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Studying Sex Differences in Animal Models of Addiction: An Emphasis on Alcohol-Related Behaviors

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Abstract

Animal models are essential for understanding the biological factors that contribute to drug and alcohol addiction and discovering new pharmacotherapies to treat these disorders. Alcohol (ethanol) is the most commonly abused drug in the world, and as the prevalence of alcohol use disorder (AUD) increases, so does the need for effective pharmacotherapies. In particular, treatments with high efficacy in the growing number of female AUD sufferers are needed. Female animals remain underrepresented in biomedical research and sex differences in the brain's response to alcohol are poorly understood. To help bridge the gender gap in addiction research, this review discusses strategies that researchers can use to examine sex differences in the context of several common animal models of AUD. Self-administration, two-bottle choice, drinking in the dark, and conditioned place preference are discussed, with a focus on the role of estrogen as a mediator of sex differences in alcohol-related behaviors.

Keywords

addiction; alcohol; ethanol; estradiol; estrogen; female

Animal models are an essential component of biomedical research. They are often our best tool for understanding biological phenomena and are routinely employed in the development of pharmacological treatments for disease. In recent years, the utility of animal models in translational research has been called into question[1, 2] because of issues with between-study reproducibility in preclinical research and the fact that only 10-20% of new therapies show efficacy in clinical trials, despite prior success in animal studies[1, 3]. While this has led some to question the validity of animal models altogether, the consensus is that improved study

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design will greatly enhance the reproducibility and translational value of animal studies in the biomedical sciences[2-4]. One crucial and long-overlooked aspect of experimental design is the sex of laboratory animals—specifically, the need to include both males and females[5-7].

Females are severely underrepresented in biomedical research[6, 8]. Since most available prescription medications were developed exclusively in male animals, it is not surprising that women experience higher incidence of adverse drug events[9, 10] and tend to have poorer health outcomes compared with men[11]. Although sociological factors such as financial resources, living conditions, and access to care play an important role in health outcomes, biological factors should not be overlooked[11].

One particularly pressing need is the development of new therapies for the treatment of drug addiction, a chronic, relapsing condition characterized by compulsive drug seeking, difficulty limiting drug intake, and the emergence of a "negative emotional state (e.g., dysphoria, anxiety, irritability) reflecting a motivational withdrawal syndrome when access to the drug is prevented"[12]. For some individuals, recreational use of alcohol and/or other drugs can lead to both acute and chronic health problems, as well as social problems, drug tolerance, craving, and withdrawal, and/or repeated, unsuccessful attempts to quit or otherwise control drug use. If a person meets some of these criteria, as listed in the *Diagnostic and Statistical Manual of Mental Disorders*, Fifth Edition (DSM-5), that individual is considered to have a substance use disorder (SUD)[13].

Incidence of drug overdose and related mortality in the United States have more than doubled since the year 2000[14], and the National Survey on Drug Use and Health (NSDUH) reported that 20.2 million Americans aged 18 or older met the criteria for SUD[15] in 2014. Of those, nearly 81% had an alcohol use disorder (AUD), making alcohol by far the most commonly abused drug in the U.S. Although there are currently three FDA-approved pharmacotherapies used to treat AUD (disulfuram, naltrexone, and acamprosate), they are not universally effective, demonstrating the need to identify new targets and compounds to treat this disorder. In order to develop the most effective therapies for those suffering from addiction, both male and female animals must be tested. This review will discuss the most common animal models used to study AUD and methods that addiction researchers can use to examine sex differences in these models. We will focus on studies that illustrate sex differences in behaviors related to AUD with particular emphasis on the role of estrogen as a mediator of such differences. While progesterone and testosterone are also known to influence some measures of AUD-like behavior, discussion of these is beyond the scope of this review.

Sex Differences in AUD

As of 2015, the lifetime prevalence of AUD in American women was just under 23%, compared to 36% in men[16]. Across the globe, men consume significantly more alcohol than women, drink more often, and are more likely to be heavy drinkers[16, 17]. Women are more likely to abstain from alcohol use altogether and have a lower overall risk of developing AUD[16, 17]. The reasons for this gender gap in alcohol use (and misuse) are complex, as drinking behavior is heavily influenced by cultural and socio-economic factors[18-20]. Recent

cohort analyses demonstrate that this gap is rapidly closing, with younger generations of women consuming more alcohol and having a higher incidence of AUD than women in previous generations [17, 21-23]. In light of these data, it seems unlikely that women are simply less prone to AUD than men by virtue of biological sex. In fact, women who develop AUD (and SUD in general) tend to exhibit a so-called "telescoping" pattern of addiction[24-26]. These women progress more rapidly from initiation of substance use to onset of physical and psychological health complications and, despite seeking treatment sooner, tend to report equal or more severe symptoms of dependence at the time of treatment entry than those reported by male users[24]. This is particularly concerning since the physiological effects of alcohol abuse are more severe in females than in males[27]. Women develop comparable or more pronounced alcohol-related liver and cardiovascular disease at lower levels of alcohol consumption than their male counterparts and are also more vulnerable to alcoholic brain damage and related cognitive impairment [27].

Psychological reasons for alcohol use also differ between the sexes. Notably, women are more likely than men to engage in heavy alcohol use as a way to alleviate psychological distress, and female alcoholics are more likely to cite negative emotions and stressful life experiences as reasons for substance use and relapse[28-30]. Women who binge drink—defined as the consumption of any quantity of alcohol that generates a blood ethanol concentration (BEC) of 80 mg/dL or greater, usually ≥4 standard drinks for a woman or ≥5 standard drinks for a man[31]—report more mentally unhealthy days (dealing with stress, depression, and emotional problems) and physically unhealthy days than their male counterparts at both low (≥4 drinks) and high (≥7 drinks) intensities of binge drinking[32]. Adolescent girls take longer to recover from high-dose drinking than boys do, experiencing negative affective states for longer periods after heavy drinking episodes[33]. Women with AUD are also more likely to have comorbid psychiatric disorders, particularly anxiety and/or depression.[30, 34] On the other hand, men tend to report drinking to enhance positive emotions or in response to peer pressure, and male alcoholics are more likely to cite external temptations as reasons for relapse[28-30]. This is not to say that women do not enjoy the experience of alcohol intoxication or that men never drink to alleviate negative affective states. In fact, the subjective alcohol experience seems to be quite similar between men and women, and some have even reported higher ratings of mental and physical wellbeing ("feeling good") in females than in males who were given alcohol in a laboratory setting[35]. Furthermore, while women suffering from AUD are more likely to have a comorbid mood disorder, men certainly experience anxiety and depressive disorders in conjunction with AUD, especially in vulnerable populations such as veterans of military service[36-38].

Use of oral contraceptives containing estrogens is positively correlated with increased ethanol intake, especially if contraceptive use begins at an early age (< 20 years)[39], and increased serum levels of 17β-estradiol (E2), the primary circulating form of estrogen, have been associated with higher levels of ethanol consumption in premenopausal women[40, 41]. Some researchers have also reported subtle differences in subjective response to ethanol across the menstrual cycle. For example, increases in negative mood during the luteal phase are more pronounced in women with a family history of alcoholism (a prominent risk factor for AUD development[42]), particularly after drinking ethanol[43]. Most studies have been unable to detect subjective differences in ethanol response across menstrual cycle phase,

however, perhaps due to the confounding effects of expectation and learned associations from previous ethanol experience[44].

Sex Differences Defined

When designing experiments to study sex differences in the laboratory, it is useful to have an understanding of the types of sex differences that one may find in nature. Two excellent articles have put forth some useful terms and definitions[45, 46]. McCarthy *et al.* describe three basic ways to categorize differences between males and females: *sexual dimorphism, sex differences,* and *sex convergence/divergence*[45]. Examples of sexual dimorphism (referred to as *qualitative* sex differences by Becker and Koob[46]) include sex-specific copulatory behavior and courtship displays. In these cases, the behavioral or physiological measure has two distinct forms, such as lordosis in the female and mounting/intromission in the male. The term *sex differences* (or *quantitative* differences[46]) applies when a behavioral or physiological measure exists on a continuum, present in both sexes to varying degrees. Examples of this second type include: pain thresholds, food preferences and intake, baseline anxiety levels, stress responses, and responses to various drugs of abuse[45]. The term *sex convergence* or *divergence* refers to situations when the endpoint manifests in the same way/to the same degree in males and females but is brought about by different biological mechanisms in one sex versus the other, such as pair bonding in prairie voles, which is mediated by different neural mechanisms in females than in males[47]. Finally, Becker and Koob include a fourth category, *population differences*, in which the incidence or distribution of individual traits varies between males and females. An example of this kind of difference would be the relatively greater frequency of AUD in human males than in females.

Sex differences are primarily brought about by *organizational* and/or *activational* effects of sex hormones. The traditional theory of sexual differentiation, both of the brain and of other bodily tissues, revolves around the notion that sex genotype (XX vs. XY) guides embryonic differentiation of the gonads (testes or ovaries), which then produce hormones that organize bodily tissues into male- or female-typical patterns of development[48, 49]. According to this theory, the appropriately organized brain of an individual will be activated by gonadal hormones later in life (i.e. after puberty) and respond by producing either male- or female-typical patterns of behavior[48, 49]. In the simplest of terms, this theory is a fairly accurate description of the sexual differentiation process. However, we now understand that the endpoints of sexual differentiation are determined by numerous factors, including genes on the X and Y chromosomes that may promote sex differences independently of the sex hormones [49-51]. It is important to remember that, though gonadal hormones are certainly crucial mediators of the process, they are not the only factors influencing sexual differentiation in either humans or laboratory animals.

Determining if Sex Differences Exist

The simplest way to look for sex differences in an animal model is to compare gonadally intact males and females across measures of interest. Many researchers assume that any study of sex differences must involve tracking the female estrous or menstrual cycle (in

rodents and non-human primates, respectively) and looking for effects of ovarian hormones[48]. It is true that many traits, both behavioral and physiological, can be influenced by the cyclic hormone fluctuations experienced by females of reproductive age. However, recently published meta-analyses demonstrate that female rats and mice are not more inherently variable than males across a range of measures[52, 53]. Therefore, it is often unnecessary to track estrous or menstrual cycle when comparing males and females in the laboratory solely to determine if there is a sex difference in a particular measure. That said, tracking the estrous cycle in rats and mice, which are commonly used to model a wide range of disease states (including AUD), is quite simple and inexpensive[54, 55]. It is useful to have such data available for analysis, especially if sex differences are known to exist in the parameters being studied.

Determining if Sex Hormones Are Responsible for Sex Differences

Once a sex difference has been discovered by a straightforward male-female comparison, researchers can then determine what factors may be responsible for the difference observed (Figure 1). The most obvious factors are sex hormones, such as the estrogens and progesterone produced by the ovaries or the androgens (e.g. testosterone) produced by the testes. To determine if sex hormones are responsible for sex differences, two complementary methods are used. The first is to examine the reproductive cycle in females and the second is to remove the gonads from males and females, known as gonadectomy (GDX).

In rats and mice, each of the four phases of the estrous cycle (proestrus, estrus, metestrus, and diestrus) is associated with distinctive changes in vaginal cell types (nucleated epithelial, cornified epithelial, and leukocytes, respectively). Therefore, cycle phase can be determined by analyzing vaginal cellular content by light microscopy [54, 55]. This is easily done using readily available supplies: cotton swabs or fine-tipped plastic pipettes, water or saline solution, and microscope slides. The vagina is either gently swabbed with a pointed, moistened cotton applicator tip and the collected cells smeared onto pre-cleaned glass microscope slides, or a fine-tipped disposable pipette is used to flush the vagina with saline solution that is then transferred to microscope slides for viewing. By collecting vaginal samples over a period of several days and examining cell types, estrous cycle phase can be determined for a given day. Correlations can then be made between cycle phase and the behavior (or other parameter) measured on that day. This method has pros and cons. It is affordable and easy to perform, requiring neither specialized equipment nor intensive training. On the other hand, it can be difficult to draw conclusions from estrous cycle data for a number of reasons. There is individual variation in the length of the reproductive cycle in female mice and rats, especially in young adult mice, making it difficult to coordinate experiments so that all the animals are in the desired cycle phase when measuring parameters of interest. An alternative method is to synchronize the estrous cycle by treating animals with a gonadotropin-releasing hormone (GnRH) receptor agonist, which regulates estrous cycle progression by inducing release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary[56]. This ensures that animals will be in the same cycle phase on a given day, up to about 4 days (the length of a “standard” estrous cycle in mice and rats). For shorter experiments, this method is useful. However, there can

still be individual variability in circulating levels of ovarian hormones and animals will eventually diverge in their estrous cycle phase over time.

In some cases, researchers may want to control hormone levels in their experimental animals. This allows for a more direct determination of whether specific hormones are responsible for the phenotype of interest. GDX allows researchers to control circulating levels of androgens, progesterone, and estrogens by surgically removing the primary endogenous source of sex hormones (i.e. testes or ovaries). While this does not eliminate local hormone synthesis (for instance in the brain), it does remove circulating hormones. GDX animals can then be compared to intact controls and/or those treated with hormone receptor ligands—either exogenous forms of naturally occurring hormones or synthetic analogues that are selective for receptor(s) of interest. One challenge associated with this kind of experimental design is choosing the correct type, treatment schedule, delivery method, and dose of ligand(s). For example, E2 has been administered exogenously in numerous behavioral studies. Optimally, E2 treatments would result in physiological levels of circulating E2, to approximate hormone levels in the intact animal. However, E2 levels in mice are very low and are thus difficult to measure accurately. E2 peaks at ~8 pg/mL in mice during proestrus, compared with ~35 pg/mL in rats at this stage[57]. The lower limit of detection of commercially available E2 immunoassay kits ranges from 2.5-9 pg/ml E2 [58]. The gold standard method for measuring E2 levels is gas chromatography-tandem mass spectrometry, but this method is more expensive and requires specialized equipment that may not be readily available. Many studies have used different methods to administer E2 with doses ranging from near-physiological to extremely supraphysiological, and research shows that the dose, administration method, and length of time between GDX and the start of hormone replacement can all impact the outcome of behavioral studies[59-61]. Therefore, these factors must be considered carefully when designing experiments involving hormone replacement.

Determining if Sex Chromosomes Are Responsible for Sex Differences

As discussed previously, sex hormones are not the only factors that cause sex differences[49]. One useful tool for researchers who wish to dissociate the effects of sex chromosome genes from the effects of gonadal hormones is the four core genotypes (FCG) mouse model[51, 62, 63]. This model separates animals into four “core genotypes”—XX animals with female-typical gonads (ovaries), XX animals with male-typical gonads (testes), XY animals with female-typical gonads, and XY animals with male-typical gonads—by moving the sex-determining region (*Sry*) of the Y chromosome to an autosomal chromosome. Since *Sry* causes masculinization of the genitalia, this helps researchers determine which sex differences result from gonadal hormones and which are produced by genes on the X and Y chromosomes. Barker *et al.* used these mice to examine sex differences in ethanol consumption and habit formation in the form of operant responding[64]. In the absence of reinforcement (no ethanol received) or in the case of reinforcer devaluation (ethanol adulterated with lithium chloride) sex chromosome complement, not gonadal phenotype, determined levels of habit-like nose poke responding. On the other hand, voluntary ethanol consumption was determined by gonadal phenotype, with gonadal females consuming more than gonadal males, consistent with findings from other rodent models.

Modeling Behaviors Related to AUD in Animals

The addiction cycle is conceptualized as having three stages: *binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation* [65]. The interplay between positive and negative reinforcement—in the forms of achieving the drug "high" and alleviating aversive withdrawal symptoms, respectively—is thought to drive this cycle. While it is impossible to capture every aspect of human addiction behavior in an animal model, there are several useful ways to model various stages of the addiction cycle. Many of the studies comparing sexes in animal models have involved behavioral tests that model the binge/intoxication or preoccupation/anticipation stage. These include operant self-administration, the conditioned place preference (CPP) test, and voluntary ethanol drinking behavior. Figure 2 illustrates these behavioral tests.

Operant Self-Administration

Self-administration is one of the methods used to study addiction-like behavior that most closely models the motivation to consume drugs. This method emphasizes the action of drugs of abuse as positive reinforcers, meaning that if an animal receives a dose of drug after performing a certain action (e.g. pressing a lever), then the animal is more likely to perform that action again [66]. The most common routes of drug administration in these studies are intravenous and oral, but many other routes are possible, including: intracerebroventricular, intracranial, inhalation, intragastric, and intramuscular[66].

Most studies conducted in non-human primates rely on self-administration techniques, although *ad libitum* drinking is also common[67]. In primates, self-administration has typically been done intravenously (i.v.), intragastrically (directly into the stomach), or through a tube from which the animals are able to consume the alcohol by mouth (p.o)[67]. Since ethanol naïve primates will generally drink only small quantities of alcohol, p.o. self-administration generally requires some kind of induction procedure (e.g. flavoring the alcohol with palatable fruit juice). The earliest published study on alcohol consumption in non-human primates was conducted in two rhesus monkeys (one male and one female) in 1960[68]. While monkeys are the most common type of non-human primate used in alcohol research, at least one other study from the 1960s examined alcohol drinking behavior in great apes (chimpanzees and orangutans)[69]. In this study, males of both species drank more than females, on average—though the range of individual differences in quantity of alcohol consumed was large. It has been noted that such variability in baseline alcohol consumption is common among non-human primates, which can prove useful in translational research by allowing for study of risk factors that make certain individuals more susceptible than others to heavy/risky drinking behavior[67]. In general, sex differences in alcohol drinking among non-human primates are similar to humans, though very few primate studies have examined sex differences[67]. When given long-term, unlimited access to alcohol, male cynomolgus monkeys drink more than females and attain higher blood alcohol levels[70]. Male rhesus monkeys also drink more than females of their species when given limited access to sweetened ethanol solution[71]. Furthermore, drinking in female monkeys may be influenced by ovarian hormones, as female macaque monkeys self-administered significantly more

alcohol at mid-cycle, when circulating E2 levels are high, than during menstruation, when E2 levels are low[72].

In contrast to what has been observed in human and non-human primates, female rodents tend to consume more alcohol than males across a range of measures[46, 73-75]. Operant ethanol self-administration generally uses oral alcohol delivery and is most often performed in rats because they are easier to train, although one study found that it is possible to induce operant responding for ethanol vapor in male C57BL/6J mice[76]. Oral consumption is generally preferred in alcohol studies because this is the route of administration used by humans. Rats will not readily consume unsweetened alcohol (except in strains selectively bred for high alcohol consumption, such as the alcohol preferring "P" rats, or in rats that have been made dependent on ethanol), so sucrose fading procedures are used to induce alcohol drinking, similar to the induction procedures used in non-human primates[77].

Moore and Lynch found that female "P" rats administered more alcohol than males during the first 10 days of testing, but males increased responding to levels that equaled female self-administration after the initial 10-day period[78]. More recently, Priddy *et al.* found that while females drank more alcohol than males when given access to alcohol in their home cages, no sex differences in consumption were found under operant conditions in either Wistar or Long-Evans rats[75]. Others have reported higher levels of operant responding for alcohol by females[79, 80]. Although Randall *et al.* reported that male Long-Evans rats tend to have greater numbers of alcohol-reinforced responses throughout self-administration training, females show similar or greater alcohol intake after correcting for differences in body weight[81]. After a period of forced abstinence or extinction, Male Long-Evans rats showed greater reinstatement (i.e. relapse) responding to alcohol cues than females[81], but in another set of studies in Sprague Dawley rats, females responded more for alcohol in response to the combination of cues and a stressor[79, 80]. Increased stress plus cue-induced reinstatement of alcohol seeking in females is consistent with evidence showing that women are more likely than men to relapse to drinking in response to stress[30].

A small number of studies have also looked for estrous cycle effects on alcohol self-administration. Three studies found no effect of estrous cycle on operant responding for ethanol in freely cycling female rats [56, 75, 80], but Roberts *et al.* reported a modest effect of cycle phase in animals whose cycles were synchronized with a GnRH receptor agonist, with highest intake levels occurring in diestrus, when E2 levels are rising, suggesting that it may be possible to unmask estrous cycle effects under certain testing conditions[56]. Evidence that E2 promotes ethanol self-administration was recently demonstrated in ovariectomized (OVX) rats that had been treated for several weeks with E2. The E2-treated rats lever-pressed at a higher rate and drank more ethanol compared with control OVX and gonadally intact female rats[80]. Interestingly, in this same study, E2 treatment had no effect on the combination of cue- and stress-induced reinstatement of operant responding[80], suggesting that the sex difference observed in reinstatement may primarily be driven by organizational and/or sex chromosome effects.

Conditioned Place Preference

Place conditioning tests are a well-established method of measuring the “rewarding” (pleasurable or appetitive) or aversive effects of a given stimulus in laboratory animals[82-85]. Place conditioning tests use a classical conditioning paradigm to form an association between a stimulus of interest (e.g. ethanol) and a contextually distinct environment. After the conditioning procedure, the subject animal can choose to spend time in or choose to avoid the stimulus-paired environment. If the animal chooses to spend more time in the stimulus-paired environment than it did before conditioning, then the stimulus is considered to be rewarding; this is called conditioned place preference (CPP). Conversely, if the animal chooses to avoid the stimulus-paired environment, the stimulus is described as aversive. In this case, the phenomenon would be called conditioned place aversion (CPA).

Mice and rats are the most commonly used animals in CPP testing, although some researchers have used zebrafish[84]. Establishing CPP for ethanol in male rats that have not been specifically bred for high alcohol consumption is difficult. The Wistar rat strain may be more amenable to developing ethanol CPP because several researchers have reported significant ethanol CPP in males of this strain[86-90]. Interestingly, however, obtaining ethanol CPP in male rats seems to depend on pretreatment with low-dose ethanol for an extended period (~15 days) before the start of the actual conditioning procedure, suggesting that a sensitization period is necessary for males to find alcohol rewarding in this test[89]. This is not commonly done for other drugs in the CPP test.

Very few studies have examined ethanol CPP in female rats, but Torres *et al.* demonstrated that female Wistar rats are more sensitive to ethanol reward than males across a range of doses[91]. In this study, which did not use a pre-conditioning sensitization period, neither adult nor adolescent males developed ethanol CPP. On the other hand, adult females developed CPP at both low (0.5 g/kg) and moderate (1.0 g/kg) doses of ethanol, and adolescent females developed CPP at the moderate dose[91]. When adult females were ovariectomized (OVX), their preference did not differ from males, suggesting that ovarian hormones play an important role in increasing ethanol reward in female rats. We have found that E2 treatment of OVX female mice enhances ethanol CPP (Hilderbrand and Lasek, manuscript under review).

In addition to its usefulness as a measure of drug reward, CPP, like operant self-administration, can also be used to assess vulnerability to a relapse-like state in animal models (Figure 2)[92], known as reinstatement. A typical reinstatement model will begin with a normal CPP conditioning procedure, followed by a period of “extinction” during which the animal is exposed to the drug-paired environment in the absence of drug. CPP is extinguished when the time spent in the drug-paired environment is roughly equal to the time spent in that environment before the conditioning period. Reinstatement of CPP is induced by re-exposure to the drug or by exposure to a stressor. Little is known about sex differences in reinstatement of ethanol CPP, as few studies have tested for such differences, but the available data suggest that sex differences in this behavior do exist. For example, one study found that, while early adolescent female mice required higher doses of ethanol to induce CPP than early adolescent males, females continued to be responsive to ethanol

reward into late adolescence[93]. Late adolescent males, on the other hand, did not develop ethanol CPP at any doses tested. This same study found that reinstatement of CPP occurred in early adolescent males and both early and late adolescent females.

Two-Bottle Choice Ethanol Consumption

The two-bottle choice test of ethanol consumption is a method of measuring voluntary ethanol drinking in the home cage and is one of the simplest tests to perform. In this test, animals are given access to two drinking bottles, one filled with normal drinking water and the other filled with an ethanol solution that ranges from 3-20%. As mentioned above, most rat strains do not readily consume alcohol without the addition of sweeteners, so sucrose or saccharin is generally used at the start of these experiments to encourage drinking. “Fading” procedures, in which the amount of sweetener is gradually decreased and alcohol concentration is gradually increased, can be used to transition rats to higher levels of alcohol consumption. Mice, especially the C57BL/6 inbred strain, will readily consume ethanol in this procedure without the addition of sweetener. For a comparison of two-bottle choice drinking behavior by different inbred mouse lines, see Belknap *et al.* and Yoneyama *et al.* [94, 95]. By providing animals with a choice between water and alcohol, this method also allows researchers to measure preference for one liquid over the other. Typically, two-bottle choice studies in rats have been used to examine preference for alcohol over a 24-hour period. One drawback of this model is that animals tend to consume relatively low (sub-intoxicating) quantities of alcohol in two-bottle choice tests, unless strains specifically bred for high alcohol consumption (e.g. “P” rats) or ethanol-dependent animals are used. However, Long-Evans and Wistar rats will consume large amounts of ethanol without sucrose fading using a 24-hr intermittent access procedure pioneered by Wise in the 1970s[96, 97].

Females tend to drink more than males and show higher preference for alcohol over water in two-bottle choice tests[95, 98-107]. Some studies have reported equal consumption between males and females, however[78, 108, 109]. This may be related to animal strain differences; CD (derived from Sprague-Dawley) or alcohol-preferring “P” rats were used in these studies, and strain is known to be an important determining factor of voluntary alcohol consumption in both rats and mice[73, 95, 100]. Some have reported different findings even within the same strain. Vetter-O’Hagan reported that adolescent males of the Sprague-Dawley strain consume more alcohol relative to their body weights than adolescent females and adults of both sexes, whereas adult females generally consume more than adult males[110]. In contrast, Lancaster *et al.* found that adolescent Sprague-Dawley females drank more than males, although their consumption decreased up until puberty[101]. However, it was noted by Lancaster *et al.* that the type of alcohol (beer vs. ethanol in water) and the delivery method (graduated drinking vial vs. standard water sipper) differed from other studies[101]. These inconsistencies likely explain the different results obtained from this study. After puberty, females drank more than males, especially when animals were exposed to stress by pair-feeding, suggesting that ovarian hormones may partly contribute higher levels of drinking in females. Marco *et al.* also found that female Wistar rats subjected to chronic mild stress (CMS), a widely accepted animal model for depression, showed significantly higher ethanol consumption and preference for ethanol over water, compared to CMS males[111]. This is

consistent with results from other animal studies and with evidence from the human literature, which shows that women are more susceptible than men to stress-induced drinking behavior. Several studies have demonstrated that OVX, which depletes circulating ovarian hormones, reduces ethanol intake in female rats and mice to levels similar to those seen in males[112-114]. This effect is not universal, as others have reported unaltered ethanol consumption after OVX in females[103, 115, 116]. Several factors—such as the timing of OVX (adolescence vs. adulthood), strain of animal used, degree of ethanol availability (i.e. limited vs. continuous access), and correction for baseline levels of consumption prior to OVX—may explain these discrepancies. The hypothesis that ovarian hormones promote ethanol consumption in females is also supported by studies that have used supplemental E2 treatment in OVX animals. For example, Ford *et al.* demonstrated a positive correlation between E2 dose and ethanol consumption in the two-bottle choice test[117].

The drinking in the dark (DID) test is a variation on the two-bottle choice procedure and models binge-like alcohol consumption[118, 119]. In this test, mice are given limited access (2-4 hours) to a single bottle of alcohol during the dark portion of the light-dark cycle. This is when mice, being nocturnal animals, are most likely to be awake and naturally engaging in feeding and drinking behavior. One advantage of this procedure over others (such as 24 hour two-bottle access) is that mice will routinely drink to intoxication and achieve blood EtOH concentrations (BECs) greater than 100 mg% [119]. Because of the high levels of drinking and pharmacologically relevant BECs obtained by this test, DID has become a popular model in alcohol research and is commonly used to test for effects of genetic and pharmacological manipulations on binge-like drinking[118]. Similar to other alcohol consumption models, females consume more than males in the DID test[73] and we recently found that E2 promotes higher levels of drinking in the DID test[120].

CONCLUSIONS

Several studies have determined that sex differences in AUD exist during different phases of the addiction cycle. In humans, more men abuse alcohol than women, but the number of women with problematic drinking has increased in recent years. Women also experience higher comorbidity of mood disorders and AUD and may be more vulnerable to developing an AUD. Studies in rodents have demonstrated a potential role for E2 in promoting behaviors during the binge/intoxication phase of the addiction cycle, indicating that this hormone may be involved in increasing the risk for women to develop AUD. More preclinical and clinical studies are clearly needed to determine sex differences and the role of sex hormones in the withdrawal/negative affect and preoccupation/anticipation (“craving”) aspects of AUD so that effective pharmacotherapies can be developed to treat AUD in both sexes.

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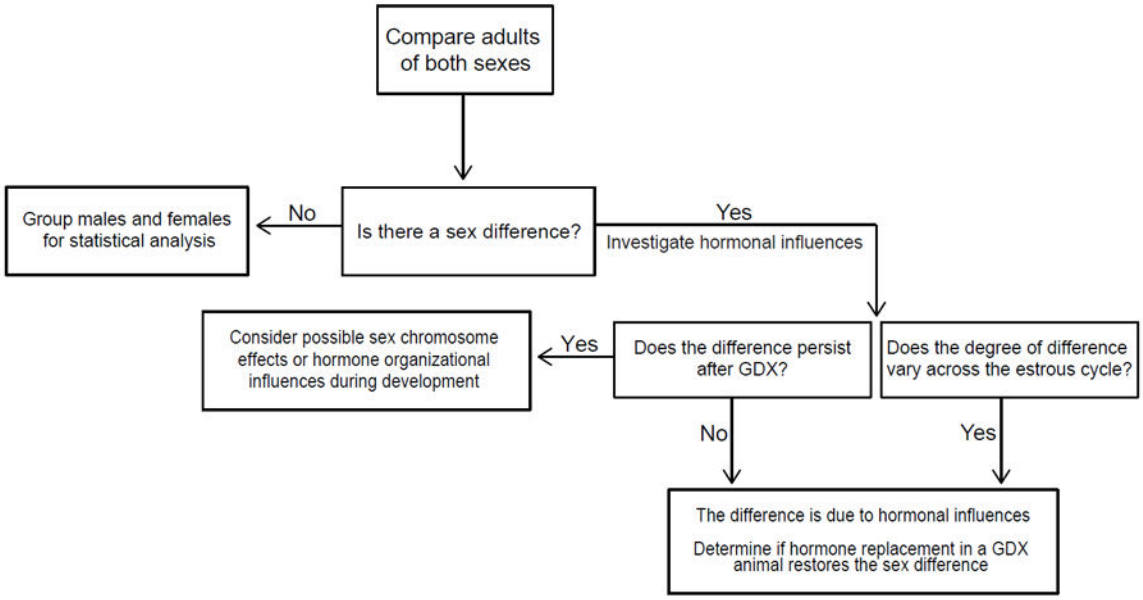

Figure 1.

Flow chart showing the process for studying sex differences in behavioral or physiological measures in rodents. The starting point is to examine first if there are sex differences by comparing adult animals and then investigating in more detail if hormones, sex chromosome complement, or organizational differences during development are responsible for sex differences in a particular measure.

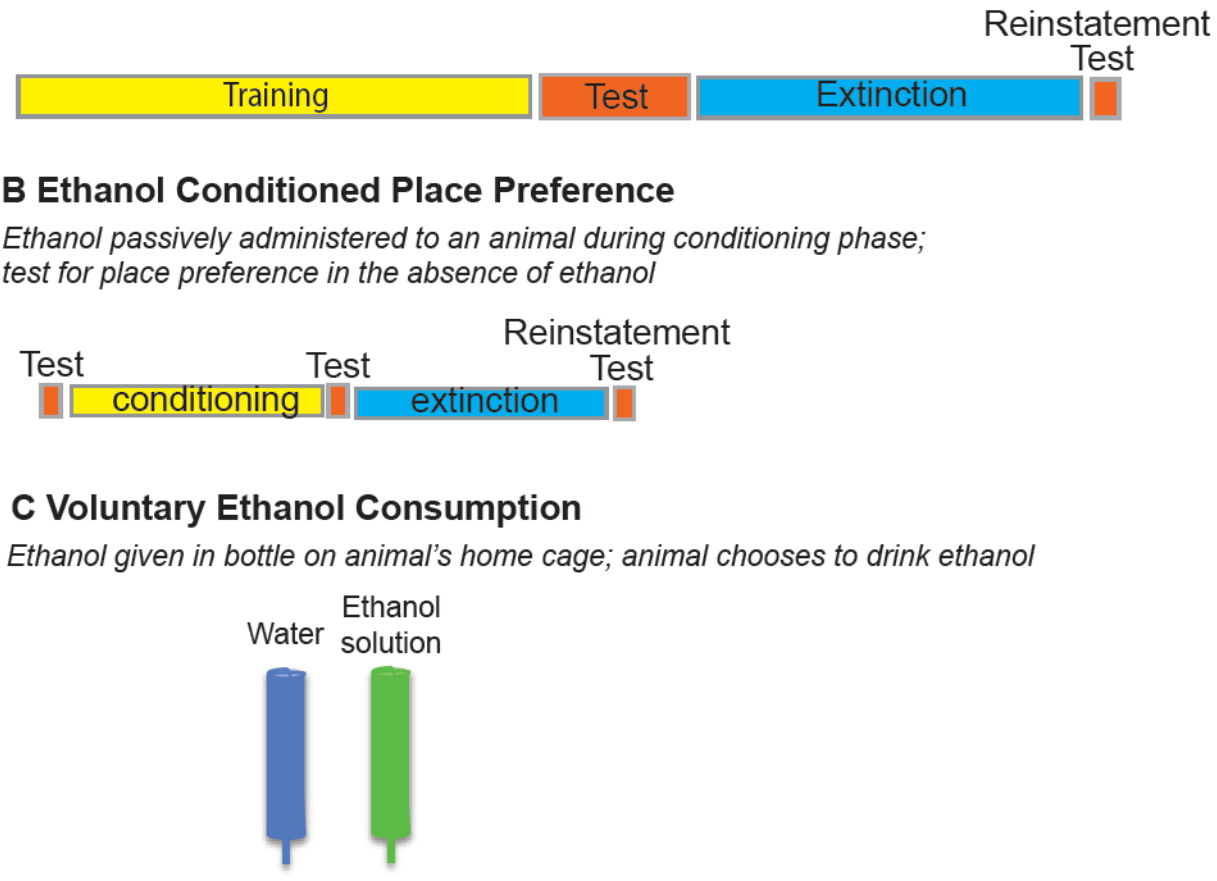

Figure 2.

Behavioral tests used in rats and mice to model aspects of alcohol use disorder. (A) Operant self-administration test, in which animals are trained to press a lever to obtain access to an ethanol solution. This test consists of the training phase, in which animals learn to press the lever for ethanol delivery, followed by the testing phase, in which they actively press the lever to obtain ethanol. In the extinction phase, animals press the lever but are not rewarded by ethanol delivery, so they learn to stop pressing the lever. The reinstatement test measures relapse-like behavior and is triggered by a priming injection of ethanol, cues that predict ethanol delivery, or by stress. Animals will begin to actively press the lever again in expectation of obtaining the ethanol reward. (B) The conditioned place preference (CPP) test, which measures the rewarding properties of ethanol. In this associative conditioning experiment, animals are first tested for preference for a particular context. They are then conditioned with injections of ethanol in one context, or saline in a different context. Animals learn to associate the ethanol injection with the context. During the post-conditioning test, animals will spend more time in the ethanol-paired context in the absence of ethanol. Similar to the operant self-administration test, extinction is done by placing the animals back into the context but not giving the ethanol injection. Reinstatement is induced by an ethanol injection, exposure to an ethanol cue, or by a stressor. (C) Voluntary ethanol

consumption, which measures the amount of ethanol that an animal drinks in the home cage. Generally, a choice is given between ethanol and water over a 24-hour period, but one bottle limited access procedures such as drinking in the dark are also used to measure binge-like ethanol drinking. Ethanol concentrations can range from 3-20%.