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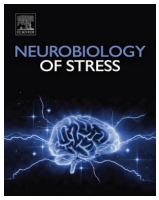

Inter- and transgenerational heritability of preconception chronic stress or alcohol exposure: Translational outcomes in brain and behavior

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ABSTRACT

Chronic stress and alcohol (ethanol) use are highly interrelated and can change an individual's behavior through molecular adaptations that do not change the DNA sequence, but instead change gene expression. A recent wealth of research has found that these nongenomic changes can be transmitted across generations, which could partially account for the "missing heritability" observed in genome-wide association studies of alcohol use disorder and other stress-related neuropsychiatric disorders. In this review, we summarize the molecular and behavioral outcomes of nongenomic inheritance of chronic stress and ethanol exposure and the germline mechanisms that could give rise to this heritability. In doing so, we outline the need for further research to: (1) Investigate individual germline mechanisms of paternal, maternal, and biparental nongenomic chronic stress- and ethanol-related inheritance; (2) Synthesize and dissect cross-generational chronic stress and ethanol exposure; (3) Determine cross-generational molecular outcomes of preconception ethanol exposure that contribute to alcohol-related disease risk, using cancer as an example. A detailed understanding of the cross-generational nongenomic effects of stress and/or ethanol will yield novel insight into the impact of ancestral perturbations on disease risk across generations and uncover actionable targets to improve human health.

1. Introduction

High (≥30%) heritability is observed in alcohol use disorder (AUD) and other stress-related neuropsychopathologies such as anxiety disorders, depressive disorders, and post-traumatic stress disorder (PTSD) (Duncan et al., 2017, 2018; Sartor, 2012; Gottschalk and Domschke, 2022; Purves et al., 2019; Verhulst et al., 2014). Until recent years, heritability studies of stress-related disorders and their phenotypes focused exclusively on genomic inheritance described by classical Mendelian genetics, wherein the DNA sequence carried by germ cell alleles for individual genes confers phenotypes observed in the progeny (Hayden and Nichols, 1983; Clé et al., 1997; Day and Bonduriansky, 2011). Genome-wide association studies have identified many genetic variations associated with stress-related disorders, (Purves et al., 2019; Coleman et al., 2020; Malan-Mü et al., 2014; Morimoto et al., 2020; Lewis et al., 2010; Stoychev et al., 2021) including AUD, (Stoychev et al., 2021; Johnson et al., 2023; Deak et al., 2022; Zhou et al., 2022) but these variations only account for a small fraction of heritability, leaving a substantial amount of missing heritability or unexplained variance (Malan-Mü et al., 2014; Morimoto et al., 2020; Trerotola et al., 2015; Manolio et al., 2009; Nadeau, 2009; Eichler et al., 2010). Consequently, research on stress-related disorder inheritance has increasingly delved into nongenomic inheritance, which considers transmission of environment-induced, epigenetic effects on gene expression that do not alter the underlying DNA sequence (Bird, 2007). These epigenetic mechanisms include DNA methylation and histone

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modifications as well as noncoding RNA regulation of gene expression that are hypothesized to account for missing heritability (Morimoto et al., 2020; Trerotola et al., 2015; Manolio et al., 2009; Nadeau, 2009).

Nongenomic inheritance can be divided into intergenerational and transgenerational inheritance (Baratta et al., 2021). These terms are often confused, even within the scientific community. When a founder generation (F0) individual is exposed to an environmental perturbation such as chronic stress or ethanol, its germ cells (Fig. 1A) and in the case of pregnant females (Fig. 1B), the primordial germ cells of any fetus concurrently in gestation, are also exposed (Baratta et al., 2021). Intergenerational inheritance refers to phenotypic changes in individuals derived from directly exposed germ cells. In contrast, transgenerational inheritance refers to phenotypes observed in individuals that were not derived from directly exposed germ cells, but instead inherited changes in gene expression from a previous generation that was directly exposed (Baratta et al., 2021). Thus, with preconception exposure of male or female F0 individuals, intergenerational inheritance occurs in the F1 generation and transgenerational inheritance occurs in the F2 generation and beyond (Fig. 1A). If female F0 exposure occurred while pregnant (gestational exposure), transgenerational inheritance occurs only after the F3 generation, with intergenerational inheritance in F1 and F2 individuals (Fig. 1B). This review specifically focuses on chronic preconception exposure to stress and ethanol. Cross-generational (an umbrella term encompassing both inter- and transgenerational-) inheritance of prenatal/gestational stress and ethanol exposure are reviewed elsewhere (Mead and Sarkar, 2014; Mbiydzenyuy et al., 2022; Ambeskovic et al., 2020; Saboory et al., 2019; Taouk and Schulkin, 2016).

Some of the first studies demonstrating nongenomic inheritance investigated the effects of maternal care on offspring stress reactivity (Francis et al., 1999; Rosenblatt, 1975; Champagne and Meaney, 2001; Liu et al., 1997). Since then, a large body of research has been dedicated

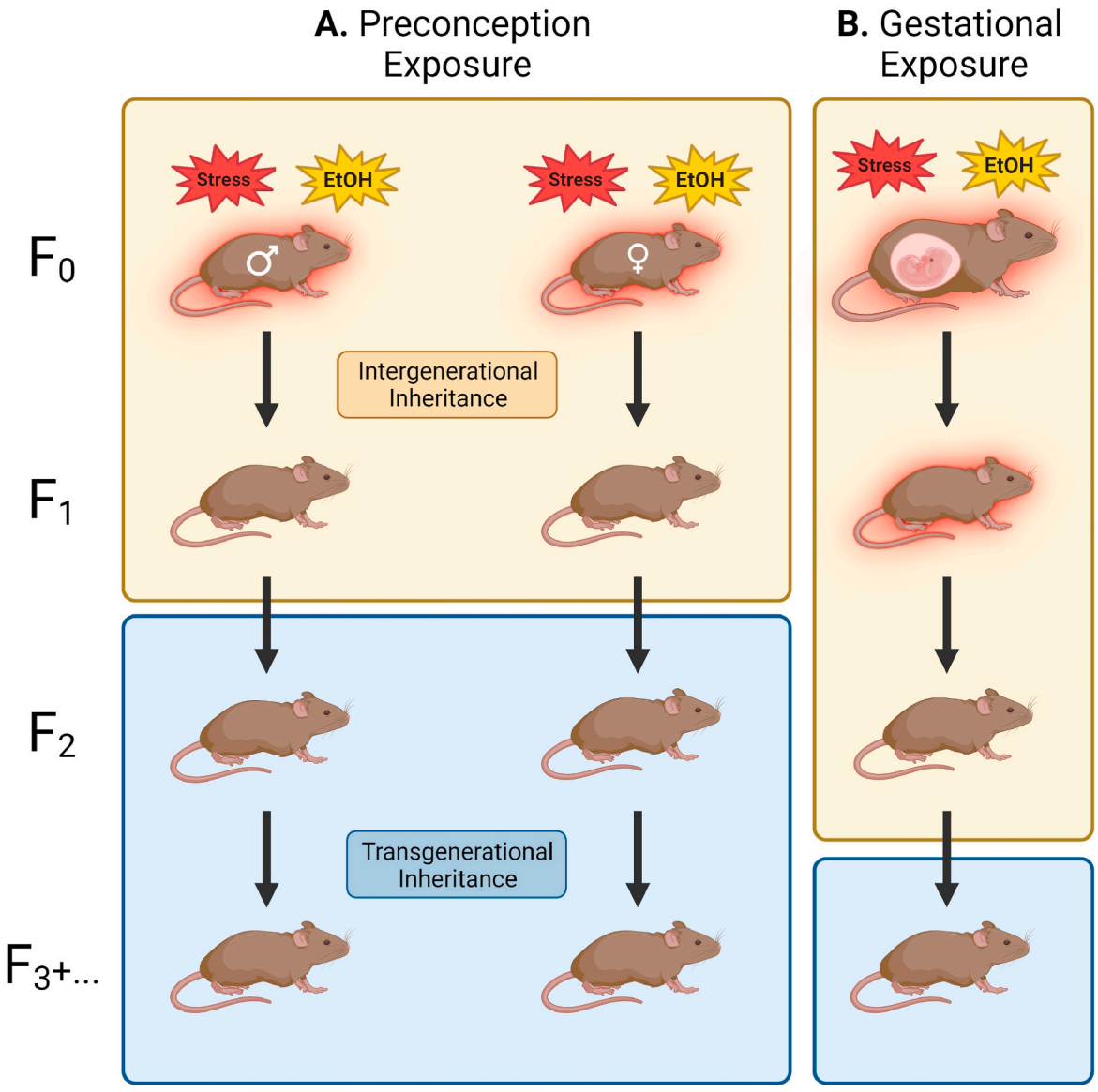

Fig. 1. Comparison of nongenomic inheritance patterns, using mice as an example. (A.) Preconception ethanol or chronic stress exposure in a male or female F0 individual also exposes its germ cells. Inheritance of phenotypes in F1 offspring due to direct exposure of germ cells in the previous generation is referred to as intergenerational inheritance, while inheritance of phenotypes in subsequent generations (which are not derived from directly exposed germ cells) is referred to as transgenerational inheritance. (B.) Ethanol or chronic stress exposure during pregnancy exposes both the germ cells of the F0 individual and the primordial germ cells of the fetus. Thus, intergenerational inheritance is observed in F1 and F2 individuals, and transgenerational inheritance is observed in F3 and subsequent generations. *Created using Biorender.com.*

to understanding nongenomic inheritance mechanisms of stress-related phenotypes. Stress heritability research is more extensive than ethanol heritability research and provides valuable insight into potential mechanisms of nongenomic inheritance that can guide future cross-generational ethanol studies. Indeed, chronic heavy alcohol consumption itself is a stressor that dysregulates the stress response, (Becker, 2017) and AUD and other stress-related disorders (*i.e.,* anxiety and depressive disorders, PTSD) display high comorbidity as well as shared pathogenetic mechanisms (Anker, 2019; McHugh, 2019; Castillo-Carniglia et al., 2019; Suh and Ressler, 2018).

The following literature review summarizes evidence across species for inter- and transgenerational inheritance of chronic preconception stress- or ethanol exposure effects, including alcohol use disorder-related phenotypes. In conducting this review, we hypothesized that cross-generational chronic preconception stress and alcohol exposure share common mechanisms and neurobiological outcomes that can be further studied to elucidate the pathogenesis of AUD, its comorbid conditions, and health consequences using cancer as an example. We outline stress- and alcohol-related behaviors observed in subsequent generations, the central nervous system adaptations that may give rise to these behaviors, and the germline transmission mechanisms that could confer these behavioral and molecular phenotypes. Finally, we suggest avenues for future research on cross-generational heritability of chronic stress- and ethanol-induced phenotypes.

2. Animal models of inter- and transgenerational chronic preconception stress exposure

2.1. Behavioral outcomes of chronic preconception stress across generations in animals

The glucocorticoid-mediated stress response is centrally regulated by the hypothalamic-adrenal-pituitary (HPA) axis (Herman et al., 2016; Smith and Vale, 2022). HPA axis stimulation releases glucocorticoids into the bloodstream, which act on multiple organ systems to mobilize the organism against the stressor; This includes the central nervous system, where glucocorticoids additionally serve to "shut off" the stress response through negative feedback inhibition of the HPA axis (Myers et al., 2012, 2014; Herman et al., 2012; Keller-Wood and Dallman, 1984). The glucocorticoid stress response is a homeostatic mechanism, and the HPA axis adapts to better prepare the organism for future stressors (Herman et al., 1995, 2016). However, this is a double-edged sword, as repeated or prolonged HPA axis activation by chronic stress leads to dysregulation in its functioning, which can be passed down to offspring along with stress-related behaviors (Herman et al., 1995, 2016; Dhabhar et al., 1997; Chrousos, 2009; Fisher and Reason, 1988; Federenko et al., 2004). As we discuss in this review, heritability of preconception stress-related phenotypes is context-dependent. It is important to note that the HPA axis is one of multiple brain stress-responsive systems (Godoy et al., 2018; Morris et al., 2020). However, the majority of preconception stress and ethanol exposure studies focus on HPA axis- and glucocorticoid-related mechanisms, as reviewed here.

The term "stressor" refers to any stimulus, endogenous or exogenous, that has the capacity to change the homeostatic state (Herman et al., 2016; Chrousos, 2009; Selye, 1956). Thus, there are many preclinical models of stress that encompass myriad types of stressors, from social factors to disease. The current review focuses specifically on chronic, behavioral preconception stress induction paradigms commonly used to study stress-related psychopathology such as anxiety and depression. In animal studies, these include chronic unpredictable stress (Monteiro et al., 2015; Katz, 1982; Willner et al., 1987; D'Aquila et al., 1994; Tannenbaum et al., 2002) (CUS, also sometimes referred to as chronic variable stress), chronic social defeat stress (CSDS), (Krishnan et al., 2007; Golden et al., 2011; Kudryavtseva et al., 1991) and early life stress specifically induced by maternal separation with unpredictable stress (MSUS) (Mansuy et al., 2011; Franklin et al., 2010). For further reviews that investigate other forms of stress (*e.g.,* nutrient deficiencies or pathogen infections) and trauma exposure, we recommend the following: Chan et al. (2018); Duffy et al. (2021); Tan et al. (2023); Batchelor and Pang (2019); Siddeek et al. (2018). For clarity and consistency both within the literature and across paradigms discussed, this review will refer to the directly stressed animal as the parental/founder generation (F0), and following mating, the initial generation of offspring produced will be referred to as the first filial generation (F1).

Paternal preconception stress (PPS) induces inter- and transgenerational anxiety- and depression-like phenotypes as well as alterations in HPA axis responsivity measured via plasma corticosterone (CORT; Table 1). However, these phenotypes can differ based on the PPS induction paradigm utilized as well as the sex of individuals in subsequent generations. For example, in mice, plasma CORT in adult male and female PPS offspring exposed to acute restraint stress was decreased in a chronic unpredictable stress (CUS) paradigm, but no effects of CUS were found in anxiety-like behavior observed in the light-dark box (Rodgers et al., 2013). In a paternal CSDS paradigm, basal CORT increased only in adult male offspring, with no effects in females nor in response to acute restraint stress (Dietz et al., 2011). Adult male and female offspring sired post-CSDS displayed decreased open arm time in the elevated plus maze (EPM) and decreased latency to immobility in the forced swim test (FST) relative to their siblings sired pre-CSDS (Dietz et al., 2011). Male, but not female, offspring showed increased locomotor behavior in a novel environment, decreased sucrose preference, and increased basal plasma CORT (Dietz et al., 2011).

Sex- and generation-dependent behavioral phenotypes have been observed in the progeny of sires exposed to maternal separation with unpredictable stress (MSUS). The MSUS paradigm of PPS can produce transgenerational alterations in behavior up to the fifth generation in male mice (Boscardin et al., 2022). Interpretation of these effects requires a synthesis of multiple studies (Table 2). In the first generation, males and females have shown decreased latency to first enter an open arm in the EPM (Franklin et al., 2010; Gapp et al., 2014a). Female F1s have additionally demonstrated increased floating time in the forced swim test (FST) as well as decreased latency to enter an unfamiliar area in a free exploratory paradigm or enter the center in the open field test (OFT) (Franklin et al., 2010). In the second generation, males displayed increased FST floating time (Franklin et al., 2010) and decreased latency to first enter the EPM open arm, (van Steenwyk et al., 2018) whereas females continued to have decreased latency to enter an unfamiliar area or to enter the OFT center (Franklin et al., 2010). In the third generation, increased open arm time and decreased latency to first enter the open arm of the EPM was observed in males and females (van Steenwyk et al., 2018). F4 and F5 effects were specific to males. Interestingly, F4 males showed decreased EPM open arm time, but increased EPM open arm time was restored in F5 males (Boscardin et al., 2022). While many of the above phenotypes (*i.e.,* increased EPM open arm time, decreased latency to enter EPM open arm or OFT center time) may appear to suggest that the parental exposure resulted in reduced anxiety-like behaviors in the progeny, there have been other interpretations of these effects. Repeated OFT in one study led authors to interpret their findings as a behavioral control deficit in response to novelty (Franklin et al., 2010). Similar to this interpretation, a 2023 study using maternal separation (not the full MSUS paradigm) found the mice displayed impaired risk assessment in response to a predator odor and increased dominance in the social dominance tube task, both in male offspring (Thivisol Ulysse et al., 2023). One study conducted delay-discounting and behavioral sequencing tasks, which revealed enhanced goal-directed behaviors and behavioral flexibility in adult female offspring (Gapp et al., 2014a). Another study further showed memory deficiencies in offspring of MSUS-exposed males in fear-conditioning and novel object recognition paradigms (Bohacek et al., 2014). These findings suggest MSUS can foster both protective and vulnerable phenotypes across generations. Indeed, one study found male-specific first and second generation deficits in social interaction and social memory, but male

Table 1
Behavioral and molecular (in brain) outcomes of paternal preconception stress (PPS) in subsequent generations across published literature.

Study	Species/Strain	Paradigm	Time between Exposure & Mating	Key Behavioral Outcomes	Key Molecular Outcomes
Franklin et al. (2010)	C57BL/6J mice	MSUS PND 1-14	–	–	Altered *Mecp2, Cnr1, & Crfr2* DNA methylation & mRNA expression preserved from F0 sperm to female F1 brain: *Mecp2* & *Cnr1:* ↑ methylation, ↓ mRNA expression *Crfr2:* ↓ methylation, ↑ mRNA expression *Mecp2* & *Crfr2* methylation patterns preserved in male F1 sperm
Franklin et al. (2011)	C57BL/6J mice	MSUS PND 1-14	–	**F1 Males:** ↓ social exploration ↓ avoidance of aggressor odor cues during CSDS ↓ anhedonia during CSDS **F2 Males:** ↓ social exploration	**F1 Males:** ↓ 5-HT_{1A}R binding lateral periaqueductal gray, dorsal raphe, and hippocampus (CA1 & DG)
Bohacek et al. (2014)	C57BL/6JRj mice	MSUS PND 1-14	–	**F1 Females:** ↑ FST floating time **F1 (sex not specified):** ↓ freezing time after fear conditioning ↓ long-term object memory	**F1 (both sexes):** Altered hippocampal and lateral amygdalar LTP/LTD **F1 Females:** downregulated plasticity-related genes in hippocampus
Gapp et al. (2014a)	C57BL/6 mice (Substrain not specified)	MSUS PND 1-14	–	**F1 Females:** ↓ EPM open arm entry latency ↑ goal-directed behaviors in delay-discounting task ↑ behavioral flexibility in behavioral sequencing task **F1 Males:** ↑ behavioral flexibility in differential reinforcement of lower rates paradigm	–
Gapp et al. (2014b)	C57BL/6J mice	MSUS PND 1-14	–	–	**F0:** Alterations in sperm miRNA Changes in serum & hippocampus miRNA expression preserved from F0 to male F1 Injecting purified RNA from F0 sperm into fertilized wild-type oocytes recapitulates changes in miRNA expression in serum and hippocampus of directly-sired male F1s
Gapp et al. (2016)	C57BL/6J mice	MSUS PND 1-14	–	-	**F0:** ↓ *Nr3c1* methylation in sperm, ↑ expression in hippocampus **F1 Males:** ↓ *Nr3c1* methylation, ↑ expression in hippocampus
Thivisol Ulysse et al. (2023)	C57BL/6J mice	Maternal separation PND 3-14	–	**F1 Males:** ↓ reward latency following predator scent	–
Niknazar et al. (2017)	Wistar rats	Forced swim stress for 3 weeks	–	**F1 (both sexes):** ↓ EPM open arm time ↑ CORT	**F1 (both sexes)** ↑ *Nr3c1* methylation, ↓ expression in hippocampus
Mashoodh et al. (2023)	Balb/C mice	Restraint or forced swim stress for 6 weeks	–	**F1 Males:** ↑ OFT center time ↓ FST immobility **F1 Females:** ↑ FST immobility	**F1 Males:** ↓ *Bdnf* in hypothalamus on PND6 **F1 Females:** ↑ *Crh* in hypothalamus on PND6
Kong et al. (2021)	Kunming mice	Restraint or CSI 8 weeks beginning in adolescence (PND 26–28)	5 days	–	Identified "candidate anxiety genes" with differential methylation & gene expression in F0 sperm and adult female F1 hippocampus: *Adora2a, Bdnf, Itpr3, Gata2.* These candidate anxiety genes have no methylation/ expression changes in male hippocampi.
Rodgers et al. (2013)	C57BL/6:129 mice	CUS 6 weeks either beginning in adolescence (PND 28) or adulthood (PND 56)	2 weeks	**F1 (both sexes):** ↓ CORT in response to restraint stress observed in both adolescent and adult CUS sire groups	**F0:** Alterations in sperm miRNA abundance overlapping between adolescent & adult-CUS exposed sires: *miR-193-5p, miR-204, miR-29c, miR-30a, miR-30c, miR-32, miR-375, and miR-532-p.* **F1:** 13 enriched gene sets in PVN & BNST, many responsive to transcription factors (*e.g.*, glucocorticoid receptor) and miRNAs.
Manners et al. (2019)	C57BL/6NTac mice	CUS 12 days beginning in adolescence (PND 28)	1 week	**F2 Males:** ↓ marble burying & acoustic startle response	**F2 Males:** Alteration of notch signaling pathway in amygdala
Ord et al. (2020)	London wild-type zebrafish	CUS 12–14 days	2 days	**F1 (sex not specified):** ↓ thigmotaxis & cortisol in response to alarm substance	**F0:** Differential expression of piRNAs, tsRNAs, and miRNAs in spermatozoa
Dietz et al. (2011)	C57BL/6J mice	CSDS 10 days	1 month	**F1 (both sexes):** ↓ EPM open arm time ↓ Latency to immobility in FST	–

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Table 1 (*continued*)

Study	Species/Strain	Paradigm	Time between Exposure & Mating	Key Behavioral Outcomes	Key Molecular Outcomes
				(also seen in IVF- derived offspring) ↑ Levels social avoidance following submaximal defeat **F1 Males:** ↑ Locomotor activity in novelty exposure ↑ baseline CORT levels in plasma ↓ baseline VEGF levels in plasma	

-: not applicable or not specified.
BNST: bed nucleus of the stria terminalis.
CUS: chronic unpredictable stress.
CSDS: chronic social defeat stress.
CSI: chronic social instability.
MSUS: maternal separation with unpredictable stress.
PND: postnatal day.
PVN: paraventricular nucleus of the hypothalamus.

Table 2
Behavioral effects of paternal preconception stress (PPS) via maternal separation with unpredictable stress (MSUS) in up to the fifth generation, synthesized across studies. All studies used C57BL/6JRj mice and performed MSUS from postnatal days 1–14.

	Franklin et al. (2010)	van Steenwyk et al. (2018)	Boscardin et al. (2022)
F1	**Males:** did not examine **Females:** ↓ EPM open arm entry latency (Gapp et al., 2014a) ↑ FST floating time ↓ latency to enter unfamiliar area in free exploratory paradigm ↓ OFT center entry latency	**Males:** ↑ EPM open arm time ↓ EPM open arm entry latency	–
F2	**Males:** ↑ FST floating time **Females:** ↓ latency to enter unfamiliar area in free exploratory paradigm ↓ OFT center entry latency	**Males:** ↓ EPM open arm entry latency ↑ FST floating time	–
F3	–	**Males:** ↑ EPM open arm time ↓ EPM open arm entry latency **Females:** ↑ EPM open arm time ↓ EPM open arm entry latency	–
F4	–	–	**Males:** ↓ EPM open arm time
F5	–	–	**Males:** ↑ EPM open arm time

-: not applicable or not specified.
EPM: elevated plus maze.
FST: forced swim test.
OFT: open field test.

offspring showed resilience to the anhedonic and defeat cue avoidance effects of CSDS relative to their control-sired counterparts (Franklin et al., 2011). Protective behavioral phenotypes have also emerged transgenerationally in an adolescent CUS PPS paradigm. Paternal adolescent CUS did not produce changes in affective behavior in offspring measured in the marble burying, acoustic startle response, or elevated zero maze assays, nor in the forced swim task in one study (Manners et al., 2019). However, F2 male adolescents buried fewer marbles and displayed a blunted acoustic startle response, demonstrating a decreased anxiety-like phenotype in males but not females (Manners et al., 2019).

Maternal preconception stress (MPS) vis the MSUS paradigm has similarly resulted in apparent protective phenotypes that reduce anxiety-like behaviors (Table 3) in offspring. In one study, male offspring of MSUS-exposed mouse dams had latency to enter unfamiliar areas in the free exploratory paradigm (Mansuy et al., 2011). Both offspring sexes displayed increased stretch-attend posture in the open arms of the EPM, which was defined by the rodent extending its body while lowering its back. While a naturally-occurring behavior in rodents, (Grant and Mackintosh, 1963) this posture commonly increases in behavioral assays such as the EPM that induce an exploratory-anxiety conflict and is generally interpreted to indicate increased anxiety-like behavior (Holly et al., 2016). In tandem with the exploratory behavior these offspring showed, increased stretch-attend posture might indicate a risk assessment process, (Holly et al., 2016) and the authors interpreted their findings as an indication that the mice were showing reduced caution (Mansuy et al., 2011; Rodgers and Dalvi, 1997). This interpretation is consistent with the previously-discussed findings of impaired risk assessment in PPS offspring from a maternal separation paradigm (Thivisol Ulysse et al., 2023) and suggests a greater pattern of intergenerational early life stress-induced effects on risk-taking behavior. Female offspring of MSUS-exposed mouse dams have also shown increased stretch-attend posture in the open arms of the EPM, as well as increased percent distance traveled in open arms of the EPM, but no differences were found in the free exploratory paradigm (Mansuy et al., 2011). No differences in offspring plasma CORT were detected in either sex, neither basally nor following restraint stress. Taken with observed findings of decreased CRFR2 binding in the amygdala and hypothalamus, this suggests central adaptations in stress-responsive brain regions as opposed to global HPA axis adaptations may drive the cross-generational behavioral phenotypes of MSUS.

In the CUS paradigm in rats, MPS offspring have shown impairments in several forms of learning as well as in the EPM and OFT. In the EPM, CUS rat dam offspring exhibited hyperlocomotion; female offspring in particular had significantly increased distance traveled in the open arms of the maze (Zaidan et al., 2013). Female CUS rat dam offspring also displayed abnormal "rim-climbing behavior" in the OFT, where they would balance on the walls of the arena and sniff the surrounding area (Zaidan et al., 2013). CUS rat dam offspring showed nonlinear acoustic startle responses, and only male CUS rat dam offspring displayed increased freezing behavior during conditioned stimulus presentations in a fear conditioning task (Zaidan et al., 2013). Both male and female

Table 3
Behavioral and molecular (in brain) outcomes of maternal preconception stress (MPS) in subsequent generations across published literature.

Study	Species/ Strain	Paradigm	Time between Exposure & Mating	Key Behavioral Outcomes	Key Molecular Outcomes
Huang et al. (2016)	Sprague-Dawley rats	CUS 21 days	1 day	–	**F1:** Altered GABA & glutamate metabolism in right hippocampus during adolescence (PND 30) Males: glutamate & GABA metabolism dysregulated after FST Females: dysregulated basal glutamate & GABA metabolism
Wei et al. (2018)	Wistar rats	CSDS 21 days	7 days	**F1 Males:** ↓ locomotion in OF, EPM, LDB ↑ anxiety-like behaviors in OF, EPM, LDB OF: ↓ time & distance spent in center, ↓ rearings ↑ depression-like behaviors in FST ↓ sucrose consumption & preference ↑ plasma CORT ↓ spatial memory in Morris water maze ↓ object recognition memory	**F1 Males:** ↓ 5-HT in hippocampus, hypothalamus & PFC NE & DA ↑ in hippocampus & PFC ↓ Bdnf & pCREB protein in hippocampus, PFC ↑ SERT in hippocampus & PFC
Zaidan et al. (2013)	Sprague-Dawley rats	CUS Duration not specified	2 weeks	**F1 Males:** ↑ freezing in fear conditioning paradigm **F1 Females:** ↑ EPM locomotion	–
Zaidan et al. (2021)	Sprague-Dawley rats	CUS 7 days beginning in adolescence (PND 45)	7 days	**F1 Males:** ↓ in OFT center duration, ↓ exploration in novel object recognition test ↓ freezing in fear conditioning paradigm No changes observed in F1 female behavior. **F2 Males:** ↓ exploration in novel object recognition test ↓ freezing in fear conditioning paradigm **F2 Females:** ↑ OFT locomotion ↓ OFT center entry latency ↓ sociability ↑ EPM open arm time ↓ EPM open arm entry latency	**F0:** ↑ *Crf1r* in mPFC, blood & oocytes ↓ expression of *Crf1r* regulators (*miR-34a* and *miR-34c*) in blood and oocytes **F1 & F2 Neonates (merged across sex):** ↓ *Crfr1*, ↑ *miR-34a* expression in mPFC ↑ *Crfr1*, ↓ *miR-34a* expression in amygdala **F1 Males:** ↓ mPFC *Crh1* in high and low stress conditions **F1 Females:** ↑ mPFC *Crf1* in low stress conditions; ↓ mPFC *Crfm1* & *miR-34a* in high stress conditions **F2 (both sexes):** ↓ basal mPFC *Crh1* and miR-34a
Zaidan et al. (2018)	Sprague-Dawley rats	CMS Duration not specified	–	–	**F0 & F1:** Alteration of A-to-I RNA editing in PFC and amygdala ↑ A-to-I editing at *Gria4* gene in PFC **F0 & F2** ↑ A-to-I editing at *Htr2c* and *Gria3* genes in PFC **F1:** ↓ A-to-I editing at *Htr2c* gene in PFC F1 results merged across sex.
Roth et al. (2009)	Long-Evans rats	Maternal maltreatment PND 1-7	–	–	**F0 & F1:** ↑*Bdnf* DNA methylation in PFC F1 results merged across sex.
Huang et al. (2010)	Sprague-Dawley rats	CUS 3 weeks	1 day	**F1 (both sexes):** ↓ platform crossings in Morris water maze, ↑ escape latency on d4 of testing ↑ serum CORT ↓ sucrose consumption at week 3 of testing	**F1 (both sexes):** ↓*Bdnf* expression in hippocampus ↓*Nr2b* expression in hippocampus (CA3 and DG)
Mansuy et al. (2011)	C57BL/6J mice	MSUS PND 1-14	–	**F1 (both sexes):** ↑ stretch-attend posture in EPM open arms **F1 Males:** ↓ latency to enter unfamiliar areas in free exploratory paradigm **F1 Females:** ↑ EPM open arm distance	**F0:** ↓ *Crf2* binding in amygdala (basomedial, medial posteroventral, & medial posterodorsal nuclei) and hypothalamus (lateral & periventricular nuclei)
Jenkins et al. (2022)	Long-Evans rats	Elevated platform stress 27 days	Immediate	–	**F1 Males:** ↓ basilar spine density in mPFC; ↓ right hippocampal volume
Bock et al. (2014)	Sprague-Dawley rats	CUS 1 week	Immediate or 2 weeks	–	**F1:** ↑ dendritic spines in left anterior cingulate cortex ↑ basal dendritic length in left anterior cingulate

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Table 3 (*continued*)

Study	Species/ Strain	Paradigm	Time between Exposure & Mating	Key Behavioral Outcomes	Key Molecular Outcomes
					cortex, prelimbic cortex, and infralimbic cortex **F1 Males:** ↑ basal dendritic length in left anterior cingulate cortex, prelimbic cortex, and infralimbic cortex **F1 Females:** ↑ basal dendritic length in prelimbic cortex and infralimbic cortex
Li et al. (2010)	Sprague-Dawley rats	CUS 3 weeks	10 days	**F1 Males:** ↓ platform crossings in Morris water maze ↓ sucrose consumption over 3 days	**F1 Males:** ↓ 5-HT in hypothalamus ↑ NE in hippocampus ↓ pCREB in hippocampus
Zaidan and Gaisler-Salomon (2015)	Sprague-Dawley rats	CUS 7 days beginning in adolescence (PND 42–49)	–	**F1 Females:** ↑ plasma CORT **F2 (both sexes):** ↓ OFT locomotion ↓ latency to enter EPM open arm ↑ freezing in response to conditioned fear stimulus **F2 Males:** ↑ open arm entries in EPM ↑ plasma CORT **F2 Females:** ↑ OFT center duration ↓ plasma CORT	–
Shachar-Dadon et al. (2009)	Sprague-Dawley rats	CUS 7 days	Immediate (PCS0) or 2 weeks (PCS2)	**PCS0 F1 (both sexes):** ↑ EPM locomotion & closed arm activity ↓ social interaction **PCS0 F1 Males:** ↑ shock avoidance **PCS2 F1 (Both Sexes):** ↓ social interaction Males ↓, Females ↑ acoustic startle response	–

-: not applicable or not specified.
5-HT: serotonin.
NE: norepinephrine.
DA: dopamine.
FST: forced swim test.
mPFC: medial prefrontal cortex.
OFT: open field test.

offspring of CUS-exposed rat dams displayed impaired spatial memory in the Morris water maze test (Li et al., 2010; Huang et al., 2010) as well as increased plasma CORT (Huang et al., 2010). Additionally, some overlap in behavioral phenotypes has been observed between the offspring of rat dams exposed to CUS and CSDS. Male F1s in both of these paradigms have had elevated plasma CORT (Huang et al., 2010; Wei et al., 2018) as well as an anhedonic phenotype in the sucrose preference test (Li et al., 2010; Huang et al., 2010; Wei et al., 2018). Like CUS, CSDS rat dam offspring showed impaired spatial memory in the Morris water maze test, but CSDS offspring additionally had impaired novel object recognition (Wei et al., 2018). Male offspring of CSDS rat dams also had anxiety-like phenotypes in the OFT, EPM, and light-dark box paradigms as well as depressive-like phenotypes in the forced swim test (Wei et al., 2018). Thus, both maternal CUS and CSDS appear to manifest in altered stress- and memory-related behaviors following intergenerational inheritance in rats. These findings suggest MPS induces robust intergenerational anxiety-related phenotypes that could culminate in apparent cognitive deficits. Indeed, recent studies have noted anxiety-related impairments in learning and memory in humans (Moran, 2016; Xia et al., 2021).

One of the earliest studies of MPS utilizing CUS in rats found the timing of the stress relative to conception was critical in conferring intergenerational behavioral phenotypes (Shachar-Dadon et al., 2009). Offspring of rat dams stressed immediately prior to conception showed increased locomotor activity and anxiety-like phenotypes in the EPM as well as decreased social interaction, whereas offspring conceived two weeks after cessation of CUS only showed social interaction deficits. Sex-dependent effects emerged with acoustic startle responses and avoidance learning. Only male offspring conceived immediately following CUS showed increased shock avoidance, and in offspring conceived two weeks after CUS, males showed decreased acoustic startle responses, whereas females had increased acoustic startle responses. Thus, the timing of chronic stress exposure relative to conception is an important factor to consider when designing cross-generational studies. In our tabulated summaries of research presented here (Tables 1, 3 and 4), we have noted this critical variable where possible. To our knowledge, the mechanisms underlying stressor-conception timing differences in stress-related phenotypes have not been investigated. However, there is the possibility that epigenetic changes in the germline could be preferentially influenced by CORT when conception more immediately follows the stress, whereas other mechanisms arise when conception is more delayed following the stress. Future studies could test this by measuring parental biological markers of HPA axis negative feedback inhibition (plasma CORT, gene expression in central brain HPA axis regulatory regions) in tandem with epigenetic analysis of parental germ cells at different timepoints following chronic stressor cessation.

Transgenerational sex-dependent behavioral phenotypes have also been present in the F2 generation derived from female rats exposed to chronic unpredictable mild stress (CMS) in adolescence (Zaidan and Gaisler-Salomon, 2015). While both male and female F2 rats displayed decreased locomotor activity in the OFT, only female offspring showed an anxiety-like phenotype. In the EPM, both male and female F2 rats

Table 4
Behavioral and molecular (in brain) outcomes of preconception ethanol in subsequent generations across published literature.

Study	Species/Strain	Parental Exposure (PPE, MPE, or BPE)	Time between Exposure & Mating	Key Behavioral Outcomes	Key Molecular Outcomes
Ceccanti et al. (2016)	CD-1 mice	PPE	2 months	**F1 Males:** ↑ EtOH CPP	–
Rompala et al. (2016)	C57BL/6J mice (PPE), mated to Strain 129S1/SvIMJ mice	PPE	Immediate	**F1 Males:** ↓ in HPA axis response to restraint stress	–
Beeler et al. (2019)	C57BL/6J mice	PPE	2 days	**F1 Males:** ↓ DID binge-like EtOH drinking Complex sex-specific effects in basal & EtOH-induced OFT behavior	–
Conner et al. (2019)	CD-1 mice	PPE	Immediate	**F1 Males:** deficits in sensorimotor integration during adolescence (PND 30): ↓ short term motor learning ↓ balance on RotaRod	**F1 Neonates (merged across sex):** Dysregulation of spatial expression patterns of RZRβ and Id2
Hollander et al. (2019)	Sprague Dawley rats	PPE	2 days	**F1 Males:** ↑ T maze arm entry latency	–
Ferraguti et al. (2020)	CD-1 mice	PPE	2 months	–	**F1 Males:*** Altered gene expression in brainstem: ↑ *Sl6a4* ↓ *Htr2c* ↓ *Bdnf* ↑ $p75^{NTR}$
Rathod et al. (2020)	C57BL/6J mice	PPE	Immediate	**F1 (both sexes):** ↓ midazolam LORR duration **F1 Females:** ↓ EtOH consumption on first day of DID **F1 Males:** ↓ stress-induced hyperthermia ↓ recovery from the second injection of ethanol during AFT	–
Nieto et al. (2021)	Wistar rats	PPE	8 weeks	**F1 (both sexes):** ↓ operant EtOH self-administration	**F1 (both sexes):** Altered *Bdnf* DNA methylation in NAc; specific CpG sites differed by sex.
Nieto and Kosten (2023)	Wistar rats	PPE	8 weeks	**F1 (both sexes):** ↓ operant EtOH self-administration	–
Jabbar et al. (2016)	Fischer-344 rats	MPE	3 weeks	**F1 Males:** ↑ in stress/anxiety-like behavior in OF & EPM, despite ↑ CORT (basally & during LPS challenge) in both sexes	**F1 Males:** ↑ *Crf* expression in hypothalamus; ↓ methylation, ↑ expression of *Crfr1* in hippocampus & amygdala ↑ methylation, ↓ expression of *Pomc* in hypothalamus
Collier et al. (2020)	AB Strain, *hcrt:EGFP*, and *HuC:GFP* zebrafish	MPE	Immediate	**F1 (both sexes):** ↑ voluntary EtOH intake ↑ locomotion	–
Suresh et al. (2021)	AB and TL Strain zebrafish	BPE	9 h	**F1 (both sexes):** ↓ swim speed, ↑ duration of immobility ↑ turn angles ↑ thigmotaxis	–
Asimes et al. (2018)	Wistar rats	BPE	24 h	**F1 Males:** ↓ basal plasma CORT	
Gaetani et al. (2014)	Wistar rats	BPE	24 h	–	**F1 (both sexes):** Altered hypothalamic gene expression related to epigenetic regulation of gene expression and neurogenesis/synaptic plasticity. DEG clusters differed by sex, more DEGs in females than males.
Asimes et al. (2017)	Wistar rats	PPE MPE BPE	24 h	–	**F1 Males:** Differential DNA methylation signatures in hypothalamus based on parental exposure PPE & MPE: Hypermethylation of *Esam* promoter PPE: few global methylation changes; *Esam* only differentially methylated gene MPE: *Fam110a* promoter hypermethylation, *Olr286* promoter hypomethylation BPE: *Arrdc1, Ephb3, & miR6216* hypermethylation; *Golt1b, Gpank1, & Sparcl1* hypomethylation

PPE: paternal preconception ethanol.
MPE: maternal preconception ethanol.
BPE: biparental preconception ethanol.
AFT: acute functional tolerance assay.
CPP: conditioned place preference.

DEG: differentially expressed gene.
DID: drinking in the dark assay.
EtOH: ethanol.
LORR: loss of righting response assay.
LPS: lipopolysaccharide.
OFT: open field test.
NAc: nucleus accumbens.
* Ferraguti et al. (2020) exposed some F1s to ethanol. Results from ethanol-naive animals presented here.

showed a decreased latency to first enter the open arm, but only male F2 rats showed an increase in open arm entries relative to controls. Both male and female F2 rats had increased freezing in response to a conditioned fear cue. Only F2 rats were tested in the behavioral paradigms, but plasma CORT analyses found that F1 females, but not males, showed increased plasma CORT relative to controls, mirroring elevated CORT in their F0 mothers. However, in the F2 generation, males showed elevated plasma CORT, whereas females showed decreased plasma CORT (Zaidan and Gaisler-Salomon, 2015). These results demonstrate complex cross-generational HPA axis reprogramming with maternal CMS that could preferentially inoculate male progeny against basal anxiety-like phenotypes while honing fear association learning.

We were only able to find two studies investigating biparental preconception stress (BPS). The first was performed in offspring mice following both parents being exposed to chronic social instability in adolescence, implemented by randomly interchanging cage mates twice a week for 7 weeks (Saavedra-Rodrí et al., 2013). This study found sex-specific outcomes in offspring (Saavedra-Rodrí et al., 2013). Female, but not male BPS offspring had elevated plasma CORT, decreased open arm time in the EPM, impaired adaptation to the novel environment of the OFT, and diminished social memory relative to their control female counterparts. This study then dissected the individual effects of PPS and MPS in female offspring from these outcomes. Both PPS and MPS female offspring had elevated plasma CORT levels comparable to those of BPS female offspring, but some behavioral phenotypes were parent specific. Decreased open arm time in the EPM occurred in both PPS and MPS groups. Significant impairments in OFT and social interaction behaviors were only observed in PPS female offspring, but the effects were smaller than those in BPS female offspring. MPS offspring had no significant impairments in these assays. Thus, the stress-related behavioral outcomes of BPS appear to be more than the sum of their PPS and MPS parts. When male and female BPS F1s were interbred, F1 female offspring phenotypes persisted into F2 females despite CORT plasma levels roughly normalizing to control levels (Saavedra-Rodrí et al., 2013). However, when F1 BPS mice were mated to control F1 mice, only the female offspring of male BPS F1s inherited the anxiety-like and impaired social interaction phenotypes. As F1 males did not show these impairments, these results suggest differential cross-generational BPS inheritance based on sex. The second BPS study exposed rats to 50 days of predatory stress before mating (Azizi et al., 2019). Male and female F1 offspring were tested in EPM before plasma CORT levels were measured; a subgroup of F1s were exposed to acute predatory stress before EPM. In unstressed F1s, the percentage of EPM open arm time and entries were decreased in both males and females, while the percentage of closed arm time and entries were increased relative to controls in PPS, MPS, and BPS groups. This resulted in an increased index of open arm avoidance in all groups. However, in female F1s, these changes (*i.e.,* decreased percentage of open arm time/entries, increased percentage of closed arm time/entries, increased index of open arm avoidance) were increased in magnitude in the PPS group than the MPS group. In male F1s, the directionality changed for percentage of open arm and closed arm time, where PPS male offspring spent more time in the open arms (less time in the closed arms) than MPS male offspring. Interestingly, CORT levels were only increased in BPS female offspring and MPS male offspring. In F1s that were exposed to acute predatory stress before EPM, a decreased percentage of open arm time, increased percentage of closed arm time, and subsequent index of open arm avoidance were seen in the MPS and BPS (but not PPS) groups for females and in the PPS and BPS (but not MPS) groups for males. CORT increased after EPM in only the BPS stressed female F1s, but in PPS and BPS stressed male F1s. The findings from this study further suggest sexually dimorphic effects of intergenerational preconception stress, where the sex of the stressed parent(s) and the offspring interact to produce alterations in anxiety-related behaviors.

In summary, PPS and MPS induce inter- and trans-generational alterations in anxiety- and depression-like behaviors that can differ based on the stress induction paradigm as well as the sex of the progeny. Memory impairments are also shared across modes of stress inheritance. Early life stress in particular appears to confer cross-generational protective phenotypes against anxiety. However, upon closer inspection, apparent reductions in anxiety-like behaviors may instead reflect cognitive deficits in offspring that could manifest as impulsivity and risky behavior. Particularly given some ambiguity/inconsistency in interpreting stretch-attend postures in the EPM, (Mansuy et al., 2011; Grant and Mackintosh, 1963; Holly et al., 2016; Rodgers and Dalvi, 1997) further studies can validate impaired risk assessment in MPS and PPS using established paradigms for risky behavior in rodents (Simon and Setlow, 2012; Orsini et al., 2019). Additionally, more research is needed to determine the cross-generational effects of BPS, especially utilizing popular stress-induction paradigms such as CUS and MSDS. Behavioral results from these future studies can be compared to the robust PPS and MPS literature on CUS and MSDS discussed here. Finally, we synthesized MSUS PPS behavioral outcomes across studies to determine sex- and generation-dependent effects (Table 2); Additional research should validate these results by producing male and female progeny to the fifth generation in individual studies.

The discussed behavioral phenotypes resulting from chronic preconception stress clearly establish that stress effects can be epigenetically transmitted intergenerationally, and in some instances even transgenerationally. These behavioral effects in PPS, MPS, and BPS offspring result from epigenetically inherited changes in brain, which are discussed in detail in the following section.

2.2. *Molecular outcomes of chronic preconception stress across generations in animals*

Various molecular phenotypes have been observed in brain of PPS descendants (Table 1). One MSUS mouse study found decreased 5-HT_{1A}R binding in multiple F1 male brain regions, including the periaqueductal gray, dorsal raphe, and hippocampus, suggesting broad deficits in serotonergic functioning (Franklin et al., 2011). Another study found altered hippocampal and lateral amygdalar plasticity in F1 in both sexes as a result of MSUS in mice (Bohacek et al., 2014). PPS via CUS and MSUS exposure has also been shown to produce robust inter- and transgenerational alterations in RNA expression in offspring brain tissue in both rats and mice (Franklin et al., 2010; Rodgers et al., 2013; Bohacek et al., 2014; Gapp et al., 2014b; Mashoodh et al., 2023; Kong et al., 2021; Niknazar et al., 2017). In addition, differential epigenetic modifications such as DNA and histone methylation and acetylation have also been consistently linked to PPS, with differential effects across the sexes in subsequent generations. For example, female offspring of mouse sires exposed to MSUS have shown altered histone epigenetic alterations and increased DNA methylation in the mineralocorticoid receptor (MR) gene coinciding with decreased expression of MR mRNA

and protein in hippocampus (Gapp et al., 2014a). While glucocorticoid receptor (GR) expression remained unaffected in female offspring, male offspring showed decreased GR gene methylation and increased expression in hippocampus. The remainder of molecular evidence on PPS inheritance is highly mechanistic and indicates the preservation of epigenetic changes from sire sperm to offspring brain. These data are discussed in the next section on germline mechanisms.

MPS has been found to induce alterations in neurotrophic, neurotransmitter, and neuropeptide signaling systems across brain regions. Molecular outcomes of MPS are summarized in Table 3. In a CUS paradigm in rats, offspring sex and brain region-specific differences in *Crf1* expression emerged with high stress (but not low stress) exposure in adulthood; Only females showed increased amygdala *Crh1* expression, whereas both sexes showed significantly decreased *Crf1* expression in frontal cortex (Zaidan et al., 2013). A follow-up study (found further diverging effects in F1 and mPFC in *miR-34a* (a regulator of *Crfr1* expression) (Zaidan et al., 2021); In F1 male rats, mPFC *Crfr1* decreased in both high- and low-stress conditions, with no effects observed on *miR-34a*. In F1 MPS females, mPFC *Crfr1* increased with low stress, but decreased with high stress. A decrease in mPFC *miR-34a* was selectively observed in high-stress F1 MPS female rats. Basal (no stress exposure) *Crfr1* and *miR-34a* were decreased in adult male and female MPS F2 mPFC. In a maternal maltreatment model of early life stress in rats, increased *Bdnf* DNA methylation in PFC persisted from early life stress dams to their offspring (Roth et al., 2009). Conversely, decreased *Bdnf* mRNA (Huang et al., 2010) and protein (Wei et al., 2018) levels were observed in the hippocampus of offspring derived from rat dams exposed to CUS (Huang et al., 2010) and CSDS, (Wei et al., 2018) respectively. CUS in rat dams resulted in decreased expression of the *Nr2b* (NMDA receptor subunit) gene in CA3 and DG of F1 hippocampi across the sexes (Huang et al., 2010). In mice, decreased Crfr2, but not Crfr1, binding was observed in several amygdalar and hypothalamic nuclei of MSUS-exposed dams, including the basomedial, medial posteroventral, and medial posterodorsal amygdala and the lateral and periventricular nucleus of the hypothalamus (Mansuy et al., 2011). Offspring of CUS-exposed rat dams have also displayed altered glutamate and GABA metabolism in the right hippocampus in adolescence, with sexually dimorphic effects (Huang et al., 2016). Female CUS offspring displayed dysregulated basal glutamate and GABA metabolism in early adolescence, whereas male CUS offspring glutamate and GABA metabolism were dysregulated only after being exposed to the forced swim test as adolescents (Huang et al., 2016). Monoamine signaling has also been implicated in the effects of MPS in male offspring. Male offspring of CUS- and CSDS- exposed rat dams had decreased serotonin levels in the hypothalamus (Li et al., 2010); CSDS F1 males additionally had reductions in serotonin in hippocampus and prefrontal cortex, and increased norepinephrine, dopamine and serotonin reuptake transporter protein (Wei et al., 2018). CUS also intergenerationally altered adenosine-to-inosine RNA editing in PFC and amygdala across generations in rats, with opposing effects in multiple genes encoding neurotransmitter receptor subunits (Zaidan et al., 2018). Most notably, at one site in the serotonin receptor 2c (*Htr2c*) gene, adenosine-to-inosine editing significantly increased in PFC of F0 dams, decreased in F1 offspring, and increased in F2, where the most robust effects on editing were observed. Further differential effects were seen in genes encoding AMPA receptor subunits. Adenosine-to-inosine editing at a site in the *Gria3* gene increased in PFC of F0 dams and F2 offspring but was not affected in F1 offspring. Conversely, adenosine-to-inosine editing at a site in the *Gria4* gene was increased in F0 dams and F1 offspring, but not in F2.

Several studies have documented MPS-induced alterations in dendritic spine density in frontal and limbic cortical brain regions in rats. In one study, chronic elevated platform MPS decreased basilar spine density in the medial prefrontal cortex and resulted in a reduction in the right hippocampal volume in male offspring (Jenkins et al., 2022). However, these effects were modest, perhaps due to low sample size (n = 4–5 offspring/group). In another study, (Bock et al., 2014) offspring of CUS-exposed female rats showed alterations in dendritic spines in the anterior cingulate and prelimbic/infralimbic cortex. Offspring collapsed across sex had increased dendritic spine numbers in the left anterior cingulate cortex. Male offspring in this group, but not female offspring, showed increased basal dendritic length in the left anterior cingulate cortex. Both male and female offspring showed increased dendritic length and complexity in the prelimbic and infralimbic cortex. In the former study, dendritic spine alterations were associated with impaired spatial memory in the Morris water maze, (Jenkins et al., 2022) though the latter study was restricted to morphology only. Electrophysiological studies are required to elucidate the functional implications of MPS-induced changes in dendritic morphology in prefrontal and hippocampal brain regions and could expand these findings into other HPA axis-associated brain regions.

In our review of the literature, we were only able to find one molecular BPS study, which utilized CSI in both parents. Behavioral outcomes were the focus of this study and were discussed above, but the authors also performed a microarray analysis on CA1 tissue from hippocampus and found a significant upregulation of *regulator of calcineurin 1* and *2* (*Rcan1* and *Rcan2*) mRNA selectively in female BPS offspring (Saavedra-Rodrí et al., 2013). *Rcan1* upregulation persisted to F2 females, whereas *Rcan2* upregulation was observed in females up to the F3 generation (Saavedra-Rodrí et al., 2013). Overexpression of *Rcan* genes, particularly *Rcan1,* in hippocampus has been previously associated with learning and memory deficits, Down Syndrome, Alzheimer's, and aging-like phenotypes (Martin et al., 2012; Wong et al., 2022).

Thus, chronic preconception stress across paradigms appears to result in impaired monoaminergic functioning and neurotrophic signaling, though this has primarily been investigated in hippocampal, hypothalamic, and frontal cortical brain regions. The hippocampus is the most extensively studied brain region across PPS, MPS, and BPS, and displays decreased plasticity while showing signs of altered neurotransmitter functioning. *Crf* signaling is also altered in the hypothalamus in both PPS and MPS, providing molecular evidence of HPA Axis dysregulation. Epigenetic marks such as methylation are more extensively studied in PPS, though MPS does result in altered RNA editing.

The studies presented here focus primarily on HPA axis-associated brain regions due to the known impact of stress (including cross-generational exposure) on HPA axis functioning; However, future research could comprehensively map preconception stress-related molecular changes across brain regions. In addition, while providing valuable insight into cross-generational molecular outcomes of preconception stress, the literature presented here either associated or correlated these changes with observed behavioral outcomes. Further studies should use (epi)genome editing tools or pharmacological approaches to causally test whether the molecular changes observed in brain drive the behavioral outcomes of preconception stress.

2.3. *Germline mechanisms of cross-generational chronic preconception stress phenotype transmission in animals*

Bridging the gap between F0 stress and epigenetic changes in offspring is the parental germline. While most epigenetic marks such as DNA and histone modifications are erased during gametogenesis, some persist (Kovalchuk and Migicovsky, 2011; Ben Maamar et al., 2021) and could be partially responsible for preconception stress phenotypes in offspring. An increasing number of studies have also implicated non-coding RNAs in cross-generational regulation of molecular and behavioral phenotypes (Duffy et al., 2021; Yin et al., 2021; Skvortsova et al., 2018; Rompala and Homanics, 2019). The following section discusses germline mechanisms of cross-generational preconception stress inheritance.

While this review was under revision, Kretchmer, Fischer, and Gapp published a separate literature review on germline mechanisms of PPS (Kretschmer et al., 2023). We encourage readers to refer to their review

for further information on PPS phenotype transmission, particularly for a more in-depth analysis of sperm epigenetic mechanisms (*e.g.,* chromatin structure) that have yet to be investigated in PPS phenotype transmission and impacts on embryonic development. Sperm RNA content of F0 animals has been shown to be critical in conferring behavioral and molecular intergenerational phenotypes of PPS. Most studies that have demonstrated an effect of stress on the abundance of sperm RNAs have found the most consistently altered RNAs are non-coding RNAs, and it appears that the specific biotype that varies in abundance is dependent upon the type of stress and/or experimental paradigm. For example, the sperm of "stress-susceptible" mouse sires exposed to CSDS has been shown to possess a discrete transcriptomic profile compared to their "stress-resilient" counterparts, where long noncoding RNAs were more prominently altered in the sperm of stress-susceptible males (Cunningham et al., 2021). Heritable stress phenotypes from MSUS have also been linked to long noncoding RNAs in mice (Gapp et al., 2018). Other PPS studies using the CUS paradigm have found consistent alterations in small noncoding RNA in sperm, particularly in miRNAs, that could drive gene expression in offspring brain. The notch signaling pathway has emerged as a specific target of these alterations in mice (Rodgers et al., 2013; Gapp et al., 2014b) as well as other species. In zebrafish (*Danio rerio*), CUS altered small noncoding RNA in spermatozoa, including six clusters of PIWI-interacting RNAs (piRNAs) and 12 clusters of tRNA-derived small RNAs (tsRNAs) (Ord et al., 2020). Notably, 12 clusters of miRNAs were also differentially expressed, with target genes in mitophagy and notch signaling pathways. In mouse, the notch signaling pathway was similarly transgenerationally altered in the amygdala of F2 males derived from sires exposed to CUS during adolescence, (Manners et al., 2019) suggesting preconception stress confers evolutionarily conserved alterations in cell growth, death, and differentiation signaling that persist across generations. Further studies investigating sire sperm and offspring brain in tandem are needed to mechanistically verify whether altered notch signaling in sire sperm via miRNA content drives these alterations in brain. However, although many miRNAs appear to be highly evolutionarily conserved, the lack of accurate characterization of miRNAs and their targets across species hinders meaningful comparisons across studies (Fridrich et al., 2019; Ha et al., 2008; Bhaskaran and Mohan, 2013). Thus, a comprehensive understanding of cross-generational transmission of stress-related phenotypes necessitates further characterization of noncoding RNAs. Although studies of sperm RNA abundance such as those just described are informative, they are correlative in nature, and unable to establish causality that the changes in RNA abundance drive the intergenerational phenotypes observed.

Other studies have directly mechanistically tested the role of sperm RNA content in PPS phenotype transmission by injecting purified PPS sire sperm RNA into fertilized wild-type oocytes. In one mouse study, injection of RNA purified from the sperm of MSUS-exposed males into fertilized oocytes derived from control breeding pairs recapitulated molecular phenotypes observed in naturally conceived offspring of MSUS-exposed sires (Gapp et al., 2014b). In another study, injection of control zygotes with long RNAs (which included mRNA and lncRNA, among other biotypes) from the sperm of MSUS-exposed male mice resulted in offspring that displayed increased time spent in the illuminated chamber of the light-dark box, whereas small RNA (miRNAs, piRNAs, *etc.*) elicited a decrease (Gapp et al., 2018). Even more remarkable, injection of only nine differentially expressed miRNAs from the sperm of male mice exposed to CUS into wild-type zygotes recapitulated blunted HPA axis reactivity observed in directly-sired offspring (Rodgers et al., 2015).

Together, the above studies demonstrate that stress alters the abundance of specific sperm RNAs, and transfer of these stress responsive sperm RNAs to fertilized, unstressed embryos is sufficient to recapitulate intergenerational stress-induced phenotypes. However, mature sperm are generally considered to be transcriptionally quiescent (Ren et al., 2017; Santiago et al., 2022; Hosken and Hodgson, 2014). So how does PPS alter the abundance of RNAs in sperm if most genes are not actively expressed in sperm? The best evidence to date suggests that the sperm RNA payload is shaped by RNA and proteins that are transferred from extracellular vesicles released from epididymal epithelial cells as sperm mature in the epididymis (Santiago et al., 2022; Sharma et al., 2018; Sullivan, 2015; Trigg et al., 2019). Furthermore, a single pioneering study has specifically demonstrated these "epididymosomes" are sufficient to confer intergenerational stress-related phenotypes of PPS in mice via their miRNA and protein content (Chan et al., 2020). Clearly, additional studies are needed to dissect the role of this soma to germline pathway in mediating intergenerational inheritance.

The dynamics of RNA expression with spermatogenesis appear to necessitate a "recovery period" between cessation of chronic unpredictable stress exposure and conception (approx. 12 weeks in mouse) to confer intergenerational transmission. Indeed, separate studies in humans (Morgan et al., 2020) and mice (Cunningham et al., 2021; Chan et al., 2020) have elucidated unique temporal dynamics of spermatic noncoding RNA abundance. In humans, perceived stress experience over time correlates with dynamic abundance of spermatic miRNA and tRNA (but not piRNA) content (Morgan et al., 2020). A mouse study utilizing chronic social defeat stress (CSDS) similarly noted temporal effects on spermatic small noncoding RNAs (Cunningham et al., 2021). These findings reinforce the importance of chronic stressor timing relative to conception as a variable in cross-generational stress exposure studies.

Although most studies have focused on the role of RNA, DNA methylation in sperm has also been implicated in transmission of PPS phenotypes in mice. One study utilizing either chronic restraint stress or CSI identified "candidate anxiety genes" that have differential DNA methylation and expression in both F0 sperm and F1 female hippocampus, including *Adora2a, Bdnf, Itpr3,* and *Gata2* (Kong et al., 2021). Similarly, the MSUS paradigm has resulted in altered DNA methylation and expression of *Mecp2, Cnr1,* and *Crfr2* that are preserved from F0 sperm to female F1 brain (Franklin et al., 2010). In another study, decreased methylation of the GR gene in hippocampus of male offspring of MSUS-exposed sires was correlated with decreased GR gene methylation in sire sperm (Gapp et al., 2016).

A translational study across mice and humans suggested circulating metabolites in serum may serve to mediate effects of PPS by influencing the germline (van Steenwyk et al., 2020). Remarkably, chronic injection of serum from MSUS sires into adult control offspring recapitulated some of the metabolic phenotypes observed in directly-sired MSUS offspring. The authors also specifically investigated the role of peroxisome proliferator-activated receptor (PPAR) pathways in these intergenerational phenotypes, which were upregulated in F0 adipose and liver tissue as well as sperm. *In vitro* exposure of early-stage spermatogonial-like cells (GC-1 spg cells) to MSUS sire serum was sufficient to increase PPAR activity. In addition, chronic injection of sires with the PPARα/γ agonist tesaglitazar mimicked the alterations in sperm RNA abundance with MSUS (Gapp et al., 2018) and recapitulated MSUS F1 metabolic phenotypes. To our knowledge, this study is the first to causally demonstrate circulating factors as vectors for epigenetic communication of stress from somatic cells to the germline. Although the precise mechanisms of this communication are unclear and could involve other somatic cells in the testes and epididymis (as the authors discuss (van Steenwyk et al., 2020)), PPARs present a novel avenue of research on germline mechanisms of PPS phenotype transmission. Further studies could examine PPAR activity and its downstream molecular effects across gonadal cell types (*e.g.,* epithelial epididymal cells and the epididymosomes they release as well as other somatic cells such as Sertoli cells) and spermatogenesis in PPS sires to build upon these findings. In addition, these results suggest that future germline studies of preconception stress inheritance mechanisms should consider probing relevant circulating factors in serum in addition to epigenetic changes in the germline.

At least some of the phenotypes of PPS may be attributable to elevated paternal glucocorticoids in response to stress, but more

research is necessary to validate this connection. Although prenatal exposure is beyond the scope of this review, extensive research has investigated the impact of prenatal glucocorticoid exposure (both through parental stress and exogenously administered glucocorticoids) and found both immediate and cross-generational epigenetic effects on growth, development, and HPA axis programming tied to stress-related behaviors. (Mbiydzenyuy et al., 2022; Constantinof et al., 2016; Moisiadis and Matthews, 2014; Hamada and Matthews, 2019). Recent studies have begun to examine paternal preconception glucocorticoid exposure and revealed cross-generational effects on molecular functioning and behavior as well as potential germline transmissive mechanisms. Multiple mouse studies exposed sires to CORT in drinking water (25 μg/mL) for four weeks. One found increased exploratory Y-maze behavior in F1 males and impaired spatial memory in the Morris water maze in F1 females (Yeshurun et al., 2017). Another found a male-specific F1 increase in anxiety-like behavior in EPM and the light-dark box, while F2 mice of both sexes had decreased anxiety-like behaviors in these assays (Short et al., 2016). Male F2 mice additionally had increased depression-like behavior in the FST. Sex-specific F1 and F2 alterations in growth factors were detected in hippocampus and attributed to altered F0 spermatic abundance of small noncoding RNAs that were associated with growth factor pathway regulation. A third mouse study using the same paradigm found reduced social dominance in F1 males, but no associated changes in PFC gene expression (Hoffmann et al., 2023). A mouse study that instead exposed sires to 5 days of dexamethasone injections found decreased methylation and subsequent increased expression of GR, MR, and estrogen receptor 1 expression in adult F1 male hippocampus (Petropoulos et al., 2014). Chronic dexamethasone treatment has also been associated with altered miRNA abundance in sperm of guinea pigs, with affected target pathways (FoxO, Hippo, and p53) related to cell growth, proliferation, and apoptosis as well as immune (TGF-β) signaling (Casciaro et al., 2023). Further studies should directly compare the sperm RNA expression and cross-generational molecular and behavioral effects of PPS and paternal preconception glucocorticoid exposure to elucidate whether glucocorticoids mediate cross-generational stress-related phenotypes.

Studies of MPS germline transmissive mechanisms are fewer than those for PPS due to the unique technical challenges presented in examining oocytes. Far fewer oocytes are carried in dams than sperm in sires, and sperm are continuously produced and replenished whereas oocyte number is determined prior to birth (Lei and Spradling, 2013; Gemmell et al., 2011). However, one study found significantly increased *Corticotropin releasing factor type 1* (*Crf1*) mRNA in mature oocytes and frontal cortex of MPS rats (CUS exposure) as well as in whole brain of neonatal offspring, (Zaidan et al., 2013) and a follow-up study found increased *Cfr1* receptor mRNA (*Crfr1*) and decreased expression of its regulators *miR-34a* and *miR-34c* in CUS rat dam blood and oocytes (Zaidan et al., 2021). However, as mentioned in the prior section, *Crf1* and *miR-34a* expression differed in adult offspring brain depending on exposure to high-versus low-stress conditions (*miR-34c* was not differentially expressed in F1 brain) (Zaidan et al., 2013, 2021). Thus, MPS may produce sexually dimorphic intergenerational effects on stress sensitivity via *Crfr1* that are conferred by oocyte miRNA content, but further studies are necessary to characterize the causal role of oocyte epigenetics in the transmission of MPS phenotypes.

Germ cell RNA content, particularly noncoding RNAs, appears sufficient to confer at least some of the molecular and behavioral phenotypes of chronic preconception stress. It is important to note, however, that none of the above embryo microinjection studies investigated transgenerational mechanisms of germline inheritance. In addition, there are several theoretical caveats to consider regarding potential germline mechanisms across generations. First, epigenetic marks conferred by the stressed parent could be diluted/erased during the multiple reprogramming events that occur during embryogenesis (Xia and Xie, 2020; Ross and Canovas, 2016). Second, sperm contribute a very small amount of RNA to the embryo compared to the large amount of maternal-derived stores in the oocyte (Santiago et al., 2022; Lalancette et al., 2008; Pessot et al., 1989). Thus, to have such a dramatic impact on neurodevelopment after PPS, some form of amplification mechanism must be present in mammals, as has been documented in *Drosophila melanogaster* (de Vanssay et al., 2012) and *Caenorhabditis elegans* (Rechavi et al., 2011). Recently, a preprint reported one such mechanism in mice; (Champroux et al., 2023) *miR-34c* from sperm amplified its own expression and subsequently, the expression of other RNAs in pre-implantation mouse embryos, which was suppressed when the sire was exposed to CSI. Restoring *miR-34c* levels in CSI-sired pre-implantation embryos attenuated the anxiety-like phenotypes resulting from CSI PPS in the offspring as well as reinstate expression of other *miR-34c* dependent genes. This suggests that PPS phenotypes could be conferred to progeny by stress-induced suppression of sperm miRNA feedforward epigenetic regulation in fertilized embryos. Further studies should confirm and elaborate upon these findings and examine amplification of other small noncoding RNAs implicated in cross-generational stress inheritance (*e.g., miR-34a*).

The notch signaling pathway appears to be a promising candidate for further PPS studies that could causally test whether alterations of small noncoding RNAs in sperm drive specific molecular outcomes in offspring brain which, in turn, dictate behavior. Pronuclear microinjection of purified germ cell RNAs has proven invaluable in experimentally testing such questions (*i.e.,* connecting molecular alterations in germ cells to those in offspring brain) in PPS and will be critical in future studies. However, additional research is necessary to properly characterize and annotate noncoding RNAs to draw meaningful conclusions, particularly across species. In addition, causal testing (*i.e.,* pronuclear microinjection studies) has not yet been performed in the context of MPS, though *miR-34a* regulation of mPFC *Crfr1* presents a promising avenue of MPS germline research.

Germ cell DNA methylation may also assist in conferring cross-generational phenotypes, though this has only been (1) studied in the context of PPS and (2) correlated with similar changes in offspring brain. Proving that stress-induced changes in methylation of specific genes mediates intergenerational phenotypes was technically impossible until the advent of CRISPR/dCas9 DNA methylation editing (Liu et al., 2016). It is now possible to precisely add or remove methyl groups from precise genomic locations to test the sufficiency and necessity of specific methyl marks. The same technology could be used to test the involvement of specific histone posttranslational modifications in epigenetic inheritance. However, to date, this technological advance has yet to be applied to studies of the epigenetic inheritance of stress. Pharmacological modulators that can alter DNA methylation may also prove useful in further causative studies on epigenetic stress inheritance. Although only examining effects within a generation, one study utilized the DNA methyltransferase inhibitor zebularine (Zhou et al., 2002) to test the causal role of DNA methylation in early life stress-induced alterations in maternal care (Keller et al., 2019). Administration of zebularine to rat dams that experienced early life maltreatment was able to reverse their subsequent maltreatment of their pups, as well as associated epigenetic changes including *Bdnf* methylation. Conversely, control dams administered zebularine displayed maltreatment behaviors toward their offspring. While this pharmacological approach is less precise than CRIPSR/dCas9 DNA methylation editing, it could be a useful tool in causative cross-generational studies of epigenetic stress inheritance interested in global DNA methylation changes.

3. Animal models of inter- and transgenerational chronic preconception ethanol exposure

Chronic stress and alcohol exposure possess a complex relationship that is still being characterized. As introduced earlier, AUD and stress-related neuropsychiatric disorders (including anxiety disorders, depressive disorders, and PTSD) are often comorbid and display common pathogenetic mechanisms (Anker, 2019; McHugh, 2019;

Castillo-Carniglia et al., 2019; Suh and Ressler, 2018). Chronic alcohol consumption can lead to increased glucocorticoid secretion (Fan et al., 2023; Badrick et al., 2008; JH and Mendelson, 1966) as well as HPA axis adaptations that can be exacerbated with chronic stress (Becker, 2017; Wittgens et al., 2022; Rachdaoui and Sarkar, 2017; Koob, 2009; Alhaddad et al., 2020). Conversely, stress has been shown to drive alcohol consumption in both humans and preclinical alcohol models (Zhou and Kreek, 2018; Breese et al., 2011; Sayette, 1999; Miczek et al., 2022; Becker et al., 2011). Indeed, people commonly drink in an effort to reduce stress (Sayette, 1999; Sinha, 2012). However, the mechanisms that underlie these effects remain poorly characterized (McGrath et al., 2016). It is also important to note that stress and alcohol are not synonymous. The effect of stress on alcohol consumption and AUD risk depends on multiple factors, including the age of the individual and duration, nature, and severity of the stressor(s) (Keyes et al., 2012). Conversely, the stress-related effects of alcohol have only been observed with prolonged heavy drinking in humans and animal models (Becker, 2017; Fan et al., 2023; Badrick et al., 2008; Rachdaoui and Sarkar, 2017; Alhaddad et al., 2020). However, as our review of the literature will demonstrate, the relationship between stress and alcohol has not been adequately explored in the context of stress-and alcohol-related heritability across generations. Far less is known regarding the cross-generational effects of ethanol exposure as opposed to chronic stress. As evidence of this, very few studies have investigated transgenerational outcomes of preconception ethanol exposure, which have been reviewed previously but are expanded upon here with new findings (Chastain and Sarkar, 2017; Finegersh et al., 2015; Liang et al., 2014). Insights from cross-generational stress exposure studies could inform further cross-generational alcohol research. The following sections summarize the major behavioral and molecular evidence of cross-generational transmission of ethanol exposure phenotypes, as well as the potential germline mechanisms that could transfer these phenotypes.

3.1. Behavioral outcomes of chronic preconception ethanol across generations in animals

Paternal preconception ethanol (PPE) is the most widely studied preconception ethanol exposure paradigm and produces robust effects on offspring behavior, including changes in learning and activity/locomotor behavior (Abel and Lee, 1988; Abel, 1989a, 1989b, 1989c, 1993; Wozniak et al., 1991; Ledig et al., 1998; Kim et al., 2014; Finegersh and Homanics, 2014) as well as stress- and anxiety-like behaviors in mice and rats (Liang et al., 2014; Ledig et al., 1998; Finegersh and Homanics, 2014; Abel and Bilitzke, 1990; Abel, 1991a, 1991b; Meek et al., 2007; Rompala et al., 2017). We and others have previously reviewed behavioral and molecular outcomes of preconception ethanol exposure, particularly PPE (Baratta et al., 2021; Rompala and Homanics, 2019; Chastain and Sarkar, 2017; Finegersh et al., 2015). Thus, this review and summary of findings in Table 4 focuses on illustrative recent studies and is not all-inclusive.

A consistent finding is that behavioral outcomes of PPE appear to be sexually dimorphic. PPE male mice offspring were sensitized to the anxiolytic (Finegersh and Homanics, 2014; Rompala et al., 2017) properties of ethanol, displayed reduced HPA axis responsivity in response to an acute stressor, (Rompala et al., 2016) displayed reduced stress-induced hyperthermia, (Rathod et al., 2020) and had decreased ethanol consumption and preference in free-choice ethanol consumption paradigms (Finegersh and Homanics, 2014; Rompala et al., 2017). Similarly, PPE male rat offspring have shown decreased ethanol self-administration in an operant paradigm (Campbell et al., 2018). However, changes in offspring ethanol consumption behaviors vary with the specific PPE induction and offspring ethanol consumption paradigms utilized (Baratta et al., 2021; Hollander et al., 2019; Ceccanti et al., 2016). PPE male mice offspring show no differences in ethanol metabolism/clearance nor in the recovery of the righting response following acute ethanol administration, (Finegersh and Homanics, 2014; Rathod et al., 2020) though both PPE male and female offspring demonstrated faster recovery of the righting response following administration of midazolam, another $GABA_A$ receptor positive allosteric modulator (Rathod et al., 2020). Overall, female PPE mouse offspring appear resistant to the PPE-induced HPA axis dysfunction seen in male offspring, (Finegersh and Homanics, 2014; Rompala et al., 2016; Beeler et al., 2019) though mixed results were observed in female PPE mouse offspring regarding ethanol-induced anxiolysis in the elevated plus maze (Finegersh and Homanics, 2014; Rompala et al., 2017). In contrast to male PPE offspring, female PPE mouse offspring had reduced binge-like ethanol consumption in a drinking-in-the-dark paradigm (Rathod et al., 2020). Reduced operant ethanol self-administration has been observed in both PPE male and female rat offspring (Campbell et al., 2018; Nieto et al., 2021; Nieto and Kosten, 2023). One study demonstrated sensitization of PPE male mouse offspring to the rewarding effects of acute ethanol administration in the conditioned place preference paradigm (Ceccanti et al., 2016); One interpretation of these results suggests a ceiling effect of ethanol reward in PPE male animals, where acute administration results in increased ethanol reward that is outweighed by decreased ethanol seeking behavior in operant paradigms.

Numerous studies have found PPE results in decreased activity/locomotor behavior in adult ($\geq$7 weeks) male and female mice and rat offspring, (Abel, 1989a, 1989b, 1989c; Ledig et al., 1998; Kim et al., 2014) with a more recent study (Conner et al., 2019) uncovering developmental deficits in motor learning, balance, and coordination observed in mouse adolescence. Similar effects were observed in human children and adolescents with a paternal history of alcohol dependence, who have had impaired postural control and increased body sway for their age (Hill et al., 2000, 2008, 2009; Hill and Steinhauer, 1993). Ethanol-induced ataxia in the accelerating rotorod test has shown mixed results in rodent adult PPE male mouse offspring, suggesting PPE results in basal impairment of locomotor activity in offspring due to developmental motor learning deficiencies (Finegersh and Homanics, 2014; Rompala et al., 2017). Further studies could determine whether molecular dysfunction in brain motor control systems predicts locomotor behavior in PPE offspring.

Rodent studies of MPE are scarce, though one 1989 study found impaired avoidance learning in male and female MPE rat offspring with no changes in locomotor activity (Abel, 1989b). MPE male, but not female rat offspring displayed increased stress and anxiety-like behavior (Jabbar et al., 2016). Plasma CORT responses to a lipopolysaccharide challenge appeared sensitized in both sexes, suggesting MPE female offspring resistance to behavioral stress phenotypes (Jabbar et al., 2016). Although MPE offspring ethanol consumption behaviors have not been appreciably studied in rodents, offspring of MPE zebrafish mothers displayed increased ethanol consumption which positively correlated with hypothalamic hypocretin neurogenesis (Collier et al., 2020).

Similar to MPE, cross-generational outcomes of BPE have been historically understudied. The single existing study in zebrafish found that male and female BPE offspring displayed decreased swimming speeds, greater immobility, greater turn angles, and greater thigmotaxis, (Suresh et al., 2021) suggesting motor impairment similar to rodent outcomes of PPE discussed above.

3.2. Molecular outcomes of chronic preconception ethanol across generations in animals

With the historical popularity of the PPE paradigm, many studies have investigated its molecular outcomes, which have also been previously reviewed (Baratta et al., 2021; Rompala and Homanics, 2019; Chastain and Sarkar, 2017; Finegersh et al., 2015); These include dysregulation of multiple neurotrophins and their receptors in frontal cortex, (Ceccanti et al., 2016) hippocampus, (Ceccanti et al., 2016) ventral tegmental area, (Finegersh and Homanics, 2014; Rompala et al., 2017) and nucleus accumbens, (Nieto et al., 2021) as well as altered dopamine

transporter methylation and expression in cerebral cortex and striatum in mice and rats (Kim et al., 2014). Some molecular phenotypes, such as dopamine transporter methylation, possibly occur through DNA methylation in sperm that persists into adult mouse offspring brain, (Kim et al., 2014) whereas others, such as somatic methylation of the *H19* CTCF 1 and 2 imprinting control regions in mice, likely occur through alternative mechanisms such as noncoding RNA regulation or chromatin remodeling (Knezovich and Ramsay, 2012).

Findings from more recent studies support those previously reviewed, specifically regarding molecular brain growth and development and sperm epigenetic mechanisms. Molecular drivers of neocortical patterning (RZRβ and Id2) were dysregulated in their spatial expression patterns in neonatal PPE mouse offspring brain (Conner et al., 2019). In the brainstem of adult male PPE mouse offspring, changes in genes related to serotonergic and neurotrophic signaling were altered, including HTR2C, $p75^{NTR}$, BDNF, and SLC6A4 (Ferraguti et al., 2020). These genetic changes may underly disruptions in sensitivity to serotonergic and neurotrophic effects in PPE offspring (Ferraguti et al., 2020). Male and female PPE rat offspring present differential promoter methylation and expression in hypothalamus of *Esam,* which is important in cell-cell adhesion (Asimes et al., 2017).

Despite extensive literature investigating the prenatal effects of alcohol to understand Fetal Alcohol Spectrum Disorder, few studies have characterized inter- and transgenerational changes following MPE. However, such studies are vital, as a large percentage of the female United States population consumes alcohol in the period prior to conception. Indeed, a 2015 survey of nonpregnant females of reproductive age found ~54% consumed alcohol in the last 30 days, and 18% binge drank (Tan et al., 2015). Similar to PPE, male and female MPE rat offspring display altered hypothalamic methylation and expression of *Esam,* (Asimes et al., 2017) though additional changes are seen in *Fam110a* and *Olr2b6* (Asimes et al., 2017). In another study, adult male and female MPS rat offspring displayed increased basal levels of CORT and ACTH in plasma, which were sensitized relative to controls with lipopolysaccharide immune challenge (Jabbar et al., 2016). Male rat offspring-specific increases in stress- and anxiety-like behavior coincided with dysregulated expression of *Crf* in the hypothalamus and *Crfr1* in the hippocampus and amygdala, which were reversed with the DNA methylation blocker 5-azadoxycytidine. Though preconception alcohol exposure altered methylation and expression, it did not change maternal competence. Thus, it can be concluded that epigenetic inheritance is the most likely mechanism operating to change the behaviors.

Offspring of BPE have displayed robust molecular changes within the HPA axis. Basal plasma CORT levels decrease in male PPE rat offspring, indicating possible HPA axis dysfunction (Asimes et al., 2018). Unique genes are altered in hypothalamic gene promoter methylation and expression in BPE compared to PPE and MPE rat offspring, including *Arrdc1, Ephb3, Mir6216, Gpank1, Golt1b,* and *Sparcl1* (Asimes et al., 2017). Further hypothalamic gene expression changes with BPE in rats relate to transcriptional regulation, mRNA processing, synaptic plasticity, and neurodevelopment (Gaetani et al., 2014).

3.3. Germline mechanisms of cross-generational chronic preconception ethanol phenotype transmission in animals

Germline mechanisms of chronic ethanol-related epigenetic inheritance have been previously reviewed, (Rompala and Homanics, 2019; Chastain and Sarkar, 2017) though several more recent findings are of particular interest. Similar to PPS, sperm RNA content has been implicated in PPE intergenerational phenotypes in mice (Rompala et al., 2018a; Bedi et al., 2019). One study from our laboratory found altered abundance of several classes of small noncoding RNAs in PPE mouse sperm, including tRNA-derived small RNAs, mitochondrial small RNAs, and miRNAs (Rompala et al., 2018a). We also found increased post-transcriptional nucleoside modifications of mitochondrial tRNAs, as well as reduced expression of the tRNA methyltransferase *Nsun2*. Another study found a PPE-induced significant increase in miRNAs and decreased ratio of tRNA-derived small RNAs to piRNAs in mice (Bedi et al., 2019). It is important to note the PPE paradigms differed in these studies, as we did not find alterations in piRNAs (Rompala et al., 2018a); We utilized five cycles of chronic intermittent ethanol vapor exposure and collected sperm 16–19 h after the last exposure, whereas the other study carried out multiple 70-day cycles of drinking-in-the-dark (DID) interspersed with mating, then subsequently sacrificed the sires for reproductive tissue collection after the last exposure. It is possible that, like PPS, the exact ethanol exposure paradigm contributes to the sperm RNA profile. However, a 2023 study also using 70 days of DID found that, while global RNA expression signatures in caput epididymal tissue (where immature sperm cells are found (James et al., 2020)) were reverted in males four weeks after cessation of chronic ethanol exposure, the small noncoding RNA profile of mature sperm remained relatively stable (Roach et al., 2023). This suggests that, at least in mature sperm, chronic ethanol exposure results in persistent changes in sperm small noncoding RNA. However, more studies are required to determine the importance of ethanol exposure timing in epigenetic changes in germ cells and phenotypic alterations in offspring.

Another study conducted in our laboratory found that, like PPS (Chan et al., 2020), PPE also impacts the sperm RNA cargo via epididymosomes. We found similar ethanol-induced alterations in tRNA-derived small RNA abundance in mouse epididymosomes and sperm (Rompala et al., 2018a). Furthermore, coincubation of ethanol-naïve mouse sire sperm with PPE sire epididymosomes followed by *in vitro* fertilization (IVF)/embryo transfer was sufficient to impart intergenerational behavioral phenotypes (Rompala et al., 2020). These findings reinforce the vital role epididymosomes appear to play in intergenerational phenotype heritability. The sperm of PPE mouse sires has also shown increased reactive oxygen species and DNA fragmentation, as well as increased histone methylation (H3K4me3) at promoters and regulatory regions associated with neuronal development, synapse assembly, and neuron differentiation (Cambiasso et al., 2022; Bedi et al., 2022).

Mechanisms of MPE have not been appreciably researched (Chastain and Sarkar, 2017). However, one study that found increased diabetes susceptibility in MPE offspring noted distinct transcriptomic changes in MPE rat dam oocytes related to stress regulation and immune function (Al-Yasari et al., 2021). To our knowledge no literature exists on BPE mechanisms. The overall lack of MPE and BPE studies present significant gaps that should be addressed with future preclinical research. For example, the mechanistic role of the transcriptomic changes observed in MPE oocytes could be tested via embryo injection studies. Related to this, to our knowledge no studies have causally tested sufficiency of differentially expressed sperm noncoding RNAs in conferring the behavioral or molecular phenotypes of PPE. Similar approaches should be taken to PPS germline mechanism studies to test this, purifying differentially expressed sperm RNAs and injecting them into fertilized wild-type embryos.

The potential role of glucocorticoids in mediating the cross-generational transmission of chronic ethanol exposure phenotypes remains uncharacterized. Several studies reviewed here found altered basal plasma CORT in preconception ethanol exposure offspring (Jabbar et al., 2016; Asimes et al., 2018). We also previously found a blunted CORT response to restraint stress selectively in male PPE offspring (Rompala et al., 2016). However, to our knowledge, no studies have directly mechanistically investigated whether preconception ethanol exposure alters glucocorticoid secretion in parents, and if this leads to changes in stress reactivity in offspring. However, it is again critical to note that the effects of alcohol consumption on glucocorticoid secretion have historically only been seen with heavy drinking in humans (Fan et al., 2023; Badrick et al., 2008; JH and Mendelson, 1966).

4. Limitations of and considerations for preclinical research on cross-generational chronic preconception stress & ethanol phenotypes

There are some vital limitations to consider in preclinical cross-generational stress and ethanol exposure studies, both when analyzing the literature and planning future research. As discussed in the above sections, several PPS and MPS studies have found the timing between chronic stress and conception affects cross-generational molecular and behavioral outcomes, which is noted as a variable in Tables 1, 3 and 4. Similarly, rodents have displayed differential stress responsivity during the estrous cycle due to fluctuating progesterone levels (Lovick, 2012; Lovick and Zangrossi, 2021; Jaric et al., 2019; ter Horst et al., 2011). Although timing between exposure and conception and estrous cycle effects have not been directly examined in preconception ethanol studies to our knowledge, ethanol is known to affect sex hormones, sperm quality, and fertility (Guthauser et al., 2014; Jensen et al., 2014; Condorelli et al., 2015; Muthusami and Chinnaswamy, 2005; Dare et al., 2002); For this reason, many cross-generational ethanol studies involve a washout period to bolster reproductive success (Table 4). Future preconception stress and ethanol research should carefully consider the period between exposure and conception as well as estrus cycle phase in light of the specific experimental question being investigated.

Another important factor to consider when interpreting and designing preclinical cross-generational stress and ethanol exposure studies is the species and strain of the model organism, though this literature is not yet comprehensive enough to draw major conclusions about the strengths or weaknesses of any given model. The majority of studies reviewed here were performed in multiple substrains of rats and mice. Several were performed in zebrafish, which are also valuable models for stress, neuroadaptations, and epigenetic regulation of gene expression (Tables 1–4). (Rosa et al., 2022; Veldman and Lin, 2008; Egan et al., 2009; Perathoner et al., 2016). Across all modes of preconception stress and alcohol inheritance, we were not able to find any studies that can be directly compared between rats and mice (*i.e.,* that use the same exposure and behavioral testing paradigms). As reviewed here, different modes of preconception stressor exposure can lead to different behavioral phenotypes. For PPS, which overwhelmingly utilized mice in the literature, this leads to opposing results both between and within species on anxiety- and depressive-like behavior in offspring (Tables 1 and 2). The same is true for MPS, which overwhelmingly utilized rats (Table 3). The two BPS studies (one in rats and one in mice) found amplified anxiety-like behaviors, particularly in female offspring (Saavedra-Rodrí et al., 2013; Azizi et al., 2019). PPE appears to decrease ethanol self-administration in male offspring (Beeler et al., 2019; Nieto et al., 2021; Nieto and Kosten, 2023). There are conflicting results on the reinforcing properties of ethanol in rats and mice that may be attributed to different behavioral testing paradigms utilized (conditioned place preference versus operant conditioning) (Ceccanti et al., 2016; Beeler et al., 2019; Nieto et al., 2021; Nieto and Kosten, 2023). To our knowledge, MPE and BPE studies have only used rats (Jabbar et al., 2016; Collier et al., 2020; Asimes et al., 2017, 2018; Gaetani et al., 2014). Similarly, the studies reviewed here are not comprehensive enough to draw conclusions on the strengths and weaknesses of rat and mouse substrains in cross-generational preconception stress and ethanol research. However, as in any scientific research, the precise experimental question and consistency with prior research are of utmost importance. Stress- and ethanol-related behaviors can differ substantially between different strains of rats and mice, (Jung et al., 2014; Van Bogaert et al., 2005; Ellenbroek et al., 2005; Belknap et al., 1993; Gauvin et al., 1993) which should be considered when designing and interpreting studies. Special attention should also be paid to substrains (*e.g.* C57BL/6J versus C57BL/6N mice), which can form across breeding colonies maintained across different laboratory suppliers or facilities (Mekada et al., 2009; Bryant et al., 2009; Zurita et al., 2010; Palm et al., 2011). Although genetics and behavior may differ between model species and substrains, multiple epigenetic mechanisms, including DNA methylation and small noncoding RNA regulation of gene expression, are highly evolutionarily conserved (Seffer et al., 2013; Zhou et al., 2017; Willbanks et al., 2016; Lee et al., 2007). The lack of methodological consistency across studies is likely due to the field of preconception stress and ethanol inheritance still being in its infancy; More studies are needed that employ consistent modes of exposure across species to enhance the generalizability and translatability of findings.

As introduced earlier, some of the first studies of nongenomic inheritance examined the effects of maternal care. This body of research found maternal care patterns persist across generations through nongenomic mechanisms (Francis et al., 1999; Champagne and Meaney, 2001; Fairbanks and McGuire, 1995; Fairbanks, 1996; Maestripieri et al., 1997; Fleming et al., 1999; Champagne, 2008; Kinnally et al., 2018; Meaney, 2001) and found increased stress reactivity in the adult offspring that were neglected by their mothers (Francis et al., 1999; Champagne and Meaney, 2001; Liu et al., 1997; Meaney, 2001; Meaney et al., 1991, 1996; Zaharia et al., 1996; Caldji et al., 1998). More recently, studies have shown that prior stress exposure influences maternal care across generations (Rosenblatt, 1975; Purcell et al., 2011; Orso et al., 2019; Nephew et al., 2017; Zaidan et al., 2023). Maternal stress also influences lactation and breastmilk composition, (Purcell et al., 2011; Ziomkiewicz et al., 2021; Juncker et al., 2023; Pfister and Muir, 2009) which significantly influences offspring development and stress-related phenotypes (Abbink et al., 2020; Walker et al., 2004). Thus, the nongenomic transmission of MPS phenotypes involves both the maternal germline and behavior, the mechanisms of which are important to dissect. Cross-fostering (Francis et al., 1999; Uchida et al., 2010; McCarty, 2017; Priebe et al., 2005) as well as embryo transfer (Rose et al., 2006) between MPS and control dams are valuable tools to accomplish this. However, many studies instead opt to monitor maternal care, which is often vaguely described and not included as a covariate in statistical analysis. Indeed, in our literature review, we could identify only three preconception stress studies that utilized cross-fostering;[73, 96] [60] Three others stated they monitored maternal care (Franklin et al., 2010, 2011; Mashoodh et al., 2023). Only one preconception ethanol study used cross-fostering (Jabbar et al., 2016) but several monitored maternal care and found no changes based on prior ethanol exposure (Jabbar et al., 2016; Asimes et al., 2017). Controlling for maternal care is important even in PPS or PPE studies, as rodent dams are known to change maternal care based on the perceived fitness of their mate (the Differential-Allocation Hypothesis), (Burley, 1988; Mashoodh et al., 2012) which could be altered based on prior stress or alcohol exposure. Related to this, the presence of the sire affects maternal care (Curley et al., 2011); Thus, it is critical to remove the sire as soon as possible after mating. The vast majority of studies reviewed here did this, either allowing a pre-specified mating period or removing the sire after confirming the presence of a vaginal plug or sperm-positive vaginal smear.

Although we could not locate any studies relevant to this review that utilized embryo transfer, one PPS study using the CSDS paradigm included IVF, in which sperm from CSDS sires was used to fertilize oocytes from control donor females. IVF recapitulated the depressive-like phenotypes observed in the FST of naturally-sired male and female offspring, while the other phenotypes observed (EPM anxiety-like behavior, hyperlocomotion, anhedonia, and increased plasma CORT) were not recapitulated (Dietz et al., 2011). The authors concluded this discrepancy between naturally-sired and IVF offspring could be due to (1) differences in maternal care due to exposure to the PPS sire and (2) the fact that the IVF process could select sperm in different stages of the maturation process, and as a result, sperm with differential genomic imprinting (Lee et al., 2002). However, seminal fluid can also confer epigenetic changes in offspring, (Donkin et al., 2018; Patlar, 2022) meaning these results could point to a role of semen composition in the nongenomic inheritance of PPS. This is an intriguing mechanism that should be investigated with further studies of nongenomic stress-related phenotype transmission utilizing IVF. It is important to note though that

collecting sperm from the epididymis requires sacrificing the sire, which could impose limitations on these studies. Mindful conductance of pre-clinical research in light of this and the other factors discussed above will bolster translatability between animal models and humans.

5. Health-related outcomes of chronic stress and ethanol exposure in animals: using cancer as a model

There is considerable public health benefit in establishing whether chronic stress or alcohol use are associated with cross-generational effects on health. Heavy chronic alcohol use and chronic stress are associated with myriad negative health outcomes (Kivimäki and Steptoe, 2017; Schmidt et al., 2008; World Health Organization, 2018; Tomiyama, 2019). In the case of alcohol consumption and cancer, we know that even light drinking (Bagnardi et al., 2013; Freedman et al., 2007) has been linked to the development of multiple cancers, (Bagnardi et al., 2013; Boffetta et al., 2006; Yoo et al., 2022) and alcohol-induced epigenetic changes have been specifically implicated in cancer risk and development (Dumitrescu, 2018; Ghantous et al., 2018; Wang et al., 2017; Slattery et al., 2010; Hlady et al., 2014). As we will review in the next section on clinical studies of cross-generational preconception stress and alcohol-related disease risk, a small body of literature has begun to investigate preconception alcohol in cancer risk in subsequent generations. While to-date no published studies have investigated cross-generational ethanol exposure and cancer risk in animal models (though our laboratories are currently undertaking such a study), animal models have been suggested as a useful strategy for investigating alcohol effects on cellular immunity for decades (Chirigos and Schultz, 1979). Preclinical studies within generations have found that both alcohol and chronic stress suppress the immune system in a way that is conducive to the development of tumors (Chirigos and Schultz, 1979; Eckerling et al., 2021; Liu et al., 2022). However, a consensus on the extent of the effects of alcohol on development of cancerous tissue has not been reached because of variation in the methods used to induce cancers in animals (Ratna and Mandrekar, 2017). Preclinical evidence has also linked chronic stress to immune alterations that promote cancer development and metastasis, specifically through enhanced glucocorticoid, epinephrine, and norepinephrine signaling (for reviews, see: Cui et al. (2021); Liu et al. (2022); Dai et al. (2020)). However, this has primarily been studied in the context of existing tumor progression and treatment outcomes, and so it is currently unclear whether chronic stress alone increases cancer risk. The study of cross-generational effects of alcohol exposure on transcriptomic changes in cancer related pathways, particularly those involving the immune system, may provide insights into how pre-conception use of alcohol in parents and even grandparents may influence cancer risk in subsequent generations. Our lab has begun these studies in humans and animals, which are discussed in the following section.

6. Integration of chronic preconception stress and ethanol exposure and translational findings

Extensive evidence has shown heritability of preconception stress-related phenotypes in humans. Initial studies examined families of Holocaust survivors, which showed greater risk of developing PTSD, depression, and anxiety disorders (Yehuda et al., 2008) as well as other adverse health outcomes such as hypertension, Type 2 diabetes, and dyslipidemia (Flory et al., 2011). Since these studies, epigenetic heritability of negative health effects due to trauma has been studied in the offspring of prisoners of war during the American Civil War (Costa et al., 2018) as well as in the children of civilians exposed to other armed conflicts (Betancourt et al., 2020; Phadera, 2021). In addition, human paternal early life stress was associated with aberrant neonatal offspring brain development (Karlsson et al., 2020), and loss of a parent in early childhood was associated with differential intergenerational health effects depending on the sex of the parent who experienced bereavement (Brew et al., 2022). Maternal bereavement was associated with early-onset asthma in the offspring, and paternal bereavement was associated with offspring autoimmune disorders (Brew et al., 2022). However, maternal effects were mediated partly by socioeconomic status and incidence of mood disorders (Brew et al., 2022). Indeed, high heritability is seen in maternal depression, (Hammen et al., 2004, 2011; Gershon et al., 2011; Murphy et al., 2022; Letourneau et al., 2018) with bidirectional and potentiating effects of depression itself as well as chronic stress induced by exposure to depression in the parent (Hammen et al., 2011; Murphy et al., 2022; Letourneau et al., 2018). This points to a larger issue with human subject research on stress-related disorder heritability, discussed further below with AUD, where the epigenetic heritability of the disorder is difficult to disentangle from the effects of chronic stress in the offspring from being exposed to the disorder in a parent. An additional issue in stress-related research is the large inter-individual variations in the perception of stress, with physiological responses often not directly related to this perception (Henckens et al., 2016).

Few studies have been performed that investigate the cross-generational effects of alcohol exposure on human health. However, those that do exist have examined cancer risk in subsequent generations. Inspired by the reports that parental pre-conception experience of famine can impact the incidence of medical conditions and longevity in offspring, (Kaati et al., 2007; Pembrey et al., 2014) we undertook a study to examine whether pre-conception parental use of alcohol that controlled for maternal prenatal use of alcohol would result in epigenetic changes in offspring. We found significant hypomethylation and increased expression of the oncogene HRAS as well as hypermethylation of the tumor suppressor gene TP53 (Hill et al., 2017). Related to this, two comprehensive reviews have assessed the literature concerning the effect of parental use of alcohol and childhood cancers (Infante-Rivard and El-Zein, 2007; Latino-Martel et al., 2010). In one review, 33 studies assessed parental behaviors and reports of any childhood cancer covering the period 1982–2003, with 10 studies reporting at least one statistically significant effect including associations between the paternal alcohol use and offspring cancer development (Infante-Rivard and El-Zein, 2007). However, it is important to note that these studies were observational and did not include the study of epigenetic effects, DNA methylation or gene expression (Infante-Rivard and El-Zein, 2007; Latino-Martel et al., 2010). In addition, these studies assessed pediatric cancers (Infante-Rivard and El-Zein, 2007; Latino-Martel et al., 2010). Due to the length of follow-up needed to make the association between parental alcohol use and adult-onset cancers, no studies to-date have shed light on this question. In view of our previous results showing epigenetic alterations in an oncogene and tumor suppressor gene evaluated in lymphocytes of offspring from parents with AUD and their parents, (Hill et al., 2017) it is likely that elevated rates of cancers during adulthood will be seen upon follow-up. We have taken the first step in evaluating such a connection, finding cross-generational effects of alcohol exposure that resulted in epigenetic changes. Currently, we are investigating cross-generational effects on genome-wide gene expression in human banked samples. This is being performed in tandem with an intergenerational mouse study on preconception ethanol exposure to address the overall lack of translational research in this field.

While maternal alcohol use during pregnancy may have influenced offspring health and cancer development in some of the studies reviewed here that assessed the incidence of pediatric cancers (though this was controlled for in our study (Hill et al., 2017)), paternal pre-conception drinking effects have been less frequently investigated (Day and Bonduriansky, 2011), meaning conclusions about causality may be misplaced. This is clear in literature on Fetal Alcohol Spectrum Disorder, where extensive evidence on the effects of maternal alcohol use during pregnancy (Dejong et al., 2019) has led to the conclusion that offspring Fetal Alcohol Spectrum Disorder is the product of prenatal use by mothers, despite the fact that up to 75% of such children have fathers with AUD, (Abel, 2004) and epigenetic inheritance of paternal

preconception ethanol exposure has been found to play a significant role in FASD-associated craniofacial growth deficiencies (Thomas et al., 2023). These findings suggest the clinical histories of both parents, and ideally their parents, need to be taken into account for a complete understanding of the relevant mechanisms promoting development of alcohol- and stress-related diseases from a cross-generational perspective.

Recently, additive effects of stress and alcohol use on epigenetic aging have been reported in humans (Jung et al., 2023). Given the interrelated nature of stress and alcohol use (Becker, 2017; Wittgens et al., 2022; Keyes et al., 2012) and the heritability of their phenotypes discussed in this review, more cross-generational research is needed to investigate the combinatorial effects of chronic preconception stress and ethanol as well as to dissect the stress-inducing effects of ethanol (Becker, 2017) (and any subsequent epigenetic modifications due to this stress) from epigenetic modifications due to ethanol itself. Indeed, the increase in childhood psychopathology seen in offspring of parents with AUD (Hill et al., 2008), though associated with genetic susceptibility within families with multiple cases of AUD (Hill et al., 2004; Hill and Hostyk, 2023), may also be the result of chronic stress experienced by children living in the home of a parent with AUD and its epigenetic effects (Hill and Sharma, 2019; Hill et al., 2022). Preclinical animal models will be a useful tool for teasing apart these effects (Hime et al., 2021). In a previous mouse study, (Rompala et al., 2018b) we found reduced binge- and free-choice ethanol consumption in male (but not female) offspring of sires exposed to CUS. However, these findings only demonstrate potential intergenerational effects of stress exposure on ethanol drinking behavior and do not suggest any molecular or germline mechanisms.

7. Conclusions and future directions

In reviewing the relevant literature, we hypothesized that cross-generational chronic preconception stress and alcohol exposure share common mechanisms and neurobiological outcomes that can be further studied to elucidate the pathogenesis of AUD and comorbid conditions. It is clear that both chronic stress and ethanol exposure result in cross-generational alterations in stress-, anxiety-, and depression-related behaviors. Preclinical studies suggest these arise from alterations in DNA methylation, gene expression, and (to some extent) synaptic plasticity in brain and are conferred by epigenetic alterations in germline cells, particularly in small noncoding RNA expression and DNA methylation. However, there exists much heterogeneity in methods across the literature that make direct comparisons between studies difficult. In addition, the role of parental glucocorticoids in response to chronic stressors in inducing cross-generational phenotypes should be further characterized.

Further translational studies are needed to determine the cross-generational effects of alcohol exposure in particular. To address this gap, we are currently investigating the intergenerational effects of PPE, MPE, and BPE on gene expression via RNA-Sequencing in adult mouse offspring. In parallel, we are performing a human RNA-Sequencing study on banked lymphocyte samples from offspring of parents with PPE and MPE for whom extensive clinical data is available along with that of the offspring's grandparents. Additional preclinical and clinical studies can dissect the effects of chronic stress from parental preconception ethanol exposure. Preclinical studies can utilize cross-fostering (Uchida et al., 2010; McCarty, 2017; Priebe et al., 2005) and/or embryo transfer (Rose et al., 2006) to specifically control for parental stress after conception. In humans, establishing a connection between molecular phenotypes in offspring and their potential for development of specific diseases due to pre-conception effects from parents requires knowledge of parental behaviors and control of several variables including the history of personal use of alcohol and any prenatal exposures. Specifically, the following data are needed: (1) Detailed clinical histories of alcohol use disorders for parents and ideally their parents in order to examine potential germ line effects; (2) Mothers' use of alcohol and other drug use during pregnancy to rule out prenatal effects; (3) Personal use histories of alcohol or other drugs by offspring (children) prior to obtaining samples for analysis. Direct evaluation of offspring for reported stress levels can be especially useful. With longitudinal assessment during childhood, opportunities for measuring the child's experience of stress as we have done (Hill et al., 2022) offers greater precision relative to presuming a uniform level of stress in offspring from disadvantaged environments such as those experienced by children of AUD parents. Other considerations include the age of offspring when sample collection occurs; collection of samples in pre-adolescent participants is an ideal condition to minimize the effect of personal exposures.

Integrated approaches to understanding cancer risk appears to require analysis of genetic and epigenetic factors along with gene expression profiles to address the complexity of cancer etiology (Thingholm et al., 2016). In addition to the direct damage induced by alcohol on tissues, (Simon et al., 2022; Jung et al., 2011) alcohol has the potential to change the methylation and expression of genes involved in tumor development (Dumitrescu, 2018; Ghantous et al., 2018; Wang et al., 2017; Slattery et al., 2010; Hlady et al., 2014). However, we have only begun to investigate these effects cross-generationally (Hill et al., 2017). Identification of the specific genes that show differential epigenetic marks and expression in association with pre-conception alcohol use is of high importance. The role of these genes in cross-generational phenotypes can then be causally investigated with pharmacological agents that can alter DNA methylation and subsequently change gene expression (Heerboth et al., 2014) such as zebularine, (Zhou et al., 2002) or with CRISPR/dCas9 DNA methylation editing (Liu et al., 2016).

Preconception stress along with prenatal and early post-natal stress and/or alcohol exposure all have the potential for elucidating important sources of variance in outcomes across generations for a number of common disorders including cardiovascular, metabolic, neurological, and developmental disorders. Recently, the additive effects of stress and alcohol use has been reported in which epigenetic aging effects were shown to be increased when both stress and heavy alcohol use were present (Jung et al., 2023). The interaction of stress and alcohol consumption across generations may provide substantial explanatory power in our understanding of disease risk.

CRediT authorship contribution statement

Rachel C. Rice: Writing – original draft, Writing – review & editing. **Daniela V. Gil:** Writing – original draft, Writing – review & editing, Visualization. **Annalisa M. Baratta:** Writing – original draft, Writing – review & editing. **Remy R. Frawley:** Visualization, Writing – review & editing. **Shirley Y. Hill:** Writing – original draft, Writing – review & editing. **Sean P. Farris:** Writing – review & editing. **Gregg E. Homanics:** Writing – review & editing.

Declaration of competing interest

None.

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