AGE AND SEX AS VULNERABILITY FACTORS FOR METHAMPHETAMINE USE AND ITS NEUROBEHAVIORAL CONSEQUENCES

BY

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DISSERTATION

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ABSTRACT

Substance users who are female and begin their use during adolescence often suffer greater consequences than male and adult-onset users. Methamphetamine (METH) use may be particularly problematic given that adolescent METH users have a higher risk of relapse than adolescents who abuse other drugs, and female METH users initiate earlier than male users. The neurobehavioral mechanisms of heightened vulnerability in females and adolescent-onset METH users remain poorly understood. Although growing evidence using rodent models suggests that sex and age-of-onset are factors that confer increased susceptibility to substance use problems, there has been limited research investigating the potential interaction of these vulnerability factors. The goal of this dissertation was to further elucidate the behavioral and neural mechanisms through which age and sex may interact to confer vulnerability to developing problems with METH use. Chapter 2 investigates age and sex differences in prefrontal cortex (PFC)-sensitive behaviors that may be antecedents to substance use. Chapter 3 examines reward-taking patterns in male and female rats that began self-administration of METH or the non-drug reinforcer, saccharin, during adolescence or adulthood. The self-administration findings are extended in Chapter 4 using a model of compulsive drug-taking, and the consequences of METH use on recognition memory and receptor expression in the corticolimbic reward pathway are also examined. In Chapter 5, the role of GluN2B-containing NMDA receptor function in extinction of METH-seeking and subsequent relapse vulnerability is assessed. Results indicate that adolescents and females are hypersensitive to rewards in most contexts tested. Furthermore, adolescents may more readily develop compulsive METH-taking and PFC-sensitive cognitive dysfunction, while females may be more prone to relapse after extinction-based treatments. Taken together, the studies in this dissertation add to our current understanding of age and sex as vulnerability factors in METH use and its consequences.

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CHAPTER 1: A GENERAL INTRODUCTION TO AGE AND SEX CONFERRING VULNERABILITY TO PROBLEMS WITH METHAMPHETAMINE

In 2014, 247 million people used illicit drugs in the past year according to a report by the United Nations Office on Drugs and Crime (UNODC, 2016). Strikingly, there were an estimated 207,400 drug-related deaths in 2014 (UNODC, 2016), demonstrating that drug use is a significant public health problem. The most severe health-related issues are experienced by individuals who inject drugs, with stimulant drug users being more likely to engage in risky sexual behaviors that increase their likelihood of contracting infectious diseases such as HIV and hepatitis C (UNODC, 2016). Especially concerning is that amphetamine-type stimulants are one of the leading abused illicit drugs worldwide (UNODC, 2016), with North America reporting the highest law enforcement seizures of METH—the largest contributor to global amphetamine seizures (UNODC, 2016).

High levels of METH trafficking and seizures likely reflect greater use of METH. Repeated drug use can lead an individual to develop a substance use disorder (SUD), characterized by its chronic, relapsing nature. The diagnostic criteria for SUD outlined in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V) include, but are not limited to, spending a great deal of time obtaining and using the drug, escalation of drug intake over time, and failure to control or cease drug use. SUD diagnosis is dependent on the severity of the symptoms, which must lead to clinically significant functional impairment such as health consequences or failure to meet major life responsibilities. Current METH users experience functional impairments in cognition (Simon et al., 2000) that worsens during abstinence (Simon, Dacey, Glynn, Rawson, & Ling, 2004). Importantly, the most severe

cognitive dysfunction is displayed by individuals who complete treatment programs for METH use but return to drug use following this abstinence, which suggests that cognitive deficits likely contribute to relapse risk (Gould, 2010; Rogers & Robbins, 2001; Simon et al., 2004).

Most drug use begins during adolescence, and adolescent-onset drug users often experience worse outcomes compared to those who initiate during adulthood (Chen et al., 2009; Poudel and Gautam, 2017). Female users also tend to experience worse outcomes of drug use, including a more rapid transition from initial use to problem drug use (Brecht et al., 2004; Haas & Peters, 2000; Piazza et al., 1989). METH use may be particularly problematic as adolescents who abuse this drug experience worse treatment outcomes than drug abusing adolescents who do not use METH (Rawson et al., 2005). In addition, females tend to initiate METH use earlier than males (Hser et al., 2005; He et al., 2013) and more adolescent females reported METH as their primary drug of choice (Rawson et al., 2005). Age-of-onset and sex appear to be factors that confer vulnerability to worse outcomes of METH use. A better understanding of the neurobehavioral basis of these vulnerability factors and how they may interact is imperative to developing appropriate treatment programs to improve outcomes in these at-risk populations.

SEX AS A VULNERABILITY FACTOR

For most drugs of abuse, drug use is more prevalent in males compared to females (Cotto et al., 2010; UNODC, 2016). Interestingly, this gender gap seems to depend on age, as the gender gap is smaller in younger drug users (Cotto et al., 2010; UNODC, 2016) but they typically have a higher prevalence of substance use than adults (UNODC, 2016). Although substance use is more common in males, females who initiate drug use often exhibit an accelerated progression to problem drug use, a phenomenon termed “telescoping” (Piazza et al.,

1989). This phenomenon is well-documented in relation to alcoholism (Piazza et al, 1989; Randall et al., 1999), and has also been reported for female stimulant users (Haas and Peters, 2000; Brecht et al., 2004; Cotto et al., 2010). In addition, rodent self-administration studies have provided further evidence for female vulnerability to the transition to problem drug use. Specifically, female rats acquire more readily (Roth and Carroll, 2004a), show greater motivation for drug (Roth and Carroll, 2004a; Cox et al., 2013), escalate drug intake more (Reichel et al., 2012; Roth & Carroll, 2004b), continue to respond when the drug is not available (Anker et al., 2011; Cox et al., 2013), and show greater reinstatement of drug-seeking (Holtz et al., 2012; Reichel et al., 2012a; Cox et al., 2013) compared to male rats.

In humans, gender differences in drug use patterns may contribute to gender differences in substance use disorders, as greater drug use has been associated with worse outcomes, including higher relapse rates (Hillhouse et al., 2007b). In a study that examined METH use patterns in the first 10 years after initiation, females constituted the majority of users who reported high METH use trajectories across this period (Brecht et al., 2013). Furthermore, individuals with high METH use patterns initiated this use at younger ages than METH users reporting low, moderate, or decreasing use (Brecht et al., 2013), suggesting that young females may be particularly susceptible to problematic drug use patterns.

Investigations into the biological bases for sex differences in drug abuse vulnerability have mostly focused on the role of ovarian hormones. In healthy women, pretreatment with estradiol increased few of the subjective effects of amphetamine (Justice & de Wit, 2000a). However, most of amphetamine's subjective effects did not differ when examined across the menstrual cycle, despite significant differences in plasma levels of estradiol (Justice & de Wit, 2000b; Munro et al., 2006). Furthermore, neurophysiological effects of amphetamine, such as

amphetamine-evoked striatal dopamine release (Munro et al., 2006), do not seem to be influenced by the menstrual cycle in women. This lack of effect of ovarian hormones in humans is similar to findings in rats using METH self-administration, extinction, and METH-primed reinstatement; no effect of the estrous cycle on any of these behaviors was observed (Reichel et al., 2012a; Cox et al., 2013). However, gonadectomy reduced acquisition, intake, and motivation in cocaine self-administration in female rats, but not male rats, and this effect was rescued with estradiol replacement in females (Jackson et al., 2006; Perry et al., 2013a). These studies suggest that male gonadal hormones do not influence vulnerability to self-administer cocaine, while ovarian hormones may contribute to female vulnerability depending on the psychostimulant drug. Overall, these studies suggest that sex hormones are unlikely to fully explain sex differences in abuse of amphetamines.

Although the mechanisms underlying sex differences in drug abuse are not fully understood, differences in corticolimbic circuitry may contribute to female vulnerability to problem drug use. Female, but not male, cocaine abusers have reduced metabolism in the frontal cortex while viewing videos of cocaine-related cues (Volkow et al., 2011). As the frontal cortex is important for executive functions, such as self-control, female drug users may experience less self-control in the presence of drug-associated cues, which may be one mechanism contributing to compulsive drug-seeking and increased relapse vulnerability. The frontal cortex undergoes considerable development during adolescence, which may make adolescent female drug users especially vulnerable to developing problem drug use.

AGE AS A VULNERABILITY FACTOR

Adolescence, estimated to begin around 12 years old and continue into the mid-twenties in humans (Dahl, 2004), is the transitional period between childhood and adulthood

characterized by considerable maturation in the brain and behavior. The typical behavioral repertoire expressed by adolescents that is reported in human studies includes increased sensation/reward-seeking (Shulman et al., 2015), greater risk-taking (Steinberg et al., 2008; Cauffman et al., 2010), and less top-down inhibitory control (Steinberg et al., 2008; Kalkut et al., 2009; Shulman et al., 2015). Increased sensation/reward-seeking is tied to pubertal development in both humans (Martin et al., 2002) and rodents (Friemel et al., 2010), and adolescent males exhibit a greater peak in sensation-seeking compared to females (Shulman et al., 2015). Higher scores of sensation-seeking are associated with drug use in adolescents (Martin et al., 2002), suggesting that sensation-seeking may lead to experimentation with drugs. Furthermore, age is negatively associated with risk taking (Gardner and Steinberg, 2005; Eshel et al., 2007; Cauffman et al., 2010), and males tend to make more risky choices than females (van Leijenhorst, Westenberg, & Crone, 2008). Increased sensation-seeking and risk-taking in adolescent males may contribute to increased prevalence of drug use in males relative to females (Cotto et al., 2010; UNODC, 2016).

Many of the behavioral changes that occur across adolescent development are conserved across species, allowing for the use of animal models in the study of adolescent neurobehavioral development. The most conservative definition of rodent adolescence is from postnatal (P) day 27-60 (Spear, 2000). However, this period encompasses a very broad range from post-weaning to well past onset of sexual maturity. The onset of puberty is a hallmark of adolescence, associated with not only the surge in gonadal hormones that underlies sexual maturation, but also adolescent neurobehavioral development in both humans and rodents (Juraska and Willing, 2017). The physical markers of pubertal onset in rodents—namely, vaginal opening in females

and preputial separation in males (Korenbrot et al., 1977; Castellano et al., 2011)—occur earlier in females (~P35) than males (~P45), further complicating the definition of adolescence.

Although the typical adolescent behavioral profile discussed above has been supported by many human studies, the sparse rodent literature in age differences in the domains of behavioral flexibility, reward sensitivity, and risk-taking has been more inconclusive. Rodent studies of age differences in behavioral flexibility that use positive (food) or negative (escape from water) reinforcement have reported mixed results (Newman & Mcgaughy, 2011; Simon et al., 2013; Willing et al., 2016), with factors such as pubertal onset potentially contributing to disparate findings (Willing et al., 2016). Studies of age differences in reward processing also highlight the importance of the peripubertal period in males, as there is a peripubertal peak in motivation for (Friemel et al., 2010) and consumption of palatable reward (Friemel et al., 2010; Marshall et al., 2017). In the only study to concurrently investigate age and sex differences in a Pavlovian approach task, our lab found that females and adolescents were less sensitive to reward devaluation (Hammerslag and Gulley, 2014). In adults, sex differences have been reported where females were insensitive to devaluation of a food reinforcer (Quinn et al., 2007). To date, a single rodent study in males reported age differences in risky choice, with adolescents exhibiting less sensitivity to reward probability compared with adults (Zoratto et al., 2013). Sex differences in risky choice have only been examined in adults, with males generally displaying more risky behavior than females (Orsini and Setlow, 2017). Thus, although there appears to be emerging evidence of age and sex differences in reward sensitivity, behavioral flexibility, and risk taking, the current literature is mostly limited to either male or adult subjects, respectively, which precludes analysis of interacting effects of age and sex in these behavioral domains.

Studies in rodents have demonstrated that the some of the behaviors typical in human adolescents were associated with drug use, as greater adolescent (P34-55) risk-taking predicted greater cocaine intake during acquisition of self-administration in adulthood in male rats (Mitchell et al., 2013) and less top-down inhibitory control in adults of both sexes predicted greater acquisition, escalation, and reinstatement of cocaine self-administration (Anker, Perry, Gliddon, & Carroll, 2009; Perry, Nelson, & Carroll, 2008). As opposed to focusing on behaviors that may predispose adolescents to problematic drug-taking, many studies using rodent models of adolescence have focused on age differences in drug-taking patterns. For example, when cocaine self-administration began at pubertal onset (~P42) in male rats, adolescents acquired self-administration more readily than adult and prepubertal rats—an effect that depended on dose (Wong et al., 2013). Duration of access to METH self-administration also appeared to influence age differences in that adolescent (P26) male rats earned more infusions and escalated their METH intake more than adults during long access, but these age differences were absent when given short access (Anker et al., 2012. Adolescents (P26) also failed to inhibit cocaine-seeking when the drug was no longer available compared to adult males (Anker and Carroll, 2010; Anker et al., 2011) and reinstated cocaine-seeking to a greater extent than adults following priming with the drug (Anker and Carroll, 2010) and various stressors (Anker & Carroll, 2010; Wong & Marinelli, 2015), but not a drug-associated cue (Anker and Carroll, 2010). Overall, it seems that both epidemiological data and rodent self-administration studies suggest adolescent-typical behaviors likely contribute to problematic drug use patterns under certain conditions.

Not only do adolescents exhibit behaviors likely to lead to experimenting with drugs, but adolescents also may be particularly susceptible to the detrimental effects of drugs on cognitive function. Drug-induced cognitive deficits are thought to contribute to problem drug use through

impaired processes such as learning/memory and PFC-dependent executive functions that interfere with treatment programs (Rogers and Robbins, 2001). A meta-analysis revealed that chronic METH abusers exhibited profound deficits in these neurocognitive domains compared to healthy controls (Scott et al., 2007). Rodent studies have also reported drug-induced cognitive impairment, and importantly these deficits sometimes depend on age of drug exposure. For example, rats exposed to METH from P41-50, compared to younger and older ages, displayed impaired spatial memory when tested in the Morris water maze 30 days later (Vorhees et al., 2005). In addition, our lab has shown that adolescent AMPH exposure impaired adult behavior in PFC-sensitive tasks, such as working memory (Sherrill et al., 2013), impulse control (Hankosky and Gulley, 2013; Hammerslag et al., 2014), and behavioral flexibility (Hankosky et al., 2013a). In some (P37-55, Sherrill et al., 2013; P27-45, Hammerslag et al., 2014) but not all (P27-45, Hankosky et al., 2013) cases, these cognitive deficits were dependent on age of AMPH exposure with greater impairments in adolescent-exposed compared to adult-exposed groups. Collectively, these studies suggest that adolescent drug exposure leads to long-lasting cognitive dysfunction, which may be due to the detrimental impact of drugs of abuse on neural developmental trajectories (Gulley and Juraska, 2013a).

DOPAMINERGIC AND GLUTAMATERGIC MECHANISMS OF VULNERABILITY

The neural bases of adolescent behavior and susceptibility to drug-induced cognitive dysfunction likely arise from the protracted development of the corticolimbic system. Many theories ultimately propose an imbalance in the maturation of limbic regions involved in reward processing and top-down prefrontal control regions as the underlying mechanism of adolescent behavioral manifestations (Steinberg, 2010; Casey et al., 2011; Shulman et al., 2015). In a

functional MRI study, Galvan and colleagues (2006) reported an adolescent peak in nucleus accumbens activation in response to a large reward, while adolescent orbitofrontal activity more closely resembled that of children rather than adults. A recent longitudinal study extended this previous report by examining pubertal development, which was linearly associated with adolescent nucleus accumbens activity (Braams et al., 2015).

Accompanying these functional changes in the corticolimbic circuitry, there are extensive structural changes across adolescent development. Studies in humans have reported gross anatomical changes, such as an adolescent peak followed by reduction in frontal gray matter volume that continues into the mid-twenties (Giedd et al., 1999), and a linear decrease with age in nucleus accumbens gray matter volume (Wierenga et al., 2014). For both of these regions, males have greater gray matter volumes than females (Giedd et al., 1999; Wierenga et al., 2014), and the adolescent peak in the frontal cortex occurs earlier in females than males (Giedd et al., 1999). In rodents, similar sex-dependent developmental events have been reported and also seem to be tied to pubertal onset. Female rats lose neurons in the medial PFC after P35 (Markham et al., 2007; Willing and Juraska, 2015), and synapse number in the mPFC of both sexes follows an inverted U-shaped curve peaking near pubertal onset (P35 for females and P45 for males; Drzewiecki et al., 2016). Furthermore, when rats were categorized by whether or not they reached puberty on P35 for females and P45 for males, prepubertal rats had significantly more synapses than postpubertal rats (Drzewiecki et al., 2016). In addition to these neuroanatomical changes, the corticolimbic system undergoes extensive changes in dopaminergic and glutamatergic neurotransmission, and disruption of these developmental trajectories by drugs of abuse may contribute to vulnerability to problem drug use.

During adolescence, there is considerable development of dopaminergic transmission in the mesocorticolimbic circuit. The ventral tegmental area sends dopaminergic projections to the PFC and these fibers increase linearly from adolescence into adulthood (Kalsbeek et al., 1988; Willing et al., 2017). In addition to changes in dopaminergic innervation, D_1R and D_2Rs exhibit an adolescent peak in expression in the PFC around P40 (Andersen et al., 2000). Studies using retrograde tracers have revealed that the D_1R and D_2R peaks are cell-type specific and occur on glutamatergic PFC neurons that project to the nucleus accumbens around P44 (Brenhouse et al., 2008; Brenhouse et al., 2013). Although D_1R and D_2Rs display similar developmental expression profiles, D_1Rs but not D_2Rs interact with the glutamatergic NMDA receptors in the PFC (Gurden et al., 2000), a function that comes online following pubertal onset in male rats (Tseng and O'Donnell, 2005).

During the same time as this peak in dopamine receptor expression, changes occur in the glutamatergic system in the PFC. NMDARs are tetrameric receptors that contain 2 obligatory GluN1 subunit and 2 other subunits—the most common in the forebrain being GluN2A and GluN2B—which confer different properties to the receptor (Cull-Candy et al., 2001; Yashiro and Philpot, 2008). The presence of GluN2B in NMDARs slows the channel kinetics allowing for longer duration excitatory postsynaptic currents (EPSCs), compared to GluN2A-containing NMDARs. In many brain regions examined, such as the visual cortex and hippocampus, an early postnatal developmental switch in NMDAR subunits was reported where GluN2A increased while GluN2B decreased into adulthood (Williams et al., 1993; Monyer et al., 1994). However, few studies investigating NMDAR subunits in the PFC across adolescence have been reported. One analysis with only a single timepoint during adolescence (1 month old; ~P30) and adulthood (3.5 months; ~P105) reported no age differences in mPFC GluN2B/GluN2A ratio in

male rats (Wang et al., 2008). Another study in male rats found that adults (samples taken at P75, 76 and 88 were collapsed) had greater GluN2B but similar GluN1 protein expression compared to adolescents (P37, P38, and P52 collapsed) in the frontal cortex (Pian et al., 2010), which may suggest that the subunit composition of NMDARs shifted towards GluN2B-containing receptors in adulthood.

Although it remains unclear whether there are changes in PFC expression of GluN2B around pubertal onset, longer duration $EPSC_{+60mV}$ emerged in mPFC slices from male rats around P45, and once present, this transmission was blocked by the GluN2B antagonist, ifenprodil, and D_1R antagonist, SCH23390 (Flores-Barrera et al., 2014). Moreover, in slices pretreated with ifenprodil, D_1R blockade failed to further reduce $EPSC_{+60mV}$ duration. The findings in this study suggest that D_1R signaling is necessary, but not sufficient, for the longer $EPSC_{+60mV}$ duration from GluN2B-containing NMDAR to appear (Flores-Barrera et al., 2014). Thus, it is likely that the ontogenetic changes in D_1Rs during adolescence provide a critical window for the functional emergence of GluN2B transmission in the mPFC, and the development of post-pubertal D_1R and NMDAR interactions (Tseng and O'Donnell, 2005). In line with this hypothesis, our lab has shown that adolescent AMPH exposure in male rats led to reduced mPFC D_1R protein expression (Kang,Wu, et al., 2016) and function (Kang et al., 2016a), and a recent study reported a reduction in GluN2B phosphorylation at serine 1303 (Ser1303) in the infralimbic (il)PFC following adolescent cocaine exposure in male rats (Caffino et al., 2018).

D_1R interaction with NMDARs emerged in post-pubertal male rats (Tseng and O'Donnell, 2005), and D_1R activation has been shown to increase Ser1303 phosphorylation of GluN2B in adult male rats (Sarantis et al., 2009), which potentiates NMDAR currents (Liao et

al., 2001). The emergence of this interaction and, ultimately, enhanced GluN2B transmission may be critical for adult-like PFC-sensitive cognitive function. In adult male non-human primates, PFC function is important for working memory and was abolished by intra-PFC administration of the GluN2B antagonist, Ro 25-6981 (Wang et al., 2013). In addition, intra-PFC infusions of Ro 25-6981 impaired trace fear conditioning in adult male rats (Gilmartin et al., 2013). Both of these PFC-sensitive tasks were impaired with GluN2B blockade, supporting the notion that GluN2B signaling is important for cognitive function in adulthood. The development of this adult-like cognitive function may be disrupted by drugs of abuse altering the ontogeny of D_1R and GluN2B function, which may contribute to heightened relapse vulnerability. In adult males, both D_1R and NMDA-GluN2B blockade have separately been shown to increase drug-seeking behavior in rat models of relapse (Fricks-Gleason et al., 2012; Otis et al., 2014), while activation of D_1R reduces drug-seeking in mice (Abraham et al., 2016). Taken together, these studies suggest that drug use during adolescence may impact the developmental trajectory of D_1R and GluN2B in the PFC leading to cognitive deficits and enhanced vulnerability to relapse.

SUMMARY

Although the drug abuse field has made great strides in understanding vulnerability in at-risk populations, the potential interaction of age and sex as vulnerability factors is largely unknown. Research investigating the combination of these factors is imperative as they do not occur in isolation. In this dissertation, I address this gap in our knowledge by examining age- and sex-dependent differences in behavioral vulnerability to drug use (Chapter 2; Westbrook et al., 2018), the patterns of drug use (Chapters 3-5; Hankosky et al., 2018; Westbrook et al., *in* press; Westbrook & Gulley, *under review*), and the cognitive consequences of drug use (Chapter

4; Westbrook et al., *in press*). I also investigated D_1Rs and GluN2B-containing NMDARs as potential neural mechanisms underlying heightened susceptibility to problem METH use (Chapters 4-5; Westbrook et al., *in press*; Westbrook & Gulley, *under review*). The knowledge gained from the collection of studies in this dissertation will further advance this field and may ultimately provide potential novel therapeutic targets to improve treatment outcomes in at-risk populations.

CHAPTER 2: AGE AND SEX DIFFERENCES IN BEHAVIORAL FLEXIBILITY, SENSITIVITY TO REWARD VALUE, AND RISKY DECISION-MAKING[1]

ABSTRACT

Compared to adults, adolescent behavior is often characterized by reduced cognitive flexibility, increased sensitivity to reward, and increased likelihood to take risks. These traits, which have been hypothesized to confer heightened vulnerability to psychopathologies such as substance use disorders (SUDs), have been the focus of studies in laboratory animal models that seek to understand their neural underpinnings. However, rodent studies to date have typically used only males and have adopted standard methodological practices (e.g., weight loss inducing food restriction) that are likely to have a disparate impact on adolescents compared to adults. Here, we used adolescent and adult Sprague-Dawley rats of both sexes to study instrumental behavior tasks that assess behavioral flexibility (strategy shifting and reversal learning), sensitivity to reward value (outcome devaluation), and risky decision making (probability discounting). We found that adolescents were faster to acquire reversal learning than adults but there were no differences in strategy shifting. Adolescents and adults were equally sensitive to changes in reward value and exhibited similar reductions in preference for a large reward when reinforcement probability was decreased. However, adolescents responded more efficiently and earned reinforcers at a higher rate than their same-sex, adult counterparts. Together, these findings provide only limited support for the existence of an "adolescent-typical" phenotype in Sprague-Dawley rats and instead suggest that age differences in the expression of these behaviors may depend on conditions such as pubertal status and motivational state.

[1] Data from this chapter are published in Westbrook, S. R., Hankosky, E. R., Dwyer, M. R., & Gulley, J. M. (2018). Age and sex differences in behavioral flexibility, sensitivity to reward value, and risky decision-making. *Behavioral Neuroscience, 132*(2), 75-87. https://doi.org/10.1037/bne0000235

RATIONALE

Leading theories of adolescent neurobehavioral development (Steinberg, 2010; Casey et al., 2011; Shulman et al., 2015) propose that the limbic reward system matures earlier than the prefrontal control system. This developmental imbalance is most pronounced during adolescence which is hypothesized to underlie to adolescent-typical behaviors, such as reward hypersensitivity, reduced cognitive control, and heightened risk-taking. Consistent with these theories, sensation/reward-seeking peaks in adolescence (Shulman et al., 2015) while behavioral flexibility increases from late childhood to young adulthood (Kalkut et al., 2009). Males tend to have higher levels of sensation-seeking (Shulman et al., 2015) and risky choices (Van Leijenhorst et al., 2008), but perform worse than females in tests of behavioral flexibility (Kalkut et al., 2009). The few studies using animal models of adolescence to study this behavioral profile have reported mixed results in behavioral flexibility (Newman & Mcgaughy, 2011; Simon et al., 2013; Willing et al., 2016) and reward sensitivity (Friemel et al., 2010; Hammerslag and Gulley, 2014; Marshall et al., 2017), and were mostly limited to male subjects, including the sole study to date of age differences in risky decision-making (Zoratto et al., 2013). Although there is some evidence for age and sex differences in these behavioral domains, few reports have investigated the potential interaction of age and sex in the same study.

In the present study we concurrently examined age and sex differences in behavioral flexibility, sensitivity to reward value, and risky decision-making with strategy shifting, outcome devaluation, and probability discounting tasks, respectively, under limited food restriction. In line with evidence from human studies and leading theories of adolescent behavior (Steinberg, 2010; Casey et al., 2011; Shulman et al., 2015) suggesting adolescent hypersensitivity to reward and poor cognitive control, we hypothesized that adolescents would display less behavioral

flexibility, greater sensitivity to reward value, and enhanced risky choices compared to their adult counterparts. We also hypothesized that these effects would be more pronounced in males, consistent with previous studies using nondrug reinforcers in rodent models (Quinn et al., 2007; Hammerslag and Gulley, 2014; Orsini and Setlow, 2017).

METHODS

Total subjects were 147 Sprague-Dawley rats (69 male, 78 female) bred in-house and divided across three tasks—strategy shifting, outcome devaluation, and probability discounting—with adolescent ages selected so that the start of testing for each task would begin around average male pubertal onset in our colony (Fig. 2.1). Within an experiment, rats were randomly assigned to adolescent or adult groups such that litters were approximately evenly distributed across groups. Rats were housed 2-3 per cage following weaning on P22. A subset of the rats (38 male, 43 female) were checked daily for physical markers to estimate pubertal onset—preputial separation for males and vaginal opening for females (Korenbrot et al., 1977; Castellano et al., 2011). Average pubertal onset in this subset was 43.6 ± 0.2 days for males (range: 41 – 47 days) and 34.9 ± 0.2 days for females (range: 32 – 39 days). During experimental testing, rats were given limited access to food (~20 hr/day), achieved by removing food approximately 2 hours prior to experimental sessions and returning food 0.5 – 2 h after the sessions ended.

In the strategy shifting task, rats were first trained to use a visual strategy using two levers, where rats were reinforced with a food pellet for a response on the lever below an illuminated cue light. Location of this light was pseudorandomly presented, and rats were required to respond within 10-sec of cue presentation. Each session consisted of up to 120 trials

separated by a 20-s intertrial interval (ITI). Trials continued until rats achieved eight consecutive correct responses and completed a minimum of 30 trials. On the day after achievement of this criterion or a maximum of five sessions, rats underwent sessions where they could complete a within-session shift from the visual strategy to an egocentric response strategy (e.g., left lever is reinforced, regardless of cue location). The shift in reinforcement would occur following eight consecutive correct choices using the visual strategy. Trials continued until rats achieved eight consecutive correct choices or a maximum of 120 trials, and rats were given up to three sessions to complete the within-session shift. Next, rats received two response strategy sessions with 75 trials each day. Following these sessions, rats underwent up to three sessions to complete a within-session response strategy reversal. First, rats had to achieve eight consecutive correct responses with the original response strategy (e.g., left lever), before the reinforced response was reversed (e.g. right lever). Trials continued until eight consecutive correct responses occurred on the response reversal or a maximum to 120 trials.

In the outcome devaluation task, rats were trained to respond on a single lever to receive either a food pellet or a dipper cup of sweetened condensed milk (SCM) counterbalanced across rats. Reinforcement occurred on a random interval 30-s (RI-30) schedule, and sessions ended after rats earned 50 reinforcers, 20 min elapsed without a lever press, or 90 total min elapsed. Rats received three RI-30 sessions prior to the first extinction test set, and an additional three RI-30 sessions before the second extinction test set. Each extinction test set consisted of two extinction test days separated by one RI-30 re-training session. During an extinction test day, rats were given access to the reinforcer (devalued condition) or an alternate reward (valued condition) for a 60 min pre-feeding phase prior to a 10 min test session in the operant chambers under extinction conditions where lever presses had no programmed consequences. After the

test, rats were given simultaneous access to the reinforcer and alternate reward for a 15 min post-test consumption phase. The order of value conditions was counterbalanced within an extinction test set across rats.

In the probability discounting task, rats were trained to nosepoke into two ports counterbalanced across rats. A response in either illuminated port resulted in reinforcers of different magnitudes and probabilities. Responses into the small, certain port resulted in delivery of one food pellet at 100% probability, while responses into the large, potentially risky port led to the delivery of three food pellets with descending probabilities across sessions—100%, 66.7%, 33.3%, and 16.7%. Each test session consisted of 6 or 12 forced choice trials where one port was illuminated randomly on each trial with an equal number of presentations of each port. Following the forced choice trials, rats received 12 free choice trials in which both ports were illuminated.

Data Analysis

For strategy shifting, trials to criterion for each strategy (visual, response, and reversal) were analyzed using two-way ANOVAs with age and sex as between-subjects factors. Dependent measures for outcome devaluation included lever press rate and reinforcement rate during RI-30 sessions, lever presses during extinction tests, and reinforcer (g/kg) consumption during feeding phases before and after extinction tests. Reinforcement rate was calculated as grams of reinforcer earned per min adjusted for bodyweight (g/kg/min) and analyzed using three-way mixed factorial ANOVA with age and sex as between-subjects factors and session (1-8) as the within-subjects factor. Four-way mixed ANOVA was conducted on lever presses during extinction tests with age and sex as between-subjects factors, while test set (first or second) and

value condition (valued or devalued) were within-subjects factors. Reinforcer consumption during pre-feeding was examined using four-way (age x sex x test set x reinforcer) mixed factorial ANOVA. Post-test reinforcer consumption was analyzed separately for pellets and SCM using four-way (age x sex x test set x value condition) mixed ANOVA. For probability discounting, risk-based decision making was assessed using the percent choice of the large port during free choice trials on the final day at each probability. Reinforcement rate (g/kg/min) was also examined during these sessions. Each dependent measure was analyzed using three-way mixed ANOVA with age, sex, and probability (within-subjects) as factors. When appropriate, Tukey post hoc tests were conducted for all analyses.

RESULTS AND DISCUSSION

In the strategy shifting task, we found no significant group differences in trials to criterion for the visual strategy or the subsequent shift to the response strategy (Fig. 2.2A, B), but adolescents learned the response reversal in fewer trials than adults (Fig. 2.2C). We found no evidence for sex differences in any phase of the strategy shifting task. The lack of sex differences and apparent adolescent enhancement in behavioral flexibility were not consistent with our hypotheses that adolescents and males would show reduced behavioral flexibility. The direction of shift from the visual strategy to the response strategy has been shown to be medial PFC-dependent due to the cost of switching from an easier to a more difficult strategy (Floresco et al., 2008). Since the visual strategy in the present study seemed to be rather difficult for our rats to acquire, the strategy shift to response in our task may not have been sensitive to medial PFC function, and this may explain the unexpected similar performance between adolescents and adults.

Our finding that adolescents displayed enhanced reversal learning compared to adults is consistent with some previous findings (Simon et al., 2013), but not others (Newman and Mcgaughy, 2011; Willing et al., 2016). Differences in study methodology, such as rat strain and stimulus complexity, may explain these discrepancies. Reports of enhanced flexibility in adolescents compared to adults have employed Sprague-Dawley rats (present study; Simon et al., 2013), while similar or worse flexibility in adolescents compared to adults have been reported using Long-Evans rats (Newman and Mcgaughy, 2011; Willing et al., 2016). Furthermore, different tasks with varying complexity of stimuli were used in each of these studies, including intradimensional/extradimensional attentional set-shifting (Newman and Mcgaughy, 2011), Morris water maze (Willing et al., 2016), Pavlovian approach to a previously inhibitory cue (Simon et al., 2013), and operant strategy shifting (present study). Future studies are required to pinpoint the precise role that these various methodological factors play in laboratory rodent assessments of age and sex differences in behavioral flexibility.

In the outcome devaluation task, adolescents had a lower response rate compared to adults during the RI-30 sessions leading up to the first test set (Fig. 2.3A). However, an age difference in reinforcement rate was evident with adolescent displaying a higher rate of reinforcement compared to adults throughout the RI-30 sessions (Fig. 2.3B). These results may suggest that adolescent rats are more behaviorally efficient under the RI-30 schedule of reinforcement as they respond less frequently during this schedule but still earned reinforcers at a higher rate than their adult counterparts. Adolescents' reduced response rate during random interval schedules is consistent with a previous study (Andrzejewski et al., 2011), although those authors did not report analysis of reinforcement rate normalized to body weight. Together, our

results highlight the importance of analyzing age differences in reinforcement in a manner that takes age differences in body size into account.

During the pre-feeding phase, there was no sex difference in adolescent consumption, but adult males consumed significantly fewer pellets and SCM compared to adult females (Fig. 2.4A). This sex difference is consistent with prior studies (Hammerslag and Gulley, 2014; Marshall et al., 2017). Despite age differences in response and reinforcement rate during RI-30 sessions leading up to the first test set, we found no group differences in sensitivity to reward devaluation during either test set (Fig. 2.4B, C). We did not find evidence for adolescent hypersensitivity to reward value when the reward value was intact, as adolescents did not respond significantly more than adults during the valued condition. Adolescents and adults were equally sensitive to a change in relative reward value, consistent with a previous study in males (Naneix et al., 2012). Adolescent and adults were also equally sensitive to reward value manipulation when consumption was measured after the extinction tests (Fig. 2.4D, E), with the exception of adolescents consuming more SCM than adults when it was devalued. As reward value sensitivity is only one aspect that may contribute to reward sensitivity, future research is needed to investigate age and sex differences in other aspects of reward sensitivity, such as Pavlovian-to-instrumental transfer and progressive ratio responding to measure motivation.

In the probability discounting task, adolescents and adults similarly reduced their large reinforcement choices as the reinforcement probabilities decreased (Fig. 2.5A), suggesting that rats of both ages and sexes were sensitive to the change in reinforcement probability. However, all rats continued to choose the large, risky option more than 50% of the time even at the lowest reinforcement probability tested (16.7%), despite the fact that the more advantageous choice was the small, certain option at this low probability of large reinforcement. In contrast to a previous

study in males (Zoratto et al., 2013), we did not find that adolescents made a greater percentage of risky choices compared to adults; however, the choices of adolescents and females during the free choice trials did lead to higher reinforcement rates per body weight in these groups than their adult and male counterparts (Fig. 2.5B). Our study employed minimal food restriction compared to the method used by Zoratto and colleagues (2013), which may contribute to discrepancies in the percent of large choices, as the probability discounting curve is less steep in adult male rats that are free-fed compared to typical ~85% free-feeding weight food-restricted rats (St Onge and Floresco, 2009). Future studies of risk taking behavior in rodent models of adolescence should examine the role of food restriction and other methodological factors as potential determinants of age differences in risky behavior.

In conclusion, the present study found that adolescent rats earned higher reinforcement rates than adults under minimal food restriction. Although this result is consistent with our *a priori* predictions that adolescents would be hypersensitive to reward, our remaining findings were contrary to expectations. Namely, adolescents exhibited greater behavioral flexibility and had similar risk preference and sensitivity to reward value compared to adults. Thus, most of our findings were not in line with theories of adolescent behavior (Steinberg, 2010; Casey et al., 2011; Shulman et al., 2015) that have been largely supported by studies in humans. Importantly, the present study suggests that adolescent behaviors that may confer vulnerability to psychopathology are not evident under all circumstances in rodent models. Future work is needed to investigate the methodological, and ultimately neural, determinants of adolescent-typical behaviors in rodent models.

FIGURES

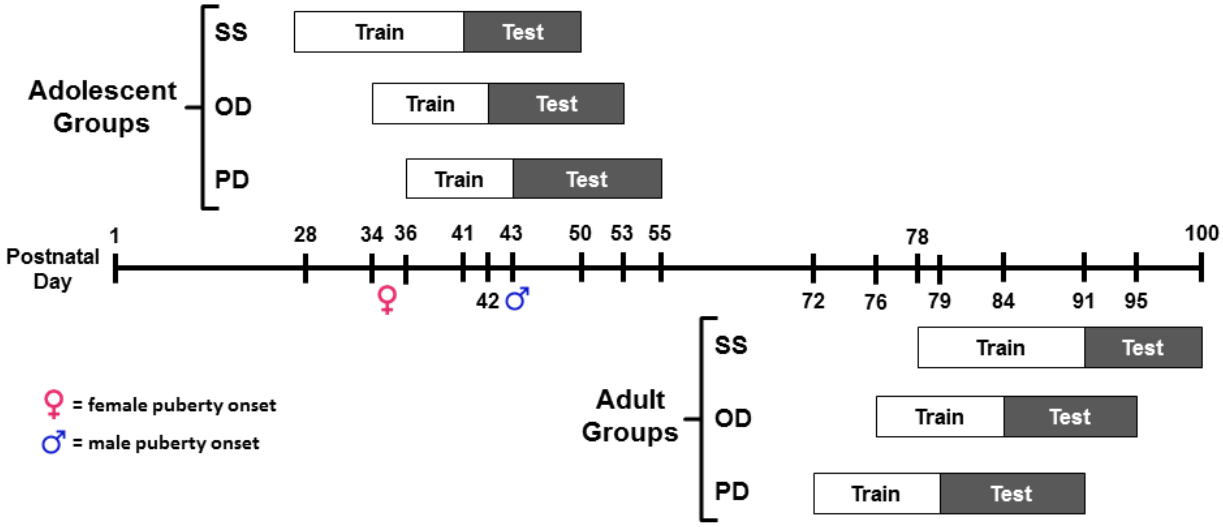

FIGURE 2.1. Experimental timeline illustrating ages during training and testing in the three tasks—strategy shifting (SS), outcome devaluation (OD), and probability discounting (PD). Male and female symbols indicate mean age of pubertal onset in the subset of rats that received puberty checks.

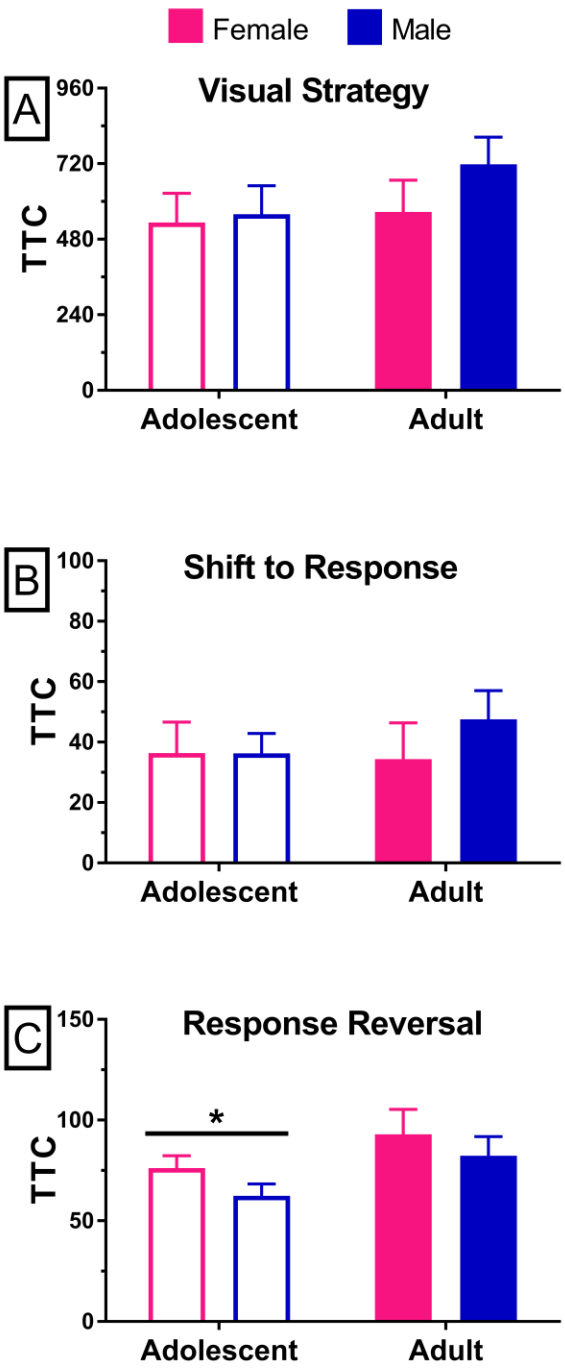

FIGURE 2.2. Trials to criterion (TTC) during acquisition of the visual strategy (A), subsequent response strategy (B), and response reversal (C) in the strategy shifting task (n = 11-12/group). Acquisition criterion was 8 consecutive correct responses using the appropriate strategy for each phase. Some rats failed to reach this criterion during the visual strategy and were assigned the total number of trials they received—960 trials. * $p < .05$ vs. adults

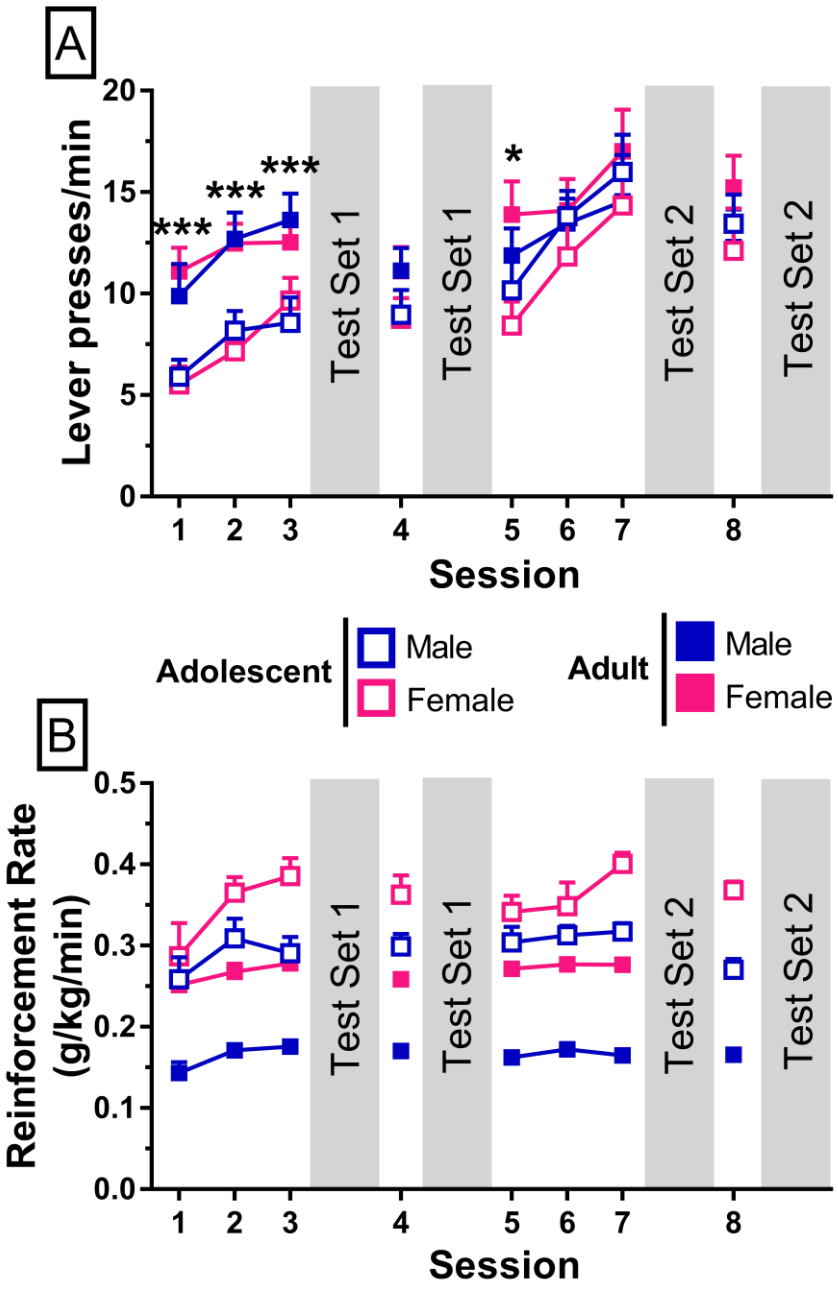

FIGURE 2.3. Lever press rate (A) and reinforcement rate adjusted for body weight (B) during RI-30 sessions in the instrumental outcome devaluation task (n = 11-14/group). Breaks in the graph correspond to extinction test days. Significant main effects of both age ($p < .0001$) and sex ($p < .0001$) reflect that reinforcement rates were greater in adolescents and females, respectively (significance not shown). * $p < .05$, *** $p < .001$ vs. adolescents collapsed across sex.

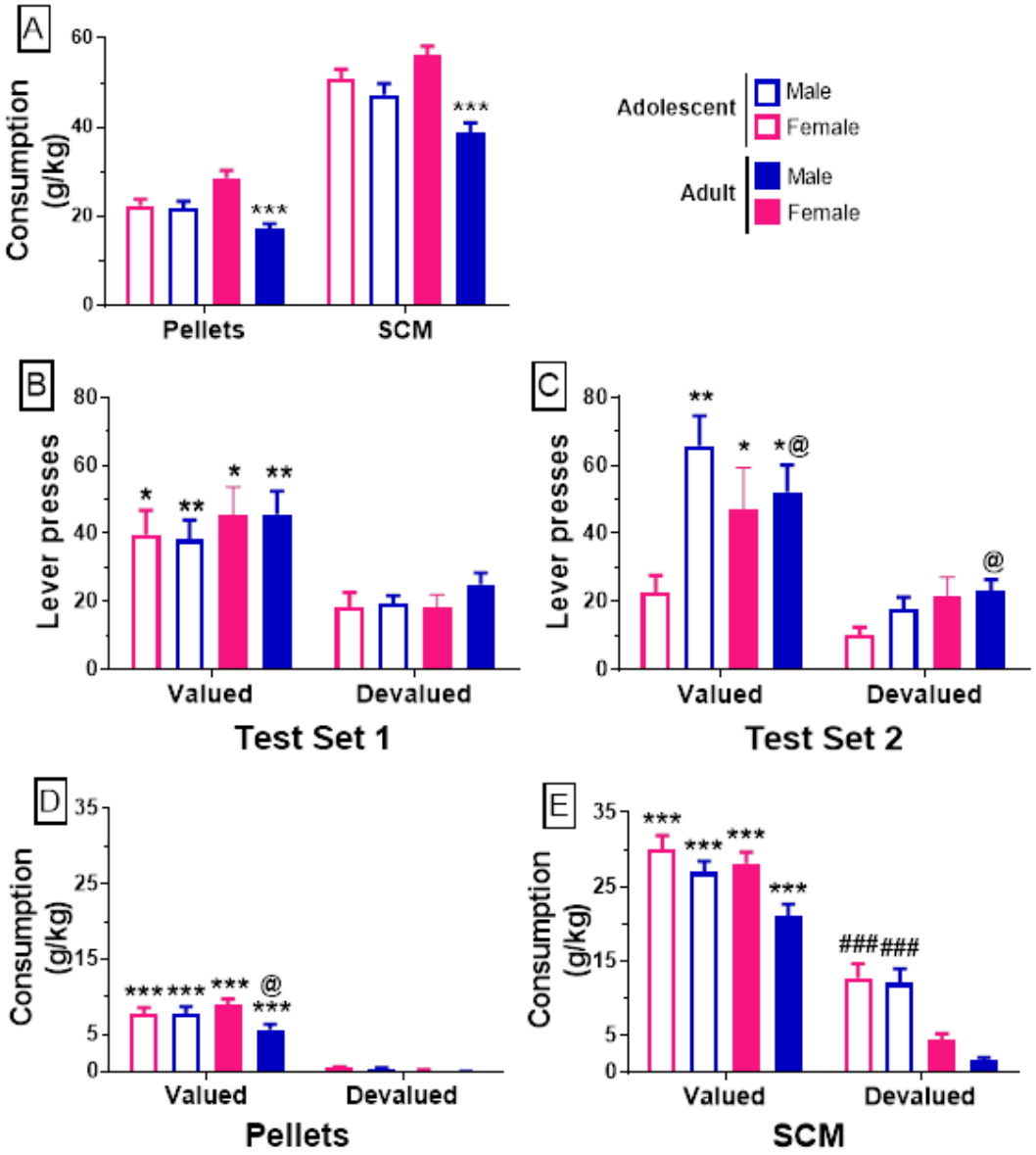

FIGURE 2.4. Consumption during 60-min pre-feeding phase of pellet and sweetened condensed milk (SCM) adjusted for body weight and averaged across the two test sets in the outcome devaluation task (A). ***$p < .0001$ vs. adult females within reinforcer type. Lever presses during the subsequent 10-min extinction tests by reinforcer value condition (B, C). Extinction test days were separated by one RI-30 session. *$p < .05$, **$p < .01$ vs. devalued within group in paired *t* test; $^{@}p < .05$ vs. adolescent males collapsed across value condition. Consumption of pellets (D) and SCM (E) by value condition during the 15-min post extinction test consumption phase. During the pre-feeding phase, rats were given the reinforcer they expected in the test session (devalued condition, which was pellets for D and SCM for E) or the alternate reinforcer (valued condition, which was SCM for D and pellets for E). ***$p < .0001$ vs. devalued within group; $^{@}p < .05$ vs. adult females with pelleted valued; $^{\#\#\#}p < .0001$ vs. adults with SCM devalued.

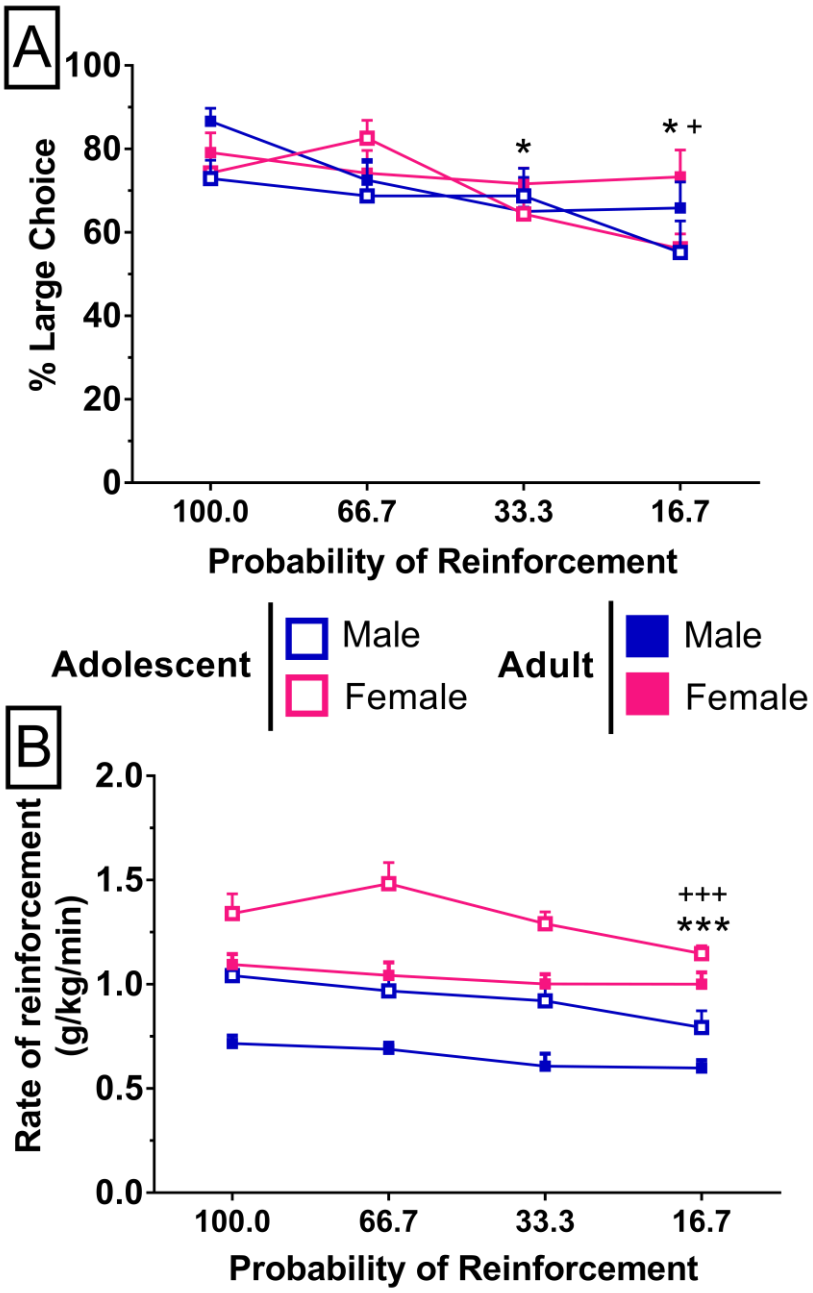

FIGURE 2.5. Responses in the large reinforcement nosepoke port during free-choice trials of the probability discounting task (n = 8-11/group). Data are represented as the percent of large port choices (A) or reinforcement rate adjusted for body weight (B) on the last session of each reinforcement probability. Main effects of age ($p < .0001$) and sex ($p < .0001$) reflect that adolescents and females had higher reinforcement rates, respectively (significance not shown). *$p < 0.05$, ***$p < 0.001$ vs. 100.0% collapsed across age and sex; $^{+}p < 0.05$, $^{+++}p < 0.05$ vs. 66.7% collapsed across age and sex.

CHAPTER 3: EFFECTS OF AGE AND SEX ON ACQUISITION AND MOTIVATION TO SELF-ADMINISTER SACCHARIN OR VARIOUS DOSES OF METHAMPHETAMINE UNDER SHORT ACCESS CONDITIONS[2]

ABSTRACT

Adolescence is a period of considerable development of brain and behavior and is the time during which most drug use is initiated. Age-dependent differences in motivated behaviors may be one of the factors that contribute to heightened vulnerability to developing substance use disorders, so we sought to compare age differences in methamphetamine (METH) and saccharin seeking. Beginning during adolescence or adulthood, male and female Sprague-Dawley rats were trained to self-administer 0.1% saccharin (via liquid dipper cup) or intravenous METH at one of three doses (0.02, 0.05, 0.08 mg/kg/inf) under increasing fixed ratio schedules of reinforcement. Subsequently, responding for METH (0.02, 0.05, 0.08 or 0.1 mg/kg/inf) under progressive ratio response requirements was assessed in rats that acquired METH self-administration at the highest dose (0.08 mg/kg/inf). We found that adult-onset rats acquired METH self-administration more readily and exhibited higher motivation compared to adolescent-onset rats, although there were no differences in METH intake during acquisition. Adult rats also acquired saccharin self-administration more readily, but in contrast to METH, there were age and sex differences in saccharin intake driven by high levels of responding in adult females. These findings challenge the prevailing notion that adolescents are hypersensitive to reward and instead raise questions about the role of methodological factors on which rodent studies often differ.

[2] Data from this chapter are published in Hankosky, E.R., Westbrook, S.R., Haake, R.M., Marinelli, M., Gulley, J.M. (2018). Reduced sensitivity to reinforcement in adolescent compared to adult Sprague-Dawley rats of both sexes. *Psychopharmacology 235*, 861–871. https://doi.org/10.1007/s00213-017-4804-5

RATIONALE

Drug users that initiate during adolescence have a greater likelihood of developing substance use disorders compared to adult-onset drug users (Chen et al., 2009). One mechanism that has been suggested to contribute to this adolescent vulnerability is adolescent hypersensitivity to reward (Casey et al., 2008; Steinberg, 2010; Shulman et al., 2015; Doremus-Fitzwater and Spear, 2016). Studies using both human and rodent subjects have provided some support for this age difference in motivated behavior. Self-reported sensation/reward-seeking peaks during adolescence in humans (Steinberg et al., 2008; Shulman et al., 2015) and this is paralleled by a peri-pubertal peak in non-drug reward consumption and motivation in rats (Friemel et al., 2010), although this age effect depends on sex (Marshall et al., 2017).

Sex differences also exist in drug use, where females exhibit a faster transition to problem drug use (Brecht et al., 2004). Rodent models also demonstrate females' increased propensity to self-administer drugs compared to their male counterparts (J. B. Becker & Koob, 2016; Reichel et al., 2012; Roth & Carroll, 2004a, 2004b). Studies of age differences have reported similar (Anker et al., 2012; Shahbazi et al., 2008), greater (Anker et al., 2012; Shahbazi et al., 2008a; Wong et al., 2013; Wong & Marinelli, 2015), or less self-administration (Doherty and Frantz, 2012; Doherty et al., 2013; Mayer-Blackwell et al., 2014) in adolescents compared to adults. These discrepant findings likely depend on drug class (i.e. adolescents self-administer less opiates but more stimulants than adults), and other methodological factors. The existing literature examining age and sex differences in self-administration are limited to male and adult subjects, respectively, as well as a single reinforcer type (Counotte et al., 2014; Myal et al., 2015).

In the present study, we sought to investigate the potential interaction of age and sex in a rodent model of self-administration of a non-drug reinforcer, or several doses of METH. We used the non-caloric sweetener, saccharin, as the non-drug reinforcer in order to mitigate the effect of potential age differences in caloric need as adolescence is a period of rapid growth (Spear, 2000). Acquisition of self-administration was assessed in adolescent-onset and adult-onset rats of both sexes, and a follow-up experiment examined motivation for various doses of METH. In line with adolescent reward hypersensitivity and previous epidemiological studies, we hypothesized that adolescent-onset and female rats would acquire self-administration more rapidly, earn more reinforcers, and exhibit greater motivation than their adult-onset and male counterparts. Furthermore, we hypothesized that these effects would be greatest in adolescent-onset females.

METHODS

Subjects were 80 male and 103 female Sprague-Dawley rats born in-house and housed 2-3 per cage following weaning on P22. Assignment to age-of-onset (adolescent or adult) and reinforcer (METH or saccharin) were counterbalanced across litters. Surgeries to implant an indwelling jugular catheter were performed on or about (± 2 days) P32 or P82 for adolescent-onset or adult-onset groups, respectively. After 6-8 days of recovery, rats were given a 90 min session in the chambers with the nosepoke ports blocked (Wong et al., 2013). The next day, rats began daily 2-hr short access self-administration sessions, wherein they were trained to nosepoke into the reinforced port (right port) in order to receive one of the following reinforcers: a 0.08 ml dipper cup of 0.1% saccharin solution or one of three unit doses of METH, 0.02, 0.05, or 0.08 mg/kg/inf. A 4-s compound light-tone cue occurred simultaneously with reinforcer delivery,

followed by a 20-s timeout period when the nosepoke ports were illuminated red and responses did not result in reinforcer delivery. Responses in the non-reinforced nosepoke port (left port) had no programmed consequences.

The 15-day acquisition period consisted of seven daily sessions of fixed ratio 1 (FR1) responding, four sessions of FR3, and four sessions of FR5. Following the acquisition period, rats trained to self-administer METH at the highest dose (0.08 mg/kg/inf) were tested for their motivation to respond for METH under progressive ratio (PR) schedule of reinforcement in four sessions at different doses (0.02, 0.05, 0.08, and 0.1 mg/kg/inf). The first PR session was the 0.08 training dose with the order of the remaining doses counterbalanced across rats. PR sessions were interspersed with one daily 2-hr FR5 maintenance session at the training dose. PR sessions terminated after 25 infusions were earned or 5-hr elapsed.

Data Analysis

Dependent measures included number of reinforced responses, number of non-reinforced responses, reinforcers earned, sessions to reach acquisition criterion, and infusions earned during PR sessions. Rats were considered to have acquired METH self-administration if two criteria were met: (1) they infused an average of at least 0.95 mg/kg METH across four consecutive sessions and (2) they maintained this level of consumption through FR5 (sessions 12-15). This second standard was used to insure "acquired" rats continued responding as the ratio requirement increased. For METH, a dose of at least 0.95 mg/kg was achieved when rats earned 48, 19 or 12 infusions at 0.02, 0.05 or 0.08 m/kg/inf, respectively. For saccharin self-administration, the acquisition criterion was set at ≥ 19 reinforcers to equate required responding with the 0.05 mg/kg/inf dose of METH. For analysis of the rate of acquisition, the final session of the first

four-session block was used. Rats that did not meet acquisition criterion were assigned a value of 16 for the sessions to criterion analyses. Sessions to criterion was analyzed separately for saccharin and each dose of METH with a two-way (age x sex) ANOVA. For PR tests, infusions were analyzed using three-way (age x sex x dose) mixed-factor ANOVAs. Significant main effects and interactions were followed up with Tukey's post-hoc tests.

RESULTS AND DISCUSSION

In contrast to our hypothesis, adult-onset rats acquired self-administration of saccharin and the low dose of METH more readily than adolescent-onset rats (Fig. 3.6A, B). Strikingly, none of the adolescent-onset rats met acquisition criterion for the low dose of METH by the end of the acquisition period. All groups trained with the moderate dose of METH acquired at similar rates (Fig. 3.6C), but sex differences were evident in self-administration of the high dose of METH with females acquiring in fewer sessions than males (Fig. 3.6D). In rats that met acquisition criteria, there were no group differences in reinforcer intake, with the exception of adult-onset females earning more saccharin than adult-onset males (Fig. 3.7). Adult-onset rats reached higher breakpoints for all doses of METH tested (Fig. 3.8), suggesting that adult-onset rats are more motivated for METH than adolescent-onset rats. Overall, we did not find evidence for adolescent-onset hypersensitivity to either drug or non-drug reinforcement in the present study.

Consistent with our hypothesis, we found that females acquired high dose METH self-administration more readily and earned more saccharin than males. Females' rapid initiation of METH self-administration at the high dose is consistent with previous reports of enhanced acquisition of psychostimulant self-administration in females compared to males (Perry,

Westenbroek, & Becker, 2013b; Roth & Carroll, 2004a). In contrast, females did not display characteristics that are linked with a transition to addiction, i.e. greater intake of, or motivation for, METH. Thus, our METH self-administration data only partially support the notion that females exhibit the "telescoping" phenomenon (Brecht et al., 2004).

Our METH self-administration findings add to a growing literature that reveals nuanced effects of age and sex on drug seeking. Evidence for heightened self-administration in adolescents and females may depend on several methodological factors, such as duration of access, and dose. Typically, when cocaine or amphetamines are available for short periods of time (≤ 2 h), adolescents (Anker et al., 2012; Harvey, Dembro, Rajagopalan, Mutebi, & Kantak, 2009; Shahbazi, Moffett, Williams, & Frantz, 2008; Wong & Marinelli, 2015) and females (Reichel et al., 2012a) self-administer similar amounts of drug as their counterparts. Consistent with findings from the current study, these age and sex differences are dependent on dose (Kantak, Goodrich, & Uribe, 2007; Schassburger et al., 2016; Shahbazi et al., 2008; Wong et al., 2013). However, under long access conditions (~4-6 h), adolescents (Anker & Carroll, 2010; Shahbazi et al., 2008; Wong et al., 2013) and females (Roth and Carroll, 2004a; Reichel et al., 2012a) have been reported to self-administer psychostimulants more readily than adults and males, respectively. Taken together, the findings suggest that propensity to self-administer drugs may not be conferred in an absolute sense and instead depends on a number of factors (Kosten et al., 2000; Walker et al., 2008; Lewis et al., 2013, 2016; Westenbroek et al., 2013; Baarendse et al., 2014).

FIGURES

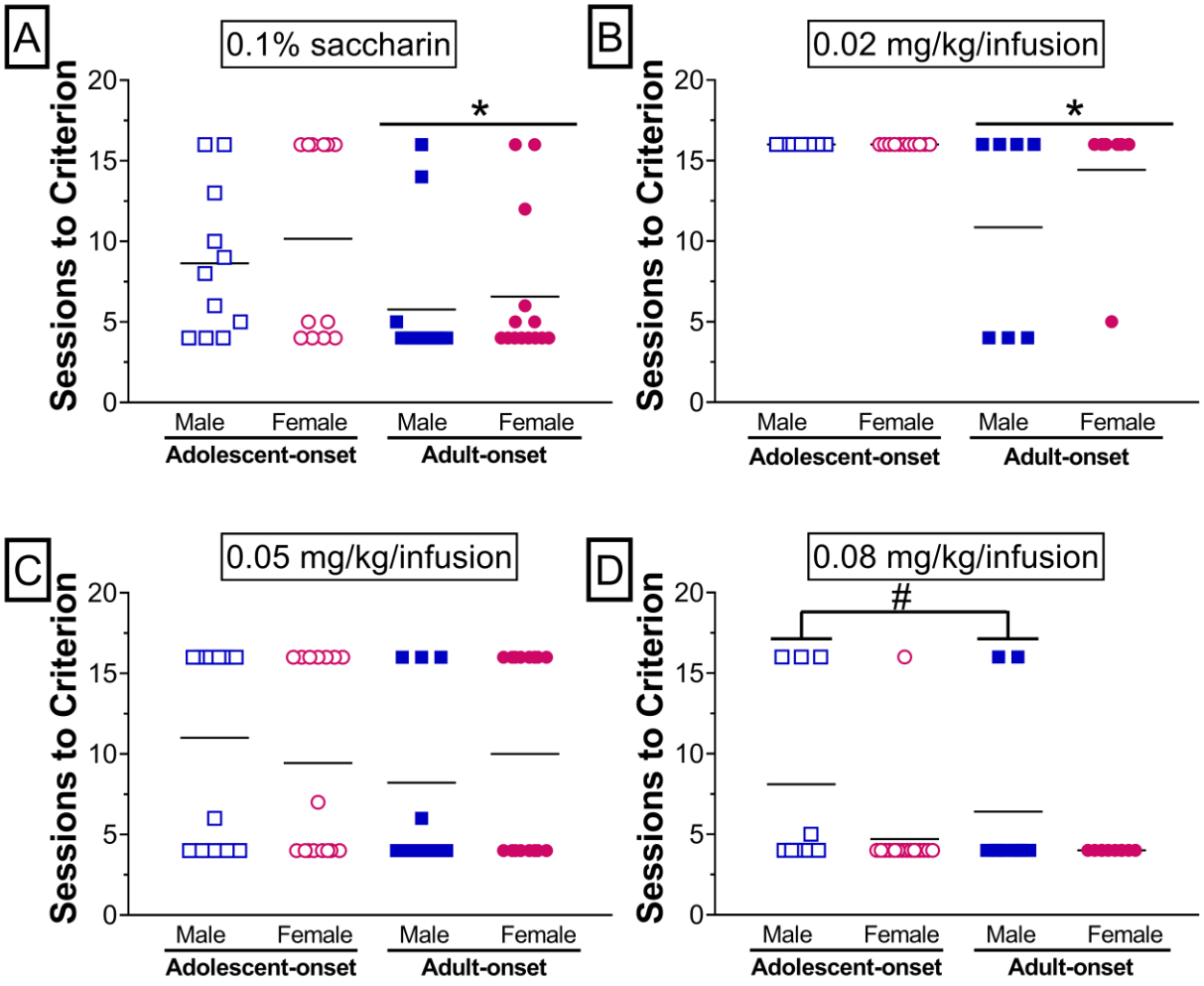

FIGURE 3.1. Sessions to reach acquisition criterion for saccharin and METH self-administration. Individual plots of number of sessions to acquisition criterion for (**A**) 0.1% saccharin (n = 11-14/group), (**B**) 0.02 mg/kg/inf METH (n = 7-11/group), (**C**) 0.05 mg/kg/inf METH (n = 9-16/group), and (**D**) 0.08 mg/kg/inf METH (n = 9-17/group). The horizontal line within each group indicates the group mean. $^{\#}$ $p < 0.05$ males vs. females; * $p < 0.05$ adolescent-onset vs. adult-onset.

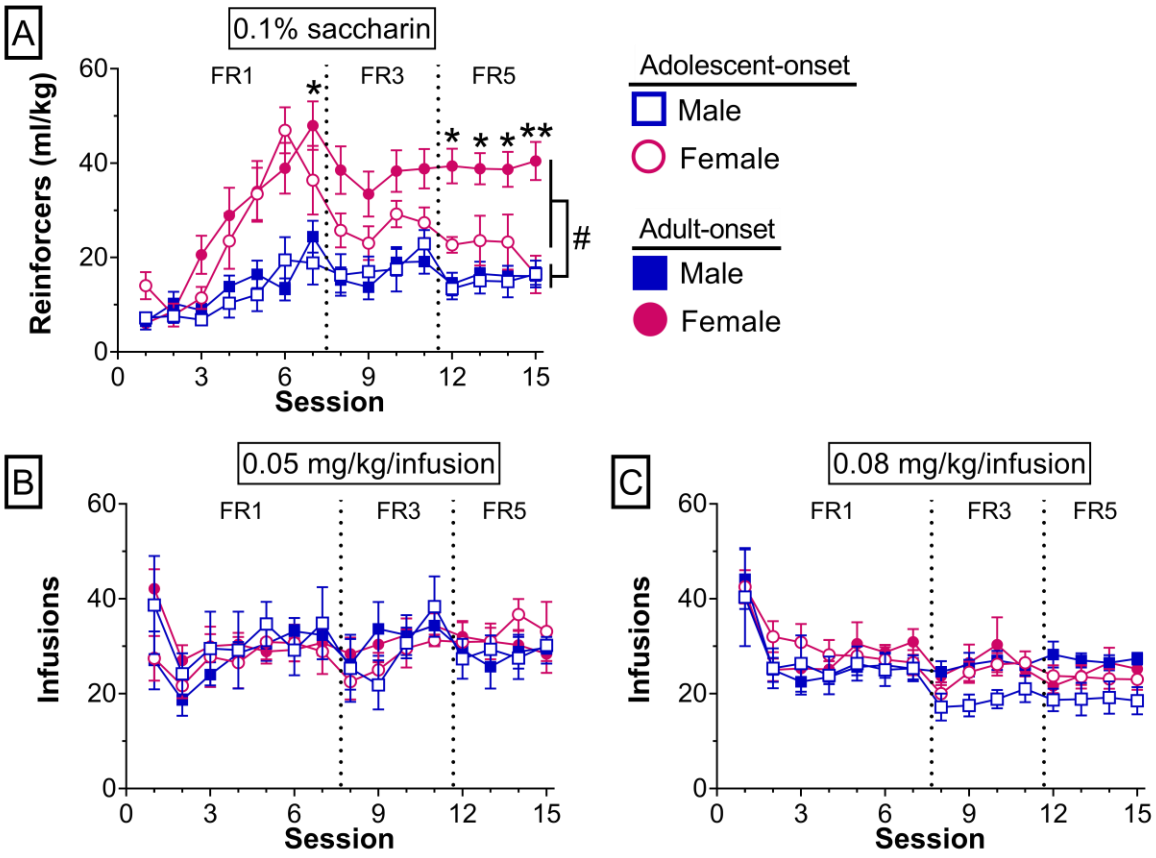

FIGURE 3.2. Number of reinforcers earned across 15 days of acquisition for (**A**) 0.1% saccharin, (**B**) 0.05 mg/kg/inf METH, and (**C**) 0.08 mg/kg/inf METH. * $p < 0.05$, ** $p < 0.01$, adolescent- vs. adult-onset; $^{\#}$ $p < 0.05$ females vs. males 3-15.

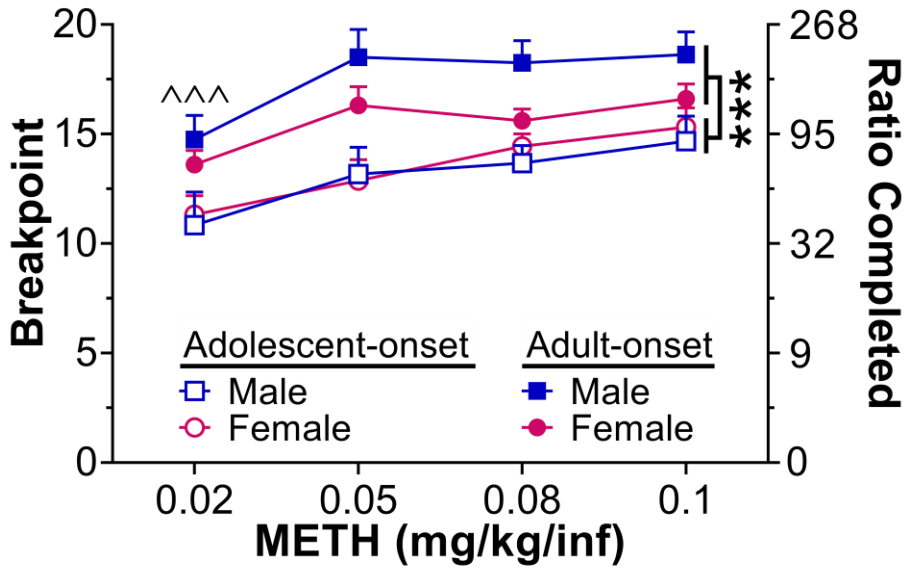

FIGURE 3.3. Responding for 0.02-0.1 mg/kg/inf METH under a PR schedule of reinforcement. Shown are breakpoint (left y-axis) and ratio completed (right y-axis) during the 5-h PR sessions. *** $p < 0.001$ adolescent-onset vs. adult-onset; ^^^ $p < 0.001$ lowest dose vs. all other doses, collapsed across groups.

CHAPTER 4: AGE AND SEX EFFECTS IN LONG ACCESS METH SELF-ADMINISTRATION, RECOGNITION MEMORY, AND RECEPTOR EXPRESSION[3]

ABSTRACT

Individuals who begin drug use during early adolescence experience more adverse consequences compared to those initiating later, especially if they are female. The mechanisms for these age and sex differences remain obscure, but studies in rodents suggest that psychostimulants may disrupt the normal ontogeny of dopamine and glutamate systems in the prefrontal cortex (PFC). Here, we studied Sprague-Dawley rats of both sexes who began METH (i.v.) self-administration in adolescence (P41) or adulthood (P91). Rats received seven daily 2-h SA sessions with METH or saccharin as the reinforcer, followed by 14 daily long access (LgA; 6 h) sessions. After 7 and 14 days of abstinence, novel object (OR) or object-in-place (OiP) recognition was assessed. One week after the final task, PFC and nucleus accumbens were collected to measure NMDA receptor subunits and dopamine D_1 receptor expression. During LgA sessions, adolescent-onset rats escalated METH intake more rapidly than adult-onset rats, with adolescent-onset females earning the most infusions. Adolescent-onset rats with a history of METH self-administration had modest deficits in OiP compared to their adult-onset counterparts, but there was no sex difference and self-administration groups did not differ from naïve control rats. All rats displayed intact novel object recognition memory. We found no group differences in D_1 and NMDA receptor expression, suggesting no long-lasting alteration of ontogenetic expression profiles. Our findings suggest that adolescent-onset drug use is more likely to lead to compulsive-like patterns of drug-taking and modest dysfunction in PFC-dependent cognition.

[3]Data from this chapter are published in Westbrook, S.R., Dwyer, M.R., Cortes, L.R., & Gulley J.M. (*in press*). Extended access self-administration of methamphetamine is associated with age- and sex-dependent differences in drug taking behavior and recognition memory in rats. *Behavioural Brain Research*.

RATIONALE

Most drug use begins during adolescence and those who initiate their use earlier in life appear to experience worse outcomes compared to those who start late in adolescence or during adulthood. For example, earlier onset of cocaine and other drug use has been associated with greater deficits in cognition in a battery of neuropsychological tests, a higher risk for psychosocial problems, and an increased risk for substance use disorder (SUD; Chen et al., 2009; Lopes et al., 2017; Poudel and Gautam, 2017). In laboratory rats, females (Reichel et al., 2012a) and those beginning drug use during adolescence (Anker et al., 2012) develop compulsive-like METH seeking more readily as evidenced by greater and more rapid escalation of METH intake during extended access self-administration sessions. Together, these studies suggest that age-of-onset and sex may be factors that confer vulnerability to adverse outcomes of METH use, including a greater likelihood to develop compulsive METH-taking behavior and a heightened susceptibility to METH-induced cognitive dysfunction.

One hypothesized explanation for this heightened vulnerability is that drug use early in life may induce delays or other significant perturbations in normal brain development. Specifically, dopamine D_1 receptor (D_1R) expression on PFC projections to the accumbens peaks during adolescence before pruning and relative decreases in expression occur as rats reach adulthood (Brenhouse et al., 2008). This D_1R remodeling may precede the late adolescent emergence of GluN2B-containing NMDA receptor transmission in the PFC, which has been shown to be mediated by D_1R signaling (Flores-Barrera et al., 2014). These ontogenetic changes are likely important mechanisms for developing adult-like cognition. In adults, intact D_1R and NMDAR transmission in the medial prefrontal cortex (mPFC) are needed for certain forms of recognition memory. Pharmacological blockade of D_1Rs in the mPFC impaired object-in-place

(OiP) recognition memory, while sparing both novel object (NOR) and object location recognition memory (Savalli et al., 2015). Non-selective NMDAR blockade in the mPFC impairs OiP (Barker and Warburton, 2008). Although the role of different NMDAR subunits in OiP memory is not entirely clear, GluN2B function in the PFC has been implicated in working memory (Wang et al., 2013). Thus, drugs of abuse taken during adolescence may disrupt the ontogeny of D_1R and/or GluN2B signaling in the developing PFC leading to greater deficits in OiP memory compared to adult-onset drug use, while sparing NOR memory.

Exposure to amphetamines during adolescence has been shown to influence the development of certain aspects of dopamine and glutamate signaling, and in turn cognitive functioning, though most of the published work to date has investigated age-of-onset or sex separately. In male rodents exposed to amphetamine non-contingently during adolescence, presynaptic sites on dopamine fiber inputs into the PFC are significantly reduced (Reynolds et al., 2015; Hoops et al., 2018), and dopamine-mediated inhibition of pyramidal cells in the PFC is significantly impaired at four- and twelve weeks after the last drug injection (Kang et al., 2016a; Paul et al., 2016). We have previously reported that these drug-induced neuroadaptations in the dopamine system are region specific, with reduced expression of D_1Rs in the mPFC but no change in the NA after adolescent AMPH exposure (Kang et al., 2016b). Moreover, the drug-induced changes in pyramidal cell function are associated with significant disruptions in cognitive function, including impaired working memory (Sherrill et al., 2013), reduced impulse control (Hammerslag et al., 2014) and reductions in behavioral flexibility (Hankosky et al., 2013b). A more recent study that examined the potential for age of exposure-dependent effects of METH on conditioned fear learning and extinction used only male rats and found adult-exposed animals to have deficits in extinction retrieval that were not apparent in their adolescent-

exposed counterparts (Luikinga et al., 2019). The impact of adolescent amphetamine exposure on glutamate signaling has not been published to date, but a recent study demonstrated reduced expression of phosphorylated GluN2B in the infralimbic PFC after adolescent cocaine exposure in male rats (Caffino et al., 2018). Notably, most of the aforementioned studies employed non-contingent experimenter administered injections, and it is currently unknown whether psychostimulant-induced reductions in PFC D_1Rs and GluN2B expression occur with contingent drug-taking during adolescence. Moreover, since most of these studies assessed adolescent drug exposure without including an adult-exposed comparison group, it is unclear whether observed adaptations are due to disruptions in the developmentally regulated processes specific to adolescence, or if they occur regardless of age of drug exposure.

The current study sought to address these gaps by using a methamphetamine (METH) self-administration paradigm to investigate the hypothesis that adolescent-onset METH-taking would disrupt the ontogenetic trajectories of D_1R and GluN2B in the PFC in a sex-dependent fashion. To this end, we trained Sprague-Dawley rats of both sexes to self-administer METH or a non-drug reinforcer, saccharin, under short access (ShA) conditions beginning during adolescence or adulthood, followed by an extended period of long access (LgA) METH self-administration. One to two weeks following cessation of self-administration, rats were tested on object recognition memory tasks to assess METH-induced memory impairments. Seven days later, tissue was collected to assess NMDAR subunits and D_1R protein expression in the PFC and NA. In line with previous work (Anker et al., 2012; Reichel et al., 2012a), we hypothesized that females and adolescent-onset rats would escalate their METH intake more rapidly during LgA compared to their male and adult-onset counterparts. Importantly, if METH-taking during adolescence indeed disrupted the ontogeny of D_1R and GluN2B function in the PFC, we

expected that adolescent-onset rats would experience greater deficits in PFC-dependent recognition memory and display greater reductions in D_1R and GluN2B protein expression in the PFC, with no changes in the NA, as we found previously (Kang et al., 2016b). We further predicted that these METH-induced neuroadaptations may be more pronounced in females. Finally, we hypothesized that the effects would be specific to METH as a reinforcer, such that these patterns would not be evident in rats that self-administered the non-drug reinforcer, saccharin.

METHODS

Subjects

Subjects were a total of 81 male and 84 female Sprague-Dawley rats that were born in-house on postnatal day (P) 1 from breeders originally obtained from Envigo (Indianapolis, IN, USA). Several rats were lost from the study due to issues with catheter patency (adolescent: males = 5, females = 3; adult: males = 3, females = 1), illness (adolescent: males = 3, females = 5), or other technical problems (adolescent females n = 2; adult: male n = 1; females = 1), yielding final subject totals of 69 males and 72 females. Rats were weaned on P22 and housed 2-3 same-sex animals per cage on a reversed 12-hour light/dark cycle (lights off at 0900). All behavioral training and testing occurred during the rats' dark cycle. Food and water were available *ad libitum* throughout the study. Rats were weighed daily beginning on P25. Daily checks for physical markers to estimate puberty onset – preputial separation in males (Korenbrot et al., 1977) and vaginal opening in females (Castellano et al., 2011) – began on P30 and continued until all rats were in puberty. Assignment to groups based on age-of-onset (adolescent or adult) and reinforcer (METH or saccharin) was counterbalanced across litters. All procedures

were approved by the Institutional Animal Care and Use Committee at the University of Illinois and followed the National Research Council's Guide for the Care and Use of Laboratory Animals.

Self-administration

The timeline for experimental procedures is shown in Figure 4.1. Intravenous catheterization surgery was performed as previously described (Chapter 3, Hankosky et al., 2018) on P32 (± 2 days) or P82 (± 7 days) for adolescent-onset and adult-onset groups, respectively. On P40 (± 2 days) or P90 (± 7 days), rats received one 90-min habituation session in the operant chambers with the nosepoke ports blocked. The following day rats were trained in 7 daily, ShA sessions (2 h duration) to nosepoke into one of two recessed ports in order to receive an intravenous infusion of METH (0.1 mg/kg/infusion) or a liquid dipper cup (0.06 or 0.08 mL) of 0.1% saccharin. Nosepoke responses into the active nosepoke port resulted in reinforcement on an FR1 schedule, whereas responses into the inactive nosepoke port had no programmed consequences. Following ShA sessions, rats received 14 daily LgA sessions that were 6 h in duration but otherwise identical to ShA sessions. In order to minimize risk of overdose, rats responding for METH could earn a maximum of 120 infusions per LgA session.

Recognition Memory

Rats with a history of METH or saccharin self-administration, as well as naïve littermates (aged P93 at first test) from the final birth cohort of rats, were tested in a counterbalanced order on novel object recognition (NOR; Fig. 4.5A) and object-in-place recognition (OiP; Fig. 4.6A) memory tasks 7 and 14 days after self-administration sessions ended. Our procedures for these

tests were adapted from our previous studies (Westbrook et al., 2014) and others (Barker et al., 2007; Reichel et al., 2012a). Each task consisted of three 10-min habituation sessions, a 5-min study phase, a 90-min delay, and a 3-min test phase. No objects were present during habituation sessions. Sessions were run over two days with one morning session followed by another session 5 h later. For the NOR task, two identical objects were present during the study phase and one of these objects was replaced with a novel object during the test phase. For the OiP task, four different objects were present during the study phase. During the test phase, the locations of two of the objects were switched with each other. All objects were of similar size and could be sanitized, but differed in shape, color, and texture. Testing occurred in open-field arenas (41 x 41 x 41 cm) with clear acrylic walls. Visual cues—pink and yellow plaid design, white paper with a black X, and blue and red vertical stripes—were adhered to the outside of the west, north, and east walls, respectively, to act as proximal spatial cues. Arenas and objects were wiped clean with 70% ethanol between all sessions in order to eliminate odor cues.

Three weeks after the last self-administration session (one week after the final recognition task), rats were anesthetized with 195 mg/kg pentobarbital and transcardially perfused with 30-40 mL of ice-cold saline. Brains were removed, chilled in saline for approximately 1 min prior to sectioning using an ice-cold metal brain matrix. Bilateral samples of the vmPFC and NA were extracted using a 2.0 mm diameter punch from 1 mm coronal slices. Prelimbic and infralimbic vmPFC were combined in accordance with our previous work that showed no differential effect of AMPH exposure on D_1R function in these subregions (Kang et al., 2016b, 2016a).

In a subset of 11 rats per group, D_1R (1:2000, ab20066, Abcam; [24]), GluN2B (1:1000, 4207, Cell Signaling, [35]), and GluN1 (1:1000, ab52177, Abcam; [36]) protein expression was

measured via Western blot as described in (Kang et al., 2016b). Although we observed multiple bands using this D_1R antibody, which is not uncommon for D_1R antibodies in general, we used the band at ~50 kDa for our analyses since we and others have previously used this approach for the current D_1R antibody (De Santis et al., 2016; Kang et al., 2016b; Zhu et al., 2017). Briefly, brain tissue was homogenized in lysis buffer, centrifuged, and protein concentration estimated in the supernatant using Precision Red Advanced Protein Assay (Cytoskeleton, CA). Samples were prepared at 25 µg protein/well with 2X laemmli loading buffer and 2-mercaptoethanol and run on pre-cast 4-15% gels (Biorad). Proteins were wet transferred onto PVDF membranes, blocked with 5% non-fat milk in TBST, and incubated overnight with one of the primary antibodies listed above. The next day, membranes were washed, incubated with secondary antibody, washed, and incubated with horseradish substrate prior to imaging. Following imaging, membranes were washed and stripped for re-probing with anti-GAPDH (1:1000, ab9484, Abcam; [24]), a house-keeping protein, used as a loading control. A homogenate of naïve adult female rats was used to normalize across gels. A single gel consisted of one well each of the naïve adult female homogenate, adolescent-onset METH male, adolescent-onset METH female, adolescent-onset saccharin male, adolescent-onset saccharin female, adult-onset METH male, adult-onset METH female, adult-onset saccharin male, and adult-onset saccharin female.

Data Analysis

Body weights. Starting on P25, body weight was measured for all rats. For each sex, body weights from P25 – P74 were analyzed with separate two-way mixed ANOVAs for adolescent-onset rats. Separate two-way ANOVAs for each sex were conducted on body

weights of adult-onset rats from P75-120. In these analyses, the between-subjects factor was reinforcer and the within-subjects factor was postnatal day.

Self-administration. Reinforcers earned were measured for each ShA and LgA session. Between-subjects factors included sex and age-of-onset, while the within-subjects factor was session. All analyses were conducted separately for rats that self-administered METH and saccharin with reinforcer intake (mg/kg or L/kg) as the dependent measure. Separate three-way mixed ANOVAs (sex x age-of-onset x session) were used to assess reinforcer intake during ShA (days 1-7) and LgA (days 8-21) sessions. Escalation during LgA sessions was assessed via separate one-way repeated measures ANOVAs within each sex/age-of-onset group with Tukey post-hoc comparisons of reinforcer intake in each LgA session (days 9-21) compared to the first LgA session (day 8). These analyses were conducted using total session intake for the entire session as well as reinforcer intake during the first hour of LgA sessions. Cumulative METH (mg/kg) and saccharin (ml/kg) intake was calculated for all self-administration sessions and analyzed using separate two-way (sex x age-of-onset) ANOVAs for each reinforcer. Tukey post-hoc tests were conducted where appropriate for all analyses.

Recognition memory tasks. Time spent exploring the objects during the study and test phase was measured. Exploration ratios were calculated as time spent exploring the novel object or switched objects divided by the total time spent exploring all objects. An exploration ratio of 0.5 indicates chance performance, i.e. equal exploration of novel and familiar objects. Separate analyses were conducted for each recognition task. Two-way factorial ANOVAs with age-of-onset and sex as factors for rats with a self-administration history (or only sex as a factor for naïve control rats) were conducted on total exploration time (sec) during the 5-min study phase to assess potential baseline differences in exploratory activity. Total exploration ratios for the 3-

min test phase were analyzed separately for rats based on self-administration history using two-way factorial ANOVAs with age-of-onset and sex as factors (only sex as a factor for naïve rats). We also analyzed test phase exploration ratios for each min of the test phase as previous studies (Dix and Aggleton, 1999; Jablonski et al., 2013) have reported that the first minute may be a more sensitive measure since this is when the novel change is the most salient. In order to assess these changes in exploration as the test phase progressed, three-way mixed ANOVAs with age-of-onset, sex, and minute (within-subjects) as factors were conducted on exploration ratios. In order to compare rats with a self-administration history to naïve control rats, two-way ANOVA with group (i.e., naïve, adolescent-onset METH, adult-onset METH, adolescent-onset saccharin, and adult-onset saccharin) and sex as factors was conducted on total exploration ratios for the test phase. Additionally, three-way ANOVA with group, sex, and minute (within-subjects) as factors was used to compare the time course of exploration ratios across the test phase. Lastly, test phase total exploration ratios and individual minute exploration ratios for each group were compared to chance performance (0.5) using one-sample t-tests as this is a common statistical convention within the recognition task literature to determine whether each group demonstrated significant preference for the novel change (Dix and Aggleton, 1999; Westbrook et al., 2014; Ramsaran et al., 2016). To account for multiple testing, we used the false discovery rate adjustment for p-values (less than 5%; [42]).

Protein expression. Optical density of the protein bands of interest (D_1R: ~50 kDa, GluN2B: ~180 kDa; GluN1: ~120 kDa) was divided by the density of GAPDH (~39 kDa). Data were normalized across gels by dividing the protein expression for each group by the expression of naïve adult female homogenate (control). The relative intensity of the bands was calculated using the following equation: [(Protein of interest/GADPH)/(Control protein of interest/Control

GAPDH)]. Relative intensities for each protein of interest (D_1R, GluN2B, and GluN1) was analyzed separately for rats with a history of METH or saccharin self-administration using two-way ANOVAs with age-of-onset and sex as factors for each brain region.

RESULTS

Body weights

Separate two-way ANOVAs by sex and age-of-onset revealed significant main effects of postnatal day [Adolescent-onset: females $F(49,1262)=90.08$, $p<0.0001$; Adult-onset: males $F(45,1211)=9.12$, $p<0.0001$, females $F(45,1435)=5.27$, $p<0.0001$], which was due to the expected weight gain as Sprague-Dawley rats age under ad libitum access to food (Fig. 4.2). In adolescent-onset males, two-way ANOVA revealed a significant reinforcer by postnatal day interaction [$F(49,1167)=1.58$, $p=0.0076$, reflecting that male rats that self-administered METH during adolescence had modest, but significant, reductions in weight gain compared to their saccharin self-administering counterparts starting on P55. These body weight differences were no longer statistically significant beginning on P67 (~1 week after self-administration ended). Notably, this weight gain suppressing effect of METH was only evident in adolescent-onset males.

Self-administration

Short access. In METH self-administering rats, three-way ANOVA of intake during ShA sessions revealed only a significant main effect of session [$F(6,55)=10.14$, $p<0.0001$], with METH intake higher on session 1 compared to all other ShA sessions (all p's<0.0002). After session 1, ShA intake of METH remained stable and did not differ statistically (Fig. 4.3A).

Three-way ANOVA of saccharin intake (Fig. 4.4A) across ShA sessions showed significant main effects of sex [$F(1,54)=50.57$, $p<0.0001$] and session [$F(6,54)=42.46$, $p<0.0001$], as well as a significant sex by session interactions [$F(6,54)=8.12$, $p<0.0001$]. During ShA sessions, females increase their saccharin intake from session 1 to 2 ($p<0.0001$) before their intake plateaued, whereas males do not exhibit any significant stepwise changes in saccharin intake across ShA sessions. Moreover, females earn significantly more saccharin than males for all ShA sessions.

Long access. During LgA sessions (Fig. 4.3A), three-way ANOVA conducted on METH intake showed significant age by session [$F(13,55)=3.88$, $p=0.0002$] and sex by session [$F(13,55)=2.79$, $p=0.0040$] interactions. Post-hoc analyses of the age by session interaction indicated that adolescents increase METH intake relative to session 1 starting on session 14 ($p=0.0022$), whereas adults did not exhibit this increase until session 20 ($p=0.0421$). Females begin increasing their METH intake earlier than males with females at session 14 ($p=0.0425$) and males at session 19 ($p=0.0038$). Three-way ANOVA conducted on saccharin intake across LgA sessions (Fig. 4.4A) revealed significant age by session [$F(13,54)=3.42$, $p=0.0007$] and sex by session [$F(13,54)=2.32$, $p=0.0156$] interactions. In addition to adolescent-onset rats having higher saccharin intake relative to adult-onset rats across all sessions (all p's<0.041), adolescent-onset rats also reduced their saccharin intake relative to session 8 earlier than adult-onset rats (adolescent-onset: session 9, $p<0.0001$; adult-onset: session 10, $p=0.0208$). A similar pattern was seen for females compared to males. Females maintained higher saccharin intake relative to males (all p's<0.001), but they reduced their intake more rapidly than males (females: session 9, $p<0.0001$; males: session 12, $p<0.0001$).

Three-way ANOVA using only the first hour of METH intake during LgA sessions (Fig. 4.3B) revealed significant age by session [$F(13,55)=1.92$, $p=0.0482$] and sex by session [$F(13,55)=2.17$, $p=0.0233$] interactions. Post-hoc analyses of the sex by session interaction showed no significant differences in first hour METH intake from session 8, whereas the age by session interaction indicated that adolescent-onset rats increased their first hour METH intake from the first to the last LgA session ($p=0.0125$). Analyses of the first hour saccharin intake (Fig. 4.4B) showed significant age by session [$F(13,702)=2.29$, $p=0.0057$] and sex by session [$F(13,702)=2.30$, $p=0.0055$] interactions. Both age groups significantly reduced their first hour saccharin intake starting on session 10 (adolescent-onset: $p<0.0001$, adult-onset: $p=0.0077$). In addition, adolescent-onset rats had higher saccharin intake for the first 2 sessions compared to adult-onset rats (all p's<0.028). Females reduce their first hour saccharin intake starting on session 9 ($p<0.0001$), while males began reducing on session 12 ($p=0.00334$). Throughout the LgA sessions, females maintained higher first hour intake of saccharin than males (all p's<0.0033).

Escalation was further assessed using a three-way ANOVA comparing METH intake on only the first and last LgA sessions (Fig. 4.3C). This test revealed a significant age by session interaction [$F(1,54)=5.92$, $p=0.0183$]. Initially, adolescent-onset and adult-onset rats' METH intake did not differ statistically on the first LgA session. However, METH intake increased in both age groups from the first to the last LgA session (adolescent-onset: $p<0.0001$; adult-onset: $p=0.0069$), with adolescent-onset rats taking significantly more METH by the last LgA session compared to adults ($p=0.0074$). A similar analysis of saccharin intake (Fig. 4.4C) revealed age by session [$F(1,54)=21.18$, $p<0.0001$] and sex by session [$F(1,54)=9.40$, $p=0.0034$] interactions. Both age groups reduced their saccharin intake from the first to the last LgA session (both

p's<0.0001), but adolescent-onset rats took more saccharin than their adult-onset counterparts during both sessions (first LgA: $p<0.0001$, last LgA: $p=0.0406$). Similarly, both sexes reduced their intake (both *p*'s<0.0001) with females maintaining higher saccharin intake than males (both *p*'s<0.0001).

Lastly, cumulative METH intake was calculated across all self-administration sessions and is illustrated in Fig. 4.3D. Two-way ANOVA indicated a significant main effect of age [$F(1,55)=4.03$, $p=0.0496$], with adolescent-onset rats having greater total intake than adult-onset rats, regardless of sex. Cumulative saccharin intake (Fig. 4.4D) significantly differed by age [$F(1,54)=19.00$, $p<0.0001$] and sex [$F(1,54)=86.41$, $p<0.0001$], but there was not a significant interaction. Adolescent-onset and female rats had higher cumulative saccharin intake than adult-onset and male rats, respectively.

Recognition memory

Novel object recognition (NOR) task. During the study phase, total object exploration time was assessed using separate two-way ANOVAs based on self-administration history (METH, saccharin, or naïve). No significant main effects or interactions were revealed for rats regardless of self-administration history. This indicates that there were no baseline differences in exploration times during the study phase that might contribute to differences seen during the test phase.

Separate two-way ANOVAs conducted on total exploration ratios during the test phase (Fig. 4.5) for rats that previously self-administered METH or saccharin revealed no significant main effects or interactions. A similar lack of significant effects was found for naive control rats. Time course analyses of exploration ratios across the three-min test phase revealed no

significant main effects or interactions in rats with a history of METH self-administration, whereas in rats with a history of saccharin self-administration there was a significant main effect of min [$F(2,54)=13.93$, $p<0.0001$] and an age by min interaction [$F(2,54)=4.38$, $p=0.0173$]. Rats in the adolescent-onset group had a significantly higher exploration ratio in min 1 compared to min 3 ($p<0.0001$). No significant main effects or interactions were reported from the two-way ANOVA comparing total exploration ratios for rats with a self-administration history to naïve control rats. Time course analyses including naïve control rats indicated a significant main effect of min [$F(2,131)=15.06$, $p<0.0001$], such that exploration ratios in the first min were significantly greater than the second and third min (p's<0.001).

Since there was no evidence of sex differences in any of the NOR task analyses, groups were collapsed across sex for the one-sample t-tests of test phase exploration ratios against chance performance (0.5). For total exploration ratios (Table S1), all groups demonstrated preference for the novel object that was significantly greater than chance (all FDR-corrected p's ≤ 0.0003). Rats also displayed significant novelty preference in the individual minute analyses (Table S2).

Object-in-place (OiP) task. Total object exploration time was examined during the study phase with separate two-way ANOVAs by self-administration history. The ANOVAs for rats from each group failed to indicate any significant main effects or interactions. Similar to the findings in the NOR task, there were no baseline differences in exploration during the study phase.

For the test phase (Fig. 4.6), two-way ANOVAs were conducted on total exploration ratios. These analyses revealed a significant main effect of age-of-onset [$F(1,55)=4.79$, $p=0.0329$] for rats with METH self-administration history and no significant main effects or

interactions for rats that responded for saccharin. Rats that self-administered METH beginning during adolescence had lower test phase exploration ratios compared to their adult-onset counterparts, regardless of sex. Time course analyses including test phase minute as a factor indicated significant main effects of age-of-onset [$F(1,55)=4.49$, $p=0.0386$] and minute [$F(2,55)=3.51$, $p=0.0369$] for rats with a history of METH self-administration and no significant main effects or interaction for rats with a history of saccharin self-administration. Two-way ANOVA comparing total exploration ratios for rats with a self-administration history to naïve control rats revealed no significant main effects or interactions. However, time course analyses including naïve control rats indicated a significant group by min interaction [$F(8,131)=2.90$, $p=0.0052$]. Exploration ratios for naïve control rats significantly decreased from the first min to the third minute ($p=0.0447$), and this effect was absent in the groups with a history of self-administration.

Groups were collapsed across sex for the one-sample t-tests of test phase exploration ratios against chance performance (0.5) due to the lack of significant effects of sex in the object-in-place recognition task analyses. Not all groups demonstrated preference for the novel targets that was significantly greater than chance performance when examining the total exploration ratios (Table S1). However, the individual minute exploration ratio analyses (Table S2) demonstrated that all groups displayed significant preference for the novel targets during the first minute of the test phase, except for rats with adolescent-onset METH or saccharin history. Moreover, rats that self-administered METH starting during adolescence did not display significant preference for the novel object during any minute of the test phase. In contrast, rats with a history of adolescent-onset saccharin self-administration demonstrated significant

preference for the switched objects during minute 2 of the test phase [t(27)=5.06, FDR-corrected p=0.045], indicating that they were able to recognize the novel change.

Receptor expression

A randomly selected subset of rats (n=11/group) were used for Western blot analyses of receptor protein expression. One rat's NA sample (a male from the adolescent-onset METH group) was lost due to an error in sample preparation. For data from the remaining samples, separate two-way ANOVAs were conducted on the relative intensity of each protein band of interest (D_1R, GluN1, GluN2B) for each brain region (PFC and NA) and by self-administration history. We observed no significant main effects or interactions for any of these measures (Fig. 4.7).

DISCUSSION

Adolescence is characterized by considerable development of D_1R and GluN2B neurotransmission in the PFC (Andersen et al., 2000; Brenhouse et al., 2008; Flores-Barrera et al., 2014), which may constitute a window of vulnerability to drug-induced neuroadaptations (Gould, 2010; Gulley and Juraska, 2013b). This vulnerability may partly explain why adolescent-onset drug users suffer worse outcomes of their drug use compared to adult-onset users. We tested this hypothesis in the current study by investigating PFC-dependent cognition and receptor expression in rats with a history of METH or saccharin self-administration. We found that adolescent-onset and female rats escalated METH intake more rapidly and maintained higher saccharin intake than their adult-onset and male counterparts. Regardless of sex, rats that initiated METH self-administration during adolescence were modestly impaired in OiP

recognition memory compared to adult-onset rats. Despite the age-of-onset difference in PFC-dependent cognition, D_1R and NMDAR subunit protein expression was not altered three weeks after the last drug-taking session. Our findings suggest that METH self-administered during adolescence impairs PFC-dependent cognition; however, METH does not appear to induce long-lasting disruptions of D_1R or GluN2B developmental expression trajectories in the PFC.

Consistent with previous reports from our lab (Hankosky et al., 2018) and others (Ahmed and Koob, 1998; Kitamura et al., 2006), METH intake was relatively stable in both adolescent- and adult-onset groups when the drug was available under ShA conditions. During ShA, METH intake was significantly higher on the first session compared to the rest of the ShA sessions. During habituation, the nosepoke ports were blocked. This practice makes the nosepoke ports more salient when the rats have access to them during the first self-administration, which likely contributes to the increased intake during this session. After the first ShA session, METH intake for all groups stabilized. Interestingly, group differences emerged across sessions when rats could self-administer METH under LgA conditions. Consistent with a previous report in males (Anker et al., 2012), adolescent-onset rats escalated more rapidly than adult-onset rats. Also in line with previous work (Reichel et al., 2012a), females began increasing their METH intake earlier than males, although this effect due largely to adolescent-onset females. Since adolescents and females weigh less than their adult and male counterparts, it could be the case that body weight influences escalation of METH intake. Our study accounted for body weight differences by adjusting the duration of the infusion for each session according to the rat's body weight each day. Since our measures are adjusted for body weight as part of the procedures, we cannot directly disentangle the issue of body weight. However, if differences in body weight-adjusted METH intake (mg/kg) were exaggerated by age or sex differences in body weight we

would predict a reduction in METH intake across sessions because rats, especially adolescents, gain weight across time. However, we observed that adolescent-onset rats (i.e., the group with the most rapid and significant weight gain) escalated the most.

One explanation for the age-of-onset difference in escalation is that adolescent rats may be less sensitive to METH and therefore need to take more METH to achieve a similar effect. We have previously reported that adolescent-onset rats are less sensitive to reinforcement with a low dose of METH (0.02 mg/kg/inf), and adolescent-onset rats reach lower breakpoints in progressive ratio (PR) compared to adult-onset rats (Hankosky et al., 2018). In the present study, we used a relatively high unit dose of METH (0.1 mg/kg/inf) and we did not see any age-of-onset differences during ShA sessions. Also inconsistent with a reduced sensitivity in adolescence is that locomotor sensitization to psychostimulants is often enhanced or equal in adolescents compared to adults (Adriani et al., 1998; Mathews and McCormick, 2007; Sherrill et al., 2013). Future studies are necessary to examine potential age differences in METH sensitivity more directly. For example, breakpoints under a PR schedule for different unit doses could be measured before and after escalation. If reduced sensitivity significantly contributes to escalation of METH intake, a downward shift in the dose-response curve for breakpoints post-escalation, as well as adolescents reaching lower breakpoints than adults, would be the likely outcomes. On the other hand, if sensitization to the reinforcing effects of METH contributes to escalation of intake, an upward shift in the dose-response curve for breakpoints post-escalation would be the most likely outcome.

An alternative explanation for the age-of-onset difference in escalation of METH intake is that adolescent-onset rats transition more readily from controlled to habitual or compulsive drug-taking. One way to investigate this idea is to measure responding when the drug is no

longer available, for example in a signaled non-availability paradigm, as continued drug-seeking may indicate that the response has become habitual or compulsive. Previous work using cocaine and food as reinforcers has shown that adolescent rats of both sexes responded more during signaled non-availability compared to adult rats (Anker et al., 2011), supporting the notion that adolescent rats may develop habitual/compulsive drug-seeking more readily than adult rats. Furthermore, age differences in compulsive drug-taking have also been reported in females when drug-taking behavior was punished with histamine (Holtz and Carroll, 2015) to model continued drug use despite negative consequences. Taken together, these findings suggest that adolescent rats exhibit greater compulsive drug-taking compared to adults, which may partly explain the greater escalation of METH intake in adolescents seen in the current study.

Both sex and age-of-onset differences were evident with self-administration of the non-drug reinforcer, saccharin. During ShA and LgA sessions, females earned more saccharin than males when normalized to body weight. In stark contrast to METH escalation during LgA, rats rapidly reduced their intake of saccharin when given access for 6 h, suggesting that escalation of intake may be specific to drug reinforcers. We chose non-caloric saccharin as our non-drug reinforcer in order to control for the influence of differing metabolic needs between adults and rapidly growing adolescents. However, when given longer access to saccharin, rats may rapidly learn the lack of nutritive effects of the reinforcer (i.e., that saccharin is non-caloric) and decrease their intake accordingly. This notion is consistent with prior studies in adult male mice (Sclafani et al., 2015). In this study, a non-caloric saccharin and sucralose mixture was preferred to caloric sucrose or glucose solutions in brief (1 min) access choice tests, but this preference switched across time when mice were given 2 days of 24-h access to these solutions. These results suggest that the post-oral nutritive effects, rather than palatability, influenced sweetener

intake across time (Sclafani et al., 2015). Despite the overall reduction in saccharin intake across time in the current study, adolescent-onset and female rats maintained higher saccharin intake than their adult-onset and male counterparts. These group differences are consistent with a body of literature that has demonstrated hypersensitivity to non-drug rewards in adolescents and females (Friemel et al., 2010; Hammerslag and Gulley, 2014; Marshall et al., 2017; Hankosky et al., 2018; Westbrook et al., 2018).

During the period when rats were engaged in daily self-administration sessions, all animals gained weight as expected for the Sprague-Dawley strain. However, adolescent-onset male rats self-administering METH exhibited suppressed weight gain starting during the second week of LgA, which coincided with escalation of their METH intake. METH is known to have appetite-suppressing effects, and human METH abusers often experience weight loss (Hawks et al., 1969). In rodents, adult male rats that are given extended access to METH at the same dose used in the current study (0.1 mg/kg/inf) have been reported to lose weight (Krasnova et al., 2010). We did not find significant weight loss in our adult-onset males, however, and this discrepancy may be due to differences in session duration. Our study used the more common 6-h duration for 14 daily sessions, whereas Krasnova and colleagues used longer duration access over fewer days (14-h for 8 days). Nonetheless, we did find METH-induced suppression of weight gain in our adolescent-onset male rats. Under normal conditions, adolescent males gain weight at the highest rate compared to the other groups, which may make it easier to detect more subtle changes in weight gain from METH self-administration in our LgA paradigm. Notably, this suppression of body weight gain is not long-lasting. About 1 week after cessation of self-administration, body weights of adolescent-onset METH rats rebounded to reach similar values as adolescent-onset rats that self-administered saccharin.

Approximately one-two weeks after their last self-administration session, rats were tested on recognition memory tasks. We saw no group differences in exploration time during the study phase for either the NOR or OiP tasks, suggesting that self-administration history did not influence exploratory behavior in general. In tests of recognition memory, we found no evidence for sex differences, regardless of self-administration history. This lack of a sex effect is consistent with a previous report in adult rats with a history of METH self-administration during adulthood (Reichel et al., 2012a). In line with our hypothesis, all rats recognized the familiar object and spent more time exploring the novel object in the NOR task. Our findings are consistent with previous studies reporting no NOR deficits after 1 week of abstinence from non-contingent, chronic binge-like METH exposure during adolescence (North et al., 2013) or adulthood (Melo et al., 2012) in male rodents. Interestingly, NOR deficits following METH may depend on dosing regimen, with chronic or escalating dosing associated with less deficits in NOR. In support of this notion, Belcher and colleagues (Belcher et al., 2008) reported NOR impairments with corresponding monoaminergic neurotoxicity in the hippocampus after a single-day binge exposure to METH; these deficits and neural changes were absent in adult rats treated with an escalating dose regimen for several days prior to the METH binge. However, Reichel and colleagues have shown NOR impairments in adult rats that escalated their intake after LgA METH self-administration (Reichel et al., 2011, 2012a, 2012b). Although the design for our current study was based on the work from Reichel and colleagues, our study did differ from these studies in one notable way. Our rats were group housed, while those in Reichel and colleagues' studies were individually housed. We chose to group house our rats in order to reduce any potential age differences in social isolation stress, as adolescence is a time of increased social interaction relative to adulthood (Douglas et al., 2004). Moreover, social

housing has been reported to enhance novel object recognition memory in mice of both sexes compared to individual housing (Liu et al., 2019). Thus, our approach of minimizing housing-induced stress may have helped protect against METH-induced deficits in NOR.

We found that OiP memory was not as robust as NOR memory in our naïve control rats, which is consistent with previous literature (Dix and Aggleton, 1999). The naïve control group did display significant preference for the novel change in OiP during the first min of the test phase, demonstrating that this group was able to recognize that the objects switched places. However, this group appeared to habituate rapidly to this change across the three-min test session, resulting in the total test phase exploration ratio not being significantly different from chance performance. This rapid habituation was not apparent in the groups with a history of self-administration. In rats that self-administered saccharin beginning during adolescence or adulthood, we did not find evidence for impaired OiP memory. Consistent with our predictions, an age-of-onset dependent difference was present in OiP recognition in rats with a history of METH use, although this effect was modest. Rats that previously self-administered METH beginning in adolescence displayed significantly lower exploration ratios than rats that began in adulthood. Furthermore, adolescent-onset METH self-administering rats failed to demonstrate significant recognition of the novel change in any of the three min in the test phase, while adult-onset METH self-administering rats recognized the change by the first min when the novelty salience of position change is at its height. However, it is important to note that neither age-of-onset group of rats with a history of METH use significantly differed in OiP performance from the naïve control rats. The specific difference in OiP in adolescent-onset compared to adult-onset METH self-administering rats suggests that METH use during adolescence may impact the PFC, hippocampus, or both brain regions (Barker and Warburton, 2011), but not the perirhinal cortex

or general sensory, motor, or motivational capacities because NOR memory was intact. Although only a modest effect, our recognition memory findings are consistent with our hypothesis that METH use during this period adversely impacts developmental events in the PFC, although more work is needed to determine whether the hippocampus may be impacted.

We measured D_1R and NMDAR subunit protein expression in the PFC and NA following three weeks of abstinence from METH or saccharin self-administration. Our findings of no significant group differences for any of our proteins of interest in either brain region are in contrast to previous reports from our lab (Kang et al., 2016b) and others (Caffino et al., 2018) using non-contingent drug exposure. It may be the case that the added stress of non-contingent injections contributes to the drug-induced neuroadaptations reported in these studies (Kang et al., 2016b; Caffino et al., 2018). The lack of change in GluN1 NMDAR subunit expression in the mPFC following LgA METH self-administration is consistent with previous studies in adult male rats that self-administered cocaine (Ben-shahar et al., 2007) or METH (Reichel et al., 2014; Mishra et al., 2017) under LgA conditions. However, previous findings for GluN2B surface expression in the mPFC were mixed with one study reporting no change (Reichel et al., 2014) and the other an increase (Mishra et al., 2017). These discrepancies may be explained by the region(s) of mPFC that were assessed. The regions of mPFC collected by Mishra and colleagues (Mishra et al., 2017) were not specified, whereas the study by Reichel and colleagues (Reichel et al., 2014) and the current report analyzed the prelimbic and infralimbic subregions of the mPFC together in a single homogenate. We chose not to separately analyze these subregions because of our lab's previous work showing no difference between them following adolescent or adult AMPH exposure (Kang et al., 2016a). While combining the two regions does not explain our lack of differences in D_1R expression, it may account for our null findings for GluN2B as

Caffino and colleagues [29] reported opposing directions of effect of cocaine injections during adolescence on phosphorylated GluN2B expression in the prelimbic versus infralimbic subregions

It is possible that the functional trajectories of D_1R and/or GluN2B in the PFC or their interactions, whether physical or functional, are disrupted without a concomitant change in total protein expression. In support of this notion, our lab previously reported long-lasting reductions in sensitivity to D_1R agonists in the PFC after non-contingent AMPH exposure during adolescence (Kang et al., 2016a). However, the current study found only a modest age-of-onset effect of self-administered METH on PFC-dependent OiP memory, suggesting that METH-induced PFC dysfunction, if present, may be less severe with contingent compared to non-contingent exposure. Further work is needed to determine whether the functions or interaction of D_1Rs and GluN2B in the PFC are negatively impacted by METH use during adolescence, perhaps by examining synaptic expression of these receptors and/or phosphorylation of GluN2B at sites known to be influenced by D_1R activation (e.g., Ser1303, (Sarantis et al., 2009)).

In conclusion, our investigation into METH-induced cognitive dysfunction and neuroadaptations in D_1Rs and NMDARs following contingent METH use revealed age-of-onset and sex differences in escalation of METH intake. In addition to these differences in drug-taking patterns, the current study may provide some evidence for modest disruptions in PFC-dependent cognitive functions when contingent METH use begins during adolescence; however, there appears to be no long-lasting impacts on the ontogeny of D_1R and GluN2B expression in the PFC. Overall, these findings suggest that adolescent-onset users may develop problematic drug-taking patterns more rapidly and may be more likely than adult-onset users to experience cognitive dysfunction in early abstinence. Both of these factors could contribute to higher drop-

out rates from treatment programs (McKellar et al., 2006), greater relapse rates (Simon et al., 2004; Poudel and Gautam, 2017), and worse treatment outcomes (Hillhouse et al., 2007a). Furthermore, adolescent-onset users may benefit more from treatment programs that focus on improving cognitive function during early abstinence.

FIGURES

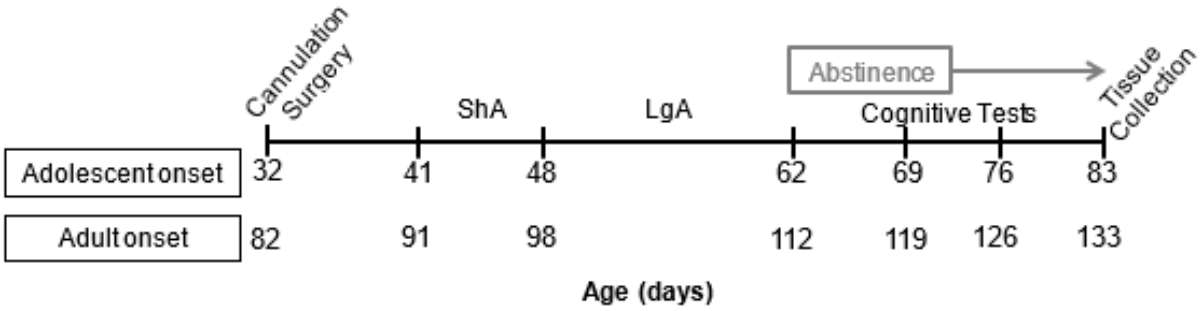

FIGURE 4.1. Experimental timeline for rats that underwent self-administration under short access (ShA) and long access (LgA) conditions. Cognitive tests included novel object recognition and object-in-place recognition tasks after 7 and 14 days of abstinence with task order counterbalanced. mPFC and NA were collected after 21 days of abstinence.

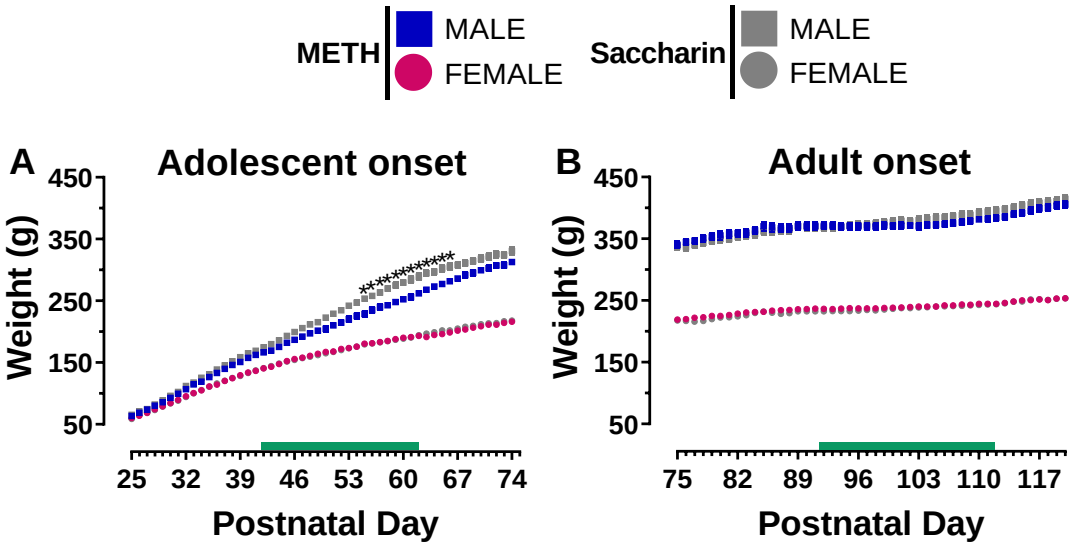

FIGURE 4.2. Body weight (g) across postnatal days (P) 25-74 for adolescent-onset rats (**A**) and P75-120 for adult-onset rats (**B**). The green bar indicates the ages when rats underwent METH or saccharin self-administration sessions. In this and all subsequent figures, data expressed as mean ± SEM. * $p < 0.05$ vs. males that self-administered METH on the noted days.

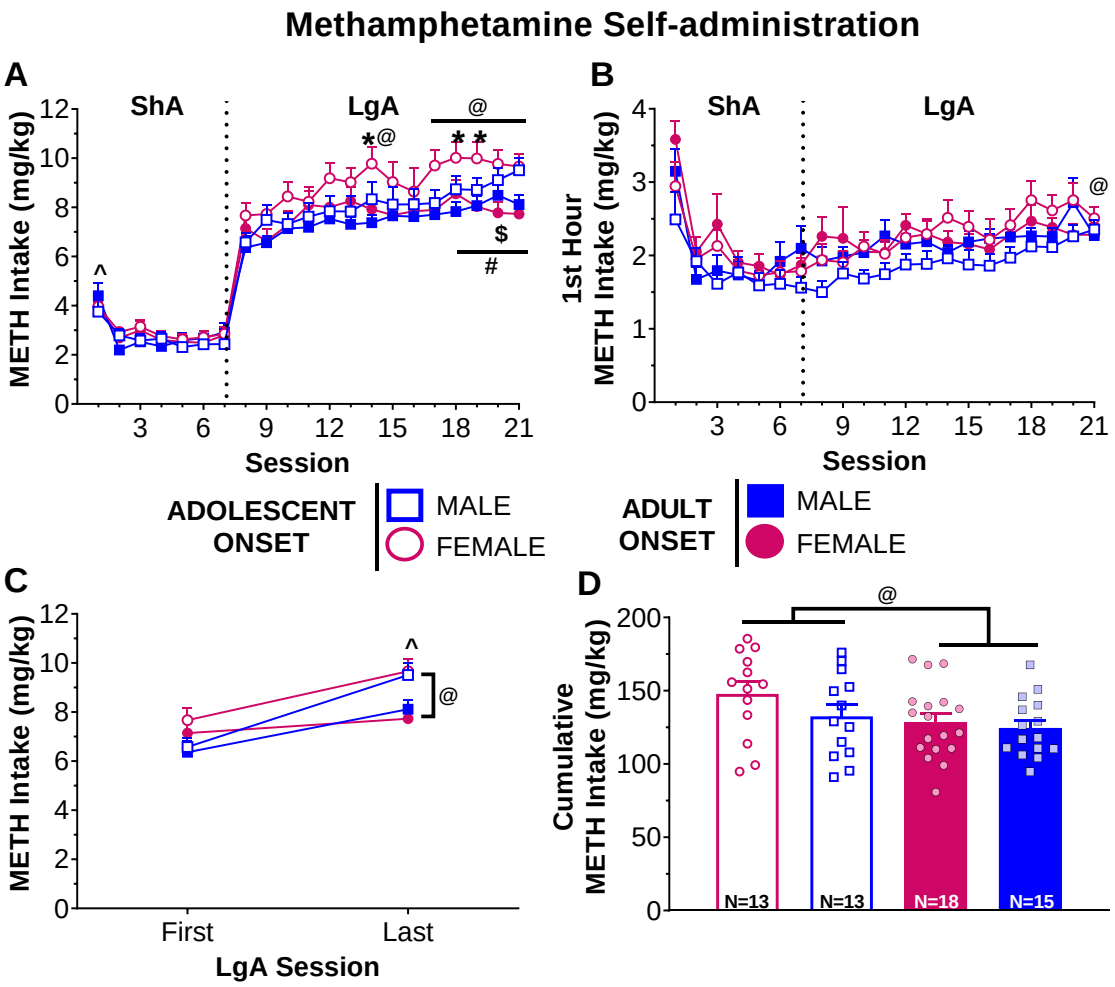

FIGURE 4.3. Adolescent- and adult-onset METH self-administration in rats of both sexes. (**A**) Daily METH intake (mg/kg) across 7 days of short access (ShA) and 14 days of long access (LgA) self-administration. ^ $p < 0.05$ vs. all other ShA sessions. Session intake vs. first LgA session: * $p < 0.05$ for females, $^{\#}$ $p < 0.01$ for males, $^{@}$ $p < 0.05$ for adolescent-onset rats, $^{\$}$ $p < 0.05$ for adult-onset rats. (**B**) Daily METH intake (mg/kg) during the first hour of each self-administration session. $^{@}$ $p < 0.05$ vs. first LgA session for adolescent-onset rats. (**C**) METH intake (mg/kg) on the first and last LgA session. $^{@}$ $p < 0.05$ age within session, ^ $p < 0.05$ vs. first LgA session. (**D**) Cumulative METH intake (mg/kg) across all self-administration sessions. Each dot represents one individual rat. $^{@}$ $p < 0.05$ age effect.

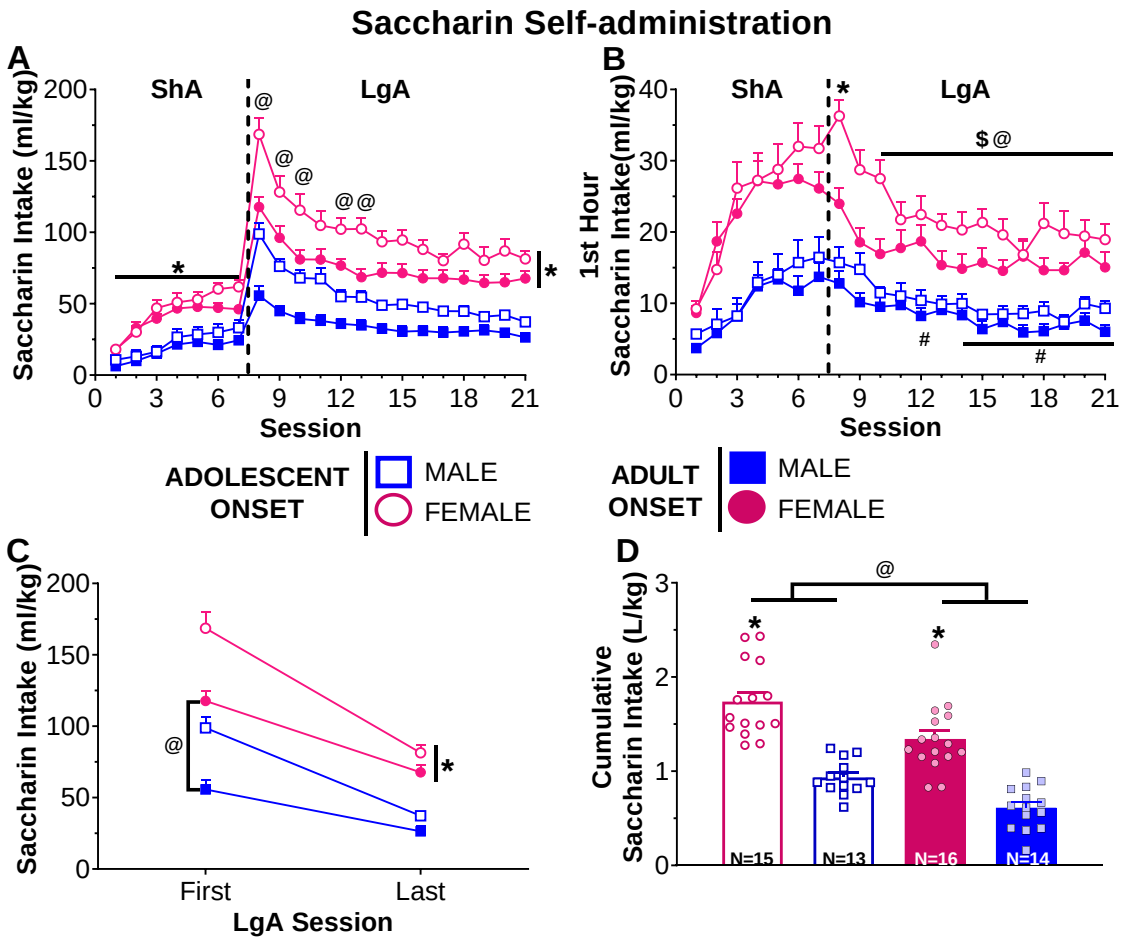

FIGURE 4.4. Adolescent- and adult-onset saccharin self-administration in rats of both sexes. (**A**) Daily saccharin intake (ml/kg) across 7 days of short access (ShA) and 14 days of long access (LgA) self-administration. * $p < 0.05$ vs. males, $^{@}$ $p < 0.05$ adolescent-onset vs. adult-onset. (**B**) Daily saccharin intake (ml/kg) during the first hour of each self-administration session. * $p < 0.05$ for females vs. all other LgA sessions, Session intake vs. first LgA session: $^{\#}$ $p < 0.01$ for males, $^{@}$ $p < 0.05$ for adolescent-onset rats, $^{\$}$ $p < 0.05$ for adult-onset rats. (**C**) Saccharin intake (ml/kg) on the first and last LgA session. * $p < 0.05$ vs. males, $^{@}$ $p < 0.05$ vs. adolescent-onset. (**D**) Cumulative saccharin intake (L/kg) across all self-administration sessions. Each dot represents one individual rat. * $p < 0.05$ vs. males, $^{@}$ $p < 0.05$ age effect.

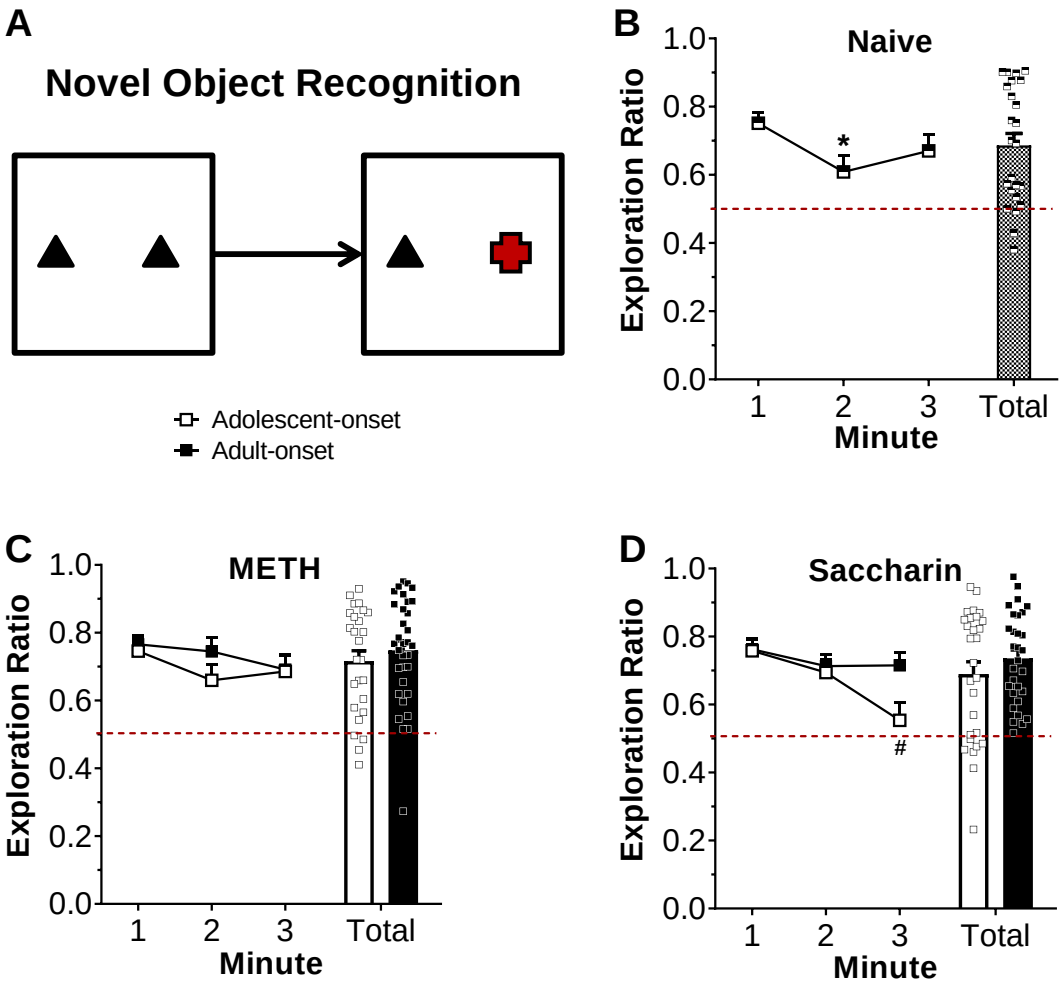

FIGURE 4.5. Novel object recognition memory assessed during abstinence from METH or saccharin self-administration. Schematic of the novel object recognition task with red object indicating the novel target (**A**). Exploration ratios during the test phase for naïve rats (**B**) or rats with a history of METH (**C**) or saccharin (**D**) self-administration. Bars represent exploration ratio averaged across the 3-min test phase with data points representing individual animal data. Exploration ratios were calculated as $time_{novel}$ / ($time_{novel}$ + $time_{familiar}$). Dotted red line indicates chance performance (0.5). We found no significant sex difference, so data are presented collapsed across sex. * $p < 0.05$ vs. min 1, $^{\#}$ $p < 0.001$ vs. min 1 within adolescent-onset.

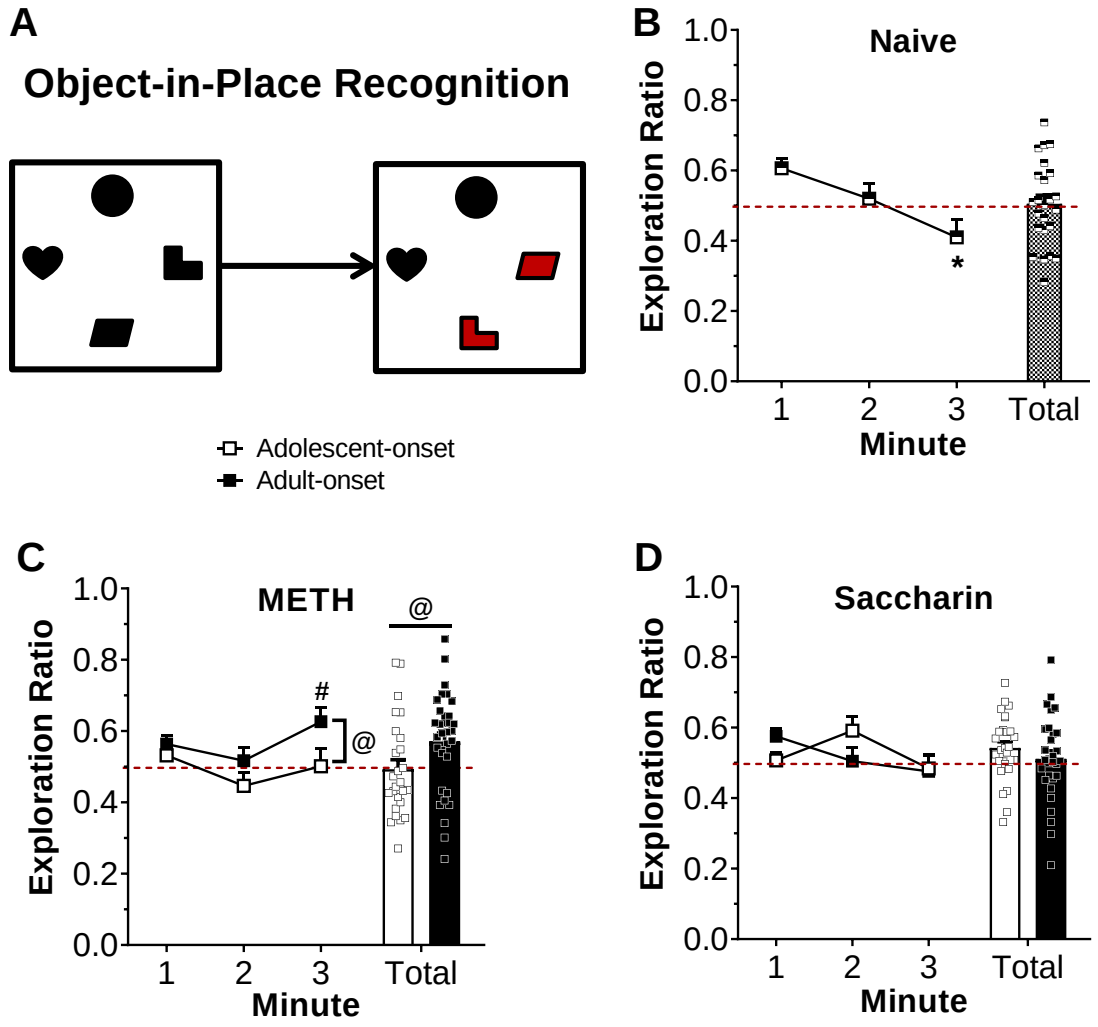

FIGURE 4.6. Object-in-place recognition memory assessed during abstinence from METH or saccharin self-administration. Schematic of the object-in-place recognition task with red objects indicating the novel targets (**A**). Exploration ratios during the test phase for naïve rats (**B**) or rats with a history of METH (**C**) or saccharin (**D**) self-administration. Bars represent exploration ratio averaged across the 3-min test phase with data points representing individual animal data. Exploration ratios were calculated as $time_{novel}$ / ($time_{novel}$ + $time_{familiar}$). Dotted red line indicates chance performance (0.5). We found no significant sex difference, so data are presented collapsed across sex. * $p < 0.01$ vs. min 1, $^{\#}$ $p < 0.05$ vs. min 2 within adult-onset, $^{@}$ $p < 0.05$ age-of-onset effect collapsed across min.

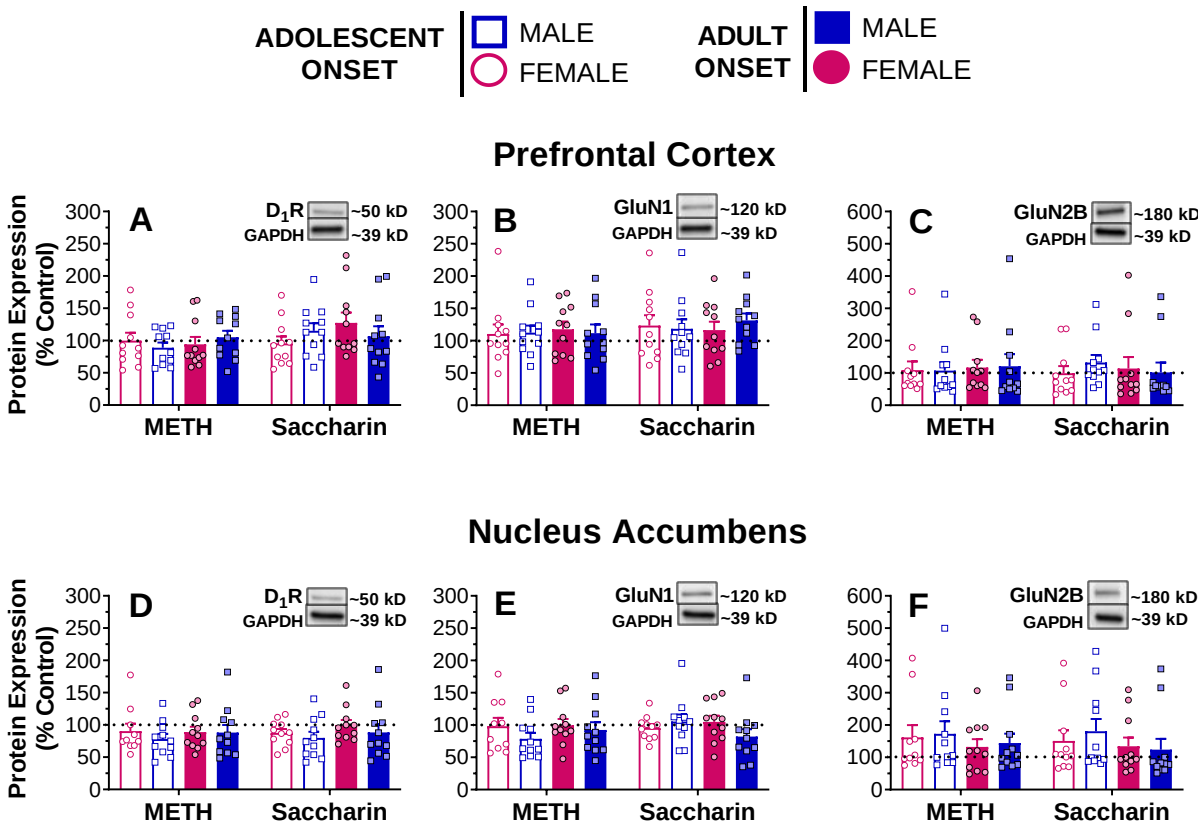

FIGURE 4.7. Dopamine D_1 receptor and NMDA receptor subunits protein expression as a percent of naïve adult female homogenate (control). Tissue was collected 21 days after the last self-administration session. Bands are shown for the control homogenate. Each dot represents one individual rat**.**

CHAPTER 5: ROLE OF GLUN2B IN AGE AND SEX DIFFERENCES IN EXTINCTION AND REINSTATEMENT OF METHAMPHETAMINE SELF-ADMINISTRATION[4]

ABSTRACT

Previous work suggests adolescent rats have deficient extinction consolidation relative to adults. Although the mechanisms of this age difference are currently unknown, studies in adult rats have implicated GluN2B-containing NMDA receptors in extinction consolidation of drug-associated memory. Importantly, GluN2B signaling emerges in adolescent development, and drugs of abuse during adolescence may delay adult-like extinction consolidation by disrupting the ontogeny of GluN2B function. Here, we trained Sprague-Dawley rats of both sexes to self-administer METH (0.1 mg/kg/infusion i.v.) starting in adolescence (P41) or adulthood (P91). Rats had 7 days of short access (2 h) sessions to self-administer METH followed by 14 sessions with long access (6 h). Subsequently, rats underwent four daily 30-min extinction sessions with immediate post-session injections of either a GluN2B antagonist (Ro25-6981; 6 mg/kg, i.p.) or vehicle. After four daily 2-h extinction sessions, a priming injection (1 mg/kg METH, i.p.) was given prior to a final 2-h reinstatement session. During LgA, adolescent-onset rats earned more METH than adult-onset rats and displayed greater drug-loading behavior. All groups similarly reduced drug-seeking across extinction sessions. Drug-seeking was reinstated following a METH priming injection, with females reinstating more than males. These results do not support our *a priori* hypothesis that METH use in adolescence disrupts the ontogeny of GluN2B function contributing to age-of-onset differences in extinction of METH-seeking. However, our findings suggest that age-of-onset contributes to compulsive METH-taking, while sex confers vulnerability to METH relapse.

[4]Data from this chapter are in a manuscript currently under review with *Behavioural Pharmacology*: Westbrook, S.R., & Gulley, J.M. Effects of the GluN2B antagonist, Ro 25-6981, on extinction consolidation following adolescent- or adult-onset methamphetamine self-administration in male and female rats. *Behavioural Pharmacology*.

RATIONALE

Adolescent-onset drug users who developed a substance use disorder had especially high relapse rats compared to those who initiated during adulthood (Poudel and Gautam, 2017). The mechanisms underlying this heightened vulnerability in adolescents are not known, but research in rodent models of relapse has implicated both D_1Rs and GluN2B receptors in extinction. Both age and sex differences have been reported in extinction, such that adolescents were impaired compared to adults across food-, fear-, and drug-related paradigms (Anker and Carroll, 2010; McCallum et al., 2010; Andrzejewski et al., 2011; Zbukvic et al., 2016) and females responded more during extinction of cocaine self-administration (Lynch & Carroll, 2000; Perry et al., 2008) and required more sessions to reduce responding during extinction of METH self-administration compared to males (Cox et al., 2013). Systemic D_1R blockade impaired extinction consolidation (Fricks-Gleason et al., 2012), while D_1R activation enhanced consolidation (Abraham et al., 2016) in cocaine conditioned place preference. Local infusion of D_1R antagonists into the infralimbic PFC (ilPFC) impaired fear extinction consolidation (Hikind and Maroun, 2008; Sotres-Bayon et al., 2009). The ilPFC also seems to be involved in extinction of drug-seeking behaviors, as pharmacological inactivation of the IL using GABA agonists resulted in increased drug-seeking behavior (LaLumiere et al., 2010). These findings suggest that intra-IL infusion of a D_1R antagonist would also impair consolidation of extinction in drug SA paradigms, but this has not been previously reported to our knowledge. With regard to GluN2B in extinction, non-specific blockade of NMDARs with CPP has been shown to reduce the retention of extinction learning in rats trained to SA cocaine (Hafenbreidel, Todd, Twining, Tuscher, & Mueller, 2014). More selective blockade of GluN2B-containing NMDARs with ifenprodil infused directly into the IL has been shown to disrupt consolidation following extinction of a cocaine conditioned

place preference (Otis et al., 2014). In sum, blockade of either D_1R or GluN2B transmission increases drug-seeking during extinction in adults, but their role in heightened relapse risk in adolescents and females remains unclear.

During adolescence, D_1Rs and GluN2B receptors in the mPFC undergo considerable development. Notably, D_1Rs exhibit their peak expression around P40-44 (Andersen et al., 2000; Brenhouse et al., 2008; Brenhouse et al., 2013). Longer duration NMDAR transmission, which is characteristic of GluN2B-containing receptors, first appears around P45, and D_1R signaling is necessary for this GluN2B transmission (Flores-Barrera et al., 2014). NMDAR transmission, particularly that of GluN2B-containing receptors (Sarantis et al., 2009), is potentiated by D_1R activation beginning after pubertal onset (Tseng & O'Donnell, 2005), and may underlie adult-like PFC functions (Gilmartin et al., 2013; Wang et al., 2013; Zhao et al., 2005). Drugs of abuse during adolescence may detrimentally impact these developmentally regulated changes in D_1Rs and GluN2B-containing receptors leading to long-lasting reductions in PFC function. Consistent with this notion, our lab has previously reported reduced mPFC D_1R function (Kang et al., 2016a) and region-specific decreased D_1R expression in the mPFC following adolescent AMPH exposure (Kang et al., 2016b). It is not known if these drug-induced changes in D_1Rs also impact the adolescent emergence of GluN2B signaling, but Ser1303 phosphorylation of GluN2B—the residue that increased in phosphorylation in response to D_1R stimulation in adults (Sarantis et al., 2009)—was decreased following adolescent (P28-42) cocaine exposure when assessed 24 hours later (Caffino et al., 2018). Taken together, these studies suggest that the reduction in D_1Rs from adolescent drug exposure may preclude GluN2B functional development in the mPFC, ultimately leading to impaired extinction consolidation and increased drug-seeking. However, no studies to date have reported investigation of GluN2B

function in relation to heightened drug-seeking in adolescents. Furthermore, the research to date has been almost exclusively limited to male subjects, preventing the study of potential interactions between age and sex in conferring vulnerability to drug-seeking.

The present study seeks to address this gap by attempting to mimic adolescent-onset impairments in extinction consolidation and increased drug-seeking by manipulating GluN2B function in adult-onset rats of both sexes. Male and female rats that will be trained to self-administer METH beginning in adolescence or adulthood will undergo extinction followed by GluN2B antagonism to assess the role of GluN2B function in extinction consolidation and subsequent METH-seeking in a reinstatement test. In line with previous reports (Sotres-Bayon et al., 2009; Hikind & Maroun, 2008; Otis et al., 2014), we predict that GluN2B antagonism in adult-onset rats will impair extinction consolidation and increase drug-seeking behaviors. We hypothesize that this manipulation will have minimal effects in adolescent-onset rats, but that adolescent-onset rats will display worse extinction consolidation and greater METH-seeking compared to adult-onset controls. Consistent with reports of sex differences (Cox et al., 2013; Lynch & Carroll, 2000; Perry et al., 2008), we expect females to exhibit greater drug-seeking than males with adolescent-onset females potentially exhibiting the worst extinction consolidation and most METH-seeking. All our *a priori* hypotheses, methods, and data analysis plan were pre-registered in March 2018 on the Open Science Framework (https://osf.io/avkhf).

METHODS

Subjects

Sprague-Dawley rats (n = 60/sex), which were the offspring of 14 litters born in our facility from breeders purchased from Envigo (Indianapolis, IN), were assigned to this study.

The final subject numbers were 52 male and 56 females (n = 108 rats total) as some rats were removed from the study due to loss of catheter patency (adolescent: males = 2, females = 1; adult: males = 5, females = 3) or abnormal development (1 adolescent male). Rats were housed in a temperature-controlled room on a reverse 12:12 light/dark cycle (lights off at 0900) with food and water available *ad libitum*. All experimental procedures occurred during the dark phase of the cycle. The day of birth was assigned postnatal day (P) 1 and rats were weaned into cages of two to three same-sex rats on P22. Assignment to age-of-onset groups (adolescent- or adult-onset) was balanced as best as possible within a litter. Rats were weighed daily beginning on P25. Daily checks for physical markers to estimate pubertal onset—vaginal opening in females (Castellano et al., 2011) and preputial separation in males (Korenbrot et al., 1977)—began on P30 and continued until these signs of puberty were observed in all rats. This experiment was conducted over the span of 18 months with 5 cohorts each consisting of 12 rats per age-of-onset group. All experimental procedures were in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Illinois, Urbana-Champaign.

Drugs

(+) Methamphetamine HCL (METH; Sigma-Aldrich, St. Louis, MO) was dissolved in 0.9% saline and self-administered i.v. at a unit dose of 0.1 mg/kg/infusion (calculated as the weight of the salt). Thirty min prior to the reinstatement session, rats were injected with METH (1 mg/kg i.p.). Ro 25-6981, an antagonist selective for the GluN2B subunit of the NMDAR, was obtained from Hello Bio, Inc (Princeton, NJ) and dissolved in 1 part dimethyl sulfoxide (DMSO)

and 2 parts 0.9% saline. Ro 25-6981 or vehicle solution was injected i.p. immediately after each of the four short extinction sessions at a dose of 6 mg/kg (1 ml/kg volume).

Self-Administration

The experimental timeline for this study is shown in figure 5.1. On the day of surgery (P32 ± 2 days for adolescent-onset or P82 ± 2 days for adult-onset), rats were implanted with an indwelling catheter in the right jugular vein as described previously (Chapter 3, Hankosky et al., 2018). The self-administration procedures were the same as previously described (Chapter 4, Westbrook et al., *in press*). On P40 ± 2 days for adolescent-onset or P90 ± 2 days for adult-onset, rats were placed in the operant chambers for a 90-min habituation session during which the nosepoke ports were covered. The next day rats began 2-h short access (ShA) self-administration sessions wherein a response in the active port (always the right port) resulted in an infusion of 0.1 mg/kg/inf METH. Rats received 7 daily 2-h ShA sessions followed by 14 daily 6-h long access (LgA) sessions, which were capped at 120 infusions in order to prevent accidental overdose during LgA.

Extinction and Reinstatement

One day following the final LgA session, rats underwent four daily 30-min extinction sessions wherein nosepoke responses in the previously reinforced port had no programmed consequence. Immediately following these sessions, rats received a challenge injection of the GluN2B antagonist, Ro25-6981 (6 mg/kg, i.p.), or an equivalent volume of vehicle (1ml/kg, i.p.). This dose was chosen based on previous studies showing impairment of behavioral flexibility and fear extinction in adult rats (Dalton et al., 2008, 2011, 2012). For the next 4 days, rats

received daily 2-h sessions to assess extinction retention. Similar procedures have been used previously with cocaine self-administration to demonstrate the effects of infralimbic prefrontal cortex inactivation on extinction consolidation (LaLumiere et al., 2010) and in studies of impairment and facilitation of extinction consolidation following systemic administration of an NMDAR antagonist and a partial agonist, respectively (Hafenbreidel et al., 2014). The following day, a priming injection of METH (1 mg/kg, i.p.) was administered 30-min prior to a 2-h session under extinction conditions. This dose was based on a previous study examining sex differences in adult rats (Reichel et al., 2012a). Responding during this METH-primed session was compared to responding during the last extinction session as a measure of reinstatement of METH-seeking.

Data Analysis

Pre-registered Analyses. These analyses were planned *a priori* and detailed in the pre-registration of this study (https://osf.io/avkhf). Nosepokes into the reinforced and unreinforced ports, as well as infusions earned were measured. The number of infusions earned was multiplied by the unit dose (0.1 mg/kg/infusion) to calculate METH intake (mg/kg). Daily METH intake and first hour METH intake were used as the dependent variables in self-administration analyses. Between-subjects factors included sex, age-of-onset, and treatment; the within-subjects factor was session. Separate three-way mixed ANOVAs (sex x age-of-onset x session) were used to assess METH intake during ShA and LgA sessions. Escalation was assessed via separate one-way repeated measures ANOVAs within each sex and age-of-onset group with Tukey post-hoc comparisons of METH intake in each LgA session compared to the first LgA session. Cumulative METH intake (mg/kg) was also calculated for all self-

administration sessions and this measure was used as a covariate in the analyses of extinction and reinstatement responding.

Nosepokes into the previously reinforced port during short extinction sessions (sessions 1-4) were analyzed using mixed ANCOVAs with sex, age-of-onset, treatment, and session as factors. To assess extinction consolidation, mixed ANCOVA (sex x age-of-onset x treatment x session) was conducted on previously reinforced nosepokes during the long extinction sessions (sessions 5-8). Differences in previously reinforced nosepokes during the first 30 min of extinction session 5 were determined via three-way ANCOVA with sex, age-of-onset, and treatment as factors. Previously reinforced nosepokes during the last extinction session and the METH-primed reinstatement session were analyzed using a mixed ANCOVA with the factors of sex, age-of-onset, treatment, and session. Finally, comparison of previously reinforced nosepokes between the final extinction session and reinstatement session was conducted within each group using pre-planned Tukey post-hoc tests.

Exploratory Analyses. Additional analyses, which were not pre-planned, were performed to compare the results of this study to those in our recent work (Westbrook et al., *in press*) demonstrating age-of-onset differences in self-administration behavior. First, body weight was analyzed across the peri-adolescent period (P25-P74) using separate two-way mixed ANOVAs (age-of-onset x postnatal day) for each sex. Second, further analysis of escalation was conducted on METH intake during the first and last LgA sessions using a three-way mixed ANOVA (sex x age-of-onset x session). Lastly, during our pre-planned analysis of extinction responding, it was noted that there was no significant effect of session after accounting for the relationship of the covariate (cumulative METH intake). Therefore, exploratory analysis of previously reinforced nosepokes was assessed in the first 30 min of these sessions—the same

duration as the first four extinction sessions—to determine if rats extinguished their previously reinforced response across sessions. This analysis was performed using a four-way mixed ANCOVA (sex x age-of-onset x treatment x session) with cumulative METH intake as the covariate.

RESULTS

METH Self-administration

Body Weight. Analysis of body weight from P25 to P74 demonstrated the expected weight gain across postnatal days in *ad libitum* fed Sprague-Dawley rats (Fig. 5.2). However, adolescent-onset males had suppressed weight gain relative to their adult-onset counterparts that remained in their homecages during this time [Age-of-onset by postnatal day interaction: $F(49,2450)=3.15$, $p<0.0001$]. Post-hoc analyses indicated that this METH-induced suppression of weight gain began on P47 near the start of LgA sessions and persisted through P74 (all p's<0.01). In females, adolescent-onset and adult-onset groups did not significantly differ on any postnatal day in post-hoc tests, despite a significant age-of-onset by postnatal day interaction [$F(49,2646)=1.85$, $p=0.0003$]. Thus, the weight gain suppressing effect of METH was only present in adolescent-onset males.

Short Access. Three-way mixed ANOVA conducted on METH intake during ShA sessions (Fig. 5.3A) indicated a significant main effect of session [$F(6,104)=25.03$, $p<0.0001$] and an interaction of sex and session [$F(6,104)=2.56$, $p=0.0237$]. METH intake on the first session was significantly greater than subsequent ShA sessions (all p's<0.0001). However, post-hoc tests did not reveal significant sex differences for any of the ShA sessions.

Long Access. During LgA sessions (Fig. 5.3A), adolescent-onset rats earned more METH than adult-onset rats [main effect of age-of-onset: $F(1,104)=10.07$, $p=0.0020$]. METH intake was significantly escalated relative to the first LgA session's intake [main effect of session: $F(13,104)=10.14$, $p<0.0001$]. This effect was apparent starting on session 10 ($p=0.0114$) and remained escalated through the rest of the LgA sessions (all p's<0.01). One-way repeated measures ANOVAs revealed significant effects of session for each group [adolescent-onset: females, $F(13,364)=2.44$, $p=0.0035$, males, $F(13,338)=2.89$, $p=0.0006$; adult-onset: females, $F(13,336)=2.47$, $p=0.0032$, males, $F(13,312)=2.72$, $p=0.0012$]. Adolescent-onset females and males began escalating their METH intake on session 15 ($p=0.0214$) and 14 ($p=0.0479$), respectively, while adult-onset females and males started escalating on session 13 (females: $p=0.0030$, males: $p=0.0066$).

METH intake during the first hour of LgA sessions was also assessed with a three-way mixed ANOVA, which revealed a significant main effect of session [$F(13,104)=5.05$, $p<0.0001$] and a significant age-of-onset by session interaction [$F(13,104)=2.63$, $p=0.0032$]. Adolescent-onset rats, but not adult-onset rats, demonstrated significant escalation of their first hour METH intake, specifically on the last LgA session ($p=0.0411$); adolescent- and adult-onset groups did not significantly differ from each other on any LgA session. One-way repeated measures ANOVAs within each group showed significant main effects of session for each group, except adolescent-onset females [adolescent-onset males: $F(13,26)=7.95$, $p<0.0001$; adult-onset: females $F(13,26)=4.89$, $p=0.0003$, males $F(13,312)=2.09$, $p=0.0148$]. Notably, post-hoc tests comparing each LgA session to the first hour intake of the first LgA session only revealed significant escalation of first hour intake in adolescent-onset males on the last LgA session ($p=0.0451$).

Exploratory analysis of escalation during LgA sessions revealed that rats significantly escalated their METH intake from the first to the last LgA session (Fig. 5.3C; main effect of session [$F(1,104)=85.49$, $p<0.0001$]). Adolescent-onset rats had significantly higher METH intake than adult-onset rats [main effect of age-of-onset: $F(1,104)=15.61$, $p=0.0001$]. Finally, age-of-onset differences in measures of escalation of METH intake across sessions was further reflected in cumulative METH intake (Fig. 5.3D) across all self-administration sessions. Adolescent-onset rats earned higher cumulative METH intake than adult-onset rats [main effect of age-of-onset: $F(1,104)=9.78$, $p=0.0023$]. There were no significant sex differences or interaction of age-of-onset and sex.

Extinction and Reinstatement

Extinction. Nosepoke responses into the previously reinforced port were analyzed using 4-way ANCOVA with cumulative METH intake as the covariate (Fig. 5.4). During the first four extinction sessions, the relationship of the covariate and nosepokes depended on group and session [Table 1; 5-way interaction with covariate: $F(3,92)=3.04$, $p=0.0327$]. After accounting for cumulative METH intake, there were no remaining significant main effects or interactions. For the last four extinction sessions, the relationship between cumulative METH intake and previously reinforced nosepokes varied by session [Table 2; $F(3,92)=3.50$, $p=0.0186$]. After accounting for cumulative METH intake, there were no significant main effects or interactions. We also assessed extinction consolidation by analyzing previously reinforced responses during the first 30 min of extinction session 5. Four-way ANCOVA revealed no significant main effects or interactions after accounting for the covariate. However, the relationship of cumulative METH intake and previously reinforced responding did vary significantly depending

on sex and drug challenge [Table 3; F(1,92)=5.43, p=0.0220]. The positive correlation only reached statistical significance for vehicle-treated females.

Exploratory analysis of the first 30 min of extinction sessions 5-8 revealed that the positive correlation between cumulative METH intake and previously reinforced nosepokes depended on drug challenge and session [Table 4; F(3,92)=2.91, p=0.0385]. After accounting for the covariate, a significant drug challenge by session interaction remained [F(3,92)=3.14, p=0.0292]. Post-hoc tests did not reveal significant differences between drug challenge groups for any of the sessions, although both drug challenge groups significantly reduced their previously reinforced responses across sessions.

Reinstatement. Previously reinforced responses following a METH-priming injection were compared to responses during the final extinction session with a four-way ANCOVA (covariate: cumulative METH intake). After accounting for the significant positive relationship of cumulative METH intake and previously reinforced nosepokes [r^2=0.0261, p=0.0174; F(1,100)=7.51, p=0.0073], a significant sex by session interaction remained [F(1,99)=4.62, p=0.0340]. Males and females increased their previously reinforced responding (Fig. 5.5) during the METH-primed session compared to the final extinction session (p's<0.0001), but females reinstated their responding to a greater extent than males (p=0.0024). Reinstatement was also assessed within each group via pre-planned comparisons between sessions. Adolescent-onset females challenged with Ro25-6981 or the vehicle solution significantly reinstated their previously reinforced responding (p<0.0001 and p=0.0116, respectively). Adolescent-onset males challenged with Ro25-6981 significantly increased their responses (p=0.0282). Reinstatement of responding was also significant in adult-onset females challenged with the vehicle solution (p=0.0082). The remaining groups—adolescent-onset males challenged with

Ro25-6981, adult-onset males regardless of drug challenge, and adult-onset females challenged with Ro25-6981—failed to reach statistical significance in the pre-planned comparisons.

DISCUSSION

GluN2B-containing NMDA receptor transmission emerges during adolescence, which may contribute to the development of adult-like extinction consolidation. Disruption of this developmentally regulated event by drugs of abuse may lead to enhanced drug-seeking behaviors, contributing to adolescents' heightened relapse rates. The goal of the present study was to investigate the potential role of GluN2B function in age-of-onset and sex differences in extinction consolidation and subsequent reinstatement following METH self-administration. We found that adolescent-onset rats have higher METH intake than adult-onset rats under long access conditions. Post-session GluN2B blockade did not impact drug-seeking during extinction and we found no age-of-onset or sex differences during extinction. In response to a METH priming injection, female rats, regardless of age-of-onset and previous drug challenge, reinstated their drug-seeking to a greater extent compared to male rats. Our hypothesis that disrupted GluN2B function contributes to age-of-onset and sex differences in drug-seeking during extinction was not supported here. However, our findings do suggest that adolescent-onset rats may more readily develop compulsive drug-taking, while females may be more susceptible to relapse above and beyond what would be predicted from their previous cumulative drug intake.

During ShA, METH intake for all groups was relatively stable except for the first session. Increased intake on the first session likely occurs because the nosepoke ports were made accessible for the first time and were thus more salient compared to the previous habituation session where the ports were covered. METH intake during subsequent sessions was consistent

with previous work reporting stable intake during ShA and age differences in escalation of intake during LgA (Anker, Baron, Zlebnik, & Carroll, 2012; Westbrook et al., *in press*). Adolescent-onset rats took more METH than adult-onset rats throughout the LgA sessions, and adolescent-onset rats showed significant escalation of intake in the first hour of the final LgA session, consistent with our recent report (Westbrook et al., *in press*). This increased drug-loading behavior during the first hour of access may indicate that adolescent-onset rats have developed habitual or compulsive drug-taking. This interpretation would be consistent with previous reports of adolescents exhibiting greater drug-taking in the face of negative consequences (Holtz and Carroll, 2015) and continued drug-seeking during periods of non-availability (Anker et al., 2011) compared to adult rats.

In the present study, we did not find evidence for sex differences in escalation during LgA. This lack of effect is not consistent with our previous report (Westbrook et al., *in press*) or others with METH (Reichel et al., 2012a) or cocaine (Algallal et al., 2019). In our previous study, the effect of sex that we reported appeared to be primarily driven by adolescent-onset females. The discrepancy in sex differences between our studies may be partly attributed to individual differences. In our first report, our adolescent-onset female group of 13 rats showed the greatest variability in LgA intake of all our groups. In contrast, all our groups in the current study have comparable variability that is reduced relative to our previous study due to the larger sample sizes (n=25-29). Therefore, it is possible that our previously reported sex effect driven by adolescent-onset females may have resulted from the sample containing a higher proportion of individuals with increased escalation. Neither of the studies from our lab, nor a recent report from another lab (Daiwile et al., 2019), are consistent with two studies that found adult females escalated their drug intake to a greater extent than adult males (Reichel et al., 2012a; Algallal et

al., 2019). This difference could be due to animal housing procedures between labs. Westenbroek and colleagues (2013) found that isolated females take more cocaine and escalate their motivation to respond for cocaine more than their isolated male counterparts, and pair-housing attenuates these effects in females. Individual housing may contribute to females' greater escalation of METH (Reichel et al., 2012a) and cocaine intake (Algallal et al., 2019), while our lab and the Cadet lab group-housed the rats and did not observe greater escalation in females (Westbrook et al., *in press*; Daiwile et al., 2019).

In the current study, we did not find evidence for any group differences in responding during extinction sessions following adolescent- or adult-onset METH self-administration. Previous reports of adolescent deficits in extinction consolidation were primarily studied in fear conditioning paradigms (for review, see Baker, Den, Graham, & Richardson, 2014); however there are some reports of greater responding in adolescents compared to adults during extinction of instrumental responding for food (Andrzejewski et al., 2011) or drug reinforcers (Anker and Carroll, 2010). The absence of an age-of-onset effect in our study may be due to the ages we tested or the housing conditions. In Anker and Carroll's study, the adolescent group began cocaine self-administration between P26 and P28 and concluded around 10 days later when extinction began. In our study, adolescent rats self-administered METH from P41-62. In the fear extinction literature, adolescent deficits in extinction consolidation have been shown to occur around P35 (McCallum et al., 2010; Kim et al., 2011). If there is a relatively short window of vulnerability for drugs of abuse to prevent the development of adult-like extinction consolidation, then it is possible that our study may have started self-administration after that window had passed while the Carroll lab's study targeted this window.

During the first four days of extinction, rats received systemic injections of a GluN2B-selective antagonist or vehicle solution. This drug challenge was given post-session, as opposed to before the session, in order to target the consolidation of the recent extinction learning that occurred in the short extinction sessions (LaLumiere et al., 2010; Hafenbreidel et al., 2014). The drug challenge did not appear to affect drug-seeking in any of our groups. One possibility is that the dose we selected for our antagonist challenge was not effective; however, this is unlikely given that we chose this dose based on previous literature showing its significant effects on fear extinction and behavioral flexibility in adult male rats (Dalton et al., 2008, 2011, 2012). Instead, the role of GluN2B transmission in extinction consolidation may depend on the type of extinction—Pavlovian vs. instrumental. In adult male rodents, extinction of Pavlovian fear conditioning was disrupted by systemic GluN2B antagonism (Dalton et al., 2012) and intra-infralimbic GluN2B blockade impaired extinction consolidation of environmental cues in a cocaine conditioned place preference paradigm (Otis et al., 2014). In contrast, GluN2B blockade did not impact extinction of instrumental responding for a drug reinforcer in the present study or another study (Hafenbreidel et al., 2017).

Following extinction sessions, rats underwent METH-primed reinstatement. Rats reinstated their drug-seeking response during the reinstatement session relative to the final extinction session and this reinstated drug-seeking behavior was positively correlated with cumulative METH intake during self-administration. Females reinstated their drug-seeking response to a greater extent compared to males consistent with the previous literature (Reichel et al., 2012a; Cox et al., 2013; Ruda-Kucerova et al., 2015), and this sex effect was still evident after controlling for the positive relationship of cumulative METH intake on reinstated drug-seeking. Since we did not find group differences in performance during extinction sessions, our

finding of sex differences in reinstatement cannot be attributed to differential extinction learning or memory. Our assessment of METH-primed reinstatement occurred on a single day, so it is possible that the estrous cycle could impact our findings and contribute to the observed sex difference. We did not monitor estrous cycle in our females because it is unclear how to control for exposure to this invasive procedure in male rats. Moreover, it is unlikely that estrous cycle phase fully explains the sex difference in our study because previous studies have failed to demonstrate a significant effect of estrous cycle phase on measures of METH self-administration, extinction, and METH-primed reinstatement (Reichel et al., 2012a; Cox et al., 2013; Ruda-Kucerova et al., 2015).

Our finding of a sex effect in reinstatement was not influenced by previous drug challenge or age-of-onset. Previous examination of age differences in cocaine-primed reinstatement have found that adolescent heightened reinstatement is dose-dependent (Anker and Carroll, 2010). We selected our dose for the METH priming injection based on the dose-response curve for sex differences in METH-primed reinstatement conducted by Reichel and colleagues (2012). We chose the 1 mg/kg dose because it produced the most pronounced sex difference in adult rats; however, this dose may not be the most appropriate dose for observing age-of-onset differences in reinstatement. Future work using additional doses is needed to determine whether age-of-onset differences in METH-primed reinstatement are dose-dependent like is seen with cocaine-primed reinstatement.

In conclusion, our findings suggest that disruption of GluN2B functional development does not contribute to heightened drug-seeking behaviors. Adolescent-onset rats may more readily develop compulsive METH-taking, which may partly explain this population's heightened vulnerability to developing problems with drug abuse. Greater METH intake during

self-administration predicted greater reinstatement of drug-seeking. Although females did not have greater METH intake compared to males during self-administration, females still displayed greater reinstatement. This sex effect above and beyond the relationship of cumulative METH intake on later drug-seeking, suggests that there are other mechanisms that underlie female vulnerability to relapse besides drug intake. Future investigations are needed to examine the underlying mechanisms of adolescent enhanced susceptibility to transition from controlled to compulsive drug-taking and female vulnerability to relapse.

FIGURES

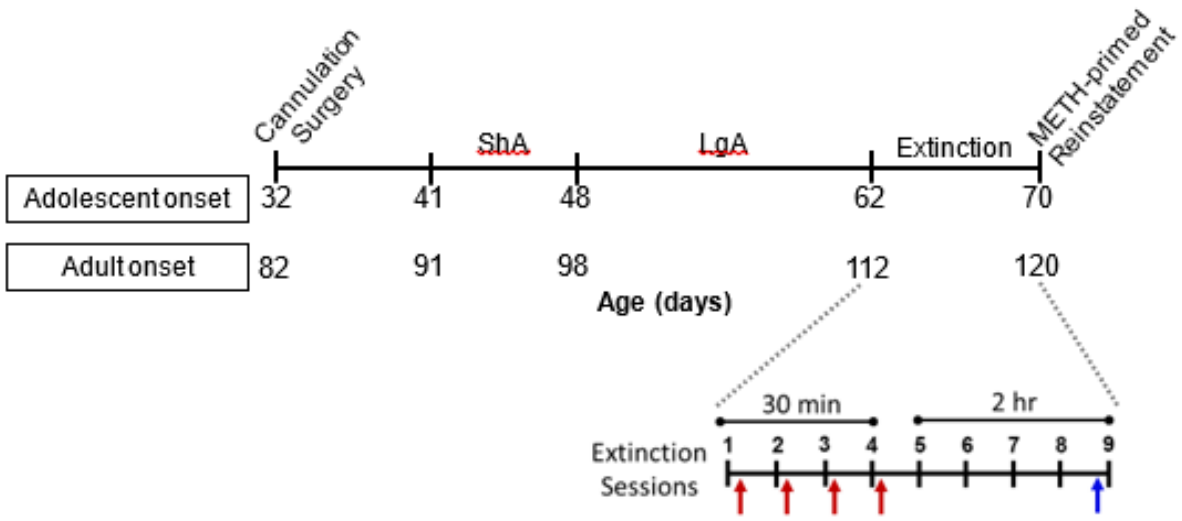

FIGURE 5.1. Experimental timeline for rats that underwent short access (ShA) followed by long access (LgA) METH self-administration. The injection schedule for the extinction sessions and METH-primed reinstatement is shown in more detail. Arrows indicate the timing of i.p. injections: red = 6 mg/kg Ro25-6981 or vehicle given after the session; blue = 1 mg/kg METH given before the session

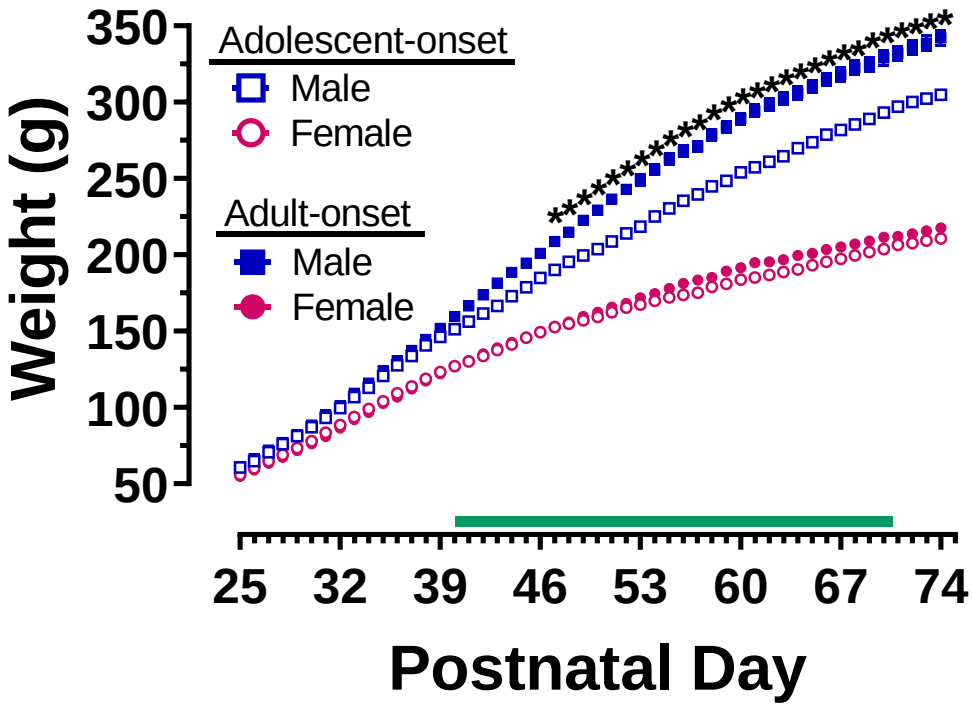

FIGURE 5.2. Body weights (g) for rats that began METH self-administration sessions during adolescence or adulthood. The horizontal green bar indicates the time period when adolescent-onset rats were performing experimental sessions. During this time, their adult-onset counterparts remained in their homecages. *$p < 0.05$ vs. adolescent-onset males on the noted days

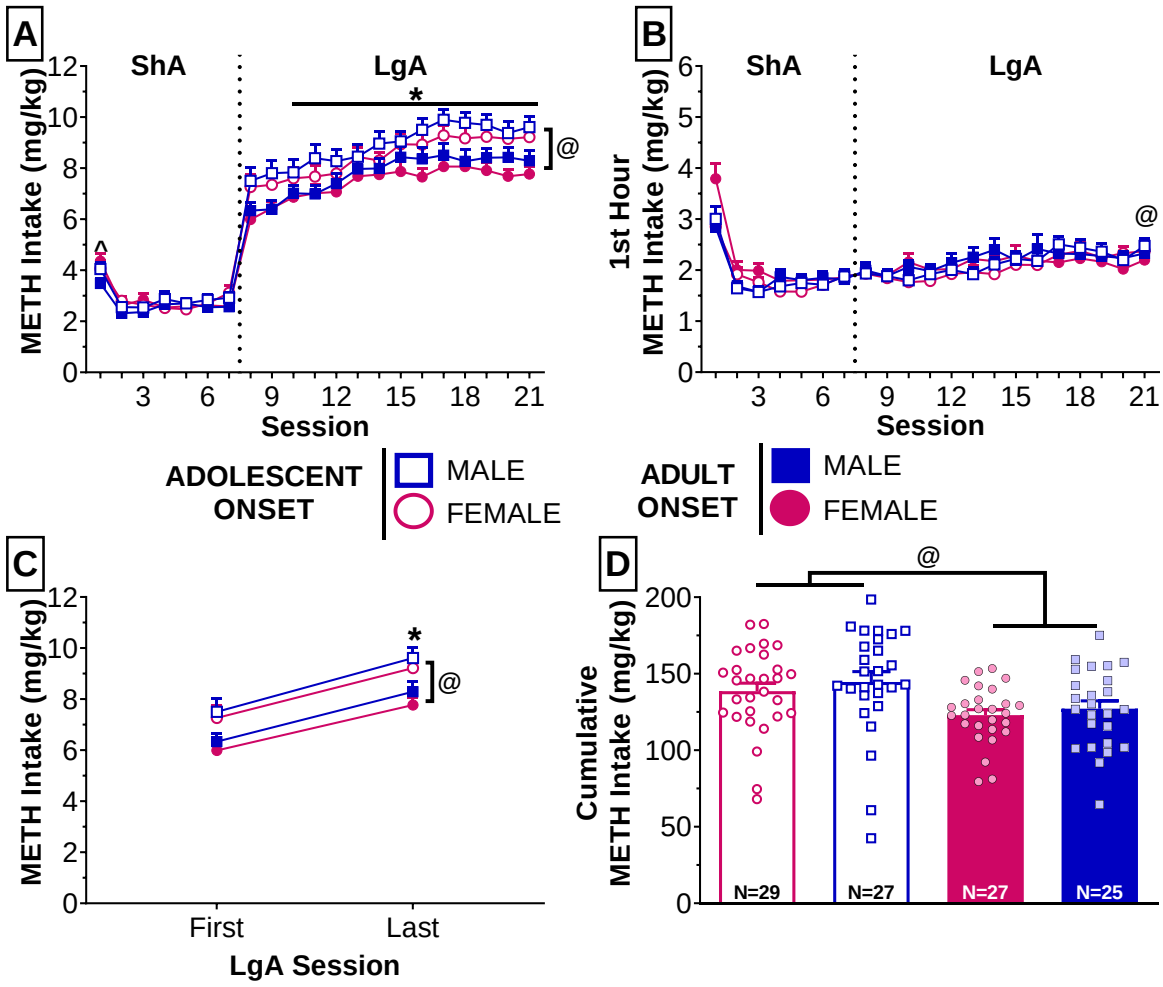

FIGURE 5.3. METH self-administration that began during adolescence or adulthood in rats of both sexes. (**A**) METH intake (mg/kg) during 7 ShA (2-h) sessions followed by 14 LgA (6-h) sessions. $^{*}p < 0.05$ vs. session 8 (first LgA session) collapsed across sex and age-of-onset, $^{@}p < 0.01$ collapsed across sex and session. (**B**) METH intake (mg/kg) during the first hour of the daily self-administration sessions. $^{@}p < 0.01$ vs. session 8 (first LgA session) for adolescent-onset rats collapsed across sex. (**C**) METH intake (mg/kg) during the first and last LgA sessions. $^{*}p < 0.05$ vs. session 8 (first LgA session) collapsed across sex and age-of-onset, $^{@}p < 0.01$ collapsed across sex and session. (**D**) Cumulative METH intake (mg/kg) summed across all self-administration sessions. $^{@}p < 0.01$ collapsed across sex

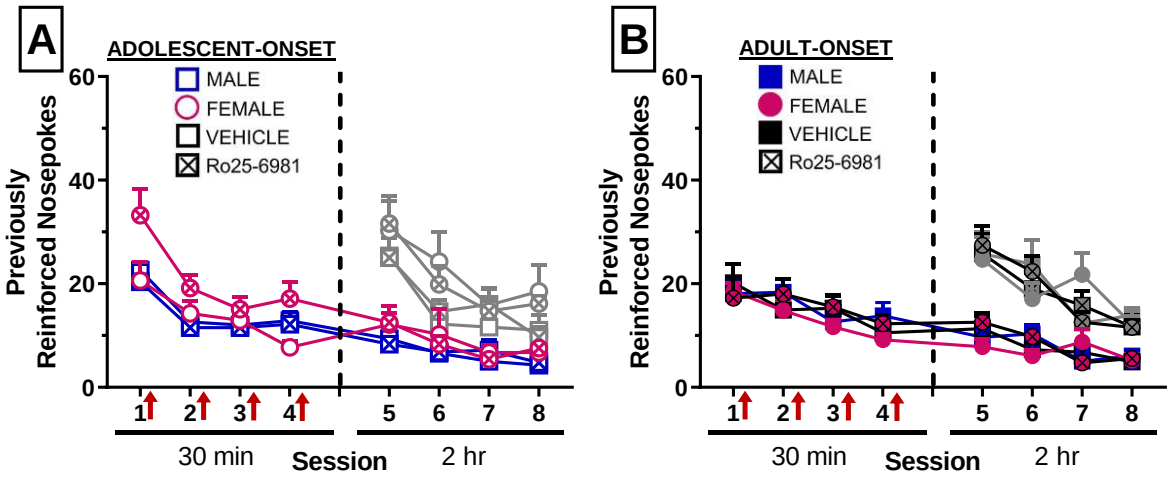

FIGURE 5.4. Responses into the previously reinforced nosepoke port during sessions under extinction conditions. For 2-h sessions, responding during the first 30 min is shown in line with the previous four sessions that were each 30 min in duration; gray symbols represent responding during the entire 2-h session. (Adolescent-onset: n = 13-15/group; Adult-onset: n = 12-14/group)

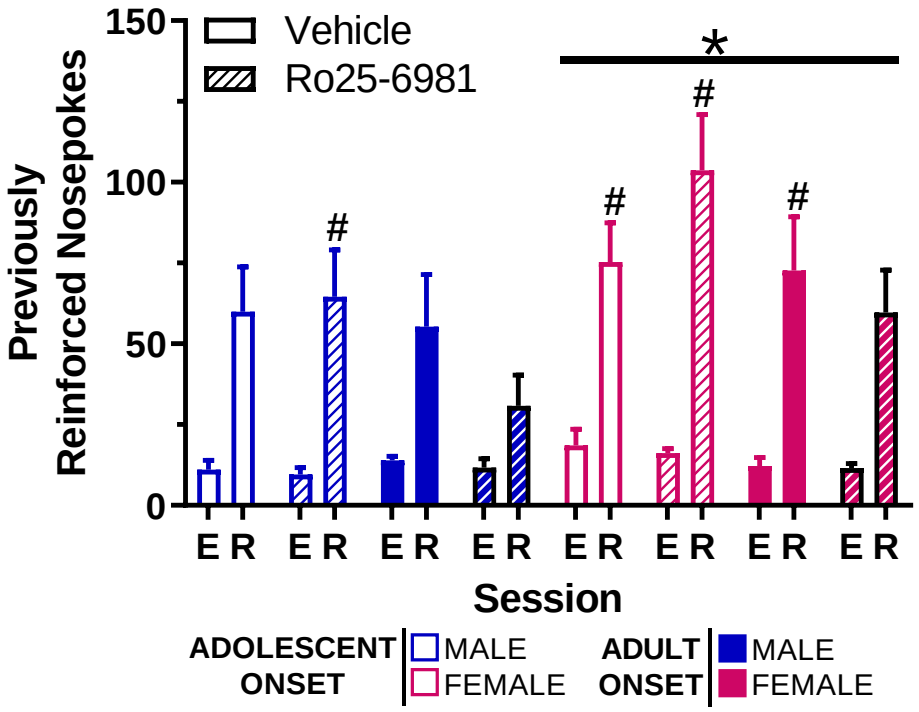

FIGURE 5.5. Previously reinforced nosepoke responses during the final extinction session (E) compared to the METH-primed reinstatement (R) session. *$p < 0.01$ vs. males for the reinstatement session collapsed across age-of-onset and drug challenge group, $^{\#}p < 0.05$ vs. last extinction session within group. (Adolescent-onset: n = 13-15/group; Adult-onset: n = 12-14/group)

CHAPTER 6: GENERAL DISCUSSION

Human epidemiological studies have shown that adolescent-onset and female drug users experience worse outcomes of their drug use than adult-onset and male users (Piazza et al., 1989; Brecht et al., 2004; Chen et al., 2009; Poudel and Gautam, 2017). More adolescent female users reported METH as their primary drug of choice compared to adolescent male users (Rawson et al., 2005), and females tend to initiate METH use earlier than males (Hser et al., 2005; He et al., 2013). These findings suggest that the vulnerability factors of age and sex may interact to confer increased susceptibility to developing problems with METH use in adolescent females. Despite growing interest in the neural and behavioral mechanisms underlying these vulnerability factors, most research to date examines these factors individually. The goal of this dissertation was to address this gap in knowledge by investigating the potential interaction of age and sex in conferring vulnerability to METH use and its neurobehavioral consequences. In four empirical investigations (Chapters 2-5), I used a rat model to assess age and sex differences in PFC-sensitive behaviors associated with drug use (Chapter 2), patterns of METH use (Chapters 3-5), and the consequences of METH use (Chapters 4-5). Each of these studies contributes to the overall objective by examining adolescent and adult rats of both sexes in order to determine whether age and sex interact when it comes to developing problems with METH use and the outcomes of that use. The findings are summarized below (Fig. 6.1).

In Chapter 2, I presented evidence for hypersensitivity to non-drug reward in the context of an outcome devaluation and probability discounting task. In adolescents, it was more difficult to devalue a palatable reward using sensory-specific satiety than in adults. Furthermore, adolescents and females responded during both tasks in a way that maximized their

reinforcement rate. These results suggest that adolescents and females are more sensitive to reward than their adult and male counterparts. This conclusion is further supported by Chapter 4, where I reported that adolescent-onset and female rats self-administer more of the non-drug reinforcer, saccharin, than their counterparts when given long access sessions. However, greater reward sensitivity is not always displayed in these groups. The study in Chapter 3 found that age-of-onset and sex differences in acquisition of METH self-administration under short access conditions depend on dose, with adults acquiring more readily at the lowest dose and females acquiring more readily at the highest dose tested. Although all groups earned similar METH intake once self-administration was acquired, adolescent-onset rats were less motivated for all doses of METH tested. When given extended access to self-administer, adolescent-onset rats of both sexes earn more METH than their adult-onset counterparts as seen in Chapters 4 and 5. The work in Chapter 4 also demonstrated a modest impairment of PFC-sensitive object-in-place recognition memory in rats with a history of METH use that began during adolescence compared to adulthood. However, these age-of-onset dependent differences in METH intake and METH-induced cognitive dysfunction were not associated with changes in expression of dopamine D1 receptor or NMDA receptor subunits in corticolimbic regions. In Chapter 5, I provide evidence for sex-dependent differences in relapse potential. Specifically, females, regardless of age-of-onset, were more susceptible to METH-primed reinstatement of a previously extinguished METH-seeking response than males. While the results of this dissertation do not support a role for disrupted ontogeny of PFC dopaminergic and glutamatergic signaling, the findings do provide novel insights into the interaction, or lack thereof, of age and sex in the behavioral mechanisms of vulnerability to problem METH use.

Work in this dissertation highlights the complexities of susceptibility to METH use and its consequences and add to a growing literature on age and sex as vulnerability factors and their neurobehavioral mechanisms. Findings with non-drug reinforcers from Chapters 2 and 4 are consistent with previous studies reporting reward hypersensitivity in adolescents and females in both humans (Galvan et al., 2006; Shulman et al., 2015) and animal models (Friemel et al., 2010; Marshall et al., 2017). Importantly, the opposing results from Chapter 3 with regard to METH self-administration calls attention to the nuanced effects of sex and age-of-onset on METH-seeking. Our findings suggest that the proclivity for these groups to self-administer drugs may not be absolute, but rather depends on many factors including methodology, such as dose (Chapter 3) and duration of access (Chapters 3-5). This conclusion is in line with findings from a number of previous studies (Kosten et al., 2000; Walker et al., 2008; Westenbroek et al., 2013; Baarendse et al., 2014; Lewis et al., 2016).

Chapter 4 and 5 elucidate some of the age-of-onset and sex-dependent consequences of METH use that may ultimately contribute to differences in relapse to drug-seeking in adolescent-onset and female users. One behavioral mechanism conferring relapse vulnerability is escalation of drug intake. In adult male rats, escalation of METH intake was associated with greater relapse risk in a METH-primed reinstatement model (Rogers et al., 2008). When given extended access in Chapter 4, rats that started METH self-administration as adolescents escalated their METH intake and earned more METH than their adult counterparts. This effect appears to be quite robust as we replicated this finding in Chapter 5 with almost double the sample size. This increased METH intake could be due to age differences in the development of tolerance, development of compulsive drug-taking, and/or sensitivity to METH. A previous study (Zombeck et al., 2009) reported similar concentrations of drug in the brain of adolescent and

adult mice given i.p. injections of 1, 2, or 4 mg/kg METH, suggesting that an age difference in pharmacokinetic tolerance is unlikely to be a major contributor to age difference in METH escalation. Rather, age differences in the development of compulsive drug use is likely to contribute to escalation as one study using METH (Anker et al., 2012) and a few others with cocaine (Anker and Carroll, 2010; Anker et al., 2011; Holtz and Carroll, 2015) report that adolescents continue drug seeking despite negative consequences to a greater extent than adults. Moreover, the study in Chapter 3 provides evidence for reduced sensitivity to METH in adolescents, which could also contribute to the age difference in LgA METH intake. More research is needed to determine the relative contribution of reduced METH sensitivity versus enhanced compulsive METH-taking in adolescents' METH escalation.

In addition to escalation of intake, other behavioral mechanisms can contribute to relapse potential, such as drug-induced cognitive dysfunction (Rogers and Robbins, 2001; Gould, 2010). Human METH users experience cognitive dysfunction during abstinence that is the most severe in individuals who relapse to drug use (Simon et al., 2004). In Chapter 4, we found only a modest deficit in PFC-sensitive recognition memory in rats, regardless of sex, with a history of adolescent- compared to adult-onset METH self-administration. Despite the reported age-of-onset differences in escalation and METH-induced cognitive dysfunction (Chapter 4 and 5), we did not find significant age differences in our model of relapse, METH-primed reinstatement of drug-seeking (Chapter 5). Instead, we found that females are more prone to relapse than males, which is in line with previous research (Reichel et al., 2012a). There are many different triggers to relapse (stress, cues, etc.), and age differences in relapse can depend on these triggers (Anker and Carroll, 2010). Studies using cocaine self-administration have reported that adolescent-onset rats show greater stress- and cue-induced relapse relative to adult-onset rats (Wong and

Marinelli, 2015; Zbukvic et al., 2016). Taken together, our findings suggest that age-of-onset and sex may contribute to relapse, but it appears these factors do so through different behavioral mechanisms.

In conclusion, the research in this dissertation suggests that adolescents and females are hypersensitive to rewards in many, but not all, contexts, with these vulnerability factors interacting and likely contributing to METH use. Once METH use is established, the vulnerability that is conferred by age-of-onset and sex appear to be dissociable. Adolescent-onset use is more likely to lead to compulsive METH-taking and cognitive dysfunction, while female users have a greater propensity for METH-induced relapse. Further research is needed to determine the neural bases of these mechanisms of vulnerability as the dopaminergic and glutamatergic mechanisms assessed in this dissertation did not appear to underlie these vulnerability factors. Importantly, the work in this dissertation suggests that treatment programs may be more effective if they are tailored to address the specific behavioral mechanisms of problem METH use in adolescent-onset and female populations.

FIGURE

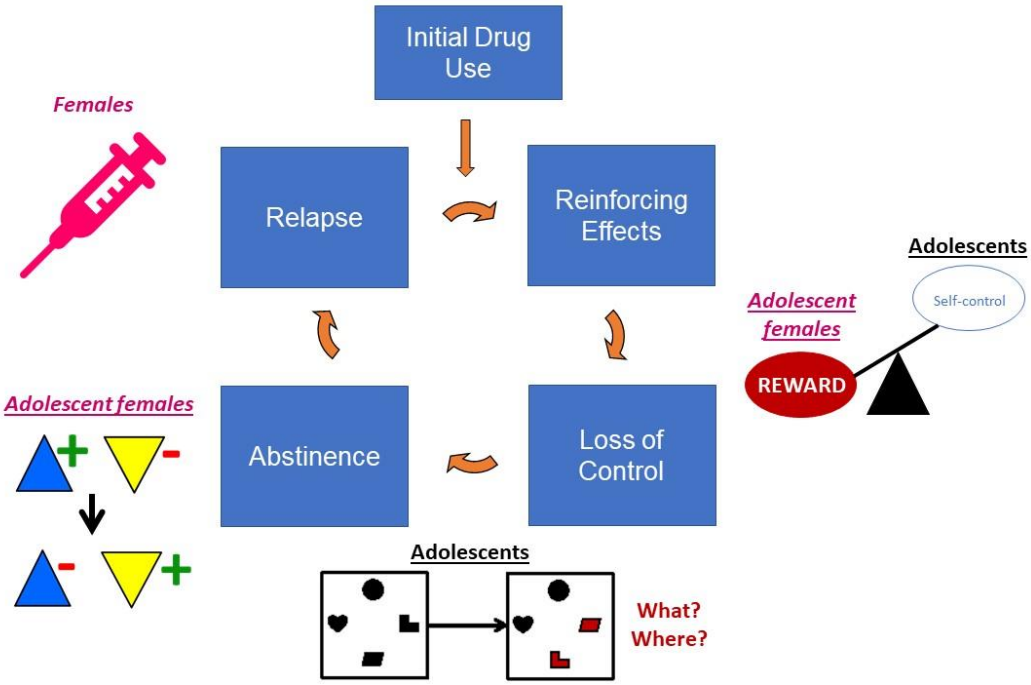

FIGURE 6.1. Summary of the findings in this dissertation. We examined the potential interaction between age-of-onset and sex as factors that contribute to different aspects of the cycle of substance use disorder in a rat model. Our findings suggest that adolescent females display heightened sensitivity to reward, while adolescents regardless of sex exhibit a greater loss of control of drug intake compared to adults, regardless of sex. During abstinence, adolescents are modestly impaired in episodic memory, while adolescent females show deficits in cognitive flexibility. Finally, females, regardless of age-of-onset, were more likely to relapse to drug-seeking compared to males.

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