2, and H3K18ac and increased abundance of H3K14ac in the nucleus accumbens (Wimmer et al., 2018). Interestingly, differential methylation in sperm exists between sires that show high cocaine motivation + high cocaine intake versus high cocaine intake alone (Le et al., 2017). Hundreds (~475) of differentially methylated CpG sites were maintained in F1 offspring, primarily at transcription start sites ($\pm$ 2,000 base pairs) and intergenic regions. Specifically, this resulted in greater methylation of *BTG family member 2* (*Btg2)* and *Nuclear receptor subfamily*

4 group A member 1 (*Nr4a1) promoters in sperm of high cocaine motivated sires and their offspring. Both genes have been implicated in neurogenesis and other brain functions (Calegari et al., 2005; Chen et al., 2014). A similar epigenetic profile was found in the nucleus accumbens of cocaine sires and offspring.* Overall, paternal cocaine exposure induces histone and DNA methylation changes that alter expression of glutamate-related, stress, neurogenesis, and neurotrophic factor genes. These epigenetic changes are accompanied by hippocampal memory deficits at baseline and lower sensitivity to the reinforcing effects of cocaine, primarily in male offspring. However, differential behavioral and DNA methylation patterns emerge when cocaine motivation in sires is considered.

Opioids

Developmental findings

Adolescent and adult paternal morphine exposure impairs offspring development in several domains. Paternal morphine treatment in Sprague-Dawley rats decreased litter size and increased offspring mortality (Cicero et al., 1991; Cicero et al., 1995). The findings on birth weight are mixed, with some studies showing greater (Wistar rats), lower (CD1 mice), and no change (Sprague-Dawley rats) in birth or adult weight (Friedler, 1985; Li et al., 2014; Vyssotski, 2011). Some developmental abnormalities are passed on over 4 generations in CD1 mice (Friedler, 1985). Paternal methadone treatment in Fischer rats does not alter litter weights or body weights prior to weaning; however, methadone-sired offspring exhibit lower body weights in adulthood (Joffe et al., 1990). Thus, there is emerging

evidence that paternal opioid exposure can have a long-lasting impact on developmental trajectories over multiple generations.

Learning and locomotor activity findings

Few studies have examined opioid-sired offspring for changes in learning and activity domains. Locomotor activity and spatial memory are unaltered in Sprague-Dawley morphine-sired offspring (Li et al., 2014); however, CD1 morphine-sired offspring show impairments in learning in active avoidance and spatial memory (Friedler, 1985). Interestingly, paternal methadone treatment in F344 rats results in changes in learning and activity (Joffe et al., 1990). Both sexes show decreased open field activity. Both sexes also showed enhanced learning in inhibitory avoidance procedures. Male offspring exhibit enhanced learning during active avoidance, while females display impaired learning. Male, but not female, offspring also have impaired motor coordination (Joffe et al., 1990). Although few studies have examined paternal opioid treatment-induced changes in learning and activity in offspring, methadone-sired offspring show greater variations in these domains, which at times occur in a sex-dependent manner.

Affective findings

The literature on paternal morphine effects on anxiety- and depression-like behavior is mixed. Sprague-Dawley morphine-sired offspring display an anxiogenic phenotype (Li et al., 2014), while anxiety-like behaviors in Wistar morphine-sired offspring are unchanged (Pooriamehr et al., 2017). Pooriamehr et al. (Pooriamehr et al., 2017) also found that depression-like behavior on sucrose preference tests are unchanged in Sprague-Dawley morphine-sired offspring. Further work using a wider

range of behavioral tests that model anxiety- and depression-like behaviors is needed.

Reward-related findings

Paternal morphine exposure alters sensitivity to morphine in adult offspring. Wistar morphine-sired offspring show increased sensitivity to the analgesic effects of morphine (Vyssotski, 2011). Sprague-Dawley morphine-sired male offspring, but not females, also exhibit increased sensitivity to morphine-induced analgesia (Cicero et al., 1995). Interestingly, paternal morphine exposure in Wistar rats results in increased morphine dependence (Vyssotski, 2011), but voluntary morphine consumption is unchanged (Pooriamehr et al., 2017). The timing of paternal treatment may influence these divergent findings, with sire treatment beginning in adolescence inducing morphine dependence in offspring. However, altered sensitivity to morphine-induced analgesia is seen in adolescent and adult paternally treated offspring. Overall, morphine-sired offspring show greater sensitivity to the analgesic effects of morphine with timing of paternal exposure determining responses to morphine reward.

Molecular and physiological findings

Paternal opioid treatment results in several physiological and molecular abnormalities. Sprague-Dawley morphine-sired male offspring have greater adrenal weights and lower luteinizing hormone and testosterone levels (Cicero et al., 1991). Paternal methadone treatment results in greater adrenal weights in adult females, and lower thymus weights in both sexes (Joffe et al., 1990). The findings on basal pain thresholds are strain-specific in males; Wistar rat offspring show greater pain

thresholds (Vyssotski, 2011) while these measures are unaltered in Sprague-Dawley offspring (Cicero et al., 1995). Greater hypothalamic beta endorphin and corticosterone levels are found in female offspring (Cicero et al., 1991). In both sexes, there is decreased hippocampal dendritic length and branching, as well as decreased *Insulin-growth factor 2* mRNA and protein levels (Li et al., 2014). Induction of long-term potentiation is also impaired in both sexes (Sarkaki et al., 2008). Interestingly, grand offspring (F2) display lower synaptophysin levels, but levels of this enzyme are unchanged in their parents (F1) (Vyssotski, 2011). Taken together, there is robust evidence that paternal opioid exposure results in changes in organ weights, synaptic activity, and several hormone levels related to growth-regulation and neurotransmitter function.

Epigenetic findings

No studies found.

Nicotine

Developmental findings

Few studies have explored the effects of paternal nicotine use on the health of subsequent generations. In C57BL/6J mice, litter size and sex ratios are unchanged in litters sired by adolescent nicotine-exposed males. Importantly, nicotine-sires were prevented from mating with a nicotine-naïve female for one week after the 5-week exposure period, well beyond the half-life of nicotine and its metabolite cotinine (Vallaster et al., 2017).

Learning and locomotor activity findings

Nicotine exposure results in hypoactivity in sires but differential changes on locomotor and learning behaviors in offspring. Specifically, C57BL/6J nicotine-sired offspring display greater locomotor activity, while recognition memory is unaltered (Dai et al., 2017).

Affective findings

Nicotine exposure induces depression-like behavior in sires but promotes resilience in offspring. For example, C57BL/6J nicotine-sired offspring show lower depression-like behaviors on the forced swim test (Dai et al., 2017), but anxiety-like behavior on the elevated plus maze is unchanged (Dai et al., 2017; Vallaster et al., 2017).

Reward-related findings

Nicotine self-administration behaviors are unaltered in nicotine-sired offspring; however, male offspring show increased survival after toxic doses of nicotine and cocaine (Vallaster et al., 2017). Thus, paternal nicotine exposure increases resilience to toxic nicotine and cocaine doses in male offspring. These findings may indicate that, in contrast to paternal cocaine studies which find cocaine-specific intergenerational effects, paternal nicotine exposure does not induce nicotine-specific reward responses in offspring.

Molecular and physiological findings

Paternal nicotine exposure alters nicotine and cocaine metabolism and signaling pathway involved in neural development. Male C57BL/6J nicotine-sired

offspring had greater expression of genes involved with hepatic metabolism and nicotine clearance (Vallaster et al., 2017), as well as thalamic *Wnt family member 4* mRNA levels (Dai et al., 2017). The *Wnt4* signaling pathway is an important regulator of neurogenesis and is associated with the pathophysiology of several neuropsychiatric disorders, including bipolar disorder and major depressive disorder (Inkster et al., 2009; Matigian et al., 2007).

Epigenetic findings

Paternal nicotine exposure alters miRNA targeting the *Wnt4* signaling pathway in offspring. Nicotine exposed sires have greater DNA methylation of mm-miR-15b in their sperm; hypermethylation of mmu-miR-15b was also maintained in the thalamus of offspring (Dai et al., 2017). Changes in mmu-miR-15b methylation levels associate with greater locomotor activity, lower depression-like behavior, and thalamic *Wnt family member 4* mRNA levels in offspring. Interestingly, viral-mediated overexpression of mmu-miR-15b induced hypoactivity and depression-like behavior in nicotine-sired offspring. Although a causal link has been demonstrated between paternal-nicotine exposure and mmu-miR-15b and the *Wnt family member 4* signaling pathway, it would be useful to investigate whether this link mediates responses to nicotine reward in offspring.

Cannabinoids

Developmental findings

Few studies have examined the role of paternal cannabinoid exposure on developmental outcomes. Offspring of adult THC-exposed sires did not differ from

control-sired offspring on litter size, sex ratio, or body weights when measured at birth and weaning. (Levin et al., 2019).

Learning and locomotor activity findings

Adolescent paternal exposure to the synthetic cannabinoid receptor agonist WIN55,212-2 (WIN) did not alter locomotor activity in adult SD offspring at baseline or after unpredictable stress (Ibn Lahmar Andaloussi et al., 2019). Adult THC-sired offspring showed more rapid habituation of locomotor activity relative to control-sired offspring; this effect was not seen in adolescent offspring (Levin et al., 2019). Additionally, episodic memory was unchanged in WIN-sired offspring relative to control offspring as measured by the object recognition test (Ibn Lahmar Andaloussi et al., 2019). No effect of adolescent paternal THC exposure was seen on the novel object recognition test of non-spatial memory (Levin et al., 2019). Although, THC-sired offspring do not show deficits on the 16-arm radial maze test of spatial memory, these offspring have impairments in sustained attention relative to control-sired offspring (Levin et al., 2019).

Affective findings

Paternal WIN exposure in adolescence alters stress-induced anxiety-like behaviors in offspring. Adolescent THC exposure in males did not alter anxiety-like behavior in offspring on the elevated plus maze (Levin et al., 2019). While WIN- and control-sired offspring do not differ on open field anxiety measures, WIN-sired offspring show greater unpredictable stress-induced anxiety-like behaviors relative to control-sired offspring (Ibn Lahmar Andaloussi et al., 2019). THC-sired offspring do

not differ from control-sired offspring in fear response as measured by the novelty suppressed feeding task (Levin et al., 2019).

Reward-related findings

No studies found.

Molecular and physiological findings

Offspring of WIN-exposed sires show differential changes on DNMT's and stress hormones prior to and after stress exposure (Ibn Lahmar Andaloussi et al., 2019). Corticosterone levels do not differ between WIN- and control-sired offspring at baseline or after chronic unpredictable stress. Prefrontal DNMT1 mRNA levels are greater in WIN-sired offspring at baseline; however, no differences are seen between groups after stress exposure. Conversely, prefrontal DNMT3a mRNA levels do not differ between the WIN- and control-sired offspring at baseline, but after stress exposure, WIN-sired offspring have higher DNMT3a mRNA levels (Ibn Lahmar Andaloussi et al., 2019).

Epigenetic findings

Stress exposure in WIN-sired offspring enhances global DNA methylation levels in the prefrontal cortex (Ibn Lahmar Andaloussi et al., 2019). Global DNA methylation levels do not differ between WIN- and control-sired offspring at baseline. However, when exposed to stress, WIN-sired offspring have greater 5-mc percentages compared to stressed control-sired offspring. Global DNA methylation levels correlate differentially with DNMT1 and DNMT3a mRNA levels in the prefrontal cortex. Specifically, global DNA methylation levels positively correlate with

DNMT1 mRNA levels, but no relationship is evident between global DNA methylation and DNMT3a mRNA levels (Ibn Lahmar Andaloussi et al., 2019).

Summary of the literature on paternal drug effects

Paternal drug exposures induce a wide range of developmental, emotional, physiological, and epigenetic consequences. Over the last few decades, paternal alcohol exposure has received more attention relative to other substances. However, given the increased rates of dependency on prescription opioid drugs, the popularity of electronic cigarettes, and decriminalization of marijuana in several U.S states, further investigation of other drug classes is warranted. Furthermore, studies vary widely in methodology across and within drug classes. Age, dose, duration and route of administration of paternal drug exposure are all important factors which may contribute to a lack of consilience between investigations. In many instances, paternal drug effects occur in a sex-dependent manner in offspring, findings that likely indicate complex interactions between sire-induced epigenetic modifications and the organizational and activational effects of gonadal hormones. In a similar vein, behavioral effects might also occur selectively in male offspring due to undetected paternal drug-induced epigenetic modifications on the Y chromosome that can modify other genes via epistasis (Kutch and Fedorka, 2018).

There is also a bourgeoning literature on paternal drug-induced effects on drug reward in offspring. There is preclinical evidence of a protective effect of paternal drug exposure on drug consumption in offspring that conflicts with studies in humans that demonstrate familial transmission of SUDs (Bierut et al., 1998; Heath et al., 1997; Kendler et al., 1999; Kendler et al., 2000; Li et al., 2003). Conversely,

many of the findings discussed above are in line with a recent longitudinal investigation that focused on paternal drug use on risk of alcohol use disorder in offspring. Maternal, but not, paternal SUD increased the risk of alcohol use disorder in offspring. Although, paternal SUD is not shown to be protective against alcohol use disorder, it does not enhance risk (Yule et al., 2018). Looking forward, it would be beneficial if preclinical and human work parallel each other when investigating the role of paternal drug consumption. For instance, the amount of drug consumed is not a criterion for a SUD; thus, there is heterogeneity in drug intake within and across drug classes. Whenever possible, it is important to measure clinical features of SUDs in sires, such as drug motivation. Rat studies showed that males with high motivation for cocaine had offspring that self-administer greater amounts of cocaine (Le et al., 2017). Thus, paternal motivation for a drug, coupled with high drug intake, may predispose offspring to develop addiction-like behaviors.

The role of maternal behaviors has also received little attention. This is unfortunate given that many paternal treatments reviewed above continued into the mating period. Furthermore, paternal environment can alter maternal behavior. For example, paternal housing conditions can alter a dam's licking and grooming behaviors toward their offspring (Mashoodh et al., 2012), supporting findings that females adjust maternal care depending on paternal quality across several species (Cunningham and Russell, 2000; Gilbert et al., 2006; Sheldon, 2000). Additionally, some paternal effects disappear after in vitro fertilization (Dietz et al., 2011) and embryo transfer likely because these effects are buffered by maternal behaviors (Mashoodh et al., 2018). These findings highlight complex maternal-paternal

interactions that may contribute to offspring phenotype. Notably, some paternal drug studies show that maternal behavior is unaltered (Fischer et al., 2017; Li et al., 2014; Vassoler et al., 2013) or use a cross-fostering protocol (Dai et al., 2017).

Paternal-drug induced epigenetic modifications in offspring are an understudied area. Future studies can focus on how epigenetic modifications may be facilitating the biological and behavioral changes observed because of paternal drug exposure. Given that some short non-coding RNA's may mediate DNA methylation processes (i.e. piRNA's) (Kuramochi-Miyagawa et al., 2008; Spadaro and Bredy, 2012), it would be beneficial to elucidate their role in the transgenerational effects of paternal drug use. Additionally, no studies examined interactions between genetic and epigenetic marks. DNA methylation commonly takes place in an allele-specific manner across the genome (Meaburn et al., 2010). Stress-induced epigenetic modifications can also occur in an allele-specific manner (Alexander et al., 2014; Duman and Canli, 2015; Klengel et al., 2013). Thus, it is likely that the intergenerational consequences of paternal drug use rely on complex interactions between genetic and epigenetic marks. For example, an allele that inactivates alcohol dehydrogenase 2 (*ALDH2*) reduces risk for developing alcohol use disorder (Reilly et al., 2017). Such variants may interact with epigenetic processes to moderate predisposition to certain alcohol drinking phenotypes. In summary, paternal drug exposure, even during periods prior to conception, can have a long-lasting impact on future generations. Further work in this area will identify novel mechanisms that underlie the paternal contribution to addiction; such findings

may lead to the development of more effective prevention and treatment strategies for substance use disorders.

Hypothesis and Specific Aims

Human and preclinical evidence suggest that epigenetic factors can be inherited via the male germ line and may explain some of the missing heritability of AUD. DNA methylation is one well studied epigenetic mechanism that is altered in sperm of alcohol drinking men. In addition, preclinical evidence suggests that these changes in DNA methylation can be passed down to subsequent offspring. However, research on behavioral sensitivity to alcohol in offspring of alcohol-exposed males remains in its infancy. Studies in mice show that paternal alcohol exposure decreases alcohol drinking and behavioral sensitivity to alcohol in a sex-dependent manner. These behavioral phenotypes are accompanied by altered DNA methylation levels in the sperm of alcohol-exposed sires that were maintained in the brains of offspring. To date, no studies have explored the influence of paternal alcohol exposure on sensitivity to unconditioned effects of alcohol or operant alcohol self-administration in rat offspring. Overall, additional research is needed exploring the epigenetic biomarkers by which paternal alcohol exposure influences addiction-like behavior in offspring. In order to address these questions and move the field forward, I developed the following specific aims:

Aim 1: Determine if paternal alcohol exposure alters behavioral sensitivity to the unconditioned effects of alcohol in offspring.

I hypothesize that alcohol-sired (A-sired) male, but not female, offspring will have blunted sensitivity to alcohol-induced anxiolysis and motor impairments compared to control-sired (C-sired) offspring.

Aim 2: **Determine whether paternal alcohol exposure alters operant alcohol self-administration in offspring.**

I postulate that A-sired male offspring, but not female offspring, will display an alcohol-resistant phenotype as indicated by slower acquisition of alcohol responding, lower motivation for alcohol, and less craving- and relapse-like behaviors compared to C-sired male offspring,

Aim 3: Determine whether paternal alcohol exposure alters *Bdnf* DNA methylation in offspring.

I hypothesize that alcohol exposure will decrease global and *Bdnf* DNA methylation in sperm, medial prefrontal cortex, and nucleus accumbens of sires. Additionally, these global and gene-specific changes in DNA methylation will be maintained in sperm and brains of offspring.

CHAPTER TWO- DETERMINE WHETHER PATERNAL ALCOHOL EXPOSURE ALTERS BEHAVIORAL SENSITIVITY TO THE UNCONDITIONED EFFECTS OF ALCOHOL IN OFFSPRING

Introduction

Level of response (LR) to alcohol is a well-established endophenotype of alcohol use disorder (AUD). LR refers to the extent to which an individual responds to a given dose of alcohol or the number of drinks a person needs to experience alcohol's psychological and physiological effects. Alcohol administration studies in humans show that LR can predict AUD risk, especially in individuals with a family history of alcohol problems (King et al., 2014; Schuckit and Smith, 1996). Schuckit and colleagues have pioneered work in this area showing that low subjective responses to the sedative and unpleasant effects of alcohol are risk factors for future alcohol dependence (Schuckit, 1984, 1994; Schuckit and Smith, 1996). This is termed the low level of response model and this theory examines response to alcohol as a unidimensional construct with a focus on the sedative and unpleasant effects of subjective responses (Schuckit and Gold, 1988).

Over the last few decades, updates to the low level of response model reflect a multidimensional construct wherein alcohol's biphasic effects are considered (Newlin and Thomson, 1990). Sons of individuals with AUD may be more sensitive to the rewarding and psychomotor stimulating effects of alcohol during the ascending blood alcohol concentration limb and less sensitive to sedative/unpleasant effects when blood alcohol concentration decline (Newlin and Thomson, 1990). For example, heavy drinkers report lower sensitivity to the sedative

effects of alcohol across the duration of BAC, which in turn associates with a higher number of AUD symptoms at 2- and 6-year follow-ups (King et al., 2014).

Preclinical work also supports relationship between LR and alcohol drinking. Much of the work in this area uses rat strains that are selectively bred to prefer and consume pharmacologically relevant amounts of alcohol. These studies find that alcohol preferring rats have lower LR compared to non-alcohol preferring rats. For example, alcohol preferring rats show less alcohol place aversion compared to non-preferring rats (Stewart et al., 1996). Alcohol-preferring rats also show less taste aversion to alcohol compared to non-preferring rats (Froehlich et al., 1988). Taken together, these studies demonstrate that rats with a genetic predisposition for consuming large amounts of alcohol are less sensitive to alcohol's aversive effects.

Family history of alcohol dependence can moderate the association between LR and risk of AUD. Newlin and Thompson (1990) originally proposed that sons of alcoholics may be more sensitive to the rewarding and stimulating effects of alcohol as blood alcohol concentrations rise and less sensitive to the negative effects of alcohol as blood alcohol concentration rise. Furthermore, a growing body of preclinical research finds that paternal alcohol exposure alters a wide range of developmental and physiological functions in subsequent generations, including behavioral sensitivity to alcohol (Finegersh et al., 2015b; Nieto and Kosten, 2019; Rompala and Homanics, 2019). Specifically, a study in mice finds that paternal alcohol exposure prior to conception increases sensitivity to alcohol's anxiolytic and locomotor enhancing effects while blunting alcohol-induced motor coordination impairments selectively in male offspring (Finegersh and Homanics, 2014).

Additionally, in contrast to the LR model in humans, these behaviors in male offspring are accompanied by lower alcohol consumption and preference that vary as a function of alcohol concentration (Finegersh and Homanics, 2014). However, to date, no studies have examined for these paternal alcohol-induced behaviors in a genetically heterogeneous rat strain.

The purpose of this study was to determine whether paternal alcohol exposure prior to conception reduced behavioral sensitivity to the unconditioned effects of alcohol. We hypothesize that paternal alcohol exposure will enhance the anxiolytic and locomotor enhancing effects of alcohol while blunting alcohol-induced impairments in motor coordination in male, but not female, offspring.

Methods

Animals

Male and female Wistar rats were purchased from Charles River and used to generate offspring used in this dissertation project. Sires (400-500 g) were pair-housed prior to mating and sacrificed shortly after dams were confirmed pregnant. Dams were group-housed prior to mating and then sacrificed after offspring were weaned. Offspring were group-housed (females) or pair-housed (males) after weaning and throughout the course of the study. Most animals were housed in amber polysulfone cages and kept in a temperature-and humidity-controlled vivarium. During chronic intermittent ethanol vapor exposure, males were placed in standard rat cages housed within vapor chambers. The vivarium was maintained on a 12:12 light/dark cycle (lights on at 7:00 AM). Animals had ad libitum access to food and water. The Institutional Animal Care and Use Committee at the University of Houston approved the experimental protocols in accordance with guidelines set forth

in the “Guide for the Care and Use of Laboratory Animals 8th Edition”. Adult (postnatal day 75) alcohol and control-sired animals (A-sired and C-sired, respectively) were tested for behavioral sensitivity to alcohol as described below.

Solution and drug preparations

Alcohol (ethyl alcohol, 190 proof, USP grade, Koptec, King of Prussia, PA) was mixed with tap water to reach concentrations of 15% (v/v) alcohol.

Paternal chronic intermittent ethanol exposure

Male rats were made dependent by chronic, intermittent exposure to vapor alcohol as previously described in (Gilpin et al., 2008). This model reliably induces alcohol dependence as indicated by the development of negative emotional-like state and somatic symptoms in withdrawal (Gilpin et al., 2008). Standard rat cages were housed inside sealed and transparent plastic chambers into which vapor alcohol was intermittently pumped. Males underwent cycles of 16 h (6pm) on and 8 h (10am) off for five consecutive days per week over six weeks. Nondependent rats were housed in similar conditions but were only exposed to room air. Blood samples were collected from the lateral saphenous vein to monitor blood alcohol levels and to adjust vapor exposure settings.

Breeding and offspring rearing

Male rats were left undisturbed for 8 weeks after their last alcohol vapor session. At the end of this period, males were housed with alcohol naïve females. Female rats were examined daily for the presence of a mating plug. Once the mating plug was confirmed by research staff, males were removed from the breeding cages and sperm and brain regions were extracted the following day. Litters from

these mating pairs were culled to 10 pups (5 pups per sex) as depicted in Figure 2.1. Pups were weighed at postnatal day (PD) 1, 4, 7, 10, 35, and then weekly into adulthood. Behavioral testing began when offspring reached adulthood ~PD 75. To control for possible litter effects, no more than 2 pups per sex per litter were used for each aim. Experimental groups and testing sequence for each aim is shown in Table 2.1.

FIGURE 2.1. BREEDING SCHEME FOR STUDY AIMS

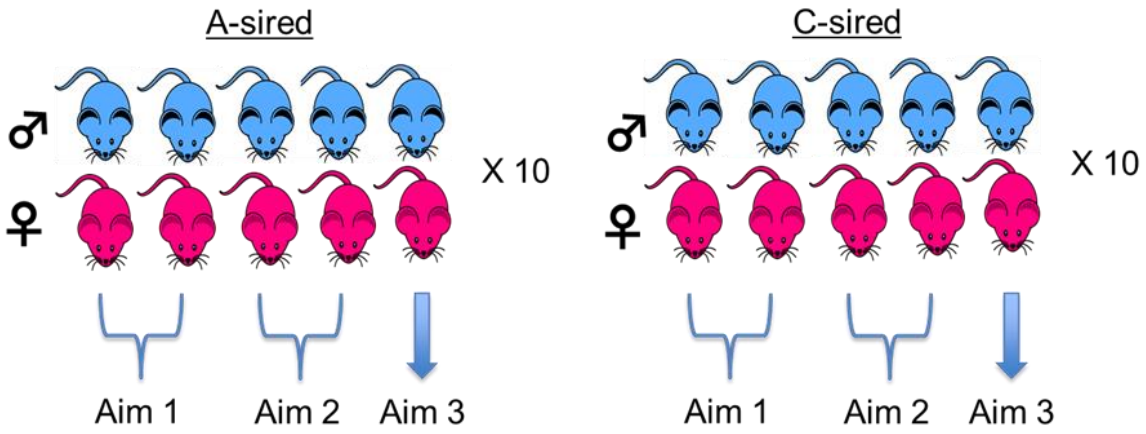

Figure 2.1. Schematic of the breeding procedure for alcohol-sired (A-sired) and control-sired offspring. No more than two offspring were used for each study aim and rats were not used for more than one study aim. All animals were left undisturbed until behavioral testing or tissue collection in adulthood (i.e., postnatal day [PD] 75).

TABLE 2.1. EXPERIMENTAL GROUPS AND TESTING SEQUENCE FOR EACH AIM

Aim	Sire treatment	N	Sex	Offspring treatment	Procedure 1	Procedure 2	Procedure 3	Procedure 4	Procedure 5
1	Alcohol	10	Female	Water	Open field	EPM	Rotarod	-	-
		9	Female	Alcohol (1.5 g/kg)	Open field	EPM	Rotarod	-	-
		10	Male	Water	Open field	EPM	Rotarod	-	-
		10	Male	Alcohol (1.5 g/kg)	Open field	EPM	Rotarod	-	-
	Control	10	Female	Water	Open field	EPM	Rotarod	-	-
		10	Female	Alcohol (1.5 g/kg)	Open field	EPM	Rotarod	-	-
		10	Male	Water	Open field	EPM	Rotarod	-	-
		10	Male	Alcohol (1.5 g/kg)	Open field	EPM	Rotarod	-	-
2	Alcohol	10	Female	Alcohol SA	Acquisition	PR tests	Extinction	Reinstatement	Reinitiation
		9	Male	Alcohol SA	Acquisition	PR tests	Extinction	Reinstatement	Reinitiation
	Control	8	Female	Alcohol SA	Acquisition	PR tests	Extinction	Reinstatement	Reinitiation
		10	Male	Alcohol SA	Acquisition	PR tests	Extinction	Reinstatement	Reinitiation
3	Alcohol	10	Female	-	*Bdnf* DNA methylation	Global DNA methylation	-	-	-
		10	Male	-	*Bdnf* DNA methylation	Global DNA methylation	-	-	-
	Control	9	Female	-	*Bdnf* DNA methylation	Global DNA methylation	-	-	-
		10	Male	-	*Bdnf* DNA methylation	Global DNA methylation	-	-	-

EPM, Elevated plus maze; SA, Operant self-administration; PR, Progressive ratio; *Bdnf*, Brain-derived neurotrophic factor

Behavioral testing

Separate groups of A-sired (n's = 9-10 per group) and C-sired (n's = 10 per group) offspring were administered alcohol (1.5 g/kg) or water via oral gavage 30 min prior to behavioral testing on the open field, elevated plus maze, and rotarod. Rats were acclimated to the gavage for one week prior to testing. The order of the tests was not counterbalanced but ordered beginning with less invasive tasks. All tests were separated by one week to ensure that alcohol was washed out of the animal's system.

Open field test

Anxiety-like behavior and general locomotor activity was assessed over 60-min in an open field apparatus that consisted of a square box with white floors and clear plexiglass walls (Med Associates Inc; 43-cm L X 43-cm W X 11-cm H) wherein rats can freely explore. Movement was tracked using infrared beam sensors over a 60-minute trial. Rats were habituated to the testing room for 30 minutes prior to testing. Anxiety-like behavior was automatically indexed by the percentage of time and entries into the center of the open field using. Locomotor activity was automatically calculated by the distance traveled (cm) within the open field. Behavioral indices were captured in six 10-min time bins.

Elevated plus maze

Anxiety-like behavior was assessed one week after the open field test. The elevated plus maze was shaped like a plus symbol and consisted of two open (45-cm long ×10-cm wide) and two closed arms (45-cm L X 10-cm W X 30-cm H), and a middle compartment (4-cm long X 4-cm wide). The floors of the maze and the walls

of the closed arms were made of black acrylic. Rats were habituated to the testing room for 30 min prior to testing. At the start of the 5 min trial, offspring were placed in the middle compartment facing one of the open arms. The maze was cleaned with disinfectant after each trial. A digital video camera mounted on the ceiling recorded the movements of each rat. Data were analyzed using an automated software program (ANY-maze; Stoellting Co.; Wood Dale, IL) connected to a computer. Times and entries in the open and closed arms were the primary measures that were tabulated.

Rotarod

Motor coordination was assessed using the accelerating rotarod test. In this test, rats were placed on an alley of a rotarod (San Diego Instruments; 66” H X 36” W X 24” D) that gradually accelerated from 4 to 50 rpm over a 5-min period. Latency to fall off was recorded and the trial ends at that time or after 6 min. Rats acclimated to the testing room 30 min prior to training and tests. One week after performance on the elevated plus maze, offspring were habituated to the rotarod over five training trials (no alcohol administration) separated by 10 minutes. The following day, a single test was conducted 30 min after water or alcohol administration.

Statistical Analysis

The average number of pups born per litter were compared using Student *t*-tests. Body weights were analyzed using a three-way mixed design analysis of variance with Sex and Sire as the between group factors and Time as the repeated measure factor. A between group analysis of variance was used for elevated plus maze data with paternal exposure, sex, and treatment (alcohol vs water) as between

group factors. A mixed design analysis of variance was used with open field and rotarod data with time/trial added as a repeated measure. Tukey *post hoc* tests were used to follow-up on statistically significant main effects and interactions. All statistical analyses were performed using SAS software 9.4 (SAS Institute) with statistical significance set at p<.05.

Results

Litter characteristics and offspring body weights

A-sired and C-sired groups did not differ on number of litters, average number of pups per litter, or number of male and female offspring (p's>.05)(Table 2.2). Body weights of A-sired and C-sired offspring are shown in Table 2.3. There were significant main effects of Sire, F(1, 92)= 49.632, p<0.001, $\eta_p^2 = .35$, Sex, F(1, 92)= 739.87, p<0.001, $\eta_p^2 = .89$, and Time, F(5, 460)= 6725.0, p<0.001, $\eta_p^2 = .98$, along with significant interactions of Time X Sire, F(5, 460)= 14.994, p<0.001, $\eta_p^2 = .14$ and Time X Sex, F(5, 460)= 527.49, p<0.001, $\eta_p^2 = .85$. Tukey *post hoc* comparisons showed that A-sired offspring (male and female) weighed less than C-sired offspring specifically at PD35 and PD75 (p's<0.001). Tukey *post hoc* comparisons also showed that male offspring weighed more than females specifically at PD35 and PD75 (p's <0.001).

TABLE 2.2. LITTER CHARACTERISTICS FOR ALCOHOL AND CONTROL SIRES

	Control sires	Alcohol sires
Number of litters	10	10
Average pups/litter (standard error)	12.4 (1.3)	10.5 (1.8)
Number of male offspring	52	46
Number of female offspring	43	49

TABLE 2.3. BODY WEIGHTS OF OFFSPRING

Sex	Group	Body Weights (Mean ± SEM)					
		PD 1	**PD 4**	**PD 7**	**PD 10**	**PD 35 ψ**	**PD 75 ψ**
Male	C-sired	8.0 ± .22	12.7 ± .08	16.9 ± .46	26.2 ± .90	147.5 ± 3.3	425.4 ± 5.1
	A-sired	6.7 ± .11	10.5 ± .16	17.3 ± .39	23.9 ± .39	126.9 ± 7.1***	387.3 ± 8.5***
Female	C-sired	8.2 ± .31	12.1 ± .17	16.5 ± .31	23.8 ± .55	125.0 ± 2.1	241.5 ± 2.7
	A-sired	6.8 ± .13	10.2 ± .15	16.7 ± .30	23.2 ± .37	108.4 ± 5.0***	224.6 ± 4.1***

Note. C-sired, control-sired: A-sired, alcohol-sired; PD, postnatal day; $\boldsymbol{\psi}$ indicates sex differences at p<0.05; *** indicates sire differences at p<0.001

Open Field

Paternal alcohol-induced effects on general locomotor activity and anxiety-like behavior on the open field test are seen in Fig. 2.2. On general locomotor activity (Fig 2.2 A & D), there were significant main effects of Sex, F (1, 71) = 10.77, p=0.0016, η_p^2 = 0.13, Time, F (5, 355) = 184.76, p <0.001, η_p^2 = 0.72, Time X Sex interaction, F (5, 355) = 8.76, p <0.001, η_p^2 = 0.11 and a Time X Sex X Treatment X Sire interaction, F (5, 355) = 2.32, p=0.042, η_p^2 = 0.03. Generally, female offspring traveled more than males throughout the 60 min period and locomotor activity decreased as time progressed. Tukey *post hoc* tests showed that locomotor activity was higher in females particularly during the first time bin (p<0.001) but did not reveal differences between A- and C-sired offspring after water or alcohol treatment (p's>0.05).

Anxiety-like behavior on the open field test is shown in Figure 2.2 Panels B-F. There were significant main effects of Sex, F (1, 71) = 11.11, p=0.001, η_p^2 = 0.14, Treatment F (1, 71) = 5.35, p=0.024, η_p^2 = 0.07, and a significant Sex X Treatment

interaction, F (1, 71) = 5.02, p=0.029, η_p^2 = 0.07. Overall, female offspring spent less time in the center of the open field relative to males. Additionally, alcohol treated rats spend more time in the center of the open field relative to water exposed rats. Tukey *post hoc* tests showed that males treated with alcohol spent more time in the center of the open field relative to water treated male (p<0.01) and female rats (p's < 0.01).

FIGURE 2.2. BEHAVIORAL SENSITIVITY TO ALCOHOL ON THE OPEN FIELD TEST

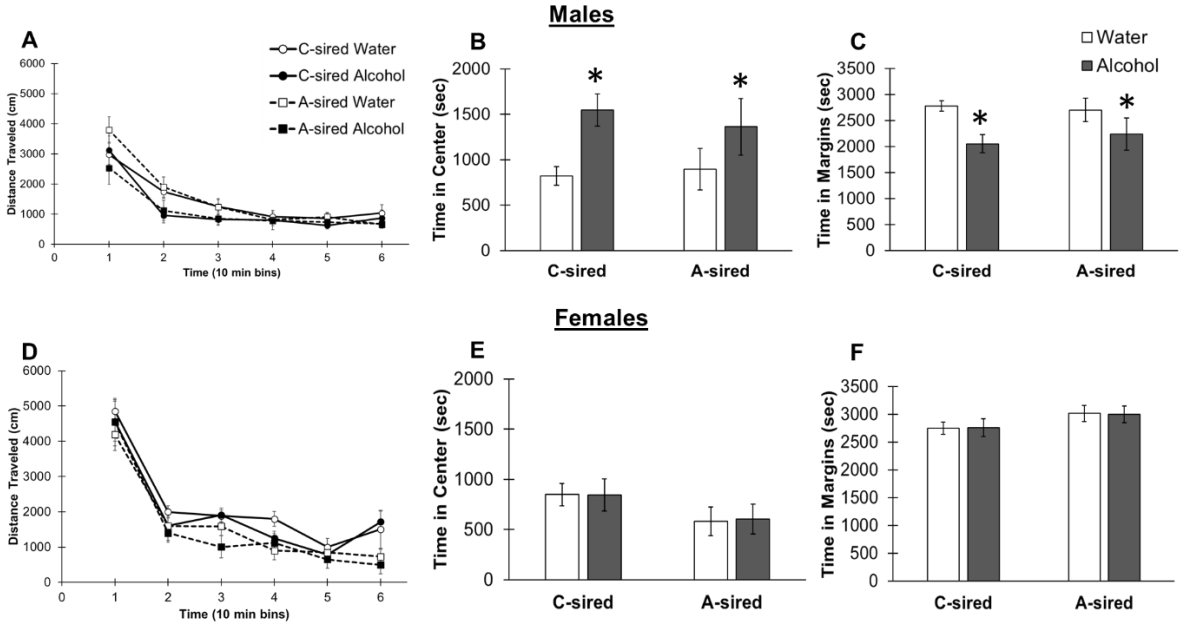

Figure 2.2. Distance traveled and time spent in the center/margin of the open field for control-sired (C-sired [n's = 10 per group]; open bars/circles) and alcohol-sired (A-sired [n's = 9-10 per group]; filled bars/circles) offspring. Distance traveled over six 10-min time bins is presented as mean (±SEM) for males (A) and females (D). Time spent in the center and margins of the open field are presented as mean (± SEM) for males (B & C) and females (E & F). An asterisk (*) represents a significant difference between water and alcohol treatment (p's<0.05).

The elevated plus maze

Anxiety-like behavior on the elevated plus maze is shown in Fig. 2.3. There was a significant main effect of Sire, F (1, 71) = 5.79, p=0.0187, η_p^2 = 0.08, as well as

a significant Sire X Sex interaction, F (1, 71) = 12.56, p<0.001, η_p^2 = 0.15 on percent time spent in open arms. A-sired offspring spent more time in the open arms, but this varied as a function of sex. Specifically, A-sired male offspring spent more time in the open arms (Panel A) compared to A-sired females (p<0.01) and C-sired offspring (p's < 0.001). For percent of open arm entries, there were significant main effects of Sire, F (1, 71) = 3.73, p<0.047, η_p^2 = 0.05, and Sex, F (1, 71) = 11.10, p=0.001, η_p^2 = 0.13, indicating that A-sired animals and male rats made more open arm entries (data not shown). There was a significant main effect of Sire, F (1, 71) = 3.90, p=0.042, η_p^2 = 0.05, and a significant Sire X Sex interaction, F (1, 71) = 9.06, p= 0.003, η_p^2 = 0.11 on percent time in the closed arms. A-sired offspring spent less time in the closed arm but varied as a function of sex. Specifically, A-sired male offspring spent less time in the closed arms (Panel B) relative to A-sired female offspring (p<0.05) and C-sired males (p<0.001). For percent of closed arm entries, there was were significant main effects of Sex, F (1, 71) = 12.19, p=0.0008, η_p^2 = 0.15, Treatment, F (1, 71) = 11.02, p=0.0014, η_p^2 = 0.13, and a Sire X Sex interaction, F (1, 71) = 7.54, p=0.0077, η_p^2 = 0.10, indicating that animals given alcohol and female rats made less entries into the closed arms. Additionally, Tukey post hoc tests revealed that A-sired female offspring made less entries into the closed arms compared to A-sired (p< 0.001) and C-sired male offspring (p<0.05; data not shown).

FIGURE 2.3. BEHAVIORAL SENSITIVITY TO ALCOHOL ON THE ELEVATED PLUS MAZE

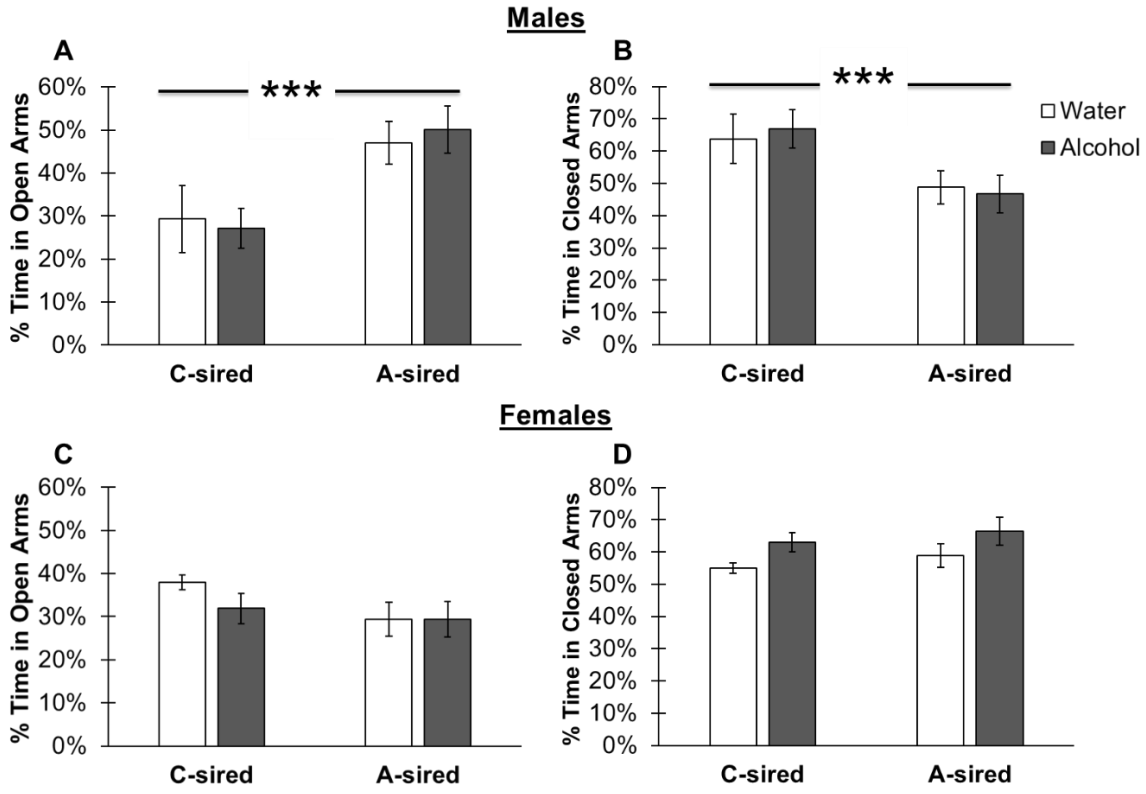

Figure 2.3. Time spent in the open and closed arms of the elevated plus maze for control-sired (C-sired [n's = 10 per group; open bars/circles) and alcohol-sired (A-sired [n's = 9-10 per group; filled bars/circles) offspring. The percent of time spent in the open and closed arms of the open field are presented as mean ($\pm$ SEM) for males (A & B) and females (C & D). *** represents a significant difference between control and alcohol-sired offspring (p's<0.001).

Rotarod

Performance on the rotarod during five training trials and on test day are shown in Fig. 2.3. For training trials, there was a significant main effect of Trial, F (4, 300) = 12.68, p = < 0.001, η_p^2 = 0.14, and a significant Trial X Sex interaction, F (4, 300) = 7.13, p <0.001, η_p^2 = 0.09. Generally, all animals improved their performance on the rotarod as trials progressed. However, female rats (Panel C) had better

rotarod performance on Trial 3 relative to males (Panel A; p<0.01). For the rotarod test, there were significant interactions, Sire X Sex, F (1, 71) = 12.45, p<0.001, η_p^2 = 0.72, Sex X Treatment, F (1, 71) = 4.63, p=0.035, η_p^2 = 0.06, Sire X Sex X Treatment, F (1, 71) = 5.66, p=0.020, η_p^2 = 0.07. A-sired males (Panel B) were less sensitive to the motor impairing effects of alcohol relative to alcohol-exposed C-sired males (p<0.05). However, A-sired females (Panel D) were more sensitive to the motor impairing effects of alcohol relative to alcohol-exposed A-sired males (p<0.05) and C-sired females (p<0.01).

FIGURE 2.4. BEHAVIORAL SENSITIVITY TO ALCOHOL ON THE ROTAROD TEST

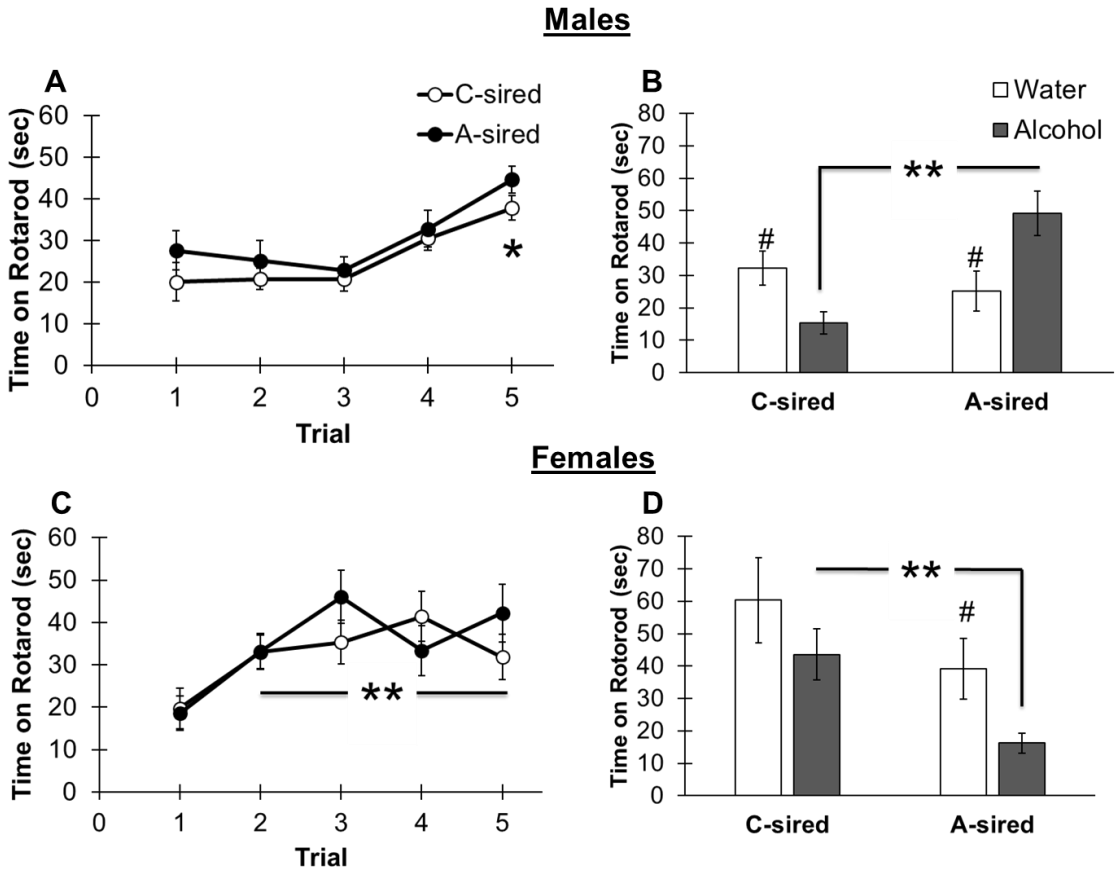

Figure 2.4. Time on the rotarod for control-sired (C-sired [n's = 10 per group; open bars/circles) and alcohol-sired (A-sired [n's = 9-10 per group; filled bars/circles)

offspring. Time spent on the accelerating rotarod during training trials and test day is presented as mean (±SEM) for males (A & B) and females (C& D). * represents a significant difference between control and alcohol-sired offspring (p's<0.05). ** represents a significant difference between control and alcohol-sired offspring (p's<0.01). # represents a significant difference between alcohol and water treatment (p's<0.05).

Discussion

The purpose of the present study is to determine whether paternal alcohol exposure alters behavioral sensitivity to alcohol. We show that A-sired male and female offspring display changes in sensitivity to the motor impairing effects of alcohol on the rotarod test. Specifically, A-sired male offspring show blunted sensitivity, while female A-sired offspring have enhanced sensitivity to alcohol's effects on motor coordination. We do not observe differences between the sire groups on the open field; however, A-sired male offspring display a more anxiolytic phenotype at baseline relative to C-sired male offspring on the elevated plus maze. This is the first study to determine the consequences of paternal alcohol exposure on behavioral sensitivity to the unconditioned effects of alcohol using an outbred rat strain.

Sensitivity to alcohol is a well-characterized predictor of AUD risk (Ray et al., 2016; Schuckit, 1984, 1994). Additionally, the heritability of an individual's level of response to alcohol is ~60% (Heath et al., 1999; Kalu et al., 2012). However, as the modified differentiator model recommends, it is important to consider the biphasic alcohol curve. That is, rising blood alcohol concentrations are primarily characterized by stimulating and rewarding subjective feelings to alcohol. As blood alcohol concentrations fall, subjective responses to alcohol reflect sedative and negative

effects of alcohol. In this context, enhanced sensitivity to the rewarding effects of alcohol and blunted sensitivity to sedative alcohol effects associate with AUD risk (Newlin and Thomson, 1990). Few preclinical studies have examined the impact of paternal alcohol on behavioral responses to alcohol.

Our study shows that paternal alcohol exposure alters sensitivity to alcohol in both male and female offspring. These divergent sex findings are only seen on the rotarod test. Specifically, alcohol-sired male offspring show blunted sensitivity while female A-sired offspring exhibit greater sensitivity to alcohol's impairing effects on motor coordination. It is important to note that altered sensitivity to alcohol is only evident on the motor coordination task, and not indices of general locomotor activity or anxiety-like behavior. In addition, paternal alcohol exposure effects are seen on baseline anxiety-like behavior on the elevated plus maze but not the open field. A-sired males show a more anxiolytic phenotype relative to A-sired females and C-sired offspring. Given that alcohol administration via oral gavage occurs 30 min prior to behavioral testing, blood alcohol concentrations are estimated to be ~125-150 mg/dl based on previous work. This blood level represents the ascending limb of the biphasic alcohol curve. Taken together, paternal alcohol exposure sex-dependently alters baseline anxiety-like behavior but changes in alcohol sensitivity are specific to motor coordination.

These findings compliment and extend work on the consequences of preconceptual paternal alcohol exposure. Consistent with prior work, we show that paternal alcohol exposure does not change basal or alcohol-induced general locomotor activity (Beeler et al., 2019; Finegersh and Homanics, 2014). The

anxiolytic phenotype observed in A-sired males is consistent with a mouse study finding that voluntary paternal alcohol consumption imparts an anxiolytic phenotype on the open field test in male offspring (Beeler et al., 2019). However, it is important to note that the open field test and elevated plus maze may be measuring different dimensions of emotionality (Carola et al., 2002). Studies in mice also show that paternal alcohol exposure blunts sensitivity to alcohol on the rotarod selectively in male mice (Finegersh and Homanics, 2014), or has null effects (Beeler et al., 2019; Rompala et al., 2017). Our results show sex-dependent effects on alcohol-induced motor coordination which likely implicate organizational/activational effects of sex hormones in the expression of paternal alcohol effects. It is important to note the procedural differences in rotarod performance across studies. Previous mouse work assesses the effects of alcohol during the training trials, while our study assesses the effects of alcohol on a single test day after training. Thus, findings in mice may be examining differences in paternal alcohol effects on procedural memory learning under the effects of alcohol in offspring. Nonetheless, blunted sensitivity to alcohol was accompanied by an alcohol resistant phenotype selectively in A-sired male offspring. Specifically, alcohol-sired male offspring consume less alcohol on two bottle choice procedures (Beeler et al., 2019; Finegersh and Homanics, 2014; Rompala et al., 2017). Continued work in our lab is examining whether paternal alcohol exposure alters operant alcohol self-administration behaviors in offspring.

It is possible that altered sensitivity to alcohol as a result of paternal alcohol exposure can be attributed to epigenetic changes transmitted through the male germ line (Finegersh et al., 2015b; Nieto and Kosten, 2019). Molecular epigenetic factors,

like DNA methylation and histone modifications, influence gene expression without changing the nucleotide sequence. Indeed, a growing body of evidence shows that paternal exposure to drugs and alcohol can influence the epigenetic profile of offspring (Nieto and Kosten, 2019). Specifically, paternal alcohol-induced blunted sensitivity and lower alcohol consumption and preference associate with greater *brain derived neurotrophic factor* (*Bdnf*) expression in the ventral tegmental area and frontal cortices (Ceccanti et al., 2016; Finegersh and Homanics, 2014; Rompala et al., 2017), key brain regions involved reward-related processes.

In summary, our results indicate that paternal alcohol exposure alters behavioral sensitivity to the motor-impairing effects of alcohol in a sex-dependent manner. Specifically, alcohol-sired offspring have a blunted response while alcohol-sired female offspring have greater sensitivity to alcohol on the rotarod test. These studies corroborate and extend previous work in mice by showing that paternal alcohol exposure several weeks prior to conception alters sensitivity to alcohol in offspring. Future work in our lab aims to identify whether these changes in alcohol sensitivity correspond to increased or decreased vulnerability to addiction-like behavior.

CHAPTER THREE- DETERMINE WHETHER PATERNAL ALCOHOL EXPOSURE ALTERS OPERANT ALCOHOL SELF-ADMINISITRATION IN OFFSPRING

Introduction

Parental alcohol use can have long-lasting intergenerational consequences, including altered sensitivity to alcohol in offspring. For example, children of parents with an AUD have lower sensitivity to the motor-impairing and stress-activating effects of alcohol, responses that increase the risk of developing AUD (Schuckit, 1985, 1994; Schuckit et al., 1987; Schuckit et al., 2005; Schuckit et al., 1996). Thus, parents who engage in pathological alcohol drinking increase the risk of AUD in their children. While maternal alcohol use is a well-studied area of clinical research, the unique role of paternal alcohol drinking has not been fully investigated (Finegersh et al., 2015a; Nieto and Kosten, 2019; Rompala and Homanics, 2019). However, preclinical studies have shown that paternal (sire) alcohol use can sex-dependently alter sensitivity to alcohol in offspring. In mice, alcohol-sired (A-sired) male offspring exhibit greater anxiolysis and less sensitivity to the motor-impairing effects of a moderate dose of alcohol (1.0 g/kg) relative to control-sired (C-sired) male offspring (Finegersh and Homanics, 2014). In addition to altered sensitivity to the unconditioned effects of alcohol, sensitivity to the rewarding effects of alcohol is also changed. A-sired male offspring show less preference and consumption for alcohol in two-bottle choice procedures (Finegersh and Homanics, 2014), and aversion to an environment paired with a moderate dose of alcohol (Ceccanti et al., 2016). These studies largely corroborate studies in rats showing that male offspring of cocaine-exposed sires exhibit reduced cocaine-seeking behaviors (Vassoler et al., 2013). Given that human and mouse studies provide evidence of altered sensitivity to the

rewarding effects of alcohol in offspring of alcohol-exposed males, it is possible that heritable risks for developing AUD are passed through the male germ line (Finegersh and Homanics, 2014). However, no studies utilizing rats have assessed sensitivity to the reinforcing effects of alcohol in A-sired offspring.

Operant self-administration procedures provide valuable information beyond what is assessed using two-bottle choice procedures and conditioned place preference, including measuring both appetitive and consummatory behaviors under increasing workloads within the same operant session (Bertholomey et al., 2016; Nieto and Kosten, 2017; Nieto et al., 2018). Acquisition of drug and alcohol self-administration is characterized by a progression from sporadic to stable levels of responding (Carroll and Meisch, 2011). The focus during this period is on how rapidly and what percentage of animals acquire self-administration. Thus, the acquisition phase provides information related to initial stages of the addiction process, i.e. initiation of drug or alcohol use, that is difficult to investigate ethically in humans. A major advantage of acquisition studies is that genetic, epigenetic, and environmental factors that underlie vulnerability or resilience to drug use can be identified, leading to individualized treatments for AUD. For example, environmental and genetic manipulations can affect acquisition of drug self-administration (Carroll and Lac, 1993; Carroll and Meisch, 2011; Deminiere et al., 1989; Horger et al., 1990; Kosten et al., 1997; Kosten et al., 2000). Additionally, different operant procedures are used to model specific phases of the addiction process (Koob et al., 2009). For example, maintenance of operant alcohol self-administration may reflect the binge-intoxication phase of drinking because the focus of this assessment is on

consumption. Procedures that employ reinstating operant behavior after its extinction may reflect the preoccupation-anticipation phase because it is generally tested in the absence of alcohol reinforcement and thus, it reflects appetitive behaviors (Koob et al., 2009).

Operant self-administration procedures vary widely, including the schedules of reinforcement used. The fixed ratio (FR) schedule provides an initial qualitative assessment of reinforcer efficacy and drug intake (Arnold and Roberts, 1997; Richardson and Roberts, 1996), whereas, the progressive ratio (PR) schedule of reinforcement provides a quantitative assessment of reinforcer efficacy (Arnold and Roberts, 1997). Under a PR schedule, the response requirement gradually increases, often after each reinforcer delivery, and, in contrast to the FR schedule, it provides a measure (break point or final ratio completed) of an animal's motivation to obtain the reinforcer (Arnold and Roberts, 1997). Whether paternal alcohol exposure alter motivation for alcohol in offspring is unknown.

The purpose of this study is to examine whether paternal alcohol exposure alters operant alcohol self-administration in rats. Specifically, we will assess acquisition, maintenance, extinction, reinstatement, and reinitiation behaviors in offspring. Based on findings from previous paternal alcohol and drug studies, we hypothesize that A-sired male offspring, but not female, will show delayed acquisition of alcohol self-administration relative to C-sired offspring. Additionally, we hypothesize that A-sired male offspring will show less motivation for alcohol in progressive ratio tests and reach extinction criteria earlier than C-sired offspring. We

also postulate that A-sired male offspring will have blunted cue-induced reinstatement and lower alcohol responding during reinitiation sessions.

Methods

Animals

Sired and dams used in Aim 1 were used in this study. Briefly, male and female Wistar rats were purchased from Charles River and used to generate offspring used in this study. Sires (400-500 g) were pair-housed prior to mating and sacrificed shortly after dams were confirmed pregnant. Dams were group-housed prior to mating and then sacrificed after offspring were weaned. Offspring were group-housed (females) or pair-housed (males) after weaning and throughout the course of the study. Most animals were housed in amber polysulfone cages and kept in a temperature-and humidity-controlled vivarium. During chronic intermittent ethanol vapor exposure, males were placed in standard rat cages housed within vapor chambers. The vivarium was maintained on a 12:12 light/dark cycle (lights on at 7:00 AM). Animals had ad libitum access to food and water except during operant procedures described below. The Institutional Animal Care and Use Committee at the University of Houston approved the experimental protocols in accordance with guidelines set forth in the “Guide for the Care and Use of Laboratory Animals 8th Edition”. Adult male rats were exposed to alcohol vapor (n=10) or room air (n=10) prior to being mated with alcohol naïve females (n = 20). A-sired (n = 9 males; n = 10 females) and C-sired (n = 10 males; n = 8 females) rats were trained to lever press for sucrose pellets and then alcohol solution as described below. These offspring were littermates of offspring used in Aim 1 and Aim 2.

Solution and drug preparations

Alcohol (ethyl alcohol, 190 proof, USP grade, Koptec, King of Prussia, PA) was mixed with tap water to reach concentrations of 2.5% (v/v), 5% (v/v), and 10% (v/v) alcohol.

Paternal chronic intermittent ethanol exposure

Male rats were made dependent by chronic, intermittent exposure to vapor alcohol as previously described in (Gilpin et al., 2008; Priddy et al., 2016). This model reliably induces alcohol dependence as indicated by the development of negative emotional-like state and somatic symptoms in withdrawal (Vendruscolo and Roberts, 2014). Standard rat cages were housed inside sealed and transparent plastic chambers into which vapor alcohol was intermittently pumped. Males underwent cycles of 16 h (6pm) on and 8 h (10am) off for five consecutive days per week over six weeks. Nondependent rats were housed in similar conditions but exposed to room air. Blood samples were collected from the lateral saphenous vein to monitor blood alcohol levels and to adjust vapor exposure settings.

Breeding and offspring rearing

Male rats were left undisturbed for 8 weeks after their last alcohol vapor session. At the end of this period, males were housed with alcohol naïve females. Female rats were examined daily for the presence of a mating plug. If the mating plug was present, males were removed from the breeding cages and sperm and brain regions were extracted the following day. Litters from these mating pairs were culled to 10 pups (5 pups per sex). Pups were weighed at postnatal day (PD) 1, 4, 7,

10, 35, and then weekly into adulthood. Self-administration training sessions began when offspring reached adulthood ~PD 75. To control for possible litter effects, no more than 2 pups per sex per litter were used in self-administration.

Self-administration apparatus

Self-administration sessions were conducted in operant chambers placed within sound-attenuating cubicles equipped with fans. Each chamber was equipped with a house light on one side of the cage and two retractable levers on the opposite wall. Above each lever was a triple cue light and in between the levers were two access areas. One area was a recessed food receptacle into which food pellets could be dispensed from a pellet dispenser. A dipper could protrude through the second recessed area. The dipper was immersed in a solution reservoir and could be activated to present 0.1 ml of solution. Both access areas were equipped with a light and with infrared sensors that were used to detect head entries. Experimental parameters and data tabulation were programmed using a software package (Graphic State Notation, Coulbourn) installed on a PC computer.

Self-administration training

Rats were first water-restricted overnight (7pm to 9am) and then trained to drink water from the dipper for two weeks. Levers were retracted during this period and each session began with two dipper presentations. After these two dipper "primes" and for the rest of the 30 min sessions, any head entry into the dipper access area triggered a dipper presentation. Dipper presentation times gradually decreased from 15 sec to 3 sec, the duration used for the rest of the study. Water-

restriction and dipper training ended after all rats met the criterion of at least 25 dipper presentations over two consecutive days. After these dipper training sessions, rats were food restricted to 85% of their free-feeding body weight and trained to press a lever for food pellets under a fixed ratio 1 (FR1) schedule of reinforcement (30 min sessions). These sessions started with the house light illuminated and protrusion of levers into the chamber. A food pellet was dispensed only after the active lever was pressed. Inactive lever presses had no programmed consequences. Operant training for food-maintained responding was continued until all rats obtained 20 reinforcers in ≤5 min over two consecutive days. Food restriction continued throughout the remainder of the study.

Alcohol self-administration sessions were conducted under similar parameters as food training sessions (30 min sessions; FR1 schedule), except that weekly ascending concentrations of alcohol (2.5%-10%) were available as a reinforcer. Animals successfully acquired operant alcohol self-administration when they achieved at least 25 active lever presses and responding was consistent (< 20% variability of active lever presses over 2 consecutive days). To decrease the group differences between alcohol and control sired offspring, specifically within female offspring, a 5% alcohol concentration was the final alcohol solution used during the remaining weeks of alcohol training. The number of active and inactive lever presses and alcohol deliveries were measured.

Maintenance and progressive ratio sessions

Rats were maintained on operant alcohol self-administration under FR2 and FR4 schedule of reinforcement for two weeks, respectively. After this period, motivation for alcohol was assessed over two weeks (4 days per week) under 3-hr progressive ratio test sessions as described previously (Kosten, 2011; Nieto and Kosten, 2017; Nieto et al., 2018; Walker and Koob, 2008). Under this schedule of reinforcement, the response requirement gradually increases over the 3 hr period demanding the organism to respond at higher levels in order to receive an alcohol reinforcer. We utilized a slow growth progressive ratio schedule in the following steps: 1, 1, 2, 2, 3, 3, 4, 4, 5, 5, 7, 7, 9, 9, 11, 11, 13, 13, 15, 15, 18, 18, 21, 21, 24, 24, etc. The number of active and inactive lever presses and alcohol deliveries were measured.

Extinction, cue-induced reinstatement, and reinitiation sessions

Extinction sessions were conducted in the absence of reinforcement under FR4 (30 min) and PR (3 hr) sessions. Animals completed FR extinction sessions 5 days per week and PR sessions 4 days per week. Offspring were exposed to two types of extinction sessions under each schedule. First, alcohol was replaced with water such that active lever presses activated cue lights and the animal was presented with water. Second, all cues and water were eliminated from extinction sessions. Rats needed to meet extinction criterion (<20% of baseline responding over 2 consecutive days) before reinstatement testing.

Two cue-induced reinstatement tests were conducted in the absence of reinforcement. Rats were tested for reinstatement, wherein responding on the active

lever produced cue lights + dipper presentations in the presence or absence of alcohol odor. Order of reinstatement tests were counterbalanced. Between tests, rats underwent at least one week of extinction sessions to ensure that the extinction criteria were met. The number of active and inactive lever presses and alcohol deliveries were measured where appropriate.

Statistical Analysis

Sessions to acquire water, food, and alcohol were analyzed using between groups analysis of variance with Sex and Sire as between groups factors. Active lever presses and alcohol deliveries during self-administration were analyzed using a three-way mixed design analysis of variance with Sex and Sire as the between groups factor and Session or Week as the repeated measure factor when appropriate. Effect sizes are reported as partial eta squared (η_p^2). Tukey *post hoc* tests were used to follow up on significant interactions. Statistical analyses were performed using SAS software 9.4 (SAS Institute, Cary, NC) with statistical significance defined as $p<0.05$. Data are presented as mean ± SEM.

Results

Alcohol self-administration

Active lever presses during alcohol acquisition for males and females are shown in Figure 3.1. There was a significant main effect of Day, $F(39, 975)= 6.777$, $p<0.001$, $\eta_p^2 = .21$, and significant interactions of Day X Sex, $F(39, 975)= 2.970$, $p<0.001$, $\eta_p^2 = .11$, and Day X Sire X Sex, $F(39, 975)= 2.254$, $p<0.001$, $\eta_p^2 = .08$. To determine the loci of sire differences, we averaged daily active lever presses into weekly blocks. Tukey *post hoc* analyses within each of these weekly blocks showed

that A-sired males (Panel B) pressed the active lever less than C-sired males during weeks 2-3 (p's<0.05). A-sired females (Panel D) pressed the active lever less than C-sired females during weeks 4-7 (p's<0.05).

FIGURE 3.1. ALCOHOL RESPONDING DURING ACQUISITION OF ALCOHOL SELF-ADMINISTRATION

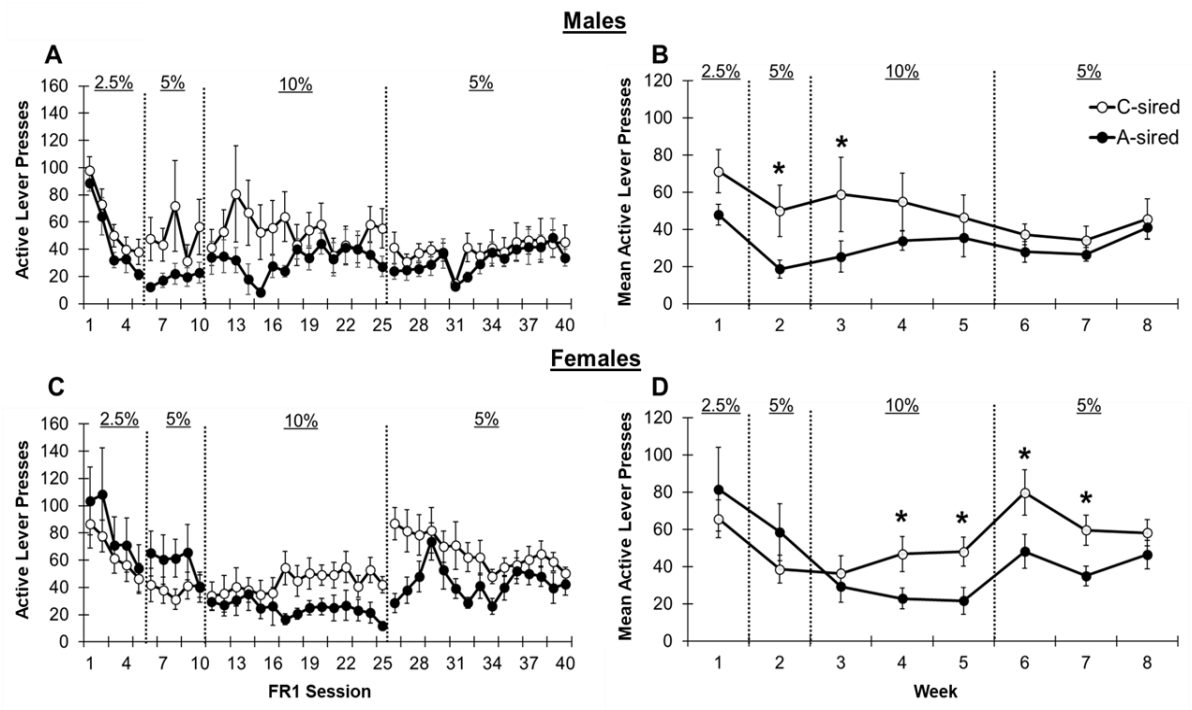

Fig 3.1. Active lever presses for control- (C-sired; open circles) and alcohol-sired (A-sired; filled circles) offspring during acquisition of alcohol self-administration under a fixed ratio 1 (FR1) schedule of reinforcement. Active lever presses are presented as mean (±SEM) for males (A) and females (C) for each day. Daily active lever presses by week are presented as mean (±SEM) for males (B) and females (D). An asterisk (*) represents a significant difference between A and C-sired offspring (p<*0*.05).

Alcohol deliveries during acquisition are shown in Figure 3.2. There was a significant main effect of Day, F(39, 975)= 4.191, p<0.001, $\eta_p^2 = .14$, and significant interactions of Day X Sire, F(39, 975)= 1.542, p=.019, $\eta_p^2 = .06$, Day X Sex, F(39,

975)= 3.557, p<0.001, $\eta_p^2 = .13$, Day X Sire X Sex, F(39, 975)= 1.977, p<0.001, $\eta_p^2 = .07$. Tukey *post hoc* analyses within the weekly time blocks revealed that A-sired males (Panel B) earned fewer alcohol deliveries compared to C-sired males during weeks 2 and 3 (p's < 0.05). A-sired females (Panel D) earned fewer alcohol deliveries compared to C-sired females during weeks 4-7 (p's<0.05). Sessions to reach acquisition criteria for water, food, and alcohol did not significantly differ by Sex or Sire group (p's>0.05; Table 3.1).

FIGURE 3.2. ALCOHOL DELIVERIES DURING ACQUISITION OF ALCOHOL SELF-ADMINISTRATION

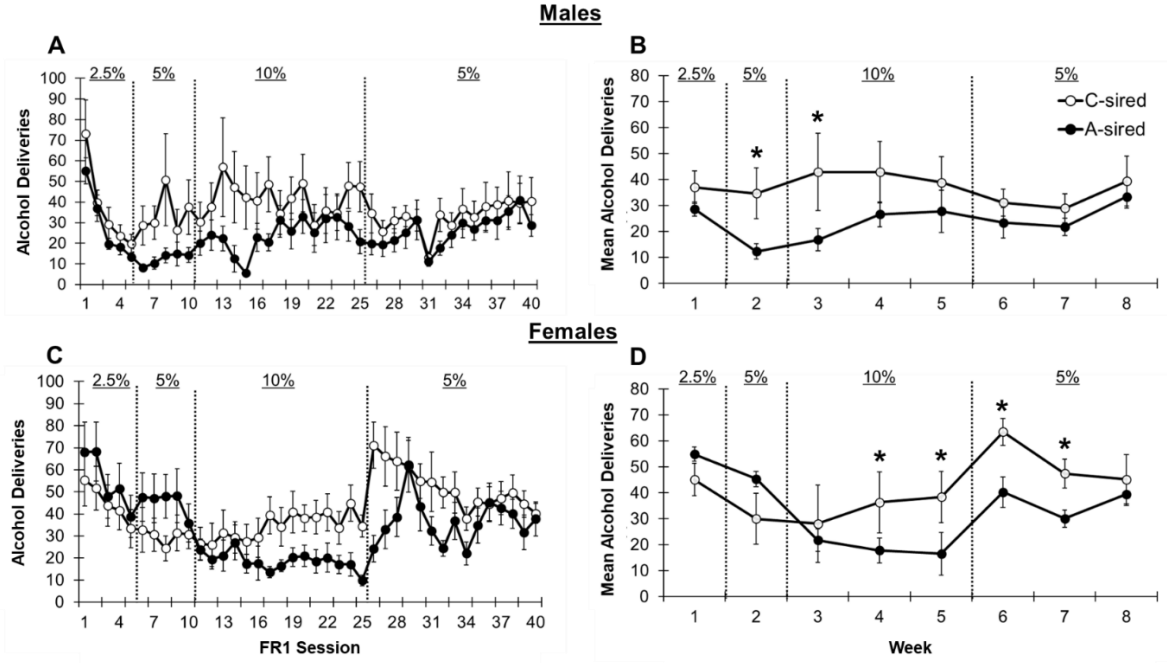

Fig 3.2. Alcohol deliveries for control- (C-sired; open circles) and alcohol-sired (A-sired; filled circles) offspring during acquisition of alcohol self-administration under a fixed ratio 1 (FR1) schedule of reinforcement. Alcohol deliveries are presented as mean (±SEM) for males (A) and females (C) for each day. Daily alcohol deliveries by week are presented as mean (±SEM) for males (B) and females (D). An asterisk (*) represents a significant difference between A- and C-sired offspring (p<*0*.05).

TABLE 3.1. SESSIONS TO REACH ACQUISITION CRITERIA FOR OPERANT SELF-ADMINISTRATION

		Sessions to acquisition (Mean $\pm$ SEM)		
Sex	**Group**	**Dipper training**	**Food training**	**Alcohol training**
Male	C-sired	7.3 $\pm$4.4	13.4 $\pm$ 4.3	9.9 $\pm$ 2.8
	A-sired	6.9 $\pm$2.8	16.8 $\pm$ 2.9	7.7 $\pm$ 2.5
Female	C-sired	6.2 $\pm$3.7	17.2 $\pm$ 3.8	6.4 $\pm$ 2.5
	A-sired	5.3 $\pm$3.1	14.5 $\pm$ 2.1	6.9 $\pm$ 2.4

Active lever presses and alcohol deliveries during maintenance are shown in Figure 3.3. For active lever presses, there was a significant main effect of Day, $F(19, 475)= 2.478$, $p<0.001$, $\eta_p^2 = .09$, indicating that animals pressed the active lever more as the schedule of reinforcement increased from FR2 to FR4. For alcohol deliveries, there was a significant main effect of Day, $F(19, 475)= 6.394$, $p<0.001$, $\eta_p^2 = .20$, indicating that alcohol deliveries steadily decreased as the schedule of reinforcement increased from FR2 to FR4. There were no significant main effects of Sire or Sex, or interactions. No significant differences were found in inactive lever presses during acquisition or maintenance sessions ($p>0.05$; data not shown).

FIGURE 3.3. ALCOHOL SELF-ADMINISTRATION DURING MAINTENANCE SESSIONS

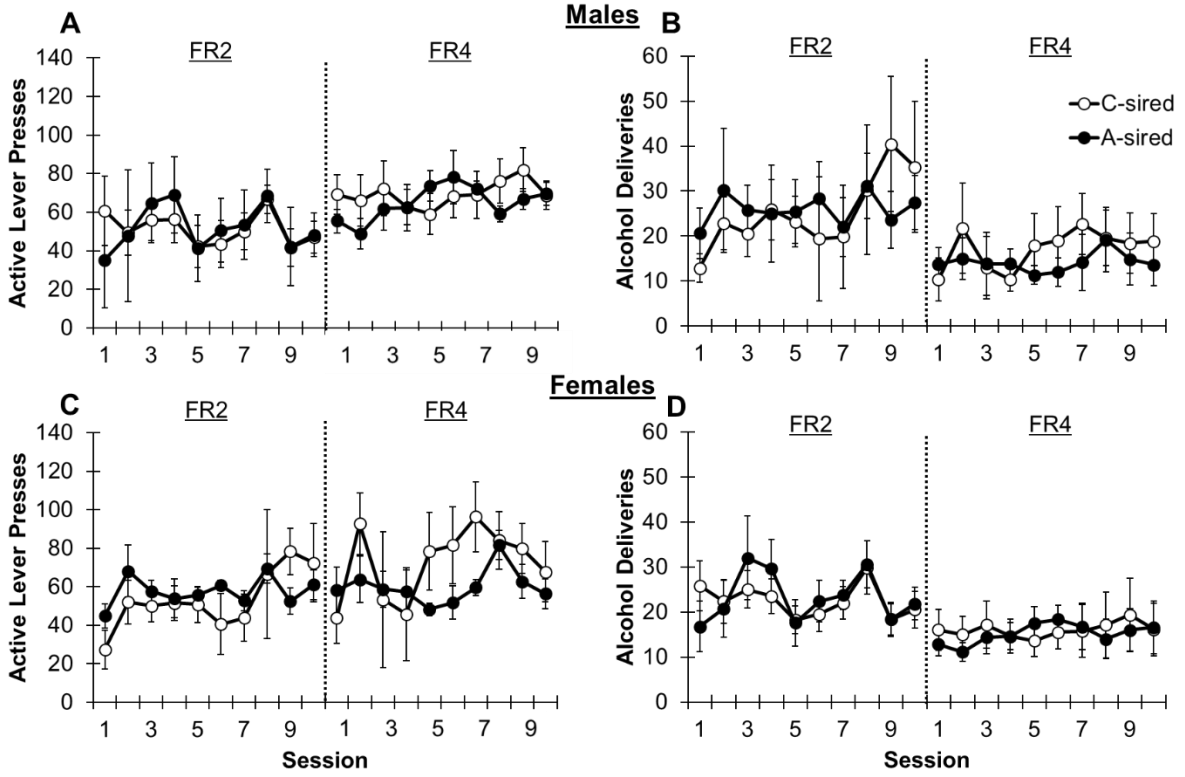

Fig 3.3. Alcohol deliveries for control- (C-sired; open circles) and alcohol-sired (A-sired; filled circles) offspring during maintenance of alcohol self-administration under fixed ratios 2 and 4 (FR2 and FR4, respectively) schedules of reinforcement. Active lever presses are presented as mean (±SEM) for males (A) and females (C) for each day. Daily alcohol deliveries by week are presented as mean (±SEM) for males (B) and females (D).

Active lever presses and alcohol deliveries during progressive ratio tests are shown in Fig 3.4. For active lever presses, there were significant main effects of Sire, F(1, 25)= 4.025, p=.041, $\eta_p^2 = .14$, Day, F(3, 75)= 29.092, p<0.001, $\eta_p^2 = .58$, and a significant Day X Concentration interaction, F(3, 75)= 2.951, p=.038, $\eta_p^2 = .11$. Overall, alcohol-sired rats of both sexes (Panels A & C) pressed the active lever less than control-sired animals. Active lever presses decreased as a function of day;

however, this decrease was slower when animals were responding for 10% compared to 5% alcohol. Comparable results were found for alcohol deliveries. There were significant main effects of Sire, F(1, 25)= 6.02, p=.021, $\eta_p^2 = .19$, Day, F(3, 75)= 35.37, p<0.001, $\eta_p^2 = .59$, and a significant Day X Concentration interaction, F(3, 75)= 2.81, p=.045, $\eta_p^2 = .10$. Alcohol-sired rats of both sexes (Panels B & D) received fewer alcohol deliveries relative to control-sired rats. In addition, alcohol deliveries decreased as a function of day, but this decrease was slower when animals were responding for 10% compared to 5% alcohol. No significant differences were found in inactive lever presses or final ratio completed during progressive ratio tests (data not shown).

FIGURE 3.4. ALCOHOL SELF-ADMINISTRATION DURING PROGRESSIVE RATIO TESTS

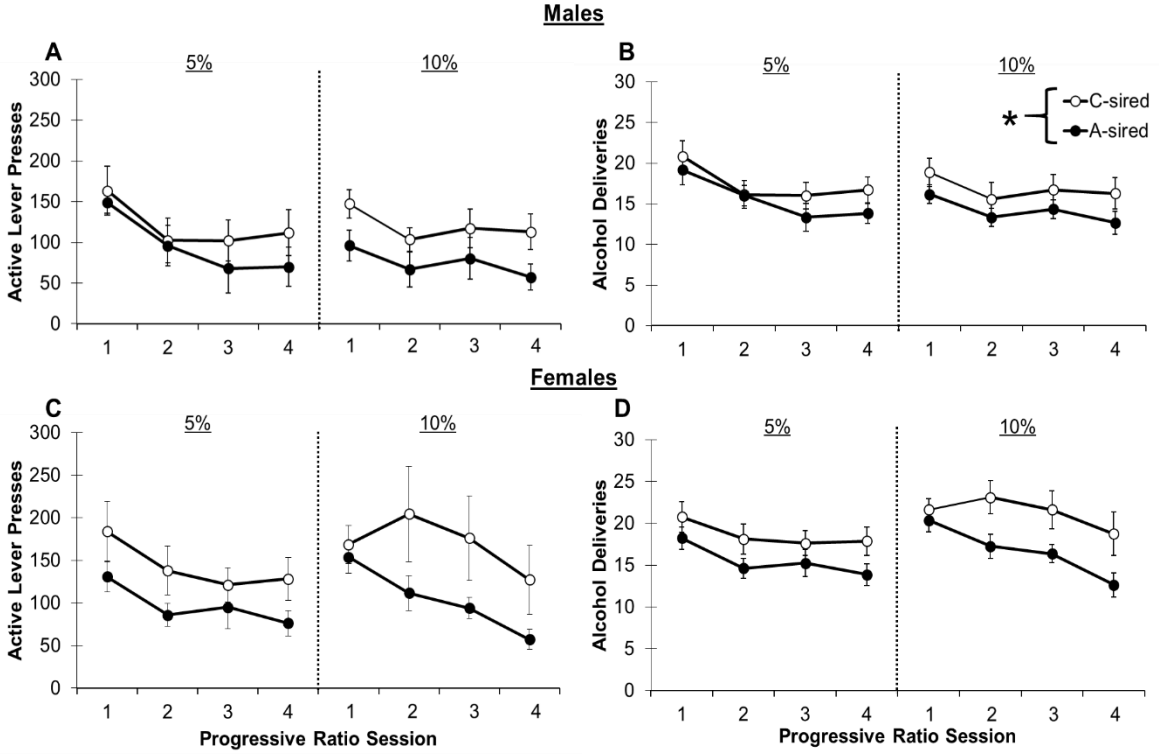

Fig 3.4. Active lever presses and alcohol deliveries for control (C-sired; open circles) and alcohol-sired (A-sired; filled circles) offspring under a progressive ratio schedule of reinforcement. Active lever presses are presented as mean ($\pm$SEM) for males (A) and females (C). Alcohol deliveries are presented as mean ($\pm$ SEM) for males (B) and females (D). An asterisk (*) represents a significant difference between control and alcohol-sired offspring on *both* behaviors (p's<0.05).

Active lever presses during extinction training under both FR and PR schedules are shown in Figure 3.5. There was a significant main effect of Sire, $F(1, 25) = 4.66$, $p=.041$, $\eta_p^2 = .16$, Sex, $F(1, 25) = 8.51$, $p=.007$, $\eta_p^2 = .25$, and Day $F(14, 350) = 8.68$, $p<.001$, $\eta_p^2 = .26$. A-sired offspring had fewer active lever presses during extinction training relative to C-sired offspring (Panels A & C). Females pressed the active lever more than males during FR extinction training. Active lever presses decreased over FR sessions with lowest levels seen during the last week of FR

sessions when cues and water were not present in the operant chamber. For PR extinction sessions (Panels B & D), there was a significant main effect of Day, F(15, 375) = 11.99, p<0.001, $\eta_p^2 = .02$, and Day X Sire, F(15, 375) = 2.51, p=0.001, $\eta_p^2 = .09$, Day X Sex interactions, F(15, 375) = 1.70, p=.049, $\eta_p^2 = .06$. Overall, active lever presses decreased over PR sessions with lowest levels seen during the last week of PR session when cues and water were not present in the operant chamber. A-sired offspring had lower active lever presses during PR extinction sessions 1-4 and 10-12 relative to C-sired offspring (p's<0.05). Female rats had more active lever presses relative to males during PR extinction sessions 6, 8, and 13 (p's<0.05). No significant differences were found in inactive lever presses during extinction sessions (data not shown).

FIGURE 3.5. EXTINCTION TRAINING SESSIONS

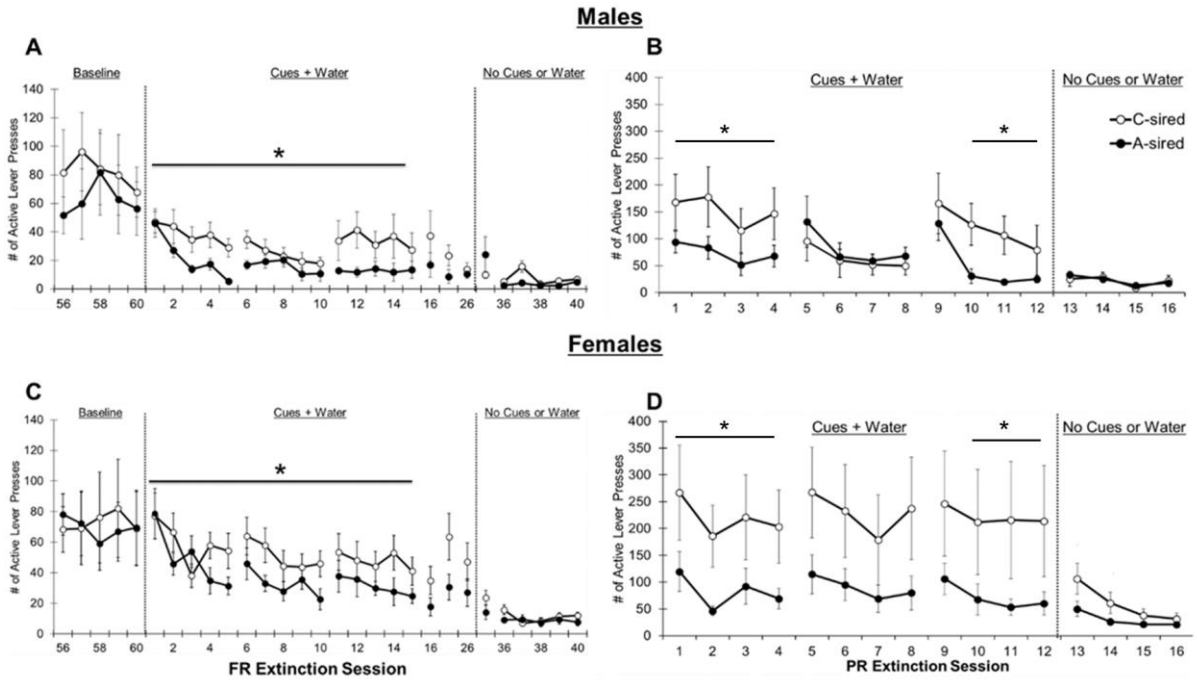

Fig 3.5. Active lever presses in control-sired (C-sired; open circles) and alcohol-sired (A-sired; filled circles) offspring during extinction sessions. Active lever

presses under fixed ratio (FR; 5 days per week) extinction sessions are presented as mean (±SEM) for males (A) and females (C). Active lever presses under a progressive ratio schedule of reinforcement (PR; 4 days per week) are presented as mean (± SEM) for males (B) and females (D). An asterisk (*) represents a significant difference between control and alcohol-sired offspring on *both* sexes (p's<0.05).

Active lever presses during cue-induced reinstatement sessions are shown in Figure 3.6. During reinstatement sessions, there were significant main effects of Sire, F(1, 25) = 8.90, p =.006, $\eta_p^2 = .26$, Sex, F(1, 25) = 11.45, p =.002, $\eta_p^2 = .31$, Session, F(2, 50) = 7.63, p =.001, $\eta_p^2 = .23$ and a Session X Sire interaction, F(2, 50) = 4.95, p =.011, $\eta_p^2 = .17$. A-sired offspring had fewer active lever presses during cue-induced reinstatment sessions relative to C-sired offspring. Females had greater active lever presses during cue-induced reinstatment sessions relative to males. Both reinstatment sessions (cues only and cues + alcohol odor) increased active lever presses compared to extinction responding, but sire group moderated this effect. Specficailly, A-sired offspring had lower active lever presses when alcohol odor was present during the reinstatment session (p<0.001; Panels A & B). No significant differences were found in inactive lever presses during reinstatment or reinitiation sessions (data not shown).

FIGURE 3.6. CUE-INDUCED REINSTATEMENT SESSIONS

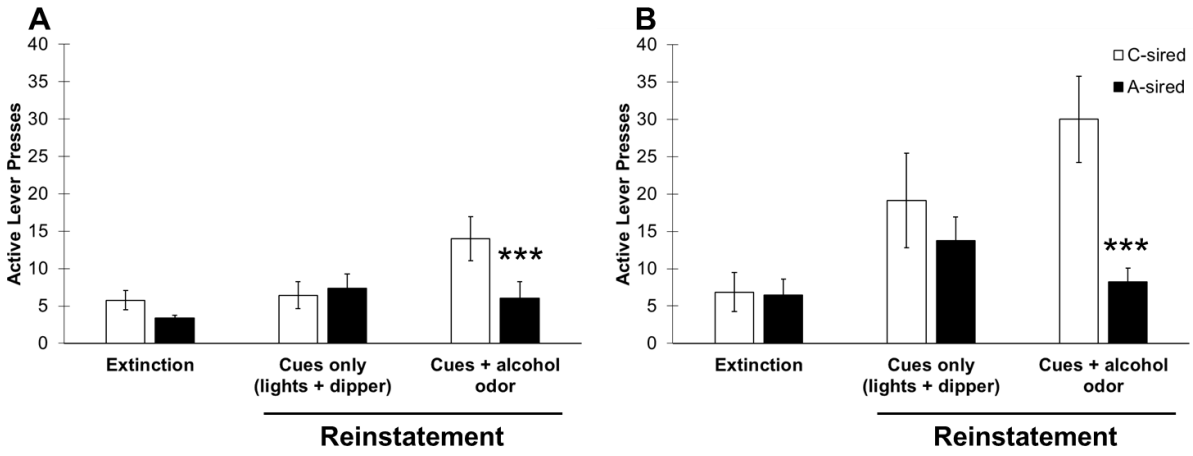

Fig 3.6. Active lever presses for control-sired (C-sired; open bars/circles) and alcohol-sired (A-sired; filled bars/circles) offspring during reinstatement sessions. Active lever presses during reinstatement sessions are presented as mean (±SEM) for males (A) and females (B). *** represents a significant difference between control and alcohol-sired offspring in *both* sexes (p's<0.001).

Active lever presses and alcohol deliveries during reinitiation sessions are shown in Figure 3.7. For active lever presses during reinitation sessions, there were significant main effects of Sire, F(1, 25) = 4.98, p=.035, $\eta_p^2 = .17$ and Day, F(4, 100) = 5.79, p<.001, $\eta_p^2 = .19$. A-sired offspring made fewer active lever presses relative to C-sired offspring (Panels B & D). Additionally, animals made more active lever presses during the sessions 4 and 5 compared to the first session. For alcohol deliveries during reinitiation sessions, there were significant main effects of Sire, F(1, 25) = 4.94, p=.036, $\eta_p^2 = .16$ and Day, F(4, 100) = 5.23, p<.001, $\eta_p^2 = .17$., and a Sex X Day interaction, F(4, 100) = 2.71, p=.034, $\eta_p^2 = .098$. A-sired offspring had fewer alcohol delivers relative to C-sired offspring. Tukey *post hoc* tests showed

that there were greater alcohol deliveries on day 4 compared to days 2 ($p<.05$) and 3 ($p<.001$) in male but not female rats.

FIGURE 3.7. ALCOHOL RESPONDING DURING REINITIATION SESSIONS

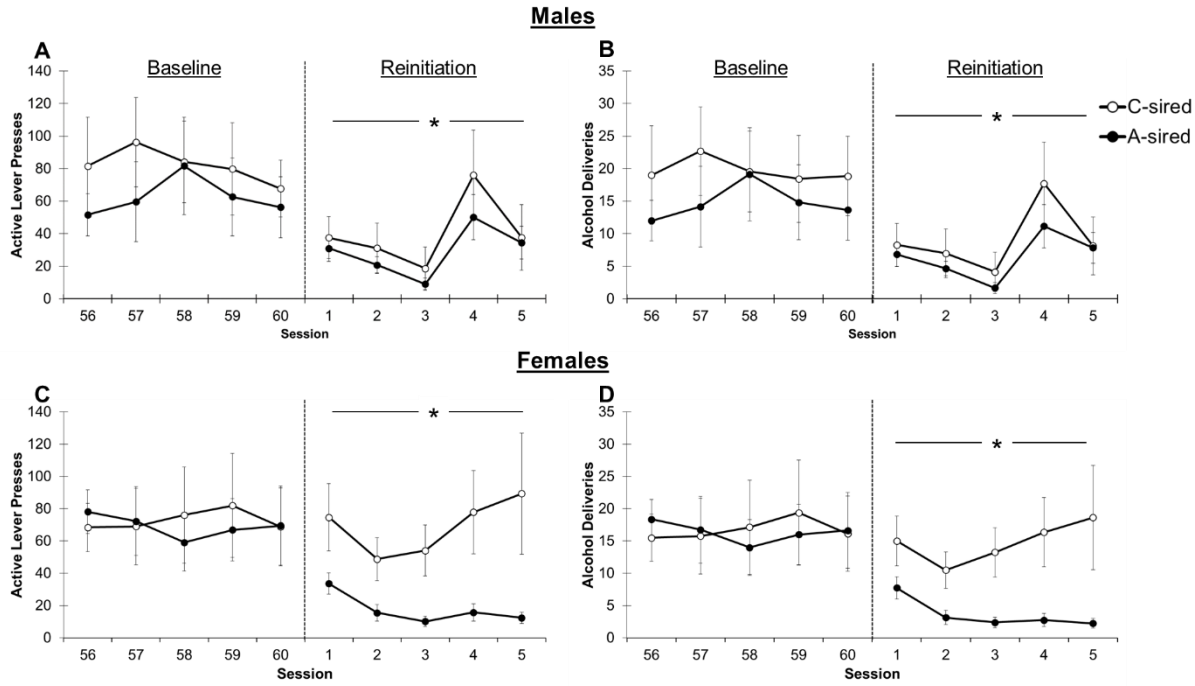

Fig 3.7. Active lever presses for control-sired (C-sired; open bars/circles) and alcohol-sired (A-sired; filled bars/circles) offspring during reinitiation sessions. Active lever presses during reinstatement sessions are presented as mean (±SEM) for males (A) and females (C). Alcohol deliveries for alcohol during reinitiation sessions under fixed ratio (FR4) schedule of reinforcement (PR) are presented as mean (± SEM) for males (B) and females (D). An asterisk (*) represents a significant difference between control and alcohol-sired offspring in *both* sexes (p's<0.05).

Discussion

The results of the present study demonstrate that paternal alcohol exposure confers an alcohol-resistant phenotype during acquisition of operant alcohol self-administration. Specifically, we observe that A-sired offspring pressed the active lever less and received fewer alcohol deliveries compared to C-sired offspring. This

phenotype is observed in both male and female A-sired offspring in a time-dependent manner. That is, differences between the sire groups appear earlier, but last for a shorter duration for males relative to females for which differences between the sire groups are seen later and last longer. No differences are seen in lever pressing for food or water. During extinction training, A-sired offspring press the active lever less during FR and PR extinction training relative to C-sired offspring; however, persistence in responding is still evident in both sire groups until cues are eliminated from extinction sessions. A-sired offspring have blunted cue-induced reinstatement, specifically during sessions when alcohol odor is present. During reinitiation sessions, A-sired offspring have lower alcohol responding relative to C-sired offspring. To our knowledge, this is the first study to show that chronic paternal alcohol has a protective effect on various aspects of the addiction cycle as modeled in operant self-administration. Importantly, by employing an operant procedure, these results provide support for paternal alcohol exposure causing a diminution of the reinforcing effects of alcohol, not merely consumption, that do not reflect impaired learning or performance.

Rodent studies have found intergenerational consequences of paternal alcohol exposure. Studies in mice and rats show several developmental abnormalities in A-sired offspring, including lower body and organ weights (Bielawski and Abel, 1997; Bielawski et al., 2002). Paternal alcohol exposure results in deficits within affective (Kim et al., 2014; Liang et al., 2014) and cognitive domains (Wozniak et al., 1991). Sensitivity to alcohol is also altered in A-sired offspring. In C57/BL6J mice, A-sired male offspring display greater alcohol-induced anxiolysis and

locomotor stimulation, as well as a blunted response to alcohol-induced impairments in motor coordination (Finegersh and Homanics, 2014). In two-bottle choice procedures, A-sired male offspring display lower preference for low alcohol concentrations and consume less of the moderate concentrations (Finegersh and Homanics, 2014; Rompala et al., 2017). In CD1 mice, A-sired male offspring exhibit greater place preference for a low dose of alcohol, while place aversion is seen at a higher dose that induced a preference in C-sired offspring (Ceccanti et al., 2016). Our results corroborate and extend these findings of altered sensitivity to the rewarding effects of alcohol by showing that male and female offspring self-administered less alcohol during the acquisition phase of self-administration. Importantly, it is unlikely that paternal alcohol-induced learning and memory impairments affected self-administration as the number of sessions to acquire dipper training, food training, and alcohol training were equivalent in both sire groups. Thus, the data from the current study and past preclinical work support the hypothesis that chronic paternal alcohol exposure confers a protective effect against the development of addiction-like behaviors for alcohol in offspring.

Paternal alcohol exposure results in decreases in motivation for alcohol in offspring. Specifically, A-sired offspring have lower responding and alcohol deliveries during progressive ratio sessions. However, the sire groups do not differ on final ratios completed. While this is the first study to examine the effects of paternal alcohol exposure on motivation for alcohol in offspring, paternal cocaine exposure reduces motivation for cocaine selectively in male, but not female, offspring (Le et al., 2017; Vassoler et al., 2013). Interestingly, these behaviors are accompanied by

paternal cocaine-induced epigenetic marks in the medial prefrontal cortex that may be transmitted via the male germ line. Taken together, the present findings support the hypothesis that paternal drug exposure decreases motivation for the same drug in offspring.

In addition to decreases in motivation, A-sired offspring show blunted craving and relapse-like behavior as evidenced by both lower sensitivity to cue-induced reinstatement tests and lower alcohol responding during reinitation sessions. In contrast to previous paternal alcohol and cocaine studies, we do not observe robust sex differences except during acquisition training. This finding might reflect a lack of interaction between paternal alcohol exposure and hormonal influences on alcohol's reinforcing effects at later stages of dependency. This is likely the case given that several weeks passed between sire's last alcohol session and mating; whereas previous paternal alcohol studies show sex differences when sires are mated immediately after their last alcohol session.

Family history of alcohol use predicts susceptibility to AUD and sensitivity to alcohol in humans. Children of individuals with AUD show decreased subjective and behavioral responses (body sway) to alcohol (Pollock, 1992; Schuckit, 1985), which negatively correlate with AUD risk (Schuckit, 1994). In addition to subjective and behavioral findings, these children also showed alterations in physiological markers. For example, children of parents with AUD exhibit blunted cortisol levels after alcohol (Schuckit et al., 1987) and decreased p300 event-related potential amplitude (Begleiter et al., 1984; Costa et al., 2000), the latter of which also negatively associates with AUD risk (Hesselbrock et al., 2001). Paternal alcohol use also

results in several abnormalities in children related to increased risk of neuropsychiatric disorders (Knopik et al., 2005; Ozkaragoz et al., 1997; Pihl et al., 1990) and cancers (Infante-Rivard and El-Zein, 2007), as well as decreased brain volume (Gilman et al., 2007) and cognitive impairments (Ervin et al., 1984). Thus, paternal alcohol use has far reaching intergenerational consequences.

There is a lack of consilience between human and preclinical studies of family history of alcohol use and the propensity to drink alcohol in children. As mentioned above, a family history of alcohol use associates with an increased risk for AUD in human children; however, paternal alcohol exposure decreases the consumption and preference for and, as we show in the current study, reinforcing effects of alcohol. There are several factors that may be responsible for the lack of agreement. First, studies in humans have not focused on the unique contribution of paternal alcohol consumption to AUD risk in children. Second, the focus of the rodent studies was on chronic alcohol exposure in sires, whereas the amount of alcohol consumption is not considered in clinical criteria for AUD. Future preclinical studies that screen sires for behaviors reflective of AUD symptomology after chronic alcohol exposure may reconcile the differences between the human and animal data. A clear example is evident in paternal cocaine studies wherein cocaine-sires that displayed high motivation for cocaine in operant self-administration had offspring that self-administered more cocaine while a cocaine-resistance phenotype was seen in the offspring of cocaine-sires with low motivation (Le et al., 2017).

While the current study has several strengths, including the use of both sexes and different alcohol concentrations during acquisition, there are some limitations. It

is possible that food restriction led to caloric compensation, wherein alcohol's caloric value maintained operant responding. If so, A-sired animals, which weighed less than C-sired offspring, should have self-administered alcohol at equivalent or greater levels as C-sired offspring. Instead, A-sired offspring self-administered less alcohol. Therefore, it is likely that the reinforcing effects of alcohol and not its caloric value maintained operant responding.

In summary, our findings demonstrate that paternal alcohol exposure decreased alcohol self-administration during acquisition. This effect is greater when animals respond for higher concentrations of alcohol (5%-10%) rather than for the lowest concentration (2.5%). Sex differences in paternal alcohol effects also occur during acquisition; sire group differences are seen at earlier sessions for males and at later sessions for females. Yet, by the end of the acquisition study, the sire groups respond for 5% alcohol at equivalent levels, suggesting that a long-term drinking history may eventually offset the protective effect of paternal alcohol exposure under low workload. A-sired offspring show less drug-seeking behaviors during FR and PR extinction sessions. In addition, A-sired offspring exhibit blunted and craving- and relapse-like behaviors during cue-induced reinstatement tests and reinitiation sessions. Taken together, our findings support the hypothesis that paternal alcohol exposure has long-lasting intergenerational consequences, including a protective effect on addiction-like behaviors in offspring that may occur as a result of transmitted epigenetic marks.

CHAPTER FOUR- DETERMINE WHETHER PATERNAL ALCOHOL EXPOSURE ALTERS GLOBAL AND *Bdnf* DNA METHYLATION LEVELS IN OFFSPRING

Introduction

Alcohol use disorder (AUD) is a highly debilitating disease and one of the most prevalent mental disorders in the United States (SAMHSA, 2015); therefore, identifying genetic and epigenetic factors that enhance vulnerability or promotoe resilience to AUD is a major initiative of NIAAA. It is well known that children of individuals with AUD may suffer from several impairments in physiological and psychosocial domains, as well as an increased risk of developing AUD. Children of individuals with AUD have decreased subjective responses and behavioral sensitivity to alcohol, sometimes occurring in a sex dependent manner (Schuckit, 1985, 1994; Schuckit et al., 1987). In fact, twin and adoption studies consistenly find that the heritability rate of AUD is ~50% (Prescott and Kendler, 1999; Young-Wolff et al., 2011; Ystrom et al., 2011). Although several genetic variants associated with AUD have been identified (Reilly et al., 2017), only a few are consistently associated with AUD. While genome wide association studies (GWAS) have yielded promising candidate genes, the genetic variants identified thus far explain only a small percentage (~0.1%) of the heritable risk for developing AUD (Heath et al., 2011). Therefore, there is a critical need to identify biological mechanisms that underlie this "missing heritability".

There are several factors that might help explain missing AUD heritability. Individual risk of developing most psychiatric disorders is the product of gene X environment interactions (Meaney, 2017). The environmental component of the equation (e.g., cultural diversity, early life stress, environmental toxins) adds a

considerable degree of heterogeneity across AUD populations (Ober and Vercelli, 2011). Additionally, AUD features complex symptomology comprised of multiple endophenotypes that further challenge efforts to identify the genetic contribution to AUD (Blanco-Gomez et al., 2016). Furthermore, there is growing evidence to suggest that a portion of the heritability of complex phenotypes may result from parental preconception experience.

Some of the earliest evidence of intergenerational effects of environmental exposures stem from the 1944 to 1945 Dutch Famine Cohort. Men exposed to famine during prenatal development are more likely to have offspring with increased body weight and more body fat in adulthood (Painter et al., 2008; Veenendaal et al., 2013). Similarly, the Överkalix study shows that the food supply of paternal grandparents negatively associates with longevity in grandchildren of both sexes (Bygren et al., 2001; Pembrey et al., 2006). In a similar vein, fathers that smoked prior to puberty are more likely to have sons with increased body mass index (Pembrey et al., 2006). Mothers and fathers exposed to traumatic stress episodes prior to conception have offspring with lower basal cortisol levels (Lehrner et al., 2014; Yehuda et al., 2007). Children of mothers that survived the Holocaust have an increased risk of developing trauma and mood-related neuropsychiatric disorders (Yehuda et al., 2008). Furthermore, sons of U.S. civil war prisoners of war are more likely to die (Costa et al., 2018). Maternal in utero effects and parental investment in child development top a long list of potential confounding variables, making a germline-specific mechanism difficult to identify. Thus, to rule out confounding variables, preclinical studies have focused on the intergenerational effects of

paternal environmental insults on a wide array of behavioral and biological measures in offspring. For instance, obese fathers confer deficits in glucose metabolism to offspring (Chen et al., 2016; Cropley et al., 2016; de Castro Barbosa et al., 2016). Numerous paternal chronic stress paradigms reshape physiological and behavioral stress vulnerability across generations (Dietz et al., 2011; Gapp et al., 2014; Rodgers et al., 2013; Short et al., 2016) and paternal cocaine exposure alters preference and hippocampal-dependent memory in male offspring (Le et al., 2017; Vassoler et al., 2013; Wimmer et al., 2017).

There are dozens of studies published on the effects of paternal preconception alcohol exposure in rodents. Most of these studies show that paternal alcohol exposure has far reaching consequences affecting developmental, physiological, cognitive, and mood-related domains in offspring (Finegersh and Homanics, 2014; Finegersh et al., 2015b; Nieto and Kosten, 2019). Few studies have examined the effect of paternal alcohol exposure on offspring alcohol drinking or alcohol sensitivity. In mice, alcohol-sired (A-sired) male offspring have increased sensitivity to the anxiolytic effects of alcohol and decreased alcohol drinking preference and consumption (Finegersh and Homanics, 2014). In addition, A-sired male offspring show enhanced place preference at low alcohol doses (0.5 g/kg) and decreased place preference at a moderate dose (1.5 g/kg) (Ceccanti et al., 2016). In rats, sires exposed to alcohol for 8 days via intragastric delivery have offspring that consume more alcohol at postnatal day 14 (Hollander et al., 2019). Taken together, these studies suggest that paternal alcohol exposure prior to conception is a heritable factor capable of driving alcohol-related phenotypes in the next generation.

Epigenetic processes can alter gene expression without changing the DNA sequence, and thus, may contribute to the heritability of AUD. Environmental factors can induce epigenetic modifications leading to diverse phenotypes in organisms. These mechanisms are reversible and can be recapitulated in successive mitotic generation of cells (Maze and Nestler, 2011). DNA methylation is a well-studied epigenetic mechanism leading to an increase or decrease in gene transcription depending on genomic location. Promoter regions of genes contain a high density of CpG dinucleotides, termed CpG islands (Larsen et al., 1992). The addition of methyl groups to cytosines near transcription start sites can decrease gene transcription and prevent binding of transcription factors (Robertson and Wolffe, 2000a). Human and mouse studies find that alcohol alters DNA methylation levels of imprinted and non-imprinted loci in sperm of males and these changes can be passed on to non-exposed offspring (Finegersh and Homanics, 2014; Knezovich and Ramsay, 2012; Liang et al., 2014; Ouko et al., 2009). For example, alcohol exposure results in lower methylation of the *brain derived neurotrophic factor (Bdnf)* gene in sire's sperm and hypomethylation is maintained in the ventral tegmental area of A-sired male and female offspring (Finegersh and Homanics, 2014). It is important to note that although offspring of both sexes inherit lower *Bdnf* methylation levels, only A-sired males consume less alcohol, suggesting that the behavioral consequences of paternal alcohol exposure can vary by sex. Interestingly, paternal cocaine reduces cocaine-seeking behavior in male offspring and this phenotype is reversible with a BDNF-receptor TrkB antagonist. Given that BDNF is a well-studied regulatior of drug and alcohol intake, the *Bdnf* gene may be an attractive target for examining

mechanistic effects of paternal alcohol exposure on offspring neurobiology across reward-related brain regions. However, paternal alcohol studies have not ruled out the contribuition of global DNA methylation changes to phenotypes seen in offspring.

The purpose of this study is to examine the effects of paternal alcohol exposure on global and *Bdnf* DNA methylation levels in adult offpspring. We hypothesize that alcohol exposure will reduce global and *Bdnf* DNA methylation levels in sperm, nucleus accumbens, and medial prefrontal cortex. We also postulate that this epigenetic profile will be maintained in male and female offspring.

Methods

Animals

Sires and dams used in Aims 1 and 2 were used for DNA methylation studies. Male and female Wistar rats were purchased from Charles River (Wilmington, MA) and used to generate offspring used in this study. Sires (400-500 g) were pair-housed prior to mating and sacrificed shortly after dams were confirmed pregnant. Dams were group-housed prior to mating and then sacrificed after offspring were weaned. Offspring were group-housed (females) or pair-housed (males) after weaning and throughout the course of the study. Most animals were housed in amber polysulfone cages and kept in a temperature and humidity-controlled vivarium. During chronic intermittent ethanol vapor exposure, males were placed in standard rat cages housed within vapor chambers. The vivarium was maintained on a 12:12 light/dark cycle (lights on at 7:00 AM). Animals had *ad libitum* access to food and water except during operant procedures described below. The Institutional Animal Care and Use Committee at the University of Houston approved the

experimental protocols in accordance with guidelines set forth in the “Guide for the Care and Use of Laboratory Animals 8th Edition”. Adult male rats were exposed to alcohol vapor (n = 10) or room air (n = 10) prior to being mated with alcohol naïve females (n = 20). A-sired (n = 10 males; n = 10 females) and C-sired offspring (n = 9 males; n = 10 females) were used for DNA methylation studies. Importantly, these animals were a separate cohort of offspring than those used in the unconditioned behaviors and self-administration studies (Chapters 2 and 3, respectively).

Solution and drug preparations

Alcohol (ethyl alcohol, 190 proof, USP grade, Koptec, King of Prussia, PA) was used to expose sires to alcohol vapor.

Paternal chronic intermittent ethanol exposure

Male rats were made dependent by chronic, intermittent exposure to vapor alcohol as previously described in (Gilpin et al., 2008; Priddy et al., 2016). This model reliably induces alcohol dependence as indicated by the development of negative emotional-like state and somatic symptoms in withdrawal (Vendruscolo and Roberts, 2014). Standard rat cages were housed inside sealed and transparent plastic chambers into which vapor alcohol was intermittently pumped according to protocols described in (Gilpin et al., 2008). Males underwent cycles of 16 h (6pm) on and 8 h (10am) off for five consecutive days per week over six weeks. Nondependent rats were housed in similar conditions but exposed to room air. Blood samples were collected from the lateral saphenous vein to monitor blood alcohol levels and to adjust vapor exposure settings.

Breeding and offspring rearing

Male rats were left undisturbed for 8 weeks after their last alcohol vapor or air session. At the end of this period, males were housed with alcohol naïve females. Female rats were examined daily for the presence of a mating plug. If the mating plug was present, males were removed from the breeding cages with sperm and brain regions extracted the following day. Litters from these mating pairs were culled to 10 pups (5 pups per sex). Pups were weighed at postnatal days [PD] 1, 4, 7, 10, 35, and then weekly into adulthood. Offspring were sacrificed and tissue was collected when they reached adulthood ~PD 75. To control for possible litter effects, no more than 1 pup per sex per litter was used in DNA methylation studies.

Tissue collection and DNA extraction

Motile sperm was collected from sires and male offspring using the double swim up assay (Anway et al., 2005). Briefly, the cauda epididymis was dissected from the testes and placed in 1% bovine serum albumin. Longitudinal cuts were made along the cauda epididymis and it was placed with 1% bovine serum albumin in a 15 mL conical tube. The tissue was incubated at 37°C for 30 minutes. Supernatant containing sperm was collected and incubated again at 37°C for 10 min. The top 1 mL of supernatant was collected and pelleted at 4°C at 4000 RPM for 5 minutes. Motile sperm was resuspended in sperm lysis buffer with Proteinase K and incubated overnight at 50°C. DNA was extracted from motile sperm using a modified guanidine thiocyanate method (Griffin, 2013). DNA samples were immediately placed on dry ice and stored at -80°C until bisulfite treatment.

Medial prefrontal cortex and nucleus accumbens were collected from sires and offspring. After rats were anesthetized with isoflurane and decapitated, brain regions were dissected using a Rodent Brain Matrix (RBM-4000C). Brain regions were immediately placed on dry ice and stored at -80°C. DNA was extracted using the Gentra Puregene DNA isolation methods (Qiagen, Valencia, CA).

DNA methylation

Global DNA methylation (5-methylcytosine quantification, 5mC) was assessed using MethylFlash Global DNA Methylation (5-mC) ELISA Easy kit (colorimetric) EpiGentek (Farmingdale, NY). DNA methylation levels within the *Bdnf* promoter region were determined using direct sequencing methods as reported previously (Hao et al., 2011; Kosten et al., 2014). Genomic DNA (300 ng) was treated with sodium bisulfite using the EZ-96 DNA Methylation Kit D5004 (Zymo Research, Irvine, CA) according to the manufacturer's instructions. Bisulfite-treated DNA was amplified using a modified step-down method with annealing temperatures of 56, 53, 50, 47, and 44°C with the primers M-RATBDNF-4F (5'-GGTAGAGGAGGTATTATATGATAGT-3') and M-RATBDNF-4R (5'-ATAACCCATATATACTCCTATTCTTCAACA-3'). Sequencing was performed at GENEWIZ, Inc. (South Plainfield, NJ) using both the forward and reverse primers used in the amplification of the *Bdnf* promoter. Trace files (.ab1) were analyzed using the Epigenetic Sequencing Methylation Analysis Software (Epigenomics AG; Berlin, Germany) version 3.2.1. Nucleotides were numbered relative to the exon IV A of the ATG translation start site. The rat *Bdnf* gene exon IV promoter region was

analyzed for predicted transcription factor binding sites using AliBaba2.1 (http://www.gene-regulation.com/pub/programs/alibaba2/index.html).

Statistical Analysis

DNA methylation data in sires were analyzed using Student's t-tests (global methylation) and two-way mixed design analysis of variance with CpG sites considered a within subject factor and Treatment (alcohol vapor vs room air) as a between groups factor. In offspring, DNA methylation levels were analyzed using two (global methylation) or three-way mixed design analysis of variance with CpG sites, Sex, and Sire as the independent variables. Tukey *post hoc* tests were used to follow up on significant interactions. Effect sizes are reported as partial eta squared (η_p^2). Statistical analyses were performed using SAS software 9.4 (SAS Institute, Cary, NC) with statistical significance defined as $p<0.05$. Data are presented as mean ± SEM.

Results

Global DNA methylation levels in sires are depicted in Figure 4.1. Alcohol-exposed sires had greater global methylation levels in sperm, $t(15) = 2.310$, $p<0.05$, Cohens' *d* = 1.10, and lower levels in the nucleus accumbens, $t(17) = -2.113$, $p<0.05$, Cohen's *d* = 0.98, relative to control sires. There was no difference in global DNA methylation levels between sire groups in the medial prefrontal cortex ($p>0.05$).

FIGURE 4.1. GLOBAL DNA METHYLATION IN SIRES

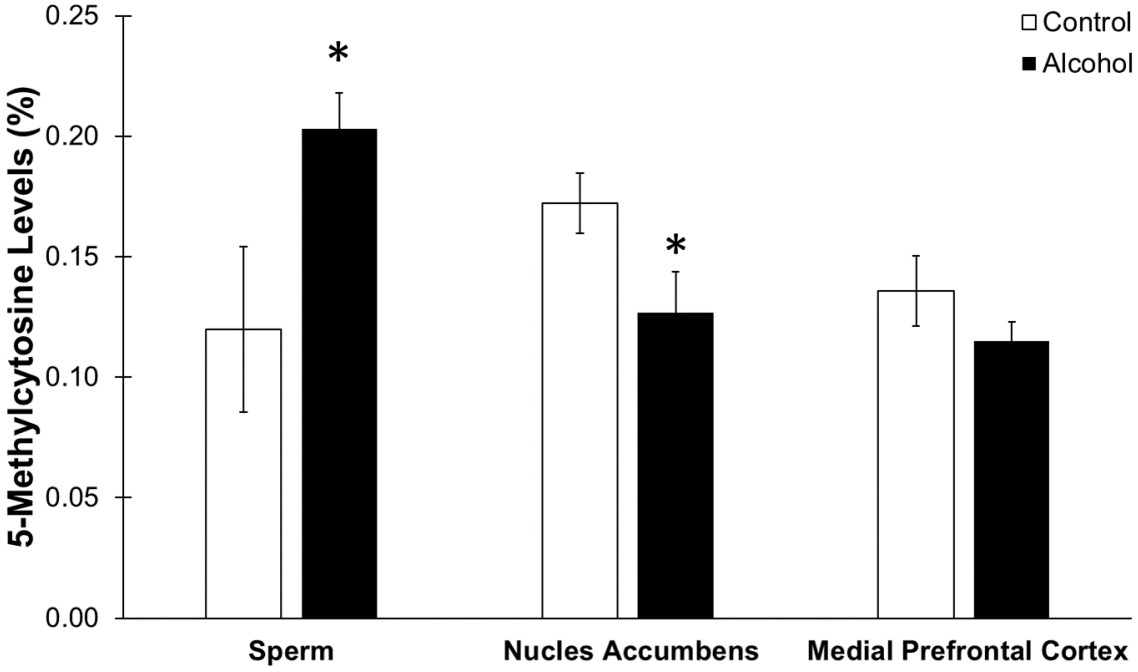

Fig 4.1. Global DNA methylation levels for control (open bars) and alcohol-treated (filled bars) sires. Percentage of Global DNA methylation is presented as mean (±SEM) within sperm, nucleus accumbens, and medial prefrontal cortex of sires. An asterisk (*) represents a significant difference between control and alcohol-treated sires ($p<0.05$).

Global DNA methylation levels in offspring are depicted in Figure 4.2. Global DNA methylation levels in sperm did not differ between A-sired and C-sired male offspring (Panel A; $p>0.05$). There were no significant main effects of Sire, Sex, or a Sire X Sex interaction in the nucleus accumbens (p's>0.05). In the medial prefrontal cortex, there was a significant main effect of Sex, $F(1, 30)= 7.077$, $p<0.05$, $\eta_p^2 = .18$, indicating that females (Panel B) had higher global DNA methylation levels relative to males, but no significant main effect of Sire or a Sire X Sex interaction (p's>0.05).

FIGURE 4.2. GLOBAL DNA METHYLATION IN OFFSPRING

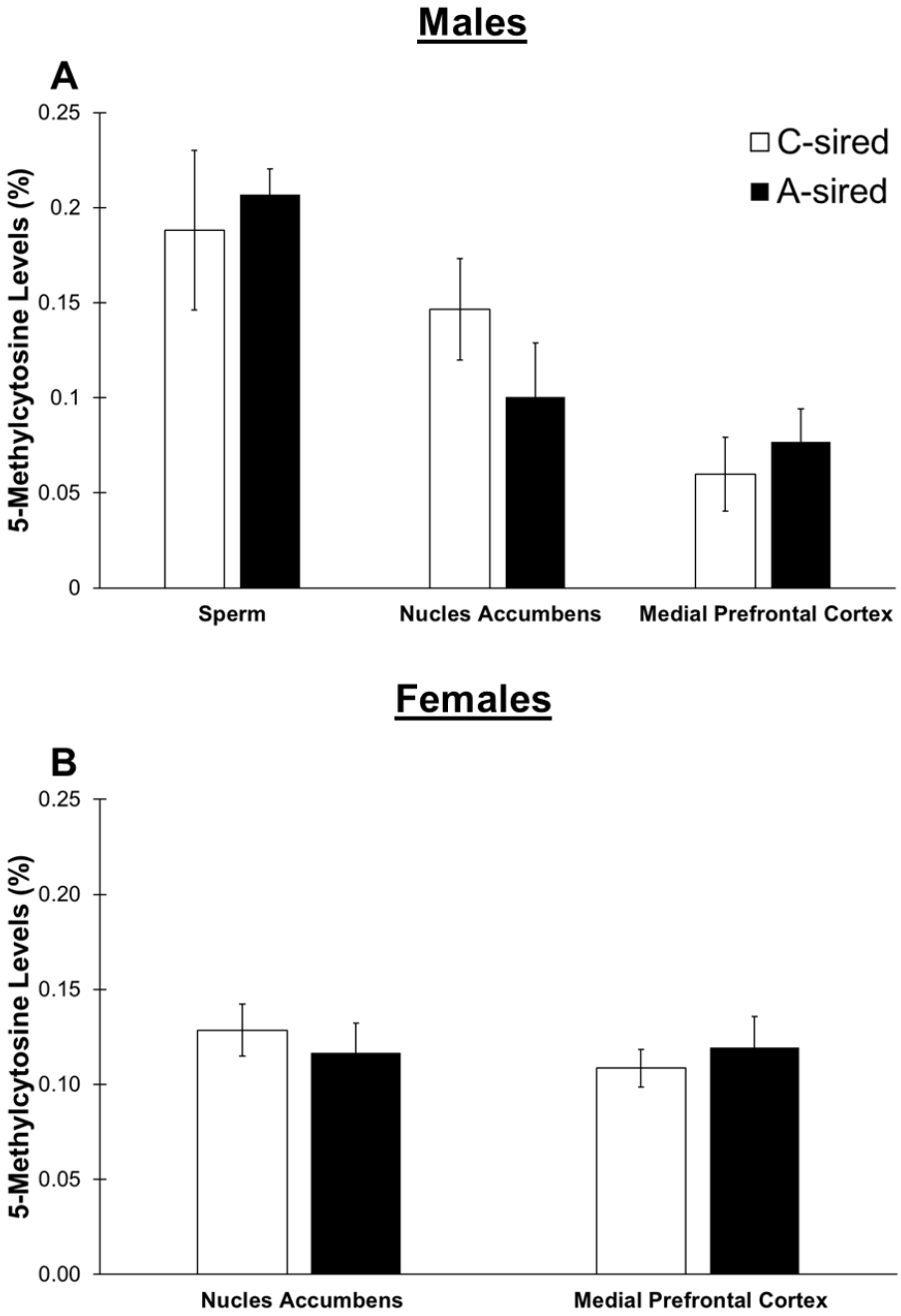

Fig 4.2. Global DNA methylation levels for control- (C-sired; open bars) and alcohol-sired (A-sired; filled bars) offspring. Percentage of Global DNA methylation are presented as mean ($\pm$ SEM) within sperm, nucleus accumbens, and medial prefrontal cortex of male (A) and female offspring (B).

Bdnf DNA methylation levels in sires are depicted in Figure 4.3. In the nucleus accumbens, there were significant main effects of Sire, F(1, 17)= 3.011, p<0.05, $\eta_p^2 = .18$, and CpG site F(11, 154) = 9.865, p<0.001, $\eta_p^2 = .41$. Alcohol-exposed sires had lower DNA methylation levels in the nucleus accumbens relative to air-exposed sires (Panel B). DNA methylation levels varied by CpG site, but there was no interaction with treatment (p>0.05). In the medial prefrontal cortex, there were significant main effects of Sire, F(1, 17)= 3.592, p<0.05, $\eta_p^2 = .20$, and CpG site F(11, 154) = 9.806, p<0.001, $\eta_p^2 = .41$. Alcohol-exposed sires had higher *Bdnf* DNA methylation levels in the medial prefrontal cortex compared to control sires (Panel D). DNA methylation levels varied by CpG site, but there was no interaction with treatment (p=0.126). In sire sperm, there was a significant main effect of CpG site, F(11, 154) = 17.44, p<0.001, $\eta_p^2 = .51$., but not Sire or their interaction (p>0.05; data not shown).

FIGURE 4.3. *BDNF* DNA METHYLATION IN SIRES

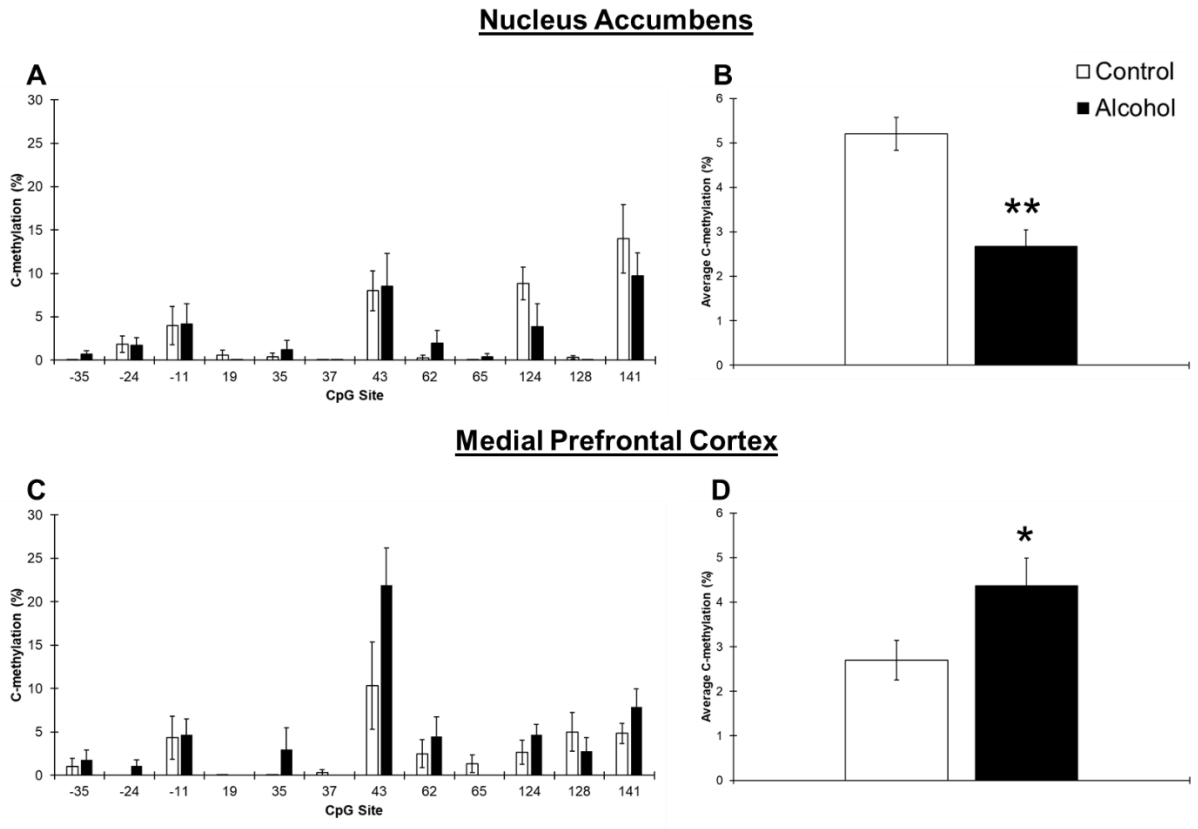

Fig 4.3. *Bdnf* DNA methylation levels for control- (open bars) and alcohol-treated (filled bars) sires. Percentage of DNA methylation at CpG sites are presented as mean (±SEM) within the nucleus accumbens (A) and medial prefrontal cortex (C) of sires. Percentage of methylation levels across CpG sites are presented as mean (±SEM) within the nucleus accumbens (B) and medial prefrontal cortex (D). An asterisk (*) represents a significant difference between control and alcohol-treated sires ($p<0.05$). ** represents a significant difference between control and alcohol-treated sires ($p<0.01$)

Bdnf DNA methylation levels in male and female offspring are depicted in Figure 4.4. In the nucleus accumbens of offspring there was a significant main effect of CpG site, $F(11, 264)= 24.465$, $p<0.05$, $\eta_p^2 = .50$, and a CpG site X Sire X Sex interaction, $F(11, 264) = 2.481$, $p<0.01$, $\eta_p^2 = .09$. Tukey post hoc tests showed that A-sired male offspring had lower methylation at CpG sites -11 and -62 ($p<0.05$) and

higher methylation levels at CpG site 43 (p<0.01; Panel A). A-sired females also had differential methylation patterns that varied by CpG sites. Specifically, A-sired females had lower methylation levels at CpG site -24 (p<0.05) and higher methylation levels at site 141 (p<0.05; Panel C). In the medial prefrontal cortex (Panels B & D), there was a significant main effect of CpG site, F(11, 286)= 14.860, p<0.001, $\eta_p^2 = .36$, but not Sire or Sex or their interactions (p's>0.05). In male offspring sperm, there was a significant main effect of CpG site, F(11, 198)= 15.261, p<0.001, $\eta_p^2 = .46$ but not Sire or their interaction (p's>0.05; data not shown).

FIGURE 4.4. *BDNF* DNA METHYLATION IN OFFSPRING

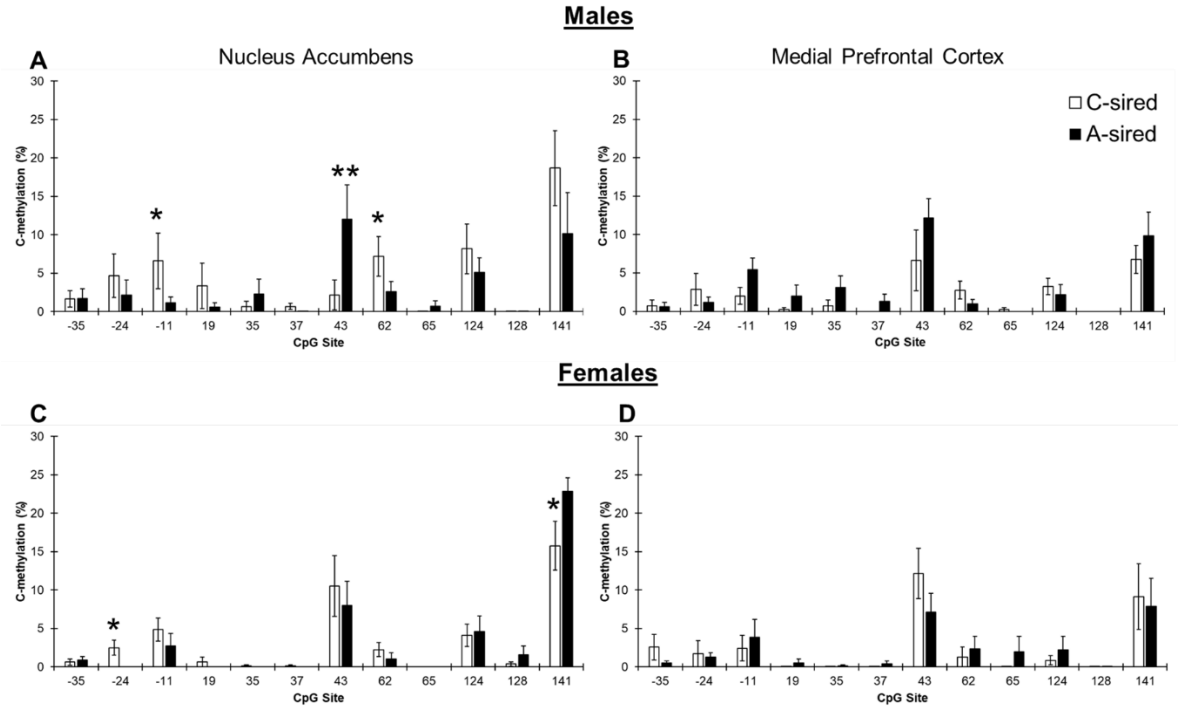

Fig 4.4. *Bdnf* DNA methylation levels for control- (C-sired; open bars) and alcohol-sired (A-sired; filled bars) offspring. Percentage of DNA methylation at CpG sites are presented as mean (±SEM) within the nucleus accumbens of male (A) and female offspring (B). Percentage of DNA methylation at CpG sites are presented as mean (±SEM) within the medial prefrontal cortex of male (C) and female (D) offspring. An asterisk (*) represents a significant difference between C- and A-sired offspring

($p<0.05$). ** indicates a significant difference between C- and A-sired offspring at $p<0.01$.

Discussion

The results of the present study demonstrate that paternal alcohol exposure prior to conception has long-lasting consequences, including the transmission of heritable epigenetic marks to offspring. Specifically, alcohol exposure decreases *Bdnf* DNA methylation levels in the nucleus accumbens but increases methylation levels in the medial prefrontal cortex of sires. Similarly, A-sired offspring of both sexes show differential methylation at specific CpG sites within the *Bdnf* promoter region. This is the first study to examine paternal alcohol-induced changes in global DNA methylation in offspring. Although alcohol exposure alters global DNA methylation levels in sperm and nucleus accumbens of sires, these changes are not maintained in the brains of offspring. Taken together, our results show that paternal alcohol exposure imparts locus-specific changes in DNA methylation levels in offspring.

Altered BDNF signaling has been implicated in neuropsychiatric disorders, including alcoholism (Ghitza et al., 2010; Ron and Messing, 2013; Russo et al., 2009). Preclinical studies support the notion that BDNF is implicated in a homeostatic pathway that influences the negative aspects of alcohol consumption (Ron and Messing, 2013). For example, studies using two bottle-choice and operant self-administration paradigms find that moderate alcohol consumption increases BDNF levels in the dorsal striatum of rodents (Jeanblanc et al., 2009; Logrip et al., 2009; McGough et al., 2004). In addition, reducing BDNF levels using pharmacological or genetic approaches increases alcohol drinking behavior

(Jeanblanc et al., 2009; Jeanblanc et al., 2006; McGough et al., 2004); however, global increases in BDNF levels reduces alcohol intake (Jeanblanc et al., 2009; Jeanblanc et al., 2006; McGough et al., 2004). Similarly, downregulation of BDNF levels in the central or medial amygdala enhances alcohol preference (Pandey et al., 2006). In a similar vein, selectively bred alcohol-preferring rats have reduced innate BDNF levels in the medial and central amygdala (Prakash et al., 2008), but increased levels in the ventral tegmental area and nucleus accumbens of relative to non-preferring rats (Raivio et al., 2014). Alcohol-preferring rats also have a blunted response to alcohol-induced increases in *Bdnf* signaling compared to non-preferring rats. Escalated alcohol intake to levels of intoxication in mice blunts alcohol-induced upregulation of BDNF levels in the dorsal striatum and medial prefrontal cortex (Logrip et al., 2009). Infusion of BDNF into the ventral tegmental area shifts alcohol place preference from a dopamine-dependent to a dopamine-independent behavior (Ting et al., 2013). Taken together, enhanced BDNF levels negatively regulate alcohol drinking behaviors in rodents (Darcq et al., 2015).

Alcohol exposure results in long-lasting changes to *Bdnf* DNA methylation levels in the brain reward circuitry of sires, while differential methylation patterns are transmitted to offspring of both sexes. Specifically, alcohol-exposed sires display lower DNA *Bdnf* methylation levels in the nucleus accumbens and higher methylation levels in the medial prefrontal cortex. It is important to note that we did not find altered *Bdnf* methylation patterns in the sperm of alcohol-exposed sires or A-sired male offspring. However, *Bdnf* DNA methylation may also be recapitulated indirectly via germ line transmission of other epigenetic processes such as

chromatin modifications and non-coding RNA's (Rompala and Homanics, 2019; Vassoler et al., 2013). Several of the CpG sites that have differential *Bdnf* DNA methylation levels in offspring encompass transcription factor binding regions that may influence *Bdnf* gene expression. For example, A-sired males compared to C-sired males have altered methylation at CpG sites -11, 43, and 62; the latter CpG sites are in the *Specificity protein 1 (Sp1)* and *Early growth response protein 1 (Egr1)* transcription factor binding sites (AliBaba2.1), respectfully. The *Sp1* transcription factor is involved in many cellular processes (e.g., cellular differentiation, cell growth, apoptosis, immune responses) and chromatin remodeling, specifically recruitment of histone acetyltransferases (Sun et al., 2009). Additionally, the *Egr1* transcription factor is involved in brain development, neural plasticity and DNA methylation, specifically in DNA demethylation by recruiting the Ten-Eleven Translocation 1 protein (Sun et al., 2019). A-sired females compared to C-sired females also have altered *Bdnf* methylation patterns at CpG sites -24 and 141 (AliBaba2.1), both are in *Sp1* transcription factor binding sites. Given that altered methylation is observed at *Sp1* binding sites in both A-sired male and female offspring, it is likely that differential *Bdnf* methylation patterns in combination with inherited histone modifications may contribute to altered *Bdnf* signaling. Indeed, previous work in mice shows that alcohol exposure in sires decreased *Bdnf* methylation in sperm (Finegersh and Homanics, 2014). Lower *Bdnf* methylation levels are recapitulated in the ventral tegmental area, a key dopamine-rich pathway involved in reward circuitry, but not the medial prefrontal cortex, of A-sired male and female offspring. Interestingly, this epigenetic profile associates with decreased

alcohol preference and consumption selectively in A-sired male offspring (Finegersh and Homanics, 2014). Similarly, epigenetic-driven intergenerational changes in BDNF signaling within reward circuitry have been noted in other paternal alcohol and cocaine studies (Ceccanti et al., 2016; Rompala et al., 2017; Vassoler et al., 2013).

The method and duration of alcohol exposure in sires likely influence the site of *Bdnf* methylation levels in sires and offspring. Vapor vs oral administration of alcohol impart differential effects on BDNF signaling. Sires that consume alcohol in drinking water have offspring with higher BDNF protein levels in the prefrontal cortex (Ceccanti et al., 2016); whereas, no changes in prefrontal *Bdnf* mRNA expression or DNA methylation levels are observed in offspring alcohol vapor-exposed sires (Finegersh and Homanics, 2014). We also show a lack of *Bdnf* DNA methylation changes in the medial prefrontal cortex in offspring of alcohol vapor-exposed males. Indeed, behavioral consequences of paternal alcohol exposure in offspring differ depending on route of alcohol administration (Beeler et al., 2019). Future work is needed to further assess the influence of voluntary vs involuntary methods of paternal alcohol administration on epigenetic mechanisms.

In summary, we find that paternal alcohol exposure results in aberrant *Bdnf* DNA methylation patterns in sires and offspring. Specifically, we observe that alcohol exposure results in higher levels *Bdnf* DNA methylation levels in the prefrontal cortex and lower methylation levels in the nucleus accumbens compared to control sires. In A-sired offspring, differential *Bdnf* DNA methylation patterns in the nucleus accumbens are also seen at certain CpG sites. Importantly, changes in *Bdnf* DNA methylation are not due to changes in global DNA methylation levels. Overall,

our work compliments and extends previous work by demonstrating that paternal alcohol exposure several weeks prior to conception has long-lasting epigenetic consequences in both male and female offspring.

CHAPTER FIVE- OVERALL CONCLUSIONS

Conclusions

The heritability of AUD is ~50%, yet the genetic basis of the disease is still poorly understood. Findings from genome-wide association studies have been questioned due to lack of replication and technical limitations. Missing heritability of AUD can be explained, at least in part, by epigenetic processes. Epigenetic mechanisms are heritable molecular factors that influence gene expression without changing the underlying DNA sequence. Ancestral environment can impact future generations through aberrant epigenetic mechanisms. In particular, paternal exposure to environmental insults in periods prior to conception (e.g., drug, stress) can have long-lasting behavioral and physiological changes in offspring that may be mediated by transmission of epigenetic factors. Whether paternal alcohol exposure alters behavioral responsivity to alcohol is an understudied area.

The purpose of this dissertation project is to use outbred rats to determine whether paternal alcohol exposure impacts offspring sensitivity to the unconditioned effects of alcohol, operant alcohol self-administration, and global and *Bdnf* DNA methylation levels in sperm and brain tissue.

Aim 1: Determine whether paternal alcohol exposure alters sensitivity to the unconditioned effects of alcohol in offspring. (Chapter 2)

We hypothesize that male A-sired offspring will show altered sensitivity to the unconditioned effects of alcohol. Specifically, A-sired male offspring, but not female, will exhibit greater alcohol-induced general locomotor activity on the open field test

compared to C-sired male offspring. In addition, A-sired male offspring will show greater alcohol-induced anxiolysis on the open field test and elevated plus maze relative to C-sired male offspring. Lastly, A-sired male offspring will be less sensitive to the alcohol-induced motor coordination impairments compared to C-sired male offspring.

The open field test provides measures of general locomotor activity and anxiety-like behavior. A-sired offspring do not differ from C-sired offspring on general locomotor activity across the 60 min test. While alcohol treatment lowers anxiety-like behavior as indicated by increases in time spent in the center of the open field area selectively in A-sired male offspring, A- and C-sired offspring did not differ in their responses to alcohol. These results agree with a previous study in mice showing that A-sired and C-sired offspring do not differ on open field behaviors (Finegersh and Homanics, 2014). Thus, paternal alcohol does not alter sensitivity to alcohol's effects on general locomotor activity or anxiety-like behavior measured on the open field test.

The elevated plus maze provides a widely-used and valid measure of anxiety-like behavior. We find that A-sired males have a more anxiolytic phenotype indicated by greater percentages of time spent in the open arms and lower percentage of time spent in the closed arms of the maze; however, contrary to our hypothesis, A-sired offspring do not differ from C-sired offspring after alcohol treatment. These results are contrary to previous studies in mice showing that A-sired male offspring are more sensitive to the anxiolytic-effects of alcohol compared to C-sired males (Finegersh and Homanics, 2014; Rompala et al., 2017). Previous work in mice used

genetically similar mouse strains, thus, a lack of agreement between studies may reflect differences in species and genetic background. Nonetheless, paternal alcohol does not alter sensitivity to alcohol's effects on anxiety-like behavior indexed on the elevated plus maze in rats.

Rotarod performance is an index of motor coordination in rodents. A-sired offspring do not differ from C-sired offspring on the training trials indicating intact procedural memory. On the test day, A-sired males given alcohol are less sensitive to alcohol's motor coordination impairing effects relative to alcohol-treated C-sired males. Conversely, A-sired female offspring are more sensitive to alcohol-induced impairments in motor coordination. These results corroborate and extend previous work using mice (Finegersh and Homanics, 2014). However, paternal alcohol effects on rotarod performance are not consistent across studies and depend on the genetic profile of mice (Rompala et al., 2017).

Sensitivity to alcohol is a candidate endophenotype of AUD. Generally, individuals with a family history of alcohol misuse display greater sensitivity to the stimulating/rewarding effects and blunted sensitivity to the sedative effects of alcohol at peak blood alcohol levels. Interestingly, this profile increases the risk of developing an AUD. In Aim 1, we show robust effects of paternal alcohol exposure specifically on alcohol-induced impairments in motor coordination which occur in a sex-dependent manner. Thus, these results likely reflect complex interactions between paternal alcohol exposure, alcohol treatment, and organizational/activational effects of sex hormones.

Aim 2: Determine whether paternal alcohol exposure alters operant alcohol self-administration. (Chapter 3)

We hypothesize that A-sired male offspring will show an alcohol-resistant phenotype on alcohol self-administration. Specifically, A-sired males will acquire alcohol self-administration slower than C-sired males. A-sired males will also show lower motivation during progressive ratio tests. Finally, A-sired males will exhibit less alcohol craving- and relapse-like behaviors as measured during extinction training, cue-induced reinstatement tests, and reinitiation sessions.

Acquisition of operant self-administration measures acute drug/alcohol taking behaviors and represent a transition from sporadic to stable levels of responding. A-sired offspring do not differ in the number of sessions to acquire food, water, or alcohol self-administration. Interestingly, during the alcohol training period, A-sired offspring have lower responding which varies by sex and time. A-sired male offspring exhibit lower responding during initial sessions while A-sired female offspring have lower responding during later sessions. By the end of the FR1 training sessions, the sire groups do not differ in level of alcohol responding. Alcohol responding does not differ when the schedule of reinforcement increases to FR2 and FR4.

Progressive tests provide a measure of an animal's motivation to obtain a reinforcer. During PR tests, the response requirement gradually increases for an animal to obtain a single alcohol reinforcer. A-sired male and female offspring show lower motivation for 5% and 10% alcohol compared to C-sired offspring. Extinction training is an index of craving-like behavior and is measured by an animal's

persistence of responding in the absence of the reinforcer. When alcohol is replaced with water, A-sired offspring have lower active lever presses compared to C-sired offspring. Yet, both sire groups do no reach extinction criteria until all cues are eliminated.

Cue-induced reinstatement and reinitiation sessions are measures of relapse-like behaviors. Two reinstatement tests are conducted in the absence of the reinforcer wherein a press on the active lever will activate light and dipper cues. Alcohol odor is present in the operant chamber in one reinstatement session; the order of reinstatement tests is counterbalanced. During cue-induced reinstatement sessions, A-sired offspring of both sexes have fewer active lever presses relative to C-sired offspring, specifically when alcohol odor is present in the operant chamber. When animals are given access to 5% alcohol for one week during reinitation sessions, A-sired offspring show lower alcohol responding relative to C-sired offspring.

Operant self-administration is the gold standard in addiction research and is used to measure various aspects of the addiction cycle. While environmental and genetic insults can alter self-administration behaviors, the effects of paternal alcohol exposure on the propensity to develop addiction-like behaviors is an understudied area of research. In Aim 2, we provide robust evidence that paternal alcohol exposure contributes to an alcohol-resistant phenotype in offspring. Although, sex moderates paternal alcohol effects during acquisition training, we do not observe a robust moderating role of sex on self-administration behaviors as previously shown for two-bottle choice tests (Finegersh and Homanics, 2014) and paternal cocaine

studies (Vassoler et al., 2013). These results are in line with previous work showing that hormonal influences are more influential during acquisition of alcohol self-administration and are less influential after stable responding is established (Becker and Koob, 2016).

Aim 3: Determine whether paternal alcohol exposure alters DNA methylation levels in offspring. (Chapter 4)

We hypothesize that paternal alcohol exposure will alter global and *Bdnf* DNA methylation levels in offspring. Specifically, alcohol exposure in sires will increase global and *Bdnf* DNA methylation levels in sperm, nucleus accumbens, and medial prefrontal cortex. Additionally, the same epigenetic profile will be maintained in sperm and brain regions of offspring.

We measure global methylation using ELISA kits and *Bdnf* DNA methylation using bisulfite-treated DNA and direct sequencing methods. Alcohol-treated males have greater global methylation levels in sperm but lower methylation levels in the nucleus accumbens relative to control males. However, paternal alcohol exposure does not alter global methylation levels in sperm, nucleus accumbens, or medial prefrontal cortex in offspring. Additionally, A-sired males have lower *Bdnf* methylation levels in the nucleus accumbens and greater methylation levels in the medial prefrontal cortex. There is no difference in *Bdnf* methylation levels in sperm. A-sired offspring also show aberrant *Bdnf* DNA methylation patterns in the nucleus accumbens that varied by CpG site and sex. A-sired female offspring have higher *Bdnf* methylation levels at CpG site 141 and lower methylation levels at CpG site -24

compared to C-sired males. A-sired male offspring have greater *Bdnf* methylation levels at CpG site 43 and higher methylation levels at CpG sites -11 and 62 compared to C-sired male offspring. No differences in *Bdnf* DNA methylation levels are seen in the medial prefrontal cortex or sperm of offspring.

Epigenetic mechanisms are likely involved in the phenotypes observed in this study. DNA methylation in the promoter region of a gene often suppresses gene activity. However, paternal alcohol exposure does not alter global DNA methylation levels in offspring, indicating that paternal alcohol use results in locus-specific changes in DNA methylation levels. Given that BDNF signaling is a well-established regulator of drug and alcohol-seeking behavior in rodents, it is pertinent to assess *Bdnf* DNA methylation levels in sperm and reward-related circuitry. In Aim 3, we provide evidence that paternal alcohol exposure alters *Bdnf* DNA methylation levels in offspring. Several of the CpG sites that have differential *Bdnf* DNA methylation levels encompass transcription factor binding regions that may influence *Bdnf* gene expression. This finding supports and extends previous paternal studies implicating altered BDNF activity in offspring's sensitivity to cocaine and alcohol (Finegersh and Homanics, 2014; Vassoler et al., 2013). Importantly, sires are mated 8 weeks after their last alcohol or control session; thus, paternal alcohol exposure has *long-lasting* effects on *Bdnf* DNA methylation levels that are transmitted to offspring.

Limitations

This dissertation project has many strengths, including the use of an outbred rat strain, inclusion of both sexes, a wide range of operant self-administration behavior, etc.; however, there are some limitations. In our study, blood alcohol levels

of sires are kept at a range previously shown to induce dependence, but sires are not screened for behaviors that reflect AUD symptomology in humans. As an example, motivation for alcohol in sires in unclear. It is possible that paternal alcohol effects in offspring may differ depending on sire's motivation for alcohol. Indeed, paternal cocaine studies show that cocaine sires with high motivation for cocaine have offspring that self-administer greater cocaine than offspring of sires with low motivation for cocaine (Le et al., 2017). Although, we did not assess for changes in metabolism between sire groups, previous work in mice demonstrates that pharmacokinetic differences are unlikely (Finegersh and Homanics, 2014). In a similar vein, it is unclear whether A-sired offspring are responding for alcohol at pharmacologically relevant levels during self-administration or if taste of alcohol influences self-administration levels. However, these may be marginal considerations given that A-sired offspring find alcohol reinforcing and by the end of the acquisition training the sire groups are responding at equivalent levels. Additionally, we did not measure *Bdnf* mRNA or protein levels; thus, it is unclear whether changes in *Bdnf* DNA methylation levels alter gene activity. Lastly, we did not monitor estrus cycle, so it is possible that some paternal effects are masked by sex hormones.

Future Directions

There is a lack of consilience between human and preclinical work investigating paternal alcohol effects. While children of alcoholics have an increased risk of developing an AUD, paternal alcohol exposure in rodents imparts an alcohol-resistant phenotype. As mentioned above, screening rodent sires for behaviors that

reflect AUD symptomology in humans may reconcile these differences. The extent to which maternal behavior is influenced by paternal environment should also be further examined. In addition, novel gene editing tools that can apply locus-specific CpG methylation in the germline will help to determine the validity of paternal alcohol effects on the sperm epigenome. It is also important that future work in this area examine paternal alcohol effects on other epigenetic mechanisms. Sperm RNA may have a causal role in paternal effects. Paternal diet and stress studies find that injecting embryos with affected paternal sperm RNA can recapitulate phenotypes seen in offspring.

Final Comments

This dissertation project examines the effects of paternal alcohol exposure. We show that paternal alcohol exposure alters sensitivity to alcohol-induced impairments in motor coordination in offspring. Furthermore, paternal alcohol exposure induces an alcohol-resistant phenotype on alcohol self-administration behaviors that model various aspects of the addiction cycle. Lastly, paternal alcohol exposure alters *Bdnf* methylation levels in offspring of both sexes. Overall, this dissertation project concludes that paternal alcohol exposure imparts long-lasting behavioral and epigenetic consequences in rat offspring.

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