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How to study sex differences in addiction using animal models

Marilyn E. Carroll[*,1] and **Wendy J. Lynch**[2]

[1]Marilyn E. Carroll, Department of Psychiatry, University of Minnesota, MMC 392, Minneapolis, MN, USA

[2]Wendy J. Lynch, Department of Psychiatry and Neurobehavioral Sciences, University of Virginia, PO Box 801402, Charlottesville, VA

Abstract

The importance of studying sex as a biological variable in biomedical research is becoming increasingly apparent. There is a particular need in preclinical studies of addiction to include both sexes, as female animals are often excluded from studies, leaving large gaps in our knowledge of not only sex differences and potential prevention and treatment strategies, but also with regard to the basic neurobiology of addiction. This review focuses on methodology that has been developed in preclinical studies to examine sex differences in the behavioral aspects and neurobiological mechanisms related to addiction across the full range of the addiction process, including initiation (acquisition), maintenance, escalation, withdrawal, relapse to drug seeking and treatment. This review also discusses strategic and technical issues that need to be considered when comparing females and males, including the role of ovarian hormones and how sex differences interact with other major vulnerability factors in addiction, such as impulsivity, compulsivity and age (adolescent vs. adult). Novel treatments for addiction are also discussed, such as competing nondrug rewards, repurposed medications such as progesterone and treatment combinations. Practical aspects of conducting research comparing female and male animals are also considered. Making sex differences a point of examination requires additional effort and consideration; however, such studies are necessary given mounting evidence demonstrating that the addiction process occurs differently in males and females. These studies should lead to a better understanding of individual differences in the development of addiction and effective treatments for males and females.

Keywords

Addiction; Animal models; Impulsivity; Sex differences; Hormonal effects; Neurobiology; Treatment

[*]Corresponding author at: Department of Psychiatry, MMC 392, University of Minnesota, Minneapolis, MN 55455, United States, Tel: +1 612 626 6289; fax: +1 612 624 8935, mcarroll@umn.edu (M. Carroll).

Contributors

Marilyn E. Carroll, Department of Psychiatry, University of Minnesota, MMC 392, Minneapolis, MN, USA

Wendy J. Lynch, Department of Psychiatry and Neurobehavioral Sciences, University of Virginia, PO Box 801402, Charlottesville, VA

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1. Introduction

Animal models have been invaluable for understanding processes that lead to drug addiction and for developing prevention and treatment strategies. Several forms of addiction, including abuse of legal drugs such as alcohol and nicotine, and illicit drugs such as cocaine, opioids and stimulants account for an estimated cost to society in the United States of more than $700 billion dollars a year (NIDA, 2015). Addiction is difficult to study prospectively in humans due to safety and ethical issues and to the long duration of the addiction process. Human laboratory studies have provided valuable information on acute drug effects and short-term drug self-administration of both legal and illicit drugs as well as treatment efficacy (Anker & Carroll 2010, 2011; Carroll & Anker 2010; Evans & Foltin 2010; Fattore *et al.* 2014; Quinones-Jenab & Jenab 2012). However, animal studies are essential to adequately study the development and progression of addictive behavior and to develop behavioral and pharmacological treatments for addiction in humans. Animal studies allow for careful observation of factors that lead to addiction under controlled conditions. This allows for vulnerability characteristics to be identified, so prevention and treatment strategies can be developed and tailored to individual vulnerabiities.

The importance of animal studies for examining sex differences in addiction is that it allows us to consider how biological factors influence addiction, and studies of sex differences in human drug addiction in the laboratory confirm the influence of some of these biological (e.g., hormonal) factors. However, societal and economic factors in humans can override biological factors, and this leads to differences in the influence of sex on drug addiction. For example, the predominant finding in animal studies, that females show more drug-seeking and –taking behavior than males for most drugs of abuse, is in contrast to the sex differences found in humans; whereby, males exceed females in addictive use of some drugs such as stimulants (cocaine, methamphetamine), while females exceed males with other drugs such as prescription opioids (World Health Organization 2010). These differences in humans are mainly due to societal factors may override basic drug-seeking behavior that is influenced by hormonal status (Becker *et al.* 2012; Kornetsky 2005). In fact, sex differences in human drug addiction that have fluctuated over the last several decades, as the cultural factors and societal norms, regulations and laws have changed over time. For example, alcohol and opioid abuse were more common in women than men in the 1800s, but in the 1900s, as the Harrison Tax Act of 1914 came into effect, there was a decrease in women's use of patent medicines and as a result, the opioids, and men began to exceed women in their abuse of drugs. Thus, it is essential to understand the biological basis of sex as a factor in preclinical studies of drug addiction and its treatment, but application of that knowledge to solving problems of drug abuse in humans is limited by social context.

There are strategic and technical considerations to be taken into account when comparing males and females such as species differences. For example, studies of sex differences in animals indicate that there are reliable and reproducible sex differences in the subjective effects of abused drugs). These sex differences are verified in humans and are influenced by ovarian hormones, estrogen and progesterone in animals and humans. In rodents, hormonal cycles are very short (4–5 days), and the lifespan (~3 yr) is more compressed than in humans. Also there is a gradual decrease in hormones as rats age that does not parallel the

more rapid change in humans. Nevertheless, the similar but shorter patterns in the hormonal cycles and lifespan of rodents allow us to compare sex and hormonal influences from adolescent to adult ages and provide valuable information about genetic and neurobiological factors in addictive behavior.

Rhesus monkeys have also been a valuable resource to more closely approximate sex and hormonal status over a lifespan that is more representative of humans. An advantage is that in female rhesus monkeys the ~28-day hormonal cycle is very close to humans, and monkeys have a lifespan of up to about 40 years in the laboratory. This allows within-subject designs with small sample sizes, and repeated, within-subject studies, sometimes with patterns of different drug and polydrug use over a lifetime that is comparable to human drug abuse patterns. Furthermore, monkeys respond to treatments for addiction in the same way that humans do (i.e., there are no effective non-agonist treatments for drug addiction). However, medical treatments are more likely to successfully reduce drug abuse in rats (e.g., Carroll et al. 1990), but they are not well-translated to humans (e.g., Grabowski et al. 1995). In contrast, behavioral treatments developed in rats seem to translate well to humans (see *section 6*). Since rats are more likely to show treatment effects than monkeys (and humans), monkeys are a critical step in treatment development. Thus, studies with nonhuman animals have been invaluable for discovering individual differences such as sex, hormonal status, and age of onset, that increase our understanding of vulnerability to drug addiction as well as the impact of environmental factors (nondrug rewards) that are critical to its development.

There are other aspects of addiction that can be studied in animals more easily than in humans, such as patterns and progression of use, environmental stress and social factors (Bardo *et al.* 2001), toxicity, and newer, rapidly changing and more toxic forms of drug abuse (e.g., designer drugs). Additionally, novel treatments such as vaccines and viral vector-delivered enzymes and relationships to other forms of addiction (food, exercise) that interact with drug addiction can be more easily studied with animal models. These factors have received less experiment attention than drug addiction, and similarly, do not often include analyses of sex differences (see Fattore *et al.* 2014). However, progress made in these areas of animal research has vastly improved our knowledge of the addiction process in humans. An important finding from animal studies over the last 15 years is that sex and ovarian hormones are major factors in drug addiction (Anker & Carroll, 2010, 2011; Becker *et al.* 2012; Carroll & Anker 2010), yet more work is needed to understand these and other important predictors of drug addiction and how they interact with sex differences.

There have been several excellent reviews of animal studies of drug addiction clearly acknowledging that sex differences and hormonal influences are heavily implicated (Evans & Foltin 2010), and there are excellent reviews of the neurobiology of the sex/hormonal differences that underlie addictive behavior (Hu & Becker, 2008; Becker *et al.* 2012). The present review focuses on the methodology that has been developed in these studies to examine behavioral and neurobiological differences across the full range of the addiction process, from initiation (acquisition) to escalation, withdrawal, abstinence, relapse or reinitiation of drug seeking and treatment. The present review focuses on methodology and novel animal models used to study sex differences in addiction. In the following review *Section 2* discusses how animal models can provide information that cannot be obtained

with humans, *Section 3* examines sex differences across all the phases of addiction and discusses the role of ovarian hormones at each phase, from the initiation of drug use to escalation and compulsive, escalating use, to withdrawal (or forced abstinence in animals) and subsequently reinstatement of drug-seeking and -taking or relapse. *Section 4* describes how sex differences are related to conditions that also predict addiction, such as impulsivity, sweet preference and adolescence, and how these genetic, environmental, and developmental individual differences interact to predict severity of addiction and responsiveness to treatment in all phases of addiction (see Carroll *et al.* 2010, 2013, 2014; Carroll & Holtz 2014; Carroll & Smethells 2015). *Section 5* compares sex and other individual differences with neurobiological changes associated with addiction, and finally, *Section 6* reviews sex and hormonal differences in treatment for addiction. *Section 7* reviews the practical aspects of conducting research on male and female animals, accounting for female hormonal cycles.

2. How animal models provide information on sex differences in addiction that cannot be obtained with human research

The main goal of this review is to explain how animal models inform our understanding of the impact of *sex* on the development, progression, and treatment of addiction in humans. A second goal is to describe how these animal models can be used to prevent drug abuse in *men and women.* The purpose of this section is to emphasize that the most important aspects of drug abuse that are essential to our understanding of this devastating disorder cannot be studied in humans in a prospective manner. Fortunately, animal models allow us to obtain these areas of information that are highly relevant to addiction, but necessarily neglected, in drug abuse studies with humans. Human laboratory studies of addiction have great value in identifying endogenous factors such as sex (Anker & Carroll 2011) and how sex interacts with other major predictors of addictive behavior, such as impulsivity (Weafer & de Wit 2014), compulsive and excessive consumption of sweets (Carroll *et al.* 2013a; Carroll and Holtz 2014), and age (adolescents vs. adults; Spear 2011) that are closely related to the development of drug addiction. However, there are many aspects of drug addiction (drug type, toxicity, individual characteristics, environmental factors) for which sex differences have a critical role in our understanding of addiction, as well as its prevention and treatment that are difficult to study prospectively in humans. Thus, the following areas are the focus of this review: A) how sex differences occur over all phases of addiction, from initiation to escalation, withdrawal, and relapse, and how addictive patterns (e.g., bingeing, repeated relapse) occur (see *section 3*), B) Additive vulnerability of sex and other individual differences such as impulsivity, and compulsive bingeing on food. Age (adolescence vs. adulthood) is another risk factor for drug seeking behavior and subsequent addiction. These factors, impulsivity, compulsive sweet intake and age, add to increase vulnerability to drug abuse (see reviews by Carroll et al. 2013a; Carroll and Holtz, 2014; Carroll and Smethells, 2016; and *Section 4*), C) how understanding the neurobiology underlying drug abuse leads to important advances in developing treatment strategies (see *section 5*), D) the importance of sex and hormonal differences in designing treatments for addiction (see *section 6*), and E) practical aspects of studying sex differences in addiction in animals that consider a new NIH mandate for use of both male and female animals in all aspects of biomedical research (see *section 7*).

3. How sex differences have been studied across all phases of addiction

Animal models have been developed to represent the major phases of the addiction process including initiation of drug use, maintenance levels of moderate, steady use, escalation and binge use, forced abstinence or extinction, and reinstatement of drug seeking (relapse). There is also a growing interest in developing models that take into consideration more of the features that are characteristic of human drug abuse including impulsive and compulsive use, enhanced motivation for the drug, continued use despite negative consequences, high vulnerability and repeated relapse. Sex differences have been reported for each of the main phases and critical aspects of addiction. The following sections discuss how sex differences and underlying hormonal influences in these critical phases of addiction are typically studied using animal models.

3.1. Initiation of drug use

Drug use initiation has been examined in animal models by measuring the rate of acquisition of drug self-administration. Methods for studying the acquisition process were recently reviewed by Carroll and Meisch (2011). Typically, animals are given i.v. or oral access to a drug for a few hours each day, with deliveries contingent upon an operant response, using a lever or nose-poke device to record a response under a fixed-ratio 1 (FR 1) schedule (i.e.,1 response = 1 drug delivery). Acquisition of drug self-administration is then measured as the number of sessions needed to reach a criterion level of intake, the number of drug deliveries taken, and/or the ratio of active to inactive (control) lever or nose-poke responses (Campbell & Carroll 2000; Carroll & Meisch 2011). The emergence of sex differences during the initiation of drug use has often been documented, but it depends on the testing conditions (Carroll & Meisch 2011) and the drug dose. Sex differences are more likely to be revealed at low drug doses, they are reliable and robust under these conditions, and they have been observed in ~~both~~ rats (Lynch & Carroll 1999), mice (Locklear *et al.* 2012; Tambour *et al.* 2008), and monkeys (Carroll et al. 2000, 2005) across a range of drugs including alcohol (Tambour *et al.* 2008), cocaine (Lynch & Carroll 1999), methamphetamine (Roth & Carroll 2004), heroin (Lynch & Carroll 1999), nicotine (Donny *et al.* 2000; Locklear *et al.* 2012) and phencyclidine (Carroll et al. 2000, 2005). Results of these studies comparing the acquisition of self-administration of low i.v. drug doses, or concentrations of phencyclidine in the case of oral intake, revealed enhanced drug self-administration in females compared to males (Carroll *et al.* 2005). In studies where high doses were used, male and female rats typically did not differ during acquisition (Caine *et al.* 2004), possibly due to a ceiling effect or motoric effects of high doses like stereotyped behavior that introduce variability and obscure sex differences. However, acquisition dose has not been systematically studied.

While it is difficult to examine the role of cyclical changes in hormones in rats on rates of acquisition, due to the short estrous cycles, hormonal influences have been examined and are related to sex differences in acquisition. This is done by manipulating absolute hormone levels using group designs (e.g., gonadectomy and replacement, pharmacological blockade). These studies indicated that gonadectomy and pharmacological blockade of the estrogen receptor reduced the otherwise elevated acquisition of drug self-administration (compared to males), and estradiol replacement restored the elevated self-administration of cocaine self-

administration (Lynch *et al.* 2001). Gonadectomy in males did not affect rates of acquisition of cocaine self-administration (Lynch et al. 2001). Acquisition studies in animals are often difficult to conduct, because they appear only at low drug doses, and rates of intake reach asymptote quickly. However, the information obtained, that females acquire drug self-administration more quickly than males, is an important point of translation to understanding the development of drug abuse in humans.

3.2 Maintenance levels of drug use

After initiation of drug self-administration, levels of drug intake are usually maintained at low to moderate rates when short access is available each day. These maintenance levels are a useful baseline against which to study escalation of use that later occurs when access is increased. Since these early phases of drug abuse cannot be prospectively studied in humans, laboratory animals are valuable models to model the progression from occasional use to escalated, out-of-control use that constitutes addiction criteria in humans. Thus, studies of the early maintenance phase limit the amount of drug that can be obtained each day such that levels of intake are low and relatively stable. For example, when sessions are limited to 1–3 hr/day, animals regulate their intake to achieve a relatively stable amount throughout the session each day (e.g., Lynch et al. 2000). Maintenance intake is typically assessed under FR-1 schedules. Sex differences in maintenance levels of drug use have been examined in numerous studies, but fewer sex differences are found during maintenance than during escalation or reinstatement. As in the case of clinical findings (Hser *et al.* 1987; Kosten *et al.* 1993), male and female animals generally take a similar levels of drugs including cocaine (Roberts *et al.* 1989; Lynch et al. 2000), methamphetamine (Reichel *et al.* 2012; Roth & Carroll 2004), nicotine (Li *et al.* 2014), heroin (Lynch & Carroll 1999), and alcohol (Moore & Lynch 2015) during the maintenance phase. While most of the work in this area has been conducted in rats, similar findings were also reported in mice (Ward & Walker 2009) and non-human primates (Carroll *et al.* 2005). However, when more challenging behavioral schedules (Anker & Carroll 2011) are used, sex differences are reported during maintenance ~~have been reported~~ (see review by Carroll & Anker 2010).

The steady levels of drug self-administration during the maintenance phase have been used to compare the reinforcing efficacy of drugs to nondrug rewards, and to compare intake of drug and nondrug substances in males and females. In humans, as drug abuse increases, the desire to obtain the drug supercedes the desire to engage in other available reinforcement alternatives (Carroll & Meisch 1984, 2011). In animals, the substitution of drug self-administration for food reward is studied by choice procedures whereby animals choose between drug and food. In these studies females expressed a greater preference for drug over food than males (Kerstetter *et al.* 2012), and they were more likely to develop a preference for drug over time compared to males (Perry *et al.* 2013). There were also sex differences in responding under a progressive-ratio (PR) schedule, in which the ratio of responses to drug deliveries progressively increased within a session (Lynch 2009). This schedule is often used to model motivation for drug use and responding under this schedule is considered a measure of compulsive behavior, and with these schedules, sex differences have been reported in the maintenance phase (Carroll & Meisch 2011). Specifically, female rats, mice and non-human primates reached higher final ratios (breakpoints) in the time allotted than

males, indicating greater motivation for the drug in females versus males (Roberts *et al.* 1989; Mello *et al.* 2007; Martini *et al.* 2014). Again, these findings were generally observed under low to moderate (e.g., Westenbroek *et al.* 2013), but not high-dose conditions (e.g., Ramôa *et al.* 2013). Studies with animals using PR schedules to assess motivation suggest that interventions for drug abuse might be more successful during the early stages when motivation for the drug and doses consumed have not yet peaked vs. later stages when dose and motivation are at the maximum.

Maintenance conditions are also ideal for examining the relationship between levels of hormones and drug taking behavior, and exogenous hormones can be administered or phases of the estrous/menstrual cycle can be examined during this steady-state phase. However, like sex differences, estrous/menstrual cycle phase effects are more likely to be revealed when more challenging behavioral schedules are used. For example, while intake generally does not differ across the estrous/menstrual cycle phase under an FR 1 schedule, it does under a PR schedule with the levels of estradiol/progesterone predicting PR break points, which are considered a measure of motivation for drug seeking (Roberts *et al.* 1989; Lynch 2009).

3.3. Transition and escalation to binge use (DSM criteria)

After drug use has initiated and reached a steady-state of maintenance, usually under limited-access conditions, a transition can occur from controlled drug use to escalation of intake leading to addiction, especially if the access period is lengthened (Ahmed and Koob 2009). This critical transition to pathological drug use in humans has been elegantly captured in a multi-symptomatic model of loss of control of drug seeking, using the development of cocaine addiction in rats as a model (Deroche-Gamonet et al. 2004; Belin & Deroche-Gamonet 2012; Deroche-Gamonet & Piazza 2014). This progression from steady daily use to escalating use over time is thought to be an important model of casual drug use/experimentation in humans that transgress to addiction. Early animal studies were invaluable for examining the behavioral economic basis that drives addiction in animals (and humans) and allows the transition from use to abuse and to develop so that interventions to stop the process can be tested (Carroll & Campbell 2000). Several changes in drug-taking behavior were identified as signs of transition from steady, regular drug use to addiction. Key features of this model are derived from DSM IV/5 criteria (Deroche-Gamonet et al. 2004; Belin & Deroche-Gamonet 2012; Deroche-Gamonet & Piazza 2014), and they include: 1) Dose escalation that involves difficulty stopping or limiting drug use, and taking increasingly large amounts (escalation). This occurs when animals have long daily access to the drug, and dysregulated intake patterns are compared to the more regular, controlled patterns during shorter-access periods that can be observed before and after long access, 2) Increased motivation to use the drug that is modeled by schedules that require more responding for each successive drug delivery (progressive-ratio - PR) and often considered compulsive drug intake, and 3) Continued use despite aversive consequences shown by persistent drug use despite punishment for using. These characteristics of addiction that develop following extended access to drug self-administration develop differently between males and females, and in females their development varies with hormonal conditions. Details of sex differences are described in the following sections regarding animal models used to examine these 3 criteria for addiction.

3.3.1 Dose escalation—During the initial maintenance phase drug self-administration remains at a steady level; however, if drug access is changed from a short (ShA) to a long (LgA) daily access period, drug taking can begin to rise or escalate within a few weeks. Also, if there is a change in the dose received, the response cost of the drug, or if tolerance occurs, whereby higher doses are taken to achieve the same effect as previously experienced, increased or escalated drug intake may result. This has been modeled in rats and monkeys, and sex differences have been reported. Three methods of modeling dose escalation have been used in the laboratory: 1) providing long daily exposure to the drug with minimal schedule requirements (e.g., FR 1), 2) allowing 24-hr drug access, but limiting infusions per minute to prevent excessive intoxication, and 3) allowing the animal to select a high vs. low dose using 2 levers, one that increases the next dose and one that decreases it, within an extended access period.

3.3.1.1. Method 1: Long access (LgA) vs. short access (ShA): In humans, bingeing on drugs is a sign of compulsive, dysregulated behavior that marks the pivotal point when regular drug use transgresses into addiction. This has been modeled in animals by exposing them to several methods that allow them to develop the compulsive, binge-like, escalating patterns of voluntary drug intake that occur in humans. In most cases stimulant drugs have been studied, and little information is available in animal models regarding escalating/bingeing patterns with other drugs of abuse. Bingeing on drugs also has characteristics in common with bingeing on palatable substances (e.g., sweets) that results in food addiction, obesity, and similar maladaptive behaviors in humans (see Bocarsly and Avena 2013). Thus, the methods are not necessarily specific to the development of drug addiction but to other nondrug addictions as well (see Carroll et al. 2013b).

The ShA vs. LgA method is the main procedure that has been used in animals to model the transition from controlled, steady levels of use to the escalating and binge patterns that characterize unstoppable human drug addiction (e.g., Ahmed & Koob 2009). Modeling binge patterns is accomplished by providing LgA (6 – 12 hr /day) under a low FR schedule (e.g., FR 1) to a drug such as cocaine and comparing intake to a short (1–2 hr/day) access group that does not significantly increase drug intake (Ahmed & Koob 1998; 2009) over the typical time period that is tested (e.g., 3 – 4 weeks). A disadvantage of this method is that LgA daily drug intake can result in adverse health consequences in animals; thus, it requires careful monitoring. If adverse effects occur, this is remedied by giving temporary access to low doses or scheduling days off (Ahmed & Koob (1998). This is a useful method for examining individual differences in bingeing to determine the type and timing of interventions to prevent the progression to drug addiction; however, this method has most often been used to study stimulant addiction, and fewer studies are available with other drug classes.

The LgA method has been used to examine sex differences in both rats and non-human primates with results consistently showing greater drug intake and greater escalation of drug self-administration in females as compared to males for cocaine (Roth & Carroll 2004), or methamphetamine in rats (Reichel *et al.* 2012), and PCP in monkeys (Carroll *et al.* 2005). Levels of intake are inherently variable under extended access conditions, and high levels can also disrupt the estrous/menstrual cycle (Mello & Mendelson 1997) making it difficult to

assess the influence of circulating hormones on drug-taking behavior. Results from studies using between-group designs, however, have shown that hormones play a critical role in levels of intake and escalation of use over time. For example, in ovariectomized (OVX) female rats, estradiol enhanced escalation of cocaine intake, and in controls progesterone or its metabolite, allopregnanolone, reduced it to levels of OVX females and males (Larson *et al.* 2007; Anker *et al.* 2010).

3.3.1.2. Method 2: Discrete trials, 24 hr access: This method allows 24-hr access to a drug with limited toxicity in a discrete trials procedure wherein rats are given 24-hr access to drug infusions that are available in discrete 10-min trials (Roberts et al. 2002; Lynch & Roberts 2004). However, there is a limit to the number of infusions that can be earned per 15-min interval to avoid toxicity. This method has been used for cocaine self-administration, and the results under extended access conditions (e.g., 4 discrete trials per hour) show that rats self-administer high levels of the drug in a binge pattern that is dysregulated from the diurnal cycle. Responding occurs at high levels during both dark and light phases, and drug intake is elevated (Lynch & Roberts 2004). Sex differences have been observed under this discrete trial method with results showing that female rats self-administered more cocaine, binged for longer initial periods of time, and showed a more diurnally dysregulated pattern of self-administration than males (Lynch & Taylor 2004). This pattern was characterized by bouts of binge responding throughout the 24-hr period vs. regularly-spaced responding in the dark vs. less responding during the light phase which is typical of rodents.

Results from these studies also revealed the importance of ovarian hormones in maintaining binge patterns of use with results showing that while OVX reduced cocaine intake, and the initial binge length, estradiol replacement restored the effects to a level comparable to those observed in intact females (Lynch & Taylor 2005; Ramôa *et al.* 2013). The high levels of drug intake found during escalation also disrupted the estrous/menstrual cycle (Lynch & Taylor, 2004), making it difficult to assess the influence of circulating hormones on drug-taking behavior. However, results from studies using between-group designs have shown that hormones played a critical role in escalation/binge use. For example, in OVX female rats, estradiol enhanced escalation of cocaine intake, and progesterone or its metabolite, allopregnanolone, reduced it to levels of males or OVX females (Larson *et al.* 2007; Anker *et al.* 2011). Sex and hormonal effects were consistently observed particularly under low dose conditions, but also under high dose conditions (Anker & Carroll 2011), suggesting that sex and hormonal conditions are particularly robust during the transition from controlled to escalated and binge use.

3.3.1.3. Method 3: Dose self-selection: Another method that was used for producing escalation of drug intake involved dose self-selection in an operant chamber with two levers, whereby responding on one lever increased the next dose, and responding on the other lever decreased it. Session length, the drug self-administered, and other factors such as genetic and other individual differences in the rats, determine the extent to which escalation occurs. With this procedure, the degree to which the drug dose is regulated (defined as a high correlation between dose self-administered and the subsequent interdose interval) can be determined during each daily session and then compared over time and between groups

(Lynch & Carroll 2001). Sex differences were examined with this procedure in one study for cocaine self-administration (Lynch *et al.* 2000), and results showed that while both male and female rats tightly regulated their intake of cocaine during the initial maintenance phase, over time the regularity of responding became disrupted, and higher doses were consumed more frequently, particularly in females. This dysregulation increased cocaine intake more in females than males. Also, females in the estrus phase of their estrous cycle showed almost exclusive responding on the high dose lever, and their ability to regulate dose was markedly reduced (i.e., a lower correlation was found between the preceding self-administered dose and the subsequent inter-dose interval). This study illustrated that within the microstructure of escalated drug self-administration, the timing of self-administration became less precise and dysregulated, and higher doses were self-administered in females compared with males and in females during estrus, compared with other phases of the estrous cycle.

In summary, sex and hormonal effects have been consistently observed under each of the different methods of extended access self-administration procedures, across several different drugs and species, and using both low and high dose conditions suggesting that sex and hormonal conditions are particularly robust during the transition from controlled use to escalation and binge use. However, given that levels of intake are inherently variable under extended access conditions with both rats and nonhuman primates, it may be necessary to use either a repeated-measures design or a larger number of animals to have enough statistical power to observe significant sex and hormonal differences in escalation/binge use. Alternatively, data could be normalized and evaluated by using smaller numbers of animals and percent change from baseline. This method was useful for evaluating highly variable behavioral economic demand functions to assess drug reward (Winger et al. 2006).

3.3.2. Enhanced motivation for the drug—Enhanced motivation for the drug is another criterion used to describe an addicted phenotype in animals. For example, extended access compared to ShA in rats self-administering cocaine (Deroche-Gamonet & Piazza 2014; Ramôa *et al.* 2013 and monkeys self-administering PCP (Carroll *et al.* 2005) resulted in enhanced motivation to obtain the drug as assessed under a PR schedule. Additional studies revealed that the use of extended access conditions coupled with an abstinence period of seven days or more was necessary to induce this phenotype in male rats (Roberts *et al.* 2007), possibly due to incubation of craving that occurs during abstinence (see section 3.5.3). Female animals develop an addictive phenotype sooner than males after an abstinence period. For example, in LgA females motivation to obtain cocaine was increased compared with ShA controls following 7 days of abstinence; whereas, in males, this increase did not occur until after 10–14 days of abstinence (Lynch & Taylor 2004). Importantly, following 14 days of abstinence, both males and females showed enhanced motivation for cocaine, and the magnitude of the increase was similar between the sexes (Ramôa *et al.* 2013). Comparable results have also been reported for sex differences in motivation for drug vs. food (Perry *et al.* 2013). Females developed a preference for drug over food sooner than males, but once the preference had developed, males and females were similar in their choice behavior. In humans women progressed more rapidly to seeking treatment for opioid-, cannabis- and alcohol dependence than men (Hernandez-Avila et al. 2004). These findings were also consistent with women taking less time than men to progress from initial

drug use to abuse, but once dependent, women and men reported similar levels of drug use (Kosten *et al.* 1993). Thus, animal and human studies show that females may develop drug seeking behavior faster than males, but after extended use of the drug, sex differences in drug intake are less apparent (see Anker & Carroll 2011; Ramôa *et al.* 2013; 2014). However, in animals, drug seeking (reinstatement – relapse) after drug use has terminated, is more likely to occur in female than male animals, but the direction of the sex difference is less clear in humans and depends on historical factors that are not controlled and studied in animal research.

3.3.3. Continued use despite adverse consequences (punishment)—Excessive use of drugs of abuse may lead to aversive consequences due to the dysphoric effects of large doses, repeated withdrawal episodes in individuals attempting multiple quit attempts or to family and social pressures placed on a drug-addicted individual. In fact, a criterion for addiction is continued use despite aversive consequences (Deroche-Gamonet & Piazza 2014). Recent data from animal studies indicate that there are sex and other individual differences in reactivity to this criterion for addiction. Also, humans report both rewarding and aversive effects of drugs such as nicotine or caffeine depending on the dose consumed (see Carroll 1998; Le Foll & Goldberg 2009). Interestingly, sex differences in reactivity to aversive drug events are related in an opposite way (e.g., M>F) to the rewarding effects (F>M). This opposite relationship between the rewarding and adverse effects of drugs is also present in other studies of individual differences such as in animals with high vs. low impulsivity (HiI vs. LoI), sweet intake (HiS vs. LoS), adolescents vs. adults, and other bred or selected phenotypes that differ on other motivational measures (see Carroll *et al.* 2013a, Holtz & Carroll 2015).

Differences in high vs. low drug abuse-vulnerable phenotypes (e.g., female vs. male, HiI vs. LoI, HiS vs. LoS, and adolescents vs. adults) have been reported in their response to aversive treatment for cocaine addiction. For example, animal studies have modeled the effect of punishment on drug seeking and self-administration, and results indicate that mild forms of punishment are effective and enduring. For example, after several months of ethanol intake rats continued to drink alcohol despite the consequences of footshock (Hopf *et al.* 2010) or bitter-tasting quinine (Seif *et al.* 2013), and this aversion-resistant alcohol intake is considered to be a model of compulsive drug abuse in humans (Everitt and Robbins 2005). Similar methods of punishment were used to reduce drug self-administration in rats with individual differences such as sex, age (adolescent vs. adult), sweet preference (HiS, LoS), and impulsivity (HiI, LoI), and the results showed that all groups reduced their responding for cocaine when histamine was added to the iv cocaine solution. However, females, LoS and adult (vs. adolescent) groups showed a slower return to baseline cocaine self-administration than their vulnerability counterparts (males, HiS, adolescents) when histamine was removed from the cocaine solution (Holtz *et al.* 2013; Holtz & Carroll 2015). The lingering suppression of drug self-administration suggests that females, LoS and adult rats were more sensitive to aversive consequences of cocaine use, and this form of treatment may be useful for the low (vs. high) drug-seeking animals such as LoS and adults (vs. HiS and adolescents). In addition, differentially vulnerable groups may require different treatment strategies, such as aversive consequences for low-addiction-prone individuals or

reward substitution for high drug-seeking individuals. Importantly, sex interacts with other major vulnerability factors to exaggerate drug abuse liability; thus, it is important to account for the combined vulnerability in treatment attempts (see Section 4). This inverse relationship between reward seeking and sensitivity to aversive events has been noted for a wide range of individual differences (see Carroll *et al.* 2013; Carroll & Smethells 2016; Riley 2011).

Similar to the individual differences in response to the aversive effects of histamine, recent findings also showed individual differences in drug withdrawal. Adult rats had more severe withdrawal effects than adolescent rats (Shahbazi *et al.* 2008), and this was in contrast to the greater sensitivity to rewarding effects of drugs such as cocaine in adolescent rats compared to adults (Perry *et al.* 2007; 2015a,b,c).

3.4 Abstinence/withdrawal

Withdrawal studies in rats initially focused on physiological disturbances that could be seen during withdrawal of experimenter-administered drugs such as barbiturates, ethanol, and opioids, and sex differences were apparent (e.g., Cicero *et al.* 2002; Devaud & Chadda, 2001). In most cases, males showed more severe withdrawal effects than females. Similarly, female rats and monkeys (Radke *et al.* 2015b; Perry *et al.* 2006), that are more avid drug seekers than males, exhibited fewer physical and behavioral withdrawal signs than males. In recent monkey studies that were based on operant conditioning models in which animals were trained to respond on devices for drug vs. water under a fixed-ratio 8 (FR 8) schedule, with food available under a high FR schedule (e.g., FR 128, 256), drug withdrawal (water substitution) resulted in disruptions in the behavior maintained by food or other nondrug rewards (saccharin), indicating subtle withdrawal effects. These withdrawal effects appeared to be a motivational deficit, because when food was freely available (FR 1 from a feeder or hand fed) monkeys consumed a normal amount. Also, at high and low FRs there were no observable signs of withdrawal. When male and female monkeys were compared on withdrawal of oral access to phencyclidine (PCP). The withdrawal-induced decrease in motivation was greater in males than females (Carroll *et al.*, 2009). In a recent study monkeys were evaluated for their impulsivity for a sweet substance, a palatable SACC solution, under a delay discounting task (DD), and PCP withdrawal affected males more than females as indicated by increased impulsive behavior directed toward SACC reward during PCP withdrawal (Carroll *et al.*, 2013). A similar study was conducted with PCP withdrawal and impulsive responding for SACC in females during their follicular phase when estrogen peaks and progesterone is low, and in the luteal phase of the menstrual cycle when progesterone peaks and estrogen is lower, and withdrawal disruptions were greater in the luteal phase (Carroll *et al.* 2013). The finding of greater withdrawal disruptions in males than females agreed with the findings discussed in section 3.3.3, that the less addiction-prone phenotype/condition (e.g., males, luteal phase) shows a greater response to aversive conditions. These results may inform the design of human treatment programs by coordinating specific treatment strategies that involve abstinence with a specific phase of the female hormonal cycle. Treatment studies for cigarette smoking in women showed that more favorable results occurred during the follicular phase when behavioral therapy (Allen *et al.* 2009) or behavioral therapy plus bupropion (Mazure *et al.* 2011) were used, but in other

studies agonist (nicotine) replacement therapy was more successful during the follicular phase (Franklin *et al.* 2007; Carpenter *et al.* 2008).

3.5. Relapse – enhanced drug seeking after cessation of use

After a forced abstinence phase, relapse or reinstatement of drug seeking occurred when an individual used the drug again or when cues associated with previous drug-taking were available to the animal. In human drug abusers relapse occurs repeatedly, alternating with periods of abstinence, and results in a chronically relapsing disorder. This is the most important phase of addiction, because after all the other phases have occurred, it is the endpoint of the addiction process. At this point behavioral and/or pharmacological interventions have been used to prevent drug-seeking behavior induced by cues or drug exposure. To study relapse in the laboratory, several models are currently used: 1) Drug- or cue-primed reinstatement, a process under which the acquisition, maintenance, extinction, and reinstatement phases are modeled in the operant chamber, and drug-priming injections (self-administered drug, other drugs, or stress-inducing agents such as yohimbine), cues, and stressful stimuli are typically used to elicit reinstatement responding (Anker & Carroll 2011), 2) Context-induced reinitiation of drug seeking (Crombag & Shaham 2002), a model in which the acquisition, maintenance, and reinstatement phases are conducted in the operant chamber, but the extinction phase occurs in a completely different environment, often the home cage, and reinitiation of drug seeking was initiated by cues in the original drug-taking environment, and 3) Incubation of craving which is an extension of the context-induced reinitiation model of relapse, only longer periods (weeks-months) are imposed before returning the animal from the home cage, or different environment, to the drug-taking environment. Longer delays resulted in higher levels of reinitiation of responding (relapse), which was assumed to represent the incubation of craving over time. The three relapse methods are described in more detail below.

3.5.1. Drug- or cue-primed reinstatement—This procedure evolved from early animal models of relapse in which 3 phases of addiction, maintenance, extinction, and drug-induced reinstatement occurred the same day (see reviews Carroll & Comer 1996; Shaham *et al.* 2003). Later, to approximate human behavior, the 3 phases were tested over several weeks, and eventually several stimuli such as different drugs, doses, stress, auditory, olfactory, tactile, and visual cues were used to reinstate drug seeking. Typically, this form of reinstatement is done within the operant chamber, and a priming injection (ip) of the self-administered drug or cue, is administered at the beginning of the session to generate reinstatement responding. Reinstatement is then measured by the number of responses on the previously-active lever. Drug, vehicle or control priming conditions serve as control conditions. Using this procedure, studies indicated higher levels of reinstatement were found in female vs. male rats following cocaine (Lynch & Carroll 2000), methamphetamine (Holtz *et al.* 2012), and fentanyl (Klein *et al.* 1997) after priming injections of the drug that was formerly self-administered. However, sex differences were less apparent following exposure to cues formerly associated with the drug (Fuchs *et al.* 2005). When reinstatement responding was assessed following 14 days or more of abstinence from extended-access self-administration (Zlebnik & Carroll 2015b), the level of reinstatement was higher than after a short period after drug access terminates, but no sex differences were reported (Doyle

et al. 2014). The finding of no sex differences here concurred with other studies that reported no sex differences during short access or low dose conditions to drug and steady-state phases of drug addiction, such as ShA maintenance prior to extinction/reinstatement. The lack of sex differences during these phases of addiction suggests that brain mechanisms involved were not affected by sex hormones.

The effects of circulating sex hormones and phase of the estrous/menstrual cycle can be difficult to examine in reinstatement studies, since levels of extinction and reinstatement vary over the time periods that hormonal changes in the estrous cycle (4–5 days) are occurring. However, in studies that examined drug-primed reinstatement in females' hormonal cycles, cocaine-induced reinstatement was greater in estrus than nonestrus phases of the estrous cycle (e.g., Kippin *et al.* 2005). Others have examined the effects of gonadal hormones on reinstatement by gonadectomy with systematic hormone replacement, and they found that estrogen increased reinstatement, while progesterone, and its metabolite allopregnanolone, reduced it (see Anker & Carroll 2011).

3.5.2. Context-induced reinitiation of drug seeking—This method, developed by Crombag and Shaham (2002) was based on the human condition (Wikler 1973; O'Brien *et al.* 1992) in which environmental contexts previously associated with drug taking elicited relapse in human addicts. In the animal model, drug self-administration occurred in context (A) with discrete, compound, drug-related cues that are paired with each drug infusion. Drug-reinforced responding was extinguished in a different context (B) after the animal was removed from the drug-taking environment to another housing location, and after a period of time was returned to the original drug-taking environment (A). Here responding on the previously active lever led to presentation of discrete cues that were previously associated with drug, but not drug access. This method used only the original drug-taking context to examine relapse, and this procedure has been used with almost all drugs of abuse (Crombag *et al.* 2008) and with both female and male animals; however, reports of sex differences with this method are scarce. A study by Anker et al. (2012) used a modified version of this procedure to examine cocaine-induced reinitiation of drug seeking in male and female rats that were repeatedly tested at 8 time intervals over a 6 mo period with both context-induced reinitiation of drug-seeking on Days 1–3 after returning to the testing environment, and with saline- vs. cocaine priming injections on Days 4 and 5. There were small there were no significant sex differences in context-induced reinitiation of responding and cocaine-primed reinstatement, and context-induced responding was higher than drug-primed reinstatement. Also, the context-induced reinitiation paradigm generated high levels of responding that extinguished after the first day in the testing environment (see Anker et al. 2012). Thus, a ceiling effect could have precluded a sex difference.

3.5.3. Incubation/enhancement of context-induced reinitiation of drug-seeking following abstinence—Enhanced drug seeking is observed following abstinence from extended drug self-administration, and it appears to progressively increase, or incubate, over time (Grimm *et al.* 2001). In animals, drug seeking and its incubation over time are typically studied using a within-session extinction-reinstatement paradigm. With this procedure, animals are first given either short or extended access to the drug, and once the period of

drug access is complete, drug infusions are discontinued, and responding on the formerly active lever is then assessed following a period of forced abstinence under non-reinforced conditions. Once the levels of extinction responding have dissipated, levels of cue-induced reinstatement responding is determined. This incubation effect occurs after both short and extended access self-administration, but it is markedly enhanced following extended versus short access self-administration (Doyle *et al.* 2014). Recent data suggest that this incubation effect can be blocked by presenting a nondrug reward (physical exercise) to female and male rats during the incubation period (Peterson et al. 2014; Zlebnik & Carroll 2015b). While incubation of cue-induced drug-seeking has been observed in both males and females, in females, the time-course of heightened responding was more prolonged (up to 6 months in females), and during abstinence it was higher in estrus females compared to females in other estrous phases or males (Kerstetter *et al.* 2008). Notably, when responding was assessed following 14-days of abstinence from extended access self-administration, conditions known to induce an addicted phenotype such as high levels of drug-seeking, both males and females showed similar high levels of drug seeking (Doyle *et al.* 2014) suggesting that once addiction develops, males and females may be similar at a behavioral level.

4. Sex and other individual differences related to addiction: additive vulnerability

The extensive and relatively consistent findings on sex differences in addiction suggest that sex and hormonal status (estrogen, progesterone) are major determinants of drug addiction. However, it is important to consider that there are other individual differences that are genetically determined and are as important as sex in predicting drug abuse vulnerability. It is useful to compare these vulnerability factors with sex differences regarding their influence on drug addiction and its treatment and to determine whether they interact with sex differences. The individual differences that have been most frequently examined for their interaction with sex differences in drug addiction are: impulsivity (HiI vs. LoI), sweet intake (HiS vs. LoS) and novelty reactivity in outbred (HR vs. LR) and selectively bred rats (bHR vs. bLR), Lewis (LEW) vs. Fischer (F344) inbred rat strains, and rats selectively bred for high (HAC) vs. low (LAC) alcohol intake (See reviews by Carroll *et al.* 2013a; Holtz & Carroll 2015). Other traits are associated with addiction, but have not been studied as extensively, such as selectively-bred Roman High (RHA) vs. Low Avoidance rats (RLA) (Fattore *et al.*, 2009), rats selected for high (HiR) vs. low (LoR) voluntary wheel running (Larson & Carroll 2005), or for sign-tracking (ST) vs. goal-tracking (GT) for food reward, which refers to behavior directed toward stimuli that predict reward (ST) vs. the goal or location where the reward is delivered (GT) (Saunders & Robinson 2011). As with the male vs. female comparisons during different phases of addiction, the high addiction-prone phenotypes/genotypes mentioned above are more avid drug seekers and consumers, while the low phenotypes exhibit less drug seeking. Also, like males (vs. females) the low drug-consuming phenotypes/genotypes are more responsive to aversive conditions than the high-consuming animals (see reviews by Carroll & Holtz 2014; Holtz & Carroll 2015; Carroll & Smethells 2016).

Two of these vulnerability factors that have been studied the most, impulsivity (HiI vs. LoI) and excessive sweet consumption (HiS vs. LoS) are discussed further below, as they show a similar degree of influence on drug-seeking and –taking as females vs. males, respectively, and these phenotypes interact with sex differences to enhance vulnerability. Impulsive behavior (e.g., HiI rats) is a major component of drug addiction (see Perry & Carroll 2008), and food craving/sweet addiction (e.g., HiS) is a form of behavioral dysregulation that is very similar to drug addiction (Carroll & Holtz 2014).

4.1 High vs. low impulsive (HiI vs. LoI) rats

Regarding the HiI vs. LoI rats, two types of impulsivity, impulsive action and impulsive choice are typically studied (Weafer & de Wit 2014). In animal studies (and in humans) impulsive action is often assessed with a 5-Choice Serial Reaction Time Task (5-CSRTT) that requires an individual to respond for a reward when there is a Go cue, but they are required to refrain from responding in the absence of such a cue. Studies of sex differences in rats and mice using this procedure have produced mixed results. For example, normal and gonadectomized rats were compared on a modified version of the 5-CSRTT, and gonadectomized males were less impulsive than controls, suggesting a role for testosterone. Ovariectomy increased impulsivity in females, suggesting estrogen and/or progesterone played a role in reducing impulsivity (Jentsch & Taylor 2003). In other rodent studies males exceeded females in impulsive action using a 5-CSRTT (for review Weafer & de Wit 2013), but Anker *et al.* (2008), using a Go/No-go procedure, found that male and female rats were equal on this impulsivity measure for food reward. In contrast, females exceeded males on a similar Go/No-go task for iv cocaine self-administration.

In studies of impulsive choice, using a delay-discounting task, female rats were more impulsive than males for food reward (Van Haaren *et al.* 1988; Koot *et al.* 2009) and i.v. cocaine infusions (Perry *et al.* 2005). Perry *et al.* (2005) compared male and female rats on a delay discounting, impulsive choice task for food or cocaine self-administration. There were no sex or phenotype differences in the acquisition of cocaine self-administration, but in a relapse model HiI females had greater levels of cocaine reinstatement responding compared to males and to both male and female LoI animals. Generally, there is greater impulsive choice in female (vs. male) animals, and HiI vs. LoI females exhibit more vulnerability for cocaine-seeking behavior than their male counterparts (Carroll *et al.* 2010). Thus, differences in impulsivity interact with the measure used and with sex differences in predicting drug abuse, and in this respect, animal studies have many parallels to human impulsive behavior (Weafer & de Wit 2014).

4.2 High vs. low sweet-preferring rats (HiS vs. LoS)

Similar interactions of individual differences and measures of drug seeking were found in studies of sex differences in rats bred for high vs. low sweet consumption (HiS vs. LoS) (see Carroll *et al.* 2013a). HiS and LoS females exceeded HiS and LoS males, respectively, in the acquisition phase of cocaine self-administration, and in the maintenance phases when short access to drug was available (Carroll *et al.* 2002; Dess *et al.* 2005). HiS females also exceeded males on ethanol (Dess *et al.* 2005) and heroin (Carroll *et al.* 2002) intake. Female HiS rats also showed more cocaine-induced locomotor activity and cocaine-induced

locomotor sensitization, than LoS female rats, which is thought to be predictive of stimulant addiction (Carroll *et al.* 2007). Thus, initial comparisons with the HiS LoS phenotypes concur with those found in the HiI vs. LoI impulsive-choice phenotypes, and together the data suggest that these major vulnerability factors, impulsivity, sex, and sweet consumption are interrelated and additive for predicting addictive behavior for differences in drug-seeking behavior.

4.3 Age (adolescents vs. adults)

Age is another factor that is closely related to impulsive, drug-seeking behavior and sweet consumption. Adolescence is a time of major hormonal changes, and since female hormones markedly affect addictive behavior, this is a time when female and male differences in addictive behavior emerge. In addition to hormonal influences there are organizational/developmental neurobiological differences in addictive behavior that account for differences in drug-seeking behavior between adolescents and adults within the same sex. Furthermore, age differences may add to other individual differences that predict addictive behavior, such as impulsivity (Anker & Carroll 2011; Vaidya *et al.* 2004) and compulsive sweet intake (Desor and Beauchamp 1987; Friemel *et al.* 2010; Vaidya *et al.* 2004), although not much data are available regarding 3-way interactions of these major behavioral vulnerability factors, with age and sex. Differences in the rewarding and aversive aspects of drugs of abuse in adolescents vs. adults in both animals and humans have been previously reviewed (Schramm-Sapyta et al. 2009; Spear 2000a,b: 2009, 2011).

In humans, alcohol is one of the first drugs to be abused by humans; thus, it has been one of the most widely studied drugs in adolescent vs. adult humans (SAMHSA, 2013) and animals (Broadwater et al., 2011; Doremus et al., 2005; Truxell et al. 2007). Like humans adolescent rats that have consumed alcohol are more impulsive, making more risky choices than control rats that did not have adolescent access (Nasrallah et al., 2009, 2011). This finding mirrors results of in humans in which exposure to alcohol interacts with impulsive and risky behavior (Field et al. 2007; Goudriaan et al. 2007; Johnson et al., 2008; Vuchinich & Simpson 1998). In rats sex differences depend upon age. For instance, exposure to alcohol early in life may be a critical period when drug use alters prefrontal brain development leading to increased impulsivity (Crews & Boetigger 2009; Brown and Tapert 2004). In females, adults consume more alcohol than adolescents; however, in males adolescents consume more than adults (Doremus et al. 2005). The higher alcohol intake in male adolescents may be explained by a slightly higher rate of alcohol metabolism in adolescents than adults (Brasser & Spears 2002; Walker & Ehlers 2009) as well as reduced sensitivity to sedative/hypnotic (Silveri and Spear, 1998), motor disrupting (White et al. 2002), and decreased anxiolytic (Varlinskaya & Spear 2002) and anxiogenic (Doremus et al. 2003) effects. There have been few treatment studies comparing sex in adolescent vs. adult rats; however, when rats trained to self-administer cocaine were exposed to a nondrug reward, physical exercise, as a treatment, it was more effective in adolescents than adults (Zlebnik et al. 2012). These findings in rats may offer effective, nonmedical treatment options that would be viable, self-sustaining strategies for human adolescents during the critical phase of adolescence to prevent the progression of addiction. Comparisons of animal and human research on addiction suggest that age (adolescence vs. adulthood) is a significant

vulnerability factor that is influenced by other major factors such as sex, impulsivity and risk taking. The interaction of these factors is particularly important in the case of alcohol consumption, since males consume more alcohol than females during adolescence.

5. Sex and other individual differences in neurobiological changes associated with addiction

The underlying neurobiology of these sex differences during different aspects of addiction has been reviewed by others (Hu & Becker 2008; Perry & Carroll 2008; Becker et al. 2012; Bangasser & Valentino 2014). This section summarizes initial findings on the neurobiology of sex differences and hormonal influences in addiction, and recent findings that involve several neurotransmitter systems. Virtually all of the literature on mechanisms mediating sex differences in addiction has focused on dopamine signaling in the reward pathway following initial psychostimulant drug exposure. Results from these studies suggest that estradiol, by enhancing mesolimbic dopamine signaling, enhances the reinforcing effects of psychostimulant drugs in females (Bobzean *et al.* 2014; Becker 1999; Hu & Becker 2008; Becker *et al.* 2012). Specifically, evidence shows that dopamine release is enhanced in the nucleus accumbens and striatum of females as compared to males in response to psychomotor stimulants and electrical stimulation of the medial forebrain bundle (Walker *et al.* 2000; 2006). In females, psychomotor stimulant-induced dopamine release in the striatum fluctuates of the estrous cycle (Becker & Cha 1989), and in OVX females, but not gonadectomized males, is augmented by estradiol treatment (Cummings *et al.* 2014; for review see Becker *et al.* 2012). While dopamine-estrogen interactions are believed to underlie the enhanced reward sensitivity in females compared to males following initial drug exposure, it is not yet known whether similar mechanisms are involved during later phases in the transition to addiction. Such effects are possible given that estradiol also enhances locomotor sensitization following repeated cocaine administration (Cummings et al. 2014), increases drug intake under extended access (escalation) conditions (Larson *et al.* 2007), and it may be required for the development of addiction in females (Ramôa *et al.* 2013). The organization of neural systems and functions of neurotransmitter systems are involved in the transition from drug use as a positive reward to increased drug use, and eventual negative effects (negative reinforcement). These findings have been recently reviewed by Becker and colleagues (Hu & Becker 2008; Becker et al. 2012), and sex differences in neuroendocrine mechanisms were discussed for different brain regions and neurotransmitter systems. Much more work is needed to explore the role of other signaling pathways as mechanisms for sex differences in drug addiction. For example, glutamatergic signaling has been examined fairly extensively as a mechanism underlying relapse vulnerability in males (Schmidt & Pierce 2010), yet very little is known about its role in females. There is evidence of enhanced glutamatergic input in the nucleus accumbens of females compared to males suggesting that its role in the development of addiction may differ between the sexes (Forlano & Woolley 2010).

Corticotropin-Releasing Factor (CRF) -mediated signaling is another strong possible mechanism underlying sex differences in vulnerability to addiction (see review by Bangasser & Valentino, 2014). Results from studies in males indicates a critical role for this pathway

across each of the phases of the addiction process (Sarnyai *et al.* 2001), and behaviorally, males and females respond differently to the effects of stress in models of addiction (Bobzean *et al.* 2014). There are also sex differences in the effects of drugs of abuse including cocaine, methamphetamine, and alcohol on the activation of the hypothalamo-pituitary-adrenal (HPA) axis (Kuhn & Francis 1997; Logrip *et al.* 2013; Zuloaga *et al.* 2014) as well as sex differences in the effects of CRF on reinstatement of cocaine-seeking (Buffalari *et al.* 2012). Understanding sex differences in CRF-signaling is not only important for drug addiction, but for other stress-related psychiatric disorders such as posttraumatic stress disorder (PTSD), anxiety, and depression that are related to drug addiction. Sex differences exist at every level of analysis, from cellular and molecular mechanisms, to receptor trafficking and dendritic remodeling, and these findings are linked to increased stress in females compared to males. Overall, while the importance of sex differences in these areas of investigation has been documented, the important findings that have emerged underscore the need to consider sex differences in psychiatric disorders at all levels.

Further research is also needed to examine the possibility that drugs of abuse produce different long-term adaptations in the brains of males and females that cause them to respond differently to drugs of abuse. For example, there is increasing evidence from studies in male animals that histones and their covalent modifications play a major role in drug addiction, with particular support for a role for brain-derived neurotrophic factor (BDNF) in these long-term epigenetic changes. It is likely that this system is also involved in mediating the drug use in females given well documented interactions of BDNF and estrogen in the brain (Harte-Hargrove *et al.* 2012), and findings showing that both sex and estradiol regulate BDNF expression as well as its intracellular signaling pathway (e.g. ERK signaling) in several brain regions (Carbone & Handa 2013). Although less is known for addiction, there is also an accumulating body of work documenting sex and hormonal influences on epigenetic mechanisms that modulate risk for other diseases (Hodes 2013).

The neurobiological differences that have been found in females vs. males are likely not unique to sex differences. They may be tied to more general factors that relate to their relative avidity for addictive drugs, such as common reward mechanisms across vulnerable groups (e.g., HiI versus LoI or HiS versus LoS). These groups showed similar patterns of neuronal activity as females vs. males such as cocaine-induced c-Fos expression in several brain reward areas compared with saline-treated controls. These findings in selectively-bred HiS and LoS rats, that had not consumed SACC prior to testing (see Section 3.4), suggest that genetic differences in neuronal reactivity exist prior to drug exposure (Regier *et al.* 2014), and similar findings have emerged with wheel-running vs. sedentary rats, suggesting that genetic differences in neuronal reactivity (Zlebnik et al. 2014a). HiS and LoS rats also have higher orexin-positive cell counts compared to LoS rats in brain areas associated with feeding, providing further confirmation of genetically-mediated differences in compulsive, reward-driven behaviors. Overall, these initial neuromechanism studies highlight the importance of considering sex differences in addiction as one of several key individual differences that can predict various forms of addictive behavior and response to treatment. Treatment for drug abuse involves not only sex and other genetic predispositions, but there are epigenetic factors associated with environmental factors such as enriched vs. impoverished environments that have neurobiological bases as well, and they are interactive

with sex differences. Developments in this area will likely lead to more customized treatments for addiction.

6. Sex and hormonal differences in treatment for addiction

There are currently no treatments for any form of addiction, other than agonist substitutions (e.g., methadone, buprenorphine) for opioid abuse, that effectively reduce the morbidity and mortality associated with drug abuse. Epidemiological data indicate that most forms of addictive behavior are holding steady or increasing over the decades, new forms are emerging (see section 3.3) and novel treatments are needed. Furthermore, as discussed by Clayton & Collins (2014) in January, 2016 NIH now requires that both male and female animals are tested in NIH-supported research studies. In Canada it has been a requirement that sex differences be addressed for over 5 years (see: http//www.cihrirsc.gc.ca/e/8673.html). Currently, little is currently known about the role of sex and hormonal status as these factors relate to treatment effectiveness for addiction. Animal models for testing treatments for drug abuse are invaluable, as there are many limitations in studying novel treatments for drug abuse across all phases of addiction in humans. Thus, most of the studies of pharmacological and behavioral treatments for addiction have been conducted in rats or monkeys. Sex and hormonal factors have not been considered until relatively recently, but new animal models have recently been developed that will allow us to evaluate novel treatments while controlling for drug abuse history and previous treatments. They also offer a clear prediction of the treatment effect that is not influenced by sociocultural influences that are present in humans. In fact, some of the most effective treatments currently used for human drug abuse were developed in tandem with studies in rats and monkeys (e.g., Higgins *et al.* 2008; Carroll *et al.* 2001).

There are few treatment studies in humans that have had large enough data bases and sufficient power to examine sex and hormonal influences on treatment outcome, and those that have been conducted have mixed results. For example a review of 280 treatment studies published between 1975 and 2005 indicated better treatment outcomes for women than men with substance abuse disorders (Greenfield *et al.* 2007). However, recent analysis of the outcome of the 2001 – 2004 COMBINE (Combined Pharmacotherapy and Behavioral Interventions for Alcohol Dependence) study in 1383 men and women indicated that while sex differences were found in those seeking treatment for alcoholism, and in those reporting alcohol treatment, women and men responded similarly to the study's naltrexone treatment and the combination of naltrexone and medical management (Greenfield *et al.* 2010).

The earlier findings of Greenfield *et al.* (2007) are consistent with treatment studies in animals indicating that female monkeys have a better treatment outcome than males from environmental enrichment (Carroll *et al.* 2005, 2015) or pharmacological intervention (Carroll *et al.* 2000, 2009; Carroll & Smethells 2016; Cosgrove and Carroll 2003; Cosgrove *et al.*, 2004). Also, consistent with these findings, a nondrug reward, saccharin (SACC), when used as a behavioral treatment for cocaine use in monkeys had a better treatment effect in the follicular phase of the menstrual cycle (when estrogen peaks) than during the luteal phase when progesterone peaks and the progesterone/estrogen (P/E) ratio is higher than it is in the follicular phase (Schiller *et al.* 2012; Carroll *et al.* 2015). Similar sex differences

(females>males) in drug self-administration have been found in rats (see reviews by Anker & Carroll 2010; Carroll & Smethells 2015).

In addition to a modest but consistent sex difference effect, studies in women and female nonhuman primates showed that drug abuse treatment effectiveness varied with phase of the menstrual cycle (Carroll & Smethells 2016). In human cigarette smoking treatment studies, results can be optimized by considering menstrual cycle phase and the type of treatment such as behavioral therapy (Franklin et al. 2007; Allen *et al.* 2008; Carpenter et al. 2008; Mazure *et al.* 2011) vs. agonist (nicotine) therapy. Overall, information from treatment studies in animals has led to advances in treatment strategies for humans. These include the following 3 strategies (reviewed briefly below), that have recently been modeled in animals as novel treatments for human drug addiction: 1) repurposing progesterone as a medication to reduce nicotine and cocaine-seeking behavior, 2) environmental enrichment with nondrug rewards such as physical exercise and 3) combined pharmacological therapies (e.g., progesterone + physical exercise).

6.1 Repurposing progesterone as a treatment to reduce drug-seeking behavior

Several animal studies of drug-seeking behavior during all phases of the addition process have shown that progesterone, endogenous or exogenously administered, is related to reductions in drug-seeking behavior. Progesterone has been shown to be a viable treatment for drug addiction in females and to some extent in males (see Anker & Carroll 2010, 2011; Carroll & Anker 2010; Feltenstein & See 2007; Feltenstein et al. 2009). Previous animal and human studies suggest that this might be partially due to its anxiolytic effects (Llaneza & Frye 2009; Schneider & Popik 2007; Sinha *et al.* 2007) indicating its effectiveness in males. In humans, progesterone treatment has typically been used to maintain high-risk pregnancies. However, the addiction research findings in animals suggest the repurposing progesterone as a medication for drug addiction is a promising approach. Initial reports in human studies support the value of considering progesterone as a treatment for addiction in humans. For example, during the mid-luteal phase of their menstrual cycle women reported decreased stress-induced and cue-induced cigarette craving, and decreased cue-induced anxiety and blood pressure measures, and there were fewer urges to smoke tobacco in both female and male smokers during short-term abstinence (Sofuoglu *et al.* 2011). Recently DeVito et al. (2014) examined subjective, cognitive, and physiological responses to nicotine as a function of sex and menstrual cycle and found that females had diminished subjective effects but greater physiological effects than men, and luteal-phase females reported reduced subjective effects but better cognition compared to follicular-phase females. In a recent study it was reported that in women there was an additive effect of rising progesterone levels in the luteal phase of the menstrual cycle and treatment with the nicotine patch compared to treatment in the follicular phase (Saladin et al. 2015). This study indicated that higher endogenous levels of progesterone added to the treatment effect of nicotine replacement.

In other recent studies cocaine-dependent women with high progesterone levels showed reduced cocaine craving and lower physiological responses to stress than those with low progesterone levels (Sinha *et al.* 2007), and women with high levels of progesterone during the mid-luteal phase of the menstrual cycle showed lower physiological responses to stress,

reports of anxiety and cocaine craving compared to cocaine dependent women with low levels of progesterone (Milivojevic *et al.* 2013). Exogenous progesterone administration for 7 days decreased cocaine craving and negative mood in women and attenuated a cortisol response (Fox et al. 2013). This finding was consistent with a laboratory study in which progesterone attenuated subjective effects of smoked cocaine in women when administered during the follicular phase, but had not effect in men (Evans & Foltin, 2006). A recent study by Yonkers *et al.* (2014) indicated that post-partum (when progesterone is very low) cocaine abusers reported reduced cocaine use during a 12 week trial under progesterone (vs. placebo) treatment, although there were no differences in cocaine-positive urine samples. Thus, repurposing medications such as progesterone may be useful for treating cocaine and nicotine addiction.

Animal studies have allowed more in depth analysis of treatment effects such as the influence of hormonal cycles on treatment effects. Results showed similar cocaine intake in male and female monkeys in the luteal phase of their cycle (low P/E ratio) (Carroll *et al.* 2015), but cocaine deliveries were significantly higher in the females' follicular phase (high P/E ratio) than during other parts of the cycle. In monkeys when SACC was substituted for water, it reduced cocaine deliveries only in females in the follicular phase, but when sex differences in body weight were taken into account, by considering cocaine intake as mg/kg intake, SACC reduced cocaine intake in males and in females during both the follicular and luteal phases. These results concur with similar reports in rats self-administering iv cocaine and suggest that progesterone has a therapeutic effect on drug taking (see reviews by Anker & Carroll 2010, 2011; Carroll & Anker 2010).

6.2 Environmental enrichment with nondrug rewards

Previously in our rat (see review Carroll *et al.* 2013) and monkey (Carroll *et al.* 2000, 2005, 2009, 2016) studies environmental enrichment (a palatable noncaloric SACC drink was used as a nondrug reward that reduced drug seeking and intake. This was a useful prototype and an effective model to illustrate the ability of a self-administered, nondrug reward to reduce drug seeking, but more healthy forms of environmental enrichment such as social and intellectual stimulation are desirable (see Bardo *et al.* 2001; Stairs & Bardo 2009). Another consideration of environmental enrichment as an effective means of reducing drug-seeking behavior is that it interacts with the self-administered drug dose. As drug dose increases, the effects of environment enrichment may diminish (Gipson *et al.*, 2011). Accordingly, initial reports in human studies suggest that greater exposure to environmental enrichment (e.g., exercise) increased its effectiveness (Rawson *et al.* 2015).

A more feasible nondrug reward with many health advantages (compared to sweet substances) is physical exercise has been used as a treatment for all phases of addiction in rats (Carroll *et al.* 2002; Zlebnik *et al.* 2010, 2012, 2014b; Zlebnik & Carroll 2015a,b; Sanchez et al. 2015; see reviews by Smith & Lynch 2012; Zhou *et al.* 2014, 2015). However, direct comparisons of the effect of physical exercise on drug self-administration *in males and females* were examined in only one study (Carroll *et al.* 2002), and females showed a greater reduction in cocaine self-administration than males. A recent finding regarding the use of exercise to treat drug addiction (in female rats) indicated that voluntary wheel-running

suppressed incubation of context-induced reinstatement of cocaine-seeking for a month after cocaine self-administration had ended (Zlebnik & Carroll 2015b). Thus, the exercise method of reducing addictive behavior has enduring effects on preventing relapse to drug use. Since exercise is rewarding in humans, it has promise as a self-sustaining, long-term treatment. The clinical application of this approach has been recently reviewed (Bardo & Compton 2015).

Initial reports indicated that exercise produced greater reductions in females than males with cocaine in rats (Cosgrove *et al.* 2002) and alcohol intake in mice (Ehringer *et al.* 2009). In monkeys the effect of an enriched environment was tested on oral cocaine self-administration in male and female rhesus monkeys during the follicular and luteal phases of the menstrual cycle. A preferred substance, SACC, or water was used as a palatable nondrug reward, concurrently available with the oral cocaine solution for self-administration during 3-hr sessions daily (Carroll *et al.* 2016), as previous work had shown that SACC reduced PCP intake more in female than male monkeys (Campbell *et al.* 1998; Carroll *et al.* 2000). These initial results suggest a sex difference in treatment with nondrug rewards, but more work is needed.

6.3 Combination therapy for drug addiction – pharmacological + behavioral treatments

As indicated in the above sections, animal research has recently offered at least two potential novel and self-sustaining treatments (e.g., progesterone and physical exercise) that can serve as models for developing effective treatment strategies for humans. In recent studies when novel pharmacological approaches such as progesterone was combined with access to physical exercise (Zlebnik *et al.* 2014b) or atomoxetine was combined with progesterone in rats (Zlebnik & Carroll 2015a), the combination resulted in a substantial reduction in drug seeking. Combined pharmacotherapies have been used clinically over the years for stimulant use disorders, and they have not been widely effective (see review, Stoops & Rush 2014). However, our recent data suggest that combination treatments involving a rewarding behavioral component (physical exercise) are effective in animals, and a recent study of methamphetamine abusers using physical exercise (Rawson *et al.* 2015) reduced methamphetamine use in participants with more frequent use of exercise. Thus, an additional advantage of this treatment combination is that this approach may be self-sustaining. Overall, nondrug rewards such as exercise, health and wellness activities, and incentives combined with drug-specific medical approaches are currently the most promising forms of treatment for drug addiction.

7. Practical aspects of studying sex differences in addiction in animals

The preceding sections described the behavioral models and initial findings of neurobiological correlates that represent the entire range of addictive behaviors occurring in humans. The importance of studying sex as a biological variable in biomedical research is becoming increasingly apparent. However, as discussed above, the occurrence of sex differences is dependent on many factors, genetic, environmental, phase of addiction, and as such, the research approach to studying sex differences in addiction needs to be carefully selected in order to make meaningful conclusions regarding sex differences or a lack thereof.

Thus, adding females into a design constructed to be sensitive to detecting effects in males may not be the most effective design and may even be a detrimental approach. The biggest danger in such approaches is that they could potentially miss important differences, either by not including enough subjects or by not including conditions sensitive enough to detect differences. Such approaches would also not be an effective approach from a time, subject, or financial standpoint, especially if the end result is to erroneously conclude no difference.

Making sex differences the point of examination, rather than an add-on to the study design, requires careful consideration beginning with power analyses to determine the appropriate number of subjects required to obtain meaningful differences. Such designs may require more than doubling the number of subjects normally needed to study just males to account for variability between males and females. Given the initial findings in humans and nonhuman animals regarding the influence of ovarian hormones in treatment, a treatment study might include different arms and more subjects to control for phase of the hormonal cycle when treatment is initiated and/or inclusion of hormonal manipulations (e.g., progesterone). Thus, the number of subjects may increase if estrous/menstrual cycle effects are included during treatment and sometimes many more subjects will be needed if estrous/menstrual cycle effects are analyzed. Alternatively, it may be possible to use a repeated measures approach to examine estrous/menstrual cycle effects; however, males would need to be tested by similar sham handling procedures at similar time points. Such comparisons should also be done in collaboration with individuals with expertise at studying sex and hormonal influences. Once several published studies exist with adequate power and universally-used procedures, it may be possible to refer to the literature regarding sex differences in the basic behaviors rather than repeating the basic comparisons on every procedure and measure in subsequent studies. The establishment and widespread use of data sharing procedures would greatly facilitate such comparisons, since investigators would then have access to not only the published data where significant effects were observed, but to the negative findings that often go unpublished (Ferguson et al., 2014) This strategy for reducing the number of animals used would work best if procedures were first standardized, data collection methods had the same format across labs, and data summary tables were easily transportable in Excel files.

8. Summary and conclusions

Sex differences have been observed at each phase of the addiction process including initiation (acquisition), maintenance, escalation, withdrawal, relapse or re-initiation of drug seeking, as well as during treatment for addiction. Overall, females show greater vulnerability to drug addiction than males. Such differences are robust and reliable and have been observed in several species including rodents, non-human primates, and humans. Generally, females have less of a reaction to aversive effects of drugs of abuse such as drug withdrawal, which may also explain their greater vulnerability. Animal data suggest that females respond better to behavioral and pharmacological treatments than males, but human data on sex differences in treatment are limited. The occurrence and magnitude of sex differences in drug addiction are dependent on many factors, including the drug self-administered, dose, schedule of its availability, type and duration of the behavioral task, hormonal status, and phase of the addiction process. Sex differences are more likely to be

observed under threshold conditions (low doses), transition phases (initiation, transition from maintenance to escalation, and relapse), and during treatment. They are not as apparent during limited drug access, steady-state maintenance phases of addiction or when or when very high doses are being self-administered. Thus, sex differences may be occluded by floor and ceiling dose effects. Sex interacts with major vulnerability factors in drug addiction, such as environmental enrichment and feeding conditions and genetic/individual differences such as impulsivity, sweet preference, response to novelty, and levels of activity. Overall, the preponderance of sex differences in all aspects of drug addiction, as presented here, highlights the need to use research approaches specifically designed for studying sex differences in addiction in order to make meaningful conclusions regarding the prevention and management of this devastating condition. More research is also needed on male and female animals to understand the neurobiological basis of sex differences in addiction, in order to determine whether neuroadaptions that occur as a result of drug exposure may differ in males and females. Further work involving sex differences in both behavioral and neurobiological areas is crucial, since this information will be used to guide the development of sex-specific and individual customized treatments for addiction.

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HIGHLIGHTS

- How animal models inform us about sex differences in human addiction
- Sex differences in different phases of addiction are sometimes opposite
- Sex and other individual differences produce additive vulnerability to addiction
- Sex differences in neurobiological changes associated with addiction
- Sex and hormonal differences in treatment for addiction
- Practical aspects of studying sex differences in addiction