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Sexual dimorphism in the neural impact of stress and alcohol

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Abstract

Alcohol use disorder is a widespread mental illness characterized by periods of abstinence followed by recidivism, and stress is the primary trigger of relapse. Despite the higher prevalence of alcohol use disorder in males, the relationship between stress and behavioral features of relapse, such as craving, is stronger in females. Given the greater susceptibility of females to stress-related psychiatric disorders, understanding sexual dimorphism in the relationship between stress and alcohol use is essential to identifying better treatments for both male and female alcoholics. This review addresses sex differences in the impact of stressors on alcohol drinking and seeking in rodents and humans. As these behavioral differences in alcohol use and relapse originate from sexual dimorphism in neuronal function, the impact of stressors and alcohol, and their interaction, on molecular adaptations and neural activity in males and females will also be discussed. Together the data reviewed herein, arising from a symposium entitled “Sex matters in stress-alcohol interactions” presented at the Fourth Volterra Conference on Stress and Alcohol, will highlight the importance of identifying sex differences to improve treatments for comorbid stress and alcohol use disorder in both populations.

Keywords

alcohol; ethanol; stress; corticosterone; noradrenaline

Introduction

Substance Use Disorders (SUDs) are a major public health burden in the US, costing more than $400 billion annually in crime, poor health outcomes, and lost productivity (U.S. Department of Health and Human Services, 2016). Traditionally SUDs, including alcohol use disorder (AUD), have been diagnosed up to twice as frequently in males (World Health Organization, 2014). However, women are steadily increasing their use of alcohol and illicit substances, with greater prevalence of binge drinking and heavy alcohol consumption

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Conflict of interest

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(Dawson, Goldstein, Saha, & Grant, 2015; Grant et al., 2017; Keyes, Li, & Hasin, 2011), as well as psychoactive drug use (SAMHSA, 2015), than previously observed. In addition, women display greater vulnerability than men to all stages of addiction, including initiation, progression and relapse (Agabio, Campesi, Pisanu, Gessa, & Franconi, 2016; Quinones-Jenab, 2006). Given the chronically relapsing nature of AUDs, determining the neurobiological underpinnings of differential susceptibility to AUDs can promote the development of more effective treatments for both sexes. Of particular interest as a source for sexual dimorphism in disease prevalence, progression and resurgence is the impact of stress on neurobiology and behavior.

Stress-related disorders, including anxiety disorders, mood disorders and post-traumatic stress disorder (PTSD), are twice as frequently diagnosed in females as compared to males (World Health Organization, 2014). Stress and the negative emotional state it generates are primary triggers of relapse in men and women (Annis, Sklar, & Moser, 1998; Seo & Sinha, 2014; Sinha, 2007), suggesting that overlapping circuitry regulates alcohol use and stress responses in both sexes. Data indicate that females may display a more direct relationship between current or past stress exposure and relapse-related variables. Females with comorbid alcohol use and PTSD show greater sensitivity to the effects of stress on alcohol craving and relapse (Heffner, Blom, & Anthenelli, 2011), and PTSD may more commonly precede the development of AUD in females vs. males (Sonne, Back, Diaz Zuniga, Randall, & Brady, 2003). Moreover, chronic drug abuse shows differential neuroadaptations in men and women. Sex differences have been observed in physiologic, neuroendocrine, and craving responses to stress and drug cues (Back, Brady, Jackson, Salstrom, & Zinzow, 2005; Fox & Sinha, 2009). Importantly, these measures have been associated with high risk of relapse and poor treatment outcomes (Back et al., 2005; Daughters, Richards, Gorka, & Sinha, 2009; Fox & Sinha, 2009; Moeller, Bederson, Alia-Klein, & Goldstein, 2016; Sinha, Garcia, Paliwal, Kreek, & Rounsaville, 2006; Van Dam, Rando, Potenza, Tuit, & Sinha, 2014). These differential sensitivities to alcohol and stress in males and females suggest circuit dichotomies between the sexes – yet most preclinical research to date has focused on elucidating factors promoting alcohol use, relapse, and stress responses solely in males. The limited number of studies that have, to date, investigated sex differences in stress-alcohol interactions, or effects in females, are reviewed herein and summarized in Table 1. One primary intersection between stress and abused drugs like alcohol, which may generate divergent neuroadaptations in males and females, is the activation of systemic stress response systems.

Physiological stress responses and alcohol use

Sex differences in substance misuse, as well as the long-term impact of stressors on drinking or other drug use and relapse, may stem from sexual divergence in systemic stress responses. Activation of the hypothalamic-pituitary-adrenal (HPA) axis by stressors, resulting in elevated circulating corticosterone (CORT; cortisol in humans), is magnified in female rodents, relative to males, after various stressors (Rivier, 1999) and multiple drugs of abuse, including alcohol (Ogilvie & Rivier, 1996; Rivier, 1993). In male rats, alcohol dependence dysregulated HPA axis responses to alcohol (Richardson, Lee, O'Dell, Koob, & Rivier, 2008), whereas blockade of the glucocorticoid receptor, one target of CORT, impeded both

the development (Somkuwar et al., 2017; Vendruscolo et al., 2012) and the expression (Vendruscolo et al., 2015) of dependence-induced exacerbation of alcohol self-administration. At present, the effects of alcohol dependence on these parameters in females remain unknown and as such are a critical future direction of preclinical research, as polymorphisms in the glucocorticoid receptor gene were associated with earlier onset of alcohol use or misuse in females, more than in males, in a large cohort of Finnish teenagers (Desrivieres et al., 2011). Early abstinence from alcohol in humans is marked by dysregulated basal physiological and neuroendocrine tone, and stress- and cue-induced physiological, HPA axis and emotional changes are strongly associated with increased drug and alcohol craving, drug use and relapse risk (Back et al., 2005; Fox et al., 2009; Fox & Sinha, 2009). Administration of the glucocorticoid receptor antagonist mifepristone reduced alcohol craving, relative to placebo, in a mixed-sex clinical treatment population with AUD, similar to effects observed in male alcohol-dependent rats (Vendruscolo et al., 2015); however, the population was disproportionately male and thus sex differences in treatment efficacy could not be determined. Together, these data implicate stress responses, particularly CORT function via glucocorticoid receptors, as intrinsic drivers of alcohol use in both sexes and suggest overlapping neurocircuitry and similar neuroadaptations may drive the interaction between stress and drug use in males and females. Despite long-standing knowledge of increased systemic HPA axis response to stressors and alcohol in females, most research into neuroadaptations caused by alcohol, stress, or their co-occurrence, and the impact of such adaptations on alcohol-related behaviors, has focused exclusively on male subjects. To date, only a small fraction of preclinical investigations into stress, alcohol and their interaction have explored sex differences, at either the molecular or behavioral level, as detailed in the following sections.

Sexual dimorphism and differential neuroadaptations of stress- and alcohol-responsive circuitry

A reciprocal relationship exists for behavioral regulation by stress and alcohol, with alcohol modifying stress-related behaviors and stressors altering alcohol consumption (Logrip, Zorrilla, & Koob, 2012). This suggests that overlapping neurocircuits support behavioral responses to both alcohol and stress, with intrinsic sex differences in the circuitry producing different behavioral responses in males and females. Candidate regions activated by both stress and alcohol that display divergent structural or electrophysiological responses between the sexes include regions of the limbic system and extended amygdala, where stress and alcohol interact to regulate neuronal activity, as well the locus coeruleus (LC), responsible for controlling arousal.

Behavioral arousal, regulated by norepinephrine, modulates individual perception of stressful and rewarding experiences (Espana, Schmeichel, & Berridge, 2016), and sex differences in noradrenergic arousal mechanisms could contribute to differential systemic as well as neuronal stress responses. Noradrenergic neurons, whose cell bodies are found in the LC, display sexual dimorphism in both structure and function, with increased dendritic complexity (Bangasser, Zhang, Garachh, Hanhauser, & Valentino, 2011) and basal activation of corticotropin-releasing factor (CRF) receptors, measured as CRF_1-G_s-coupling, observed in unstressed Sprague-Dawley female rats, relative to males (Bangasser et al., 2010). Despite

the increased basal CRF_1 activation, administration of CRF into LC increased neuronal activity to a greater degree in female, versus male, neurons, although prior swim stress normalized this difference (Curtis, Bethea, & Valentino, 2006). Similar to CRF, chronic alcohol consumption via liquid diet activated more neurons in female versus male rat LC, as measured by c-fos immunoreactivity, and differentially affected CRF_1 localization, with more CRF_1 observed in the plasma membrane of female rats (Retson, Reyes, & Van Bockstaele, 2015). Decreased membrane CRF_1 levels, and associated reductions in LC neuron activation, likely result from sex differences in CRF_1 internalization by β-arrestin$_2$, which is observed only in males (Bangasser et al., 2010). Together these studies implicate the LC as one neuronal locus displaying intrinsic sexual dimorphism, yielding sex differences in activation by acute stressors, as well as sex differences in adaptation to the chronic stress of alcohol dependence.

A primary source of CRF input to the LC is the central extended amygdala (Van Bockstaele, Bajic, Proudfit, & Valentino, 2001), a circuit comprised of the central nucleus of the amygdala (CeA), bed nucleus of the stria terminalis and shell of the nucleus accumbens (Alheid & Heimer, 1988; Cassell, Freedman, & Shi, 1999). The extended amygdala provides an interface by which stress and reinforcers, including alcohol, interact, and adaptation of this circuit following chronic stress or alcohol underlies the negative affect believed to drive escalated alcohol use and relapse (Koob, 2015; Koob & Le Moal, 2008). In particular, the central nucleus of the amygdala (CeA), the output nucleus of the amygdala complex, has long been implicated in alcohol dependence and alcohol-stress interactions. In males, CeA neuron activity is altered by various neuropeptides whose expression is changed by substantial alcohol exposure in a direction associated with elevated anxiety-like behavior (Economidou et al., 2008; Funk, O'Dell, Crawford, & Koob, 2006; Gilpin et al., 2011; Pandey, Zhang, Roy, & Misra, 2006; Roberto, Bajo, Crawford, Madamba, & Siggins, 2006; Zhu, Bie, & Pan, 2007). While few studies have addressed sex differences in the extended amygdala, two studies investigating sex differences in CRF expression have shown sex-specific expression patterns in the CeA. During adolescence, female rats displayed fewer CRF-immunoreactive cells than males, with binge-like alcohol drinking blunting CRF expression in both sexes (Karanikas, Lu, & Richardson, 2013). Conversely, chronic alcohol liquid diet consumption activated CRF-expressing CeA neurons in females only, relative to alcohol-naïve controls, whereas swim stress in alcohol-dependent rats increased activation of CRF neurons only in males (Retson, Hoek, Sterling, & Van Bockstaele, 2015). Together these data demonstrate that alcohol and stress generate sexually divergent adaptations of similar targets, which may vary by developmental stage. Of importance is the impact of such neuroadaptations on neuronal activity in the region.

Alcohol's effects on male neurons of the medial CeA have been extensively studied. Alcohol enhances GABAergic inhibitory postsynaptic responses and reduces the magnitude of glutamatergic excitatory postsynaptic potentials and currents (EPSP/Cs) (Roberto et al., 2006; Roberto, Madamba, Moore, Tallent, & Siggins, 2003; Roberto, Madamba, Stouffer, Parsons, & Siggins, 2004), in part via CRF's actions (Bajo, Cruz, Siggins, Messing, & Roberto, 2008; Herman et al., 2013; Herman et al., 2016; Varodayan et al., 2017). Given the aforementioned sex differences in the effects of stress and alcohol on CeA CRF neurons, associated changes in neuronal activity would be expected to display different patterns in

males and females. To date, only two studies have tested sex differences in the electrophysiological impact of alcohol and stress on CeA neurons. The first investigated alcohol's effects on the circuitry connecting the basolateral amygdala (BLA), a region with greater spine density in males vs. females (Rubinow, Drogos, & Juraska, 2009), to the CeA's lateral (CeL) vs. medial (CeM) subdivisions in adult male and female Wistar rats (Logrip, Oleata, & Roberto, 2017). Alcohol was less effective in decreasing female EPSPs, particularly in the BLA-CeM circuit, as compared to male neurons that showed similar alcohol-induced reductions in BLA-CeL and BLA-CeM EPSPs. Hormonal status also impacted female responses, as BLA-CeM EPSPs were unexpectedly potentiated by alcohol during proestrus. To extend these investigations, stress-alcohol interactions were assessed by acute CORT application, a pharmacological challenge previously shown to potentiate BLA excitability through the same mechanism as acute stress in males (Duvarci & Pare, 2007; Karst, Berger, Erdmann, Schutz, & Joels, 2010). CORT significantly reduced EPSP magnitude only in the female BLA-CeL circuit, with no significant effects observed in female BLA-CeM or either male CeA subdivision (Logrip et al., 2017). Subsequent co-application of alcohol reduced EPSPs in both male CeA subdivisions similarly to alcohol alone, whereas female BLA-CeM neurons demonstrated no response to either stimulus and female BLA-CeL neurons showed no further decrease in EPSP magnitude beyond the dramatic reduction induced by CORT. Together these studies demonstrated sexually dimorphic sensitivity of CeA neurons to alcohol and CORT, with male neurons primarily inhibited by alcohol and not CORT, whereas female neurons were more sensitive to CORT than to alcohol. Recent studies in Long-Evans rats have shown reduced sensitivity of females, relative to males, to the effects of withdrawal from chronic intermittent alcohol vapor on BLA EPSCs as well as anxiety-like behavior (Morales, McGinnis, Robinson, Chappell, & McCool, 2018). Specifically, females required longer exposure to intermittent ethanol vapor to display the same phenotypes as males, which included increased glutamate release and increased EPSC amplitude in the BLA. These data are in line with the reduced acute alcohol sensitivity of female neurons to BLA-evoked CeA EPSPs (Logrip et al., 2017). While these differential sensitivities would suggest greater amygdala neuroadaptation to stressors for females and to alcohol for males, the broader molecular and behavioral impacts of these findings remain to be determined.

A subsequent study assessing sex differences in CeM neuronal activity, using local stimulation to activate glutamatergic inputs to the CeA from various sources, compared outbred Wistar rats with selectively bred Marchigian Sardinian alcohol-preferring (msP) rats (Kirson, Oleata, Parsons, Ciccocioppo, & Roberto, 2017). With local stimulation, Wistar females were shown to have comparable sensitivity to EPSP inhibition by alcohol as Wistar and msP males, whereas msP female rats' EPSPs were insensitive to acute alcohol treatment (Kirson et al., 2017). Activation of the CB1 receptor, considered anxiolytic, also produced sex-specific effects, inhibiting EPSPs significantly in neurons from msPs of both sexes, but only in Wistar male neurons. CB1 activation blocked alcohol's ability to modulate Wistar female CeM EPSPs and unmasked an alcohol sensitivity in msP female neurons, without altering alcohol's effects in male neurons of either genetic background. This contrasts with equivalent reductions in alcohol intake in C57BL/6J (B6) mice of both sexes after inhibition of FAAH, the enzyme that catalyzes breakdown of CB1's endogenous ligand anandamide

(Zhou et al., 2017), but aligns with greater female dose sensitivity to CB1 antagonist effects to reduce drinking in Long-Evans rats (Morales, McGinnis, & McCool, 2015). Yet CB1 inhibition alone did not alter CeM electrophysiological properties in rats of either sex or genetic background, suggesting that behavioral effects of cannabinoid system manipulation on alcohol drinking may be driven by brain regions outside the CeA or by neuroadaptations caused by extensive alcohol drinking history. Regardless, these studies demonstrate significant sex differences in alcohol's acute ability to modulate neuronal activity, illustrated here within the CeA, that may depend on the specific brain locus and/or circuit being studied as well as genetic background. These initial studies strongly support the need for additional investigations to elucidate how sex regulates neuronal adaptation to alcohol and stressors, in order to understand the myriad factors regulating sex differences in behavioral responses to alcohol, stressors and their interaction.

Sex differences in behavioral adaptations to alcohol and stress

As discussed above, sex differences exist in physiological responses to stress and to alcohol exposure, and are predictive of sex differences in behavioral responses to these two challenges. The effects of stress on alcohol-motivated behavior (reviewed in H. C. Becker, Lopez, & Doremus-Fitzwater, 2011; Sinha, Shaham, & Heilig, 2011) vary significantly across studies due to experimental differences in species, strain, age, type of stressor, chronicity of the stressor, and type of drinking behavior assessed. In contrast, animal models of drug-motivated behavior consistently show females take and seek alcohol and other drugs in larger amounts than males (J. B. Becker & Koob, 2016; Lancaster, Brown, Coker, Elliott, & Wren, 1996). Enhanced drinking in females, relative to males, would be predicted due to greater basal circulating and stress-induced levels of CORT in females (Kitay, 1961; Weinstock, Poltyrev, Schorer-Apelbaum, Men, & McCarty, 1998), since CORT levels positively correlated with the level of alcohol-seeking in females (Bertholomey, Nagarajan, & Torregrossa, 2016) and blockade of CORT's effects at the glucocorticoid receptor reduced alcohol intake in males (Vendruscolo et al., 2015). However, studies examining sex differences in stress-induced alcohol-related behaviors are inconsistent, likely due to variations in experimental factors, as listed above.

Only a handful of studies have measured the behavioral consequences of experimenter-administered alcohol as a function of stress exposure and sex. In one study, maternal separation produced behavioral sensitization to alcohol in female, but not male, Swiss mice tested in adulthood (Kawakami, Quadros, Takahashi, & Suchecki, 2007). In contrast, multiple studies have failed to detect sex differences in the ability of maternal separation or social isolation stress to alter alcohol conditioned place preference/aversion (CPP/CPA) or locomotor response to alcohol (Arias, Revillo, & Spear, 2012; Arias et al., 2010; Pautassi, Nizhnikov, Fabio, & Spear, 2012). Similarly, sex differences were not observed in the effects of adolescent or adult exposure to footshock stress on alcohol CPP in mice (Song et al., 2007). Taken together, it appears that age at stress exposure and behavioral testing, as well as species of rodent, contribute to the ability to detect sex- and stress-dependent effects on behavioral responses to noncontingent administration of alcohol.

A number of studies using limited, intermittent, or continuous home cage access to alcohol have examined the effects of stress, given at various times during development, on alcohol drinking and preference. With respect to early life stress, maternal separation in Wistar rats has been shown to increase adult alcohol intake in males, but not females (Ploj, Roman, & Nylander, 2003; Roman, Ploj, & Nylander, 2004), yet the same manipulation in a separate study enhanced restraint stress-induced alcohol drinking in adulthood in females compared to males (Penasco, Mela, Lopez-Moreno, Viveros, & Marco, 2015). A number of studies have also examined the effects of adolescent stress exposure on subsequent alcohol drinking, given that sex differences in alcohol intake tend to emerge during adolescence (Doremus, Brunell, Rajendran, & Spear, 2005; Lancaster et al., 1996). Repeated restraint stress in early adolescence (postnatal days [p]30–35) increased drinking in female, but not male, Wistar rats during limited, intermittent access in mid-late adolescence (p37–51) (Wille-Bille, de Olmos, Marengo, Chiner, & Pautassi, 2017). In contrast, adolescent social isolation/social instability stress has been shown to increase subsequent alcohol consumption in adulthood in male, but not female, Long-Evans rats (Butler, Carter, & Weiner, 2014; Roeckner, Bowling, & Butler, 2017; Skelly, Chappell, Carter, & Weiner, 2015). Animals exposed to acute stress in adulthood also show varying results. In high alcohol preferring (HAP2) mice, restraint stress increased drinking in males and reduced drinking in females (Chester, de Paula Barrenha, DeMaria, & Finegan, 2006), whereas female B6 mice were more sensitive to the effects of predator odor to increase limited access drinking (Cozzoli, Tanchuck-Nipper, Kaufman, Horowitz, & Finn, 2014). These findings contrast with another study showing that despite overall greater alcohol intake in female WSC mice, there were no modulations in alcohol intake as a function of restraint stress in either sex (Tambour, Brown, & Crabbe, 2008). Though female B6/129 mice drank more alcohol in both continuous access and binge-like (drinking-in-the-dark) conditions, only males demonstrated increases in both alcohol consumption and alcohol-induced increases in locomotor activity following exposure to unpredictable chronic mild stress (Quadir et al., 2017). Clearly, sex- and stress-related alterations in voluntary drinking vary significantly as a function of the parameters used.

Only two studies have examined sex differences in the effects of stress in altering alcohol-motivated behavior using operant self-administration and reinstatement of alcohol seeking techniques (Bertholomey et al., 2016; Bertholomey & Torregrossa, 2017). A number of studies (discussed below) have examined sex- and estrous cycle-related alterations in alcohol self-administration, finding enhanced drinking in females. However, investigation of vulnerability to “craving”-like behavior using reinstatement models is critical in addressing factors contributing to relapse (Bossert, Marchant, Calu, & Shaham, 2013; Epstein, Preston, Stewart, & Shaham, 2006). A recent study found that despite greater overall alcohol self-administration in female Long-Evans rats, they did not show the alcohol-cue+alcohol-primed reinstatement of alcohol seeking evident in males (Randall, Stewart, & Besheer, 2017). In contrast, a contemporaneous study showed not only that female Sprague-Dawley rats displayed enhanced alcohol cue-induced and yohimbine stress-induced reinstatement of alcohol seeking compared to males, but that these effects were additive when cues and yohimbine were given in combination (Bertholomey et al., 2016). Further, alcohol drinking and cue-related seeking were enhanced in female, but not male, rats exposed chronically to

CORT in adolescence (p30–50) and tested in adulthood, suggesting that both acute and chronic stressors may contribute to an increased vulnerability in females. Importantly, both plasma CORT and estradiol (E2) levels were positively correlated with responding during reinstatement, indicating that physiological markers of the stress response as well as circulating ovarian hormones contribute to the increased sensitivity to stress-related alcohol-motivated behavior in females (Bertholomey et al., 2016). Taken together, the impact of sex differences on stress modulation of alcohol drinking and seeking is inconsistent and complex, and substantial research is still needed to parse the role of each of the potential sources of sex differences on the behavioral response to stress and alcohol. Nonetheless, the consistent finding that females consume more alcohol than males, and tend to be more sensitive to stress, points to the importance of assessing overlapping stress and gonadal hormone systems when measuring behavioral responses to stress in males and females.

Role of gonadal hormones in regulating sex differences in alcohol- and stress-regulated behavior

Sex differences in the behavioral response to stress and alcohol can be mediated by chromosomal sex (*genetic effect*), the developmental effects of hormones on brain structure and function (*organizational effect*), and/or by the effects of circulating gonadal hormones at the time of stress or alcohol exposure (*activational effect*). Typically, the first step in determining the cause of sex differences in behavior is to determine if the activational effects of circulating gonadal hormones are sufficient to explain the observation (J. B. Becker et al., 2005). This can be achieved using a number of different approaches, including monitoring estrous cycle in gonadally intact females, removing the influence of endogenous gonadal hormones by gonadectomy (GDX), with or without subsequent hormone replacement, and/or treatment with hormone receptor modulators. Numerous studies have investigated whether estrous cycle-related alterations in ovarian hormones (namely estradiol and progesterone), or plasma levels of gonadal hormones measured on the day of a behavioral test mediate observed differences. Others have assessed whether removal of the major source of testosterone in males (via castration [CAST]) or estradiol/progesterone in females (via ovariectomy [OVX]) diminishes sex differences observed in gonadally intact animals, and if specific hormone replacement can rescue sex-specific effects.

Studies investigating these questions using slightly different models have been surprisingly equivocal with respect to alcohol-related behavior. For example, overall alcohol self-administration is not affected by estrous cycle phase in freely-cycling female rats (Bertholomey & Torregrossa, 2017; Ford, Eldridge, & Samson, 2002b; Priddy et al., 2017; Roberts, Smith, Weiss, Rivier, & Koob, 1998); however, the *pattern* of consumption differed in proestrus females (when estradiol levels are high) (Ford et al., 2002b) or when cycles were synchronized (Roberts et al., 1998). In studies targeting the activational effects of gonadal hormones using GDX, removal of testosterone has been shown to slightly reduce (Cailhol & Mormede, 2001), increase (Vetter-O'Hagen & Spear, 2011), or have no effect (Almeida et al., 1998) on alcohol drinking in male subjects. Conversely, removal of estradiol and progesterone via OVX has reduced alcohol drinking more consistently (Almeida et al., 1998; Cailhol & Mormede, 2001; Ford, Eldridge, & Samson, 2002a; Ford et al., 2002b; Ford, Eldridge, & Samson, 2004; Forger & Morin, 1982), although no effects were evident

in other studies (Vetter-O'Hagen & Spear, 2011). Despite the potential confound of implicating both organizational and activational effects of hormones, studies in prepubertally GDX animals find similar results, with CAST increasing drinking in male subjects (Sherrill, Koss, Foreman, & Gulley, 2011; Vetter-O'Hagen & Spear, 2011), and OVX decreasing (Sherrill et al., 2011) or not altering (Vetter-O'Hagen & Spear, 2011) drinking in females. Parallel findings are evident when gonadal hormones are replaced, as administration of testosterone (Vetter-O'Hagen & Spear, 2011) or the androgen dihydrotestosterone (Almeida et al., 1998) decreased alcohol consumption in CAST males, whereas estradiol treatment dose-dependently increased intake in OVX females (Ford et al., 2002a, 2004), although others have failed to observe estradiol replacement effects (Almeida et al., 1998). Estradiol treatment may alter alcohol intake through enhancement of alcohol's reinforcing properties, as OVX mice treated with estradiol displayed greater alcohol CPP than untreated OVX mice (Hilderbrand & Lasek, 2018). Despite some conflicting findings, the overall consensus is that testosterone is responsible for reduced alcohol drinking in males and ovarian hormones are responsible for increased drinking in females. However, these results can be difficult to reconcile as GDX, hormone replacement, and sham controls for *both* sexes were often not compared in the same study. Further, none of these studies examined the role of gonadal hormones in altering stress-related increases in alcohol reinforcement.

A previous report (described above) found that plasma estradiol levels positively correlated with the degree of cue+yohimbine-induced reinstatement in females (Bertholomey et al., 2016), suggesting that estradiol might be responsible for the increased alcohol seeking observed in females, relative to males. This finding is consistent with similar studies examining estradiol enhancement of cocaine seeking (Feltenstein, Henderson, & See, 2011; Larson, Roth, Anker, & Carroll, 2005). Thus, a subsequent study determined the effects of GDX, with or without hormone replacement, relative to sham-GDX controls, on both operant self-administration of alcohol and cue+yohimbine-induced reinstatement of alcohol seeking (Bertholomey & Torregrossa, 2017). Consistent with previous findings, females self-administered significantly more alcohol than males, and GDX increased self-administration in males and decreased self-administration in females, relative to gonadally intact sham surgery controls. Furthermore, replacing estradiol in females increased alcohol self-administration, while testosterone replacement reduced self-administration in males, relative to sham levels of responding. While circulating hormone levels could shift the degree of alcohol self-administration within sex, GDX in both sexes was not sufficient to eliminate sex differences, as OVX females still self-administered significantly more alcohol than GDX males. Therefore, the activational effects of hormones cannot fully explain sex differences in alcohol self-administration. Unlike alcohol-reinforced self-administration, neither GDX nor hormone replacement significantly altered cue+yohimbine-induced reinstatement of alcohol seeking, although estrogen receptor antagonists tended to reduce seeking in gonadally intact female rats. Therefore, differences between males and females in this alcohol craving-like response does not appear to be mediated by the activational effects of hormones. Nonetheless, it is possible that within sex, circulating hormones modulate individual differences in the degree of reinstatement, as suggested by prior correlational findings, but that the range of reinstatement response is greater in females regardless of hormonal state. Together these studies indicate that while hormone supplementation can

alter parameters of alcohol self-administration, adult GDX does not directly modulate the motivation to work for alcohol in an operant setting, in contrast with some findings discussed above for alcohol drinking in a free-access setting. Future studies will need to investigate how either organizational or genetic effects of sex alter neurodevelopment in a way that leads to increased risk for alcohol-motivated behaviors, such as alcohol seeking and drinking, as well as stress-induced craving in females. Identification of these mechanisms may lead to improved, sex-specific treatments for AUDs.

Clinical laboratory and epidemiological studies of sex differences in stress in addiction

Limitations exist in assessing treatment options in females given the need to better understand the molecular bases of sex differences in stress-alcohol interactions. However, components of the stress response – including HPA axis and adrenergic system activity – represent common targets implicated by both preclinical and clinical studies as possible points of differentiation between the sexes. Human studies have shown neuroadaptations in the HPA axis with chronic drug and alcohol abuse, as well as emotional changes during abstinence, which impact responses to stress and increase the risk of relapse (Back et al., 2005; Fox, Hong, Siedlarz, & Sinha, 2008; Fox & Sinha, 2009; Sinha et al., 2006). Evidence from clinical surveys and daily clinical assessments of drug craving indicate that both stress- and cue-induced drug craving states frequently lead to continued drug use and relapse (Bradley, Phillips, Green, & Gossop, 1989; Epstein, Marrone, Heishman, Schmittner, & Preston, 2010; Epstein et al., 2009; Hodgins, el-Guebaly, & Armstrong, 1995; Kowalczyk et al., 2015; Marlatt & Gordon, 1985; Preston et al., 2009; Wallace, 1989). The studies presented herein demonstrate evidence of sex differences in these HPA axis neuroadaptations, as assessed in human laboratory studies, as well as sex differences in treatment efficacy of medications targeting this stress pathophysiology. Because investigation of sex differences in AUD has been somewhat sparse, even at the clinical level, studies described herein demonstrate sex differences in mechanisms that may similarly drive craving and relapse in both alcohol- and cocaine-dependent individuals, to identify putative targets for future preclinical studies.

Exposure to stress as well as drug and alcohol cues consistently increases drug craving and stress-related arousal in individuals with SUD (Sinha, Catapano, & O'Malley, 1999; Sinha, Fuse, Aubin, & O'Malley, 2000; Sinha et al., 2003). Treatment-engaged patients with AUD show enhanced and persistent stress- and cue-induced alcohol craving and anxiety following one month of abstinence, accompanied by dysregulation of the physiological response to stress (Fox, Bergquist, Hong, & Sinha, 2007; Fox et al., 2009; Sinha et al., 2009). These patients also displayed higher severity of alcohol and other drug abuse, with elevated stress- and cue-induced craving, heightened anxiety and HPA axis dysregulation compared to those with less alcohol abuse severity (Fox et al., 2005). Furthermore, all AUD patients were *prospectively* followed for 90 days with up to 70% having relapsed. After accounting for baseline variations in demographics, drug use, and clinical variables, multiple indices of stress system dysregulation and altered emotional state – namely, stress- and cue-induced alcohol craving, higher basal cortisol, suppressed stress-induced cortisol and ACTH responses, and high levels of cortisol/ACTH ratio during neutral-relaxed state – predicted

future time to alcohol use (Blaine, Milivojevic, Fox, & Sinha, 2016; Sinha, Fox, et al., 2011).

While HPA arousal corresponds to relapse, SUDs are characterized by blunted stress-induced HPA axis activity, an effect that disproportionately affects women. Women with cocaine use disorder (CUD) exhibited significantly lower ACTH, cortisol and blood pressure responses following exposure to personalized stress, drug-cue, and neutral imagery, as compared to CUD men (Fox et al., 2006), yet both CUD and healthy control females reported significantly higher levels of anxiety and sadness following stress exposure, relative to males (Fox et al., 2008). Patients with AUD similarly display HPA axis alterations, as well as sexual dimorphism in the interaction between alcohol use and stress. In a population of individuals diagnosed with AUD, PTSD, or comorbid AUD and PTSD, sex differences were observed in HPA axis markers both at baseline and in response to the cold pressor task (Brady et al., 2006). Across all three groups, females showed significantly lower levels of ACTH compared to males. Moreover, females in either the AUD or the PTSD group showed greater ACTH blunting in response to stress compared to males in the matching diagnostic groups. Collectively, these findings suggest that stress and drug cues increase craving and anxiety, and that chronic drug use is associated with an altered HPA axis response to stress, marked by basal hyperactivity and blunted phasic response to stress, that is more severe in females. While it remains unknown whether HPA axis dysfunction predisposes individuals to SUDs or develops consequent to the SUD, these states have been shown to potently predict relapse, suggesting that improved understanding of the molecular mechanisms triggering these adaptations are important preclinical avenues of investigation to identify better treatments and reduce relapse in both sexes. Conversely, knowledge about medication responses in clinical trials addressing stress-alcohol interactions will provide additional indicators of sex differences that must be further elucidated by preclinical investigations.

Medications targeting stress pathophysiology and sex differences in addiction

Given the dysregulation of HPA axis responses in SUD, as well as the role of stress as a trigger for craving and relapse, pharmacologically targeting craving and stress-related HPA axis dysfunction could improve treatment and reduce relapse rates in SUD patients (Milivojevic & Sinha, 2017). As significant sex differences have been observed in SUD-related adaptation of these pathways, however, special attention must be paid to the development of sex-specific treatment targets. Whereas much experimental evidence has focused on treatments in male-only or male-biased populations, studies investigating multiple medications aimed at improving the HPA axis dysregulation found in SUDs in sex-balanced populations have yielded some sex-specific effects. In particular, targeting the adrenergic system via inhibition of postsynaptic (α1) or activation of presynaptic (α2) adrenoreceptors (AR) has been more effective as a treatment in females than in males. In preliminary studies of AUD patients in early abstinence, the α1-AR antagonist prazosin was found to reduce stress-induced alcohol craving and negative emotions, while reducing basal cortisol levels and increasing stress-induced cortisol responses (Fox et al., 2012). Similarly, in early abstinent CUD and AUD individuals, the α2-AR agonist guanfacine was found to reduce cue-induced craving, decrease baseline cortisol levels and normalize stress-induced cortisol responses (Fox et al., 2012). However, population sizes in these preliminary studies

precluded the performance of sufficiently powered sex-specific analyses. Subsequent investigations in a larger population of individuals with comorbid CUD and AUD demonstrated that guanfacine significantly reduced cocaine craving, alcohol craving, anxiety, and negative emotion following exposure to stress, drug/alcohol cue and neutral conditions; however, in this population, guanfacine's effects were significant only in females, not males (Fox, Morgan, & Sinha, 2014). Guanfacine has also demonstrated enhanced efficacy vs. placebo to improve CUD/AUD females' cognitive performance on a Stroop task under neutral, stress, and drug cue conditions (Milivojevic, Fox, Jayaram-Lindstrom, Hermes, & Sinha, 2017). Importantly, this effect was not observed in men. Together these studies not only implicate the adrenergic system as a medication target to treat females more successfully than males, but also highlight the need for preclinical investigations to elucidate sex differences in stress- and alcohol-induced adrenergic circuit adaptations that may underlie this differential treatment efficacy.

Given the *a priori* sex difference in hormone status that likely impacts neuronal activity, as discussed above, elevating progesterone levels has been explored as a therapeutic approach that may generate a sexually dimorphic response. Relative to placebo treatment, progesterone administration reduced cue-induced craving and cortisol responses in treatment-seeking men and women with comorbid CUD and AUD, in addition to improving prefrontal inhibitory function, as measured by the Stroop task (Fox, Sofuoglu, Morgan, Tuit, & Sinha, 2013). While main treatment effects were observed regardless of sex, progesterone treatment provided the added benefit of decreasing ratings of negative emotion and increasing ratings of relaxed mood following stress exposure in women but not men (Fox et al., 2013). One metabolite of progesterone that may produce different treatment responses in males and females is the neuroactive steroid allopregnanolone (ALLO). ALLO is found in higher concentrations in the female mouse brain, but increased after alcohol drinking only in male mice (Finn et al., 2004). In humans, the plasma concentration of ALLO was increased following severe intoxication in both females and males (Torres & Ortega, 2003, 2004). ALLO is a potent allosteric enhancer of gamma aminobutyric acid type A receptor ($GABA_A$) activity (Porcu & Morrow, 2014) that may differentially regulate alcohol intake by sex, with greater sensitivity shown in male vs. female mice (Ford, Beckley, Nickel, Eddy, & Finn, 2008; Sinnott, Phillips, & Finn, 2002). To assess the role of ALLO in progesterone's treatment effects, CUD/AUD individuals who received progesterone were grouped by their baseline ALLO levels. The high ALLO group showed reductions in craving, improved cognitive performance, reduced basal cortisol and increased phasic cortisol in response to stress in all subjects, compared to the low ALLO group, with no sex differences observed (Milivojevic, Fox, Sofuoglu, Covault, & Sinha, 2016). Together these studies suggest elevation of neuroactive steroids like ALLO may represent a biomarker of treatment efficacy in men and women, warranting future preclinical and clinical research into steroids like ALLO that may be useful biomarkers for long-term treatment efficacy in both sexes.

Conclusion

AUD, although currently more prevalent in males, afflicts both sexes, and the gender gap in disease prevalence is narrowing. Yet preclinical studies including females, which provide a fuller understanding of brain mechanisms underlying these disorders, continue to lag far

behind the vast body of literature focusing solely on male subjects. The data reviewed above demonstrate significant sex differences in the impact of stressors on AUD in both preclinical animal models and human studies. Importantly, observed sex differences in the brain mechanisms supporting alcohol-stress interactions, in the behavioral impact of past stress on alcohol use, and in drug treatment efficacy highlight the need for continued pursuit of knowledge in the preclinical realm to understand the neural basis of sex differences in stress responses and alcohol use, so that better therapeutic approaches may be developed for both sexes.

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Table 1

Summary of literature investigating sex differences or female effects of stress on alcohol-related behaviors.

Sex	Subjects	Stressor	Age (Tx)	Age (Test)	Measures	Outcome (group)	Citation
Mouse							
Behavior							
F=M	HAP	repeated RS	Adult	Adult	EtOH drinking ASR	↑ drinking (RS M), ↓ drinking (RS F); ND ASR	Chester et al., 2005
F=M	B6	Various	Adult	Adult	EtOH drinking hormone levels	↑ drinking (predator odor, F>M); ↓ drinking (other stressors); ↑ CORT (F>M)	Cozzoli et al., 2014
F=M	SW	Long/short MD	Neonatal (p2–14)	Adult	EtOH LMA Hormone levels	↑ LMA (long MD F) ↑ CORT (F>M baseline; M>F post-EtOH)	Kawakami et al., 2007
F=M	KM	Footshock (acute, chronic)	Adol (p26) Adult (p56)	Adol Adult	EtOH CPP	Chronic stress > baseline (Adol); stress = baseline (Adult); ND by sex	Song et al., 2007
F=M	WSC	RS	Adult	Adult	EtOH drinking	ND drinking (RS) ↑ drinking (F>M)	Tambour et al., 2008
F=M	B6/129	UCMS	Adult	Adult (CA; IA)	EtOH drinking	↑ drinking, LMA (UCMS M); ↑ drinking (F>M)	Quadir et al., 2017
Rat							
Molecular/Electrophysiological							
M=F	SD	SS	Adult	Adult	CRF1 IP CRF1 internalization	↑ Gs coupling, all females (HC/SS±OVX), SS males male SS ↑, female SS ↓	Bangasser et al., 2010
M=F	SD	acute CRF on slice SS	Adult	Adult	LC activity	LC activity F>M SS ↑ LC activity M not F	Curtis et al., 2006
F=M	Wi	SBSA	Adol (P28–42)		CeA CRF-IR	CRF-IR M>F; SBSA ↓ CRF-IR (M & F)	Karanikas et al. 2013
F=M	Wi, msP	EtOH (acute) CB1 Ant. (acute)			CeM EPSP amplitude local stimulation)	EtOH ↓ EPSP (Wi M/F, msP M); CB1 Ant ↓ EPSP (Wi M, msP M/F); CB1 Ant ┤ EtOH (Wi F); CB1 Ant ↑ EtOH (msP F)	Kirson et al., 2017
F>M F=M	Wi	EtOH (acute) CORT (acute)	Adult	Adult	CeL and CeM EPSP amplitude (BLA stimulation)	EtOH ↓ EPSP (M>F) CeL: CORT ↓ EPSP (F only) proe CeM EtOH ↑ EPSP	Logrip et al. 2017
F=M	LE	CIE + WD	Adult	Adult	EPM, BLA EPSC amplitude (M/L stimulation)	↑ ALB (M>F); ↓ PPR (M>F, M stimulation); ↑ EPSC (M>F, L stimulation	Morales et al. 2018
F=M	SD	EtOH (0–3 g/kg) puberty	Pre/peri/post	Adult	Hormone levels	↑ ACTH, CORT (F, GDX M > M; emerged peripuberty)	Ogilvie & Rivier, 1996

Sex	Subjects	Stressor	Age (Tx)	Age (Test)	Measures	Outcome (group)	Citation
F=M	SD	14d EtOH LD SS	Adult	Adult	Hormone levels CeA cFos-IR	LD ↑ CORT (M only); SS ↓ CORT (LD Fem only) LD ↑ cFos (F only); SS ↑ cFos in CRF neurons (LD M only)	Retson et al. 2015
F=M		EtOH (0.2–1.8 g/kg)	Adult	Adult	Hormone levels	↑ ACTH, CORT (F>M; pro/est F > di F)	Rivier, 1993
F=M	SD	FS	Adult	Adult	Hormone levels	↑ ACTH, CORT (F>M)	Rivier, 1999
Behavior							
F=M	Wi	EtOH (2.5 g/kg)+ MD ± SI or CORT manipulation	Neonatal (p15)	Neonatal (p15)	EtOH LMA	↑ LMA (MD ± SI, CORT manipulation; ND by sex)	Arias et al., 2010
F=M	SD	EtOH (2.5 g/kg) SI	Neonatal	Weanling (p15–18)	EtOH LMA (p21)	ND LMA	Arias et al., 2012
F=M	SD	Chronic CORT	Adol (p30–50)	Adult (p90+)	EtOH drinking; Cue/YOH REINSTM	↑ drinking, cue REINST (CORT F>H2O F>M); ↑ REINST (F>M; cue+YOH> cue or YOH)	Bertholomey et al., 2016
F=M	SD; GDX ±E2/T	YOH	Adult	Adult	EtOH drinking; Cue/YOH REINSTM	↑ drinking (F>M; E2>T); ↑ REINST (F>M; ND E2/T)	Bertholomey et al., 2017
F	LE	SI	Adol (p30–72)	Adult (p100+)	EtOH drinking ALB	transient ↑ drinking (SI) ND ALB	Butler et al., 2014
M=F	LE	CIE	Adult	Adult	IA2BC, CB1 Ant	CIE ↑ IA2BC (M only); CB1 Ant ↓ IA2BC (M>F)	Morales et al. 2015
F=M	SD	MD	Neonatal (p1–13)	Neonatal (p14–15)	EtOH CPP, LMA	ND EtOH CPP (MD) ↑ LMA (all; >2.0 g/kg) ND by sex	Pautassi et al., 2012
F=M	Wi	Single, long MD	Neonatal (p9)	Adol (p28–50)	EtOH drinking+ WD ± RS	WD+RS (F>M) ↑ drinking (MD+RS)	Peñasco et al., 2015)
F	Wi	Long/short MD	Neonatal (p1–21)	Adult	EtOH drinking ± RS	ND drinking acquisition ↑ drinking (RS)	Roman et al., 2004
F=M	LE	SI, social instability	Adol (p30–46)	Late Adol/Adult	EtOH drinking ALB, hormone levels	↑ drinking, ALB, ND CORT (male social instability); ND drinking, ALB, ↓ CORT (SI F)	Roekner et al., 2017
F=M	Wi	Repeated RS	Adol (p30–34) Adult (p70–74)	Adol (p30–34) Adult (p70–74)	EtOH drinking ALB	↑ drinking, ALB (adol RS F) ↓ drinking (RS M, adult F)	Wille-Bille et al., 2017
Human							
F=M	AD± PTSD	Cold pressor stress test	Adult	Adult	Subjective/hormonal stress rating	↑ subjective stress ⥗ ACTH (AD±PTSD) ↓ basal, greater ↔ ACTH (F vs. M)	Brady et al., 2006
M>F	CS+AA; TS	Stress Imagery Alcohol Cue	Adult	Adult	Drug craving Emotional response Hormone levels	↑ craving, anxiety, ACTH, CORT (more frequent users)	Fox et al., 2005

Sex	Subjects	Stressor	Age (Tx)	Age (Test)	Measures	Outcome (group)	Citation
M>F	AD; TS	Stress Imagery Alcohol Cue	Adult	Adult	Alcohol craving Emotional response Hormone levels	↑ craving, negative emotions (stress, cue) ↑ CORT (cue)	Fox et al., 2007
F=M	AD+CA; TS	Stress Imagery Alcohol Cue	Adult	Adult	Alcohol craving Hormone levels	↑ craving (cue M) ↑ ACTH, CORT (stress F) ⫫ ACTH, CORT (stress, cue M)	Fox et al., 2009
M>F	AD; TS; ABST	Stress Imagery Alcohol Cue	Adult	Adult	Alcohol craving Emotional response Hormone levels	↑ craving, negative emotions (stress, cue) ⫫ ACTH, CORT (stress, cue)	Sinha et al., 2009
M>F	AD; TS	Stress Imagery Alcohol Cue Prazosin	Adult	Adult	Alcohol craving Emotional response Hormone levels	↑ craving, anxiety; ↔ ACTH, CORT (stress, cue); Prazosin ↔ these effects	Fox et al., 2012
M>F	Human (AD; TS; ABST)	Stress Imagery Alcohol Cue	Adult	Adult	Alcohol craving Latency to Relapse Hormone levels 90-day follow-up	↑ craving and baseline CORT ↓ time to relapse	Sinha et al., 2011
M>F	AD	GR Ant	Adult	Adult	Alcohol craving,	GR Ant ↓ craving, drinking	Vendruscolo et al., 2015

Effects indicators: ↑increase, ↓ decrease, ⫫ blunted, ┤ blockade, ND no difference

Abbreviations: *Sex*: M = male; F = female; *Species/Rodent lines*: Ms = mouse; B6 = C57BL/6; HAP = high alcohol-preferring; KM = Kunming; LE = Long-Evans; SD = Sprague-Dawley; SW = Swiss Webster; Wi = Wistar; WSC = withdrawal seizure control, *Estrous cycle indicators:* pro = proestrus, est = estrus, di = diestrus; *Experimental terms:* AA = alcohol abusing; ABST = abstinent; ACTH = adrenocorticotropic hormone; AD = alcohol dependent; ALB = anxiety-like behavior; ant = antagonist; ASR = acoustic startle response; BLA = basolateral amygdala; CIE = chronic intermittent ethanol vapor; CA= cocaine abusing; CD = cocaine dependent; CeA = central amygdala; CeL = lateral CeA; CeM = medial CeA; CORT = corticosterone (rodent)/cortisol (human); CPP = conditioned place preference; CRF = corticotropin-releasing factor; EPSP/C = excitatory postsynaptic potential/current; EPM = elevated plus maze; EtOH = ethanol (alcohol); E2 = estradiol; FC = fear conditioning; FS = Footshock; GDX = gonadectomized; g/kg = grams of ethanol per kilogram of body weight; GR = glucocorticoid receptor; IA2BC = intermittent access to 2-bottle choice; IR = immunoreactivity; LC = locus coeruleus; LD = alcohol-containing liquid diet; LMA = locomotor activity; MD = maternal deprivation/separation; mPFC = medial prefrontal cortex; PTSD = post-traumatic stress disorder; p# = postnatal day; SA = self-administration; SBSA = sweetened EtOH binge-like self-administration; SI = social isolation; SS = swim stress; T = testosterone; TS = treatment seeking; REINST = reinstatement; RS = restraint stress; UCMS = unpredictable chronic mild stress; WD = withdrawal; YOH = yohimbine;