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ATTENUATED EFFECTS OF OPIATES IN ADOLESCENT VS. ADULT MALE RATS:

REINFORCEMENT, RELAPSE, AND WITHDRAWAL

by

JAMES M. DOHERTY

Under the Direction of Kyle J. Frantz

ABSTRACT

Adolescence in humans is a vulnerable period for illicit drug use, and teenage onset of drug use is associated with long-term addiction. Adolescent sensitivity to drug reinforcement, relapse, and withdrawal has not been explored thoroughly in animal models, especially considering opiate drugs such as morphine and heroin. The present series of studies profiles adolescent sensitivity to opiates using adolescent and adult male rats to test for age differences in opiate self-administration, reinstatement, withdrawal signs, locomotor sensitization, and even brain activation during drug-seeking. To test for acute sensitivity to the reinforcing effects of morphine or heroin, we compared patterns of self-administration by adolescent vs. adult male rats on various schedules of reinforcement, drug doses, and daily access conditions. Using fixed ratio schedules

and short daily access, adolescents self-administered less morphine than adults, an effect commonly interpreted as higher drug sensitivity. In contrast, escalation of morphine intake under long access conditions was similar across ages, as was heroin intake using fixed or progressive ratio schedules of reinforcement. To test for enduring effects of opiates, we compared opiate-seeking in the absence of the drug in tests of extinction responding and cue-induced reinstatement. Regardless of the acute effects of morphine or heroin, all adolescent treatment groups showed attenuated opiate-seeking compared to adults. Next we considered behavioral correlates of reinforcement, drug withdrawal and locomotor sensitization, during and after escalating doses of experimenter-administered heroin. Consistent with attenuated opiate-seeking, adolescents exhibited attenuated somatic and locomotor signs of withdrawal compared with adults, although locomotor sensitization was similar across ages. Finally, the medial prefrontal cortex (mPFC) is a brain region heavily implicated in drug reinforcement, so we used tissue levels of Fos-like immunoreactivity to compare activation of this region by heroin-seeking. Indeed mPFC activation was absent in rats that self-administered heroin as adolescents, but robust in adults. Together these behavioral and neuroanatomical results surprisingly suggest that adolescent male rats are less sensitive than adults to some acute and enduring effects of opiates, and may predict better response profiles among younger human addicts. Through future studies, adolescent rats may provide a new model to help identify treatments for drug abuse.

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DEDICATION

To my parents, family, friends, and Hallie, whose love and support have made this possible.

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1 Introduction

The Introduction provided in Chapter 1 begins with a summary of drug use trends in humans, with a focus on opiate and synthetic opioid drug.use by adolescents. Next, the clinical definition of addiction is presented, and provides a framework for our animal research. Then, we review relevant literature using validated animal models of addiction to investigate drug-related behavior in adolescent rodents. Finally, we introduce the role of a brain region of focus for our research, the medial prefrontal cortex, which is heavily implicated in control of behavioral reinforcement and relapse to drug seeking after drug abstinence.

1.1 Chapter 1

Human drug use trends

Drug addiction is prevalent in the U.S. and typically initiated in adolescence. For example, more than 4 million people are addicted to the opiate drug, heroin, costing our society billions of dollars annually (Mark et al. 2001). Adolescents in the U.S. have a high rate of heroin use, with at least 250,000 high school students reporting heroin use already at least once in their lifetime, a trend that has held steady for decades. Adolescents and young adults aged 12-25 years old are also more likely than any other age group to abuse pain relievers. In fact, opioid narcotics have become the third most frequently abused drugs among 12th graders (behind alcohol and marijuana), and adolescent use of two synthetic opioids (Oxycontin and Vicodin) has increased dramatically since 2002 (Johnston et al. 2010; SAMHSA 2009). The majority of early-onset and adult opiate addicts are boys and men, but girls and women also report high rates of opiate abuse (Johnston et al.

2010; SAMHSA 2009). Moreover, opioidergic pain killers are prescribed for pediatric

patients, despite a lack of knowledge about potential long-term detrimental effects in

young people (Carlezon and Konradi 2004; Duedahl and Hansen 2007). Perhaps of

greater concern, numerous studies suggest that such early onset of drug use increases the

chances of later drug addiction (Anthony and Petronis 1995; Clark et al. 1998; Kandel et

al. 1992; Palmer et al. 2009). Whether or not these trends can be attributed to a biologi-

cal vulnerability associated with the adolescent phase of development is unclear. There-

fore, the use of animal models of adolescent drug intake is critical for investigating the

potential role of biological vulnerability during adolescence in drug use and addiction.

Adolescent sensitivity to drug reinforcement, relapse, and withdrawal has not been ex-

plored thoroughly in animal models, especially considering opiate drugs such as mor-

phine and heroin.

Opiates are direct derivatives of the opium poppy plant, where as opioids are syn-

thesized chemicals with opiate-like action but not derived directly from the opium poppy

plant (Waldhoer et al. 2004). Heroin, morphine, and most abused opiates and synthetic

opioids clearly produce rewarding and reinforcing effects mainly through activation of

mu opioid receptors (reviewed in Pasternak 2001). Opiates and synthetic opioids are pre-

scribed clinically as pain killers, with long-term use associated with tolerance and emer-

gence of withdrawal symptoms upon cessation of use. Heroin is classified as a "Schedule

I" drug, and morphine as a "Schedule II" drug, by the U.S. Drug Enforcement Agency,

indicating they have high abuse potential and none or few therapeutic applications.

Clinical definition of addiction: A framework for animal research

It is important to frame our studies in adolescent vs. adult animals within the current clinical definitions of drug addiction, because we are investigating the proposed relationship between early-onset drug use and long-term addiction, with the goal of eventually identifying and testing potential treatments for addicts in various life stages. Drug addiction is a chronically relapsing disorder that is characterized by the following components: 1) compulsion to seek and take drugs; 2) loss of control in limiting intake; and 3) emergence of a negative emotional state reflecting a motivational withdrawal syndrome when access to the drug is prevented (i.e. "substance dependence" according to the Diagnostic and Statistical Manual of Mental Disorders [DSM-IV] of the American Psychiatric Association 1994). From these three clinical phases of human addiction, basic researchers have outlined three corresponding stages of the addiction cycle that can be modeled using animals (Koob and Volkow 2010). For each stage, validated animal models have been developed to permit investigation of specific elements of the process of drug addiction. Investigators use animal models of self-administration, locomotor sensitization, conditioned place preference, and brain stimulation reward thresholds to study the binge/intoxication stage. Investigators use signs of withdrawal, tests of anxiety, conditioned place aversion, and brain stimulation reward thresholds to study the withdrawal/negative affect stage. Investigators use reinstatement of drug-seeking to study the preoccupation/anticipation stage (Koob and Volkow 2010). Few experiments explore the effects of drug intake during adolescence within the framework of these three addiction stages. Therefore, the overall aim of this dissertation is to characterize the effects of opiates in adolescent vs. adult male rats using at least one validated model for each stage of addiction outlined above.

The present series of studies profiles adolescent sensitivity to opiates, using adolescent and adult male rats to test for age differences in opiate self-administration, extinction responding, reinstatement of drug-seeking, withdrawal signs, locomotor sensitization, and brain activation during drug-seeking. For the purposes of this work, we define "sensitivity" as the capacity to express behavioral susceptibility to the acute or long-term effects of morphine or heroin. To model the binge/intoxication stage, we tested morphine or heroin self-administration as well as a behavioral correlate of reinforcement, locomotor sensitization after repeated injections of heroin. To model the withdrawal/negative affect stage, we tested for the severity of somatic and locomotor signs of withdrawal after repeated systemic heroin injections. To model the preoccupation/anticipation stage, we first tested for extinction responding in the absence of morphine or heroin, and reinstatement of drug-seeking in rats that self-administered either morphine or heroin as adolescents (adolescent-onset groups) or adults (adult-onset groups), then we tested for a neural correlate of drug-seeking: Fos, the protein product of the immediate early gene *c-fos*, in the medial prefrontal cortex after heroin-seeking.

Adolescent rodents in validated animal models of addiction

Generally, rodent models have good face validity for studying the behavioral and physiological changes associated with human adolescence (Adriani and Laviola 2004; Smith 2003; Spear 2000). Adolescence in rodents, often termed periadolescence because a precise definition is elusive, may be limited to approximately postnatal days (P) 28 through 60, with the range for males wider and delayed compared to females (Schneider 2008; Spear 2000; Spear and Brake 1983). In many species, including both primates and ro-

dents, this transition from youth to adulthood is characterized by robust behavioral, metabolic, hormonal, morphologic and neurochemical changes (Spear 2000). For example, adolescents exhibit high levels of social interaction and play (Brown 1990; Panksepp 1981), high levels of risk-taking, sensation-seeking, or novelty-seeking (Adriani et al. 1998; Douglas et al. 2003; Zuckerman 1992), and perhaps elevated basal or novelty-stimulated motor activity (Spear and Brake 1983; Stansfield and Kirstein 2006), 2006), although not all studies confirm the latter effect (Bolanos et al. 1998; Frantz et al. 2007; Frantz and Van Hartesveldt 1999). Adolescent rodents exhibit a growth spurt and undergo gonadarche, a peri-pubertal increase in gonadal hormones associated with the process of sexual maturation, although rodents do not undergo a different developmental process associated with puberty in humans known as adrenarche, an increase in output of adrenal hormones that begins prior to other signs of impending adolescence (Spear 2000). Prominent neural alterations occur in many brain regions during adolescence. For example, a transient overexpression of dopamine receptors occurs in reward related brain areas such as the prefrontal cortex (PFC) and nucleus accumbens (NAcc) (Andersen and Teicher 2000; Andersen et al. 2000). Also, evidence exists of blunted second messenger system activity in neurons that possess dopamine receptors (Andersen 2002). Many of the distinctive characteristics of adolescence reviewed above are similar for many species, including adolescent rodents.

When some of the above mentioned characteristics of adolescents are displayed by subsets of adult rodents, they have been associated with a higher propensity to self-administer drugs of abuse (Ambrosio et al. 1995; Belin et al. 2008; Piazza et al. 1989). Coupled with high levels of drug use in humans during adolescence, these findings have

suggested that adolescence could be a critical period of heightened vulnerability to the reinforcing effects of drugs, and highlighted the need for more research on adolescent exposure to drugs using animal models (Adriani and Laviola 2004; Crews et al. 2007; Laviola et al. 1999; Spear 2000). Compared with other "gateway drugs" such as alcohol and marijuana, as well as psychomotor stimulants such as nicotine and cocaine, opiates are the least studied in adolescent subjects.

Although experiments testing opiates or opioids in adolescent subjects are rare, adolescent rodents do appear differentially sensitive to some effects of the prototypical opiate, morphine, or the synthetic opioid, oxyocodone, compared with adults. For example, weanling and adolescent male rats became tolerant to the analgesic effects of repeated morphine injections more quickly than older males (Ingram et al. 2007; Wang et al. 2005). With regard to motor activity, adolescent male rats are more sensitive to the psychomotor stimulating and motor sensitizing properties of morphine compared to adults (Spear et al. 1982; White et al. 2008; White and Holtzman 2005). Results are mixed on age differences in morphine conditioned place preference (CPP), an animal model that tests for rewarding effects of drugs; adolescent male rats completely failed to show a preference in one study (Bolanos et al. 1996), whereas adolescent and adult, male and female rats all showed similar levels of morphine place preference in another (Campbell et al. 2000). The intravenous (i.v.) drug self-administration model is the gold standard measure of drug abuse liability because of its high face validity (Meisch 1982; Schuster and Thompson 1969). However, i.v. drug self-administration has not been used adequately to test for sensitivity to opioids or opiates in adolescent rodents. In the single study testing opioid self-administration aside from research in the present dissertation,

adolescent male mice self-administered less oxycodone than adult male mice, and adolescents were more sensitive than adults to oxycodone-stimulated extracellular dopamine in the striatum after self-administration testing (Zhang et al. 2009). Together these results suggest that responsivity to opiates changes during development, and that specific tests on the reinforcing effects of opiates are necessary.

The slightly more extensive body of rodent research on psychostimulants also suggests that drug-related behaviors are age-dependent. Results vary across drugs such as nicotine-, cocaine-, amphetamine-, or methylphenidate, with regard to motor activity or conditioned place preference, with some studies suggesting more sensitivity to the effects of the drugs during adolescence than adulthood (Badanich et al. 2008; Caster et al. 2005) and other studies suggesting similar sensitivity (Badanich et al. 2008; Belluzzi et al. 2004; Caster et al. 2005; Niculescu et al. 2005; Torrella et al. 2004). For cocaine self-administration, no age differences were noted (Frantz et al. 2007; Kerstetter and Kantak 2007; Li and Frantz 2009). For nicotine self-administration, adolescent rats self-administered more nicotine compared to adults in some studies (Belluzzi et al. 2005; Levin et al. 2007; Levin et al. 2003), but not another (Shram et al. 2008a). For amphetamine self-administration, adolescent rats self-administered more drug than adults (Shahbazi et al. 2008). Clearly, results in adolescent rodents vary across drug and subject populations, and the topic requires further investigation.

One factor hypothesized to contribute to drug use and drug-seeking in humans and experimental animals is relief from aversive states associated with drug withdrawal (Frenois et al. 2005; Kenny and Markou 2005a; Kenny et al. 2006). Mounting evidence suggests, however, that adolescent subjects exhibit less aversive drug withdrawal than

adults. For example, adolescent male rats exhibit fewer physical and affective signs of nicotine withdrawal (Infurna and Spear 1979; Natividad et al. 2010; O'Dell et al. 2006; O'Dell et al. 2007; Shram et al. 2008b; Wilmouth and Spear 2004) and fewer "hangover-like" effects of ethanol (Doremus et al. 2003; Varlinskaya and Spear 2004). The drop in extracellular levels of dopamine in the NAcc during precipitated withdrawal from nicotine is also attenuated in adolescents (Natividad et al. 2010). Lastly, adolescent mice exhibit less affective withdrawal from morphine compared to adult mice, as measured in forced swim and locomotor tests (Hodgson et al. 2009). Although not tested in a state of withdrawal, adolescent male rats are also less sensitive to the aversive properties of nicotine-, amphetamine-, or ethanol as measured by conditioned taste aversion (Infurna and Spear 1979; Vetter-O'Hagen et al. 2009; Wilmouth and Spear 2004). Given that human opiate addicts report severe withdrawal symptoms and relief from withdrawal as a driving force for continued drug use (reviewed in Frenois et al. 2005; O'Connor and Fiellin 2000), it is surprising that withdrawal from opiates has not been thoroughly studied in adolescent vs. adult non-human subjects.

The medial prefrontal cortex (mPFC) is heavily implicated in control of reinforcement and relapse behavior

The medial prefrontal cortex (mPFC) is a brain area uniquely poised to influence the behavioral effects of drugs during adolescence. The mPFC may be responsible for representation of goals, assignment of value to the goals, and selection of actions based on the resulting valuation (Hyman 2005; Miller and Cohen 2001; Perry et al. 2011). The prefrontal cortex is also a critical regulator of relapse behavior in human drug addicts and in

experimental animals (Goldstein and Volkow 2002; Kalivas and Volkow 2005; Volkow et al. 2003). Processing of drug-cue associations is in part controlled by dopaminergic modulation of glutamatergic output to subcortical targets, including the NAcc (Feltenstein and See 2008; LaLumiere and Kalivas 2008; Piazza et al. 1991). Furthermore, the mPFC is suggested to impart cognitive control over goal-directed behavior by gating or inhibiting behavior (Perry et al. 2011). Developmental characteristics of the mPFC may also provide a sensitive substrate for the adverse effects of drugs during adolescence. Adolescent humans (Lenroot and Giedd 2006; Sowell et al. 1999) and rodents (Kalsbeek et al. 1988) have immature prefrontal cortex circuitry, and human and non-human adolescents exhibit mPFC-mediated behaviors exemplified by poor inhibitory control and risky judgment (Donohew et al. 1999; Spear 2000). An underdeveloped prefrontal cortex in adolescents could also increase sensitivity to relapse "cues" (Adriani and Laviola 2004; Spear 2000). Furthermore, a drug insult during maturation may alter the trajectory of development in the immature PFC of adolescents. Although no studies directly compare adolescent and adult male rats on the effects of opiates within the mPFC, developmental differences in the endogenous opioid, dopamine, cannabinoid, and GABAergic systems within the mPFC presumably influence age-dependent drug effects (Adriani and Laviola 2004; Andersen et al. 2000; Brenhouse et al. 2008; Crews et al. 2007; O'Donnell; Perry et al. 2011; Spear 2000; Talbot et al. 2005; Tseng et al. 2007; Tseng and O'Donnell 2007).

1.2   Highlights of Chapters 2-5

The findings reviewed above leave open the question that adolescence could be a developmental period associated with increased detrimental effects of drug use. Nevertheless, a paucity of studies testing the rewarding and reinforcing effects of opiates during ado-

lescence exists. This is especially surprising, give that most addicts initiate drug use during adolescence, including heroin and opioid narcotic addicts. Thus, the aim of this dissertation was to test adolescent vs. adult male rats in several specific models of addiction: 1) we tested morphine or heroin self-administration using different access and reinforcement conditions, as well as extinction and reinstatement of drug-seeking following abstinence; 2) we tested the severity of withdrawal from repeated systemic heroin injections by measuring somatic and locomotor signs of spontaneous withdrawal, as well as heroin-induced locomotor sensitization; and 3) we tested a possible neural correlate of attenuated heroin-seeking by adolescents, using the induction of Fos, the protein product of the immediate early gene, *c-fos*, as a marker of neural activity and neuroplasticity within the medial prefrontal cortex during heroin-seeking.

1.3   Introduction to Chapters 2 and 3

The goals of the experiments in Chapters 2 and 3 were to explore the binge/intoxication and preoccupation/anticipation stages of addiction. First, we used the animal model of i.v. drug self-administration, which is known as the best predictor of drug abuse liability because it has good face validity (Ator and Griffiths 2003). We specifically tested for acute sensitivity to the reinforcing effects of two of the most commonly used and abused opiates, morphine (Chapter 2) or heroin (Chapter 3), by comparing the patterns of self-administration in adolescent vs. adult male rats on various schedules of reinforcement, drug doses, and daily access conditions. For morphine self-administration, we tested an "escalation model" that might mirror the transition from recreational to compulsive drug use in humans (Ahmed et al. 2000). Specifically, we compared morphine intake between conditions of short and long daily access to morphine (1-hr vs. 8-hr per day; ShAcc vs.

LgAcc, respectively), in rats that acquired self-administration in adolescence or adulthood. For heroin self-administration, we tested the reinforcing potency and efficacy of heroin using fixed and progressive ratio schedules of reinforcement in rats that acquired self-administration in adolescence or adulthood. For both Chapters, the overarching hypothesis was that adolescent male rats would be more sensitive to the reinforcing effects of morphine or heroin than adult male rats, as based on the evidence mentioned above that adolescent male rats were more sensitive to the psychomotor stimulating or sensitizing properties of morphine (Spear et al. 1982; White et al. 2008; White and Holtzman 2005), and that adolescent male mice were more sensitive to the reinforcing effects of self-administered oxycodone and oxycodone-stimulated extracellular dopamine in the striatum (Zhang et al. 2009). For morphine, we predicted that higher sensitivity to both acute and long-term effects of morphine in adolescents would result in lower rates of self-administration under ShAcc conditions, but faster and higher levels of escalating intake under LgAcc conditions, compared to adults. For heroin, we predicted that higher sensitivity to heroin in adolescents would result in lower rates of heroin self-administration on a fixed ratio (FR) schedule of reinforcement compared to adults. Given that testing a variety of schedules of reinforcement contribute to full characterization of a drug's reinforcing effects (Arnold and Roberts 1997), we also tested a separate cohort of rats on a progressive ratio (PR) schedule in which lever-press requirements increased exponentially within each session. We predicted that higher sensitivity to heroin in adolescents would result in a higher maximum number of infusions per session compared to adults, i.e. a higher break point on the PR schedule.

The second goal of the experiments in Chapters 2 and 3 was to explore the preoc-cupation/anticipation stage of addiction, given that long-lasting vulnerability to relapse to drug-taking during periods of abstinence is a major challenge for the treatment of drug addiction (Chung and Maisto 2006; O'Brien 1997). To test for enduring effects of opiates, we examined morphine-seeking (Chapter 2) or heroin-seeking (Chapter 3) in the absence of the drug, as measured by extinction responding and/or cue-induced reinstate-ment. Drug-seeking can be defined as responding on the drug-associated lever during extinction or reinstatement testing, and is an animal model of human drug craving and relapse following abstinence (Ahmed et al. 2000; Shaham et al. 2003). Generally, drug-seeking after abstinence from i.v. drug self-administration can be triggered by re-exposure to the drug-taking environment (context), to discrete cues previously paired with drug infusions (cues), to stress, or to the drug itself (reviewed in Shaham et al. 2003). In fact, the number of lever presses during context- (Shalev et al. 2001) or cue-induced (Grimm et al. 2003) reinstatement increases over time in abstinence for adult male rats, a phenomenon known as 'incubation of drug craving' (Bossert et al. 2005). In Chapter 2, we compared age-dependent extinction of drug-seeking and cue-induced reinstatement of morphine-seeking across morphine access groups (ShAcc vs. LgAcc; Chapter 2). In Chapter 3, we compared age-dependent extinction of drug-seeking and cue-induced reinstatement of heroin-seeking across heroin abstinence periods (1 vs. 12 days abstinence from FR testing) or cue-induced reinstatement of heroin-seeking after 12 days abstinence from PR testing.

Existing evidence suggests that adolescent subjects are less sensitive than adults to some enduring effects of drugs that may drive drug-seeking behavior. For example,

rats that self-administered cocaine as adolescents exhibited attenuated 'incubation' of co-caine-seeking compared to adults (Li and Frantz 2009). Rats that self-administered nico-tine as adolescents extinguished their drug-seeking faster than those that took the drug as adults (Shram et al. 2008a) and adolescents rats, unlike adults, did not exhibit signs of nicotine-cue conditioning, and displayed less robust nicotine-induced locomotor sensiti-zation (Schochet et al. 2004). Adolescent rats also exhibited faster extinction and less "reinstatement" of cocaine conditioned place preference (Balda et al. 2006, but see Bren-house and Andersen 2008 for opposite results), a lack of cross-sensitization to cocaine self-administration following systemic MDMA exposure during adolescence (Frantz and Parsons 2001), and less severe cognitive impairment in an amygdala-dependent task after cocaine intake (Kerstetter and Kantak 2007, but see Harvey et al. 2009 for greater im-pairment after cocaine intake in a orbitofrontal task). Thus, our hypothesis was that ado-lescent male rats would be less sensitive to the long-term effects of self-administering morphine or heroin as adolescents than adults. Our prediction was that rats that self-administered morphine or heroin as adolescents would exhibit less extinction responding and less cue-induced reinstatement of drug-seeking compared to adults.

1.4   Introduction to Chapter 4

The goal of the experiments in Chapter 4 was to explore the withdrawal/negative affect stage of the addiction cycle, along with another model of the binge/intoxication stage. Thus, we considered two behavioral correlates of drug reinforcement, withdrawal and locomotor sensitization, during and after escalating doses of experimenter-administered heroin. Withdrawal from repeated opiate administration in animals is characterized by the emergence of a general negative affective state (Higgins and Sellers 1994) and by

many somatic signs (Gellert and Holtzman 1978). Aversive withdrawal contributes to drug-seeking in acute and long-term conditions, through the process of negative reinforcement (Frenois et al. 2005; Kenny et al. 2006; Kenny and Markou 2005b). However, withdrawal severity has not been specifically tested in adolescent vs. adult rats made dependent on heroin. Thus, we used an experimenter-administered, escalating dose regimen, intended to produce dependence on heroin (modified from Antonilli et al. 2005; Ventayol et al. 1997; Yang et al. 2006; Zhou and Kalivas 2008), and then tested for somatic and locomotor signs of spontaneous withdrawal after abrupt cessation of the drug injections. Our hypothesis was based on results from our lab (e.g. Chapters 2 and 3) showing attenuated long-term consequences of drug exposure in adolescents compared to adults (Doherty et al. 2009; Li and Frantz 2009), along with the mounting evidence reviewed above suggesting less severe withdrawal from nicotine, amphetamine, or ethanol in adolescents vs. adults (Chen et al. 2006; Doremus et al. 2003; Infurna and Spear 1979; Natividad et al. 2010; O'Dell et al. 2007; Shram et al. 2008b; Varlinskaya and Spear 2004; Wilmouth and Spear 2004). Additional data show less affective withdrawal from morphine, as measured during forced swim and locomotor tests, in adolescent compared to adult mice (Hodgson et al. 2009). Therefore, our hypothesis was that adolescent male rats would exhibit less severe withdrawal from heroin than adults, and we predicted that escalating doses of heroin administered to younger rats should produce fewer somatic signs of spontaneous withdrawal within five days of the last heroin injection.

The second goal of the experiments in Chapter 4 was to explore the binge/intoxication stage of addiction by testing heroin-induced locomotor sensitization in adolescent vs. adult male rats. Locomotor sensitization may be a behavioral correlate of

reward and reinforcement, and perhaps even relapse to drug-seeking behavior (Vander-schuren and Pierce 2010), and therefore is a useful model to study the neural basis of drug addiction (Hyman and Malenka 2001; Robinson and Berridge 2000; Vanderschuren and Kalivas 2000). Long-term sensitized locomotor responses occur in adult rats after repeated morphine injections (reviewed in Paolone et al. 2007; Pontieri et al. 1997; Ranaldi et al. 2009; Vanderschuren and Kalivas 2000), and adolescent male rats exhibit increased morphine-stimulated motor activity and greater long-term locomotor sensitization compared to adults (Spear et al. 1982; White et al. 2008; White and Holtzman 2005). However, to our knowledge heroin-induced locomotor sensitization, per se, has not been tested in adolescent vs. adult male rats. It is unclear if results with morphine can generalize to heroin, as we have observed some contradictory behaviors in adolescent vs. adult male rats tested with morphine vs. heroin [adolescents self-administer less morphine in Chapter 2 (Doherty et al. 2009), but slightly more or similar amounts of heroin in Chapter 3 (Doherty and Frantz, in press)]. Thus, along with testing for adolescent vs. adult withdrawal from heroin in the same rats, we tested for heroin-induced locomotor sensitization. We measured locomotor activation after challenge doses of heroin during, and 12 days after, the experimenter-administered escalating heroin dose regimen. Based on less acute and sensitized locomotor activity after repeated morphine in adolescent vs. adult male rats (Spear et al. 1982; White et al. 2008; White and Holtzman 2005), our hypothesis was that adolescent male rats would exhibit less heroin-induced locomotor sensitization than adult male rats.

1.5   Introduction to Chapter 5

The goal of the experiment in Chapter 5 was to explore the preoccupation/anticipation stage of addiction further by testing for a possible neural correlate of the attenuated drug-seeking we had observed in rats that self-administered drugs as adolescents, vs. adults. To date we have focused on the medial prefrontal cortex (mPFC) because this brain region is heavily implicated in drug reinforcement and relapse, and known to be a critical controller of relapse behavior in humans and animals (Goldstein and Volkow 2002; Kalivas and Volkow 2005; Volkow et al. 2003). We know that adolescent humans and other mammals have immature prefrontal cortex circuitry (Adriani and Laviola 2004; Lenroot and Giedd 2006; Spear 2000), and this underdeveloped prefrontal cortex could increase sensitivity to the effects of drugs (Adriani and Laviola 2004; Spear 2000). Interestingly, the prelimbic and infralimbic subregions of the mPFC may play opposing roles during drug-seeking, to promote and inhibit cocaine-seeking, respectively (Peters et al. 2009), although a recent report suggests that heroin-seeking, per se, may be promoted by activity in either subregion (Bossert et al. 2011). We determined the proportion of Fos immuno-reactive (Fos-ir) positive neurons activated within the prelimbic and infralimbic areas during a one hour heroin-seeking test that occurred after 12 days abstinence from heroin self-administration in adolescent- vs. adult-onset male rats. Fos is the protein product of the immediate early gene (IEG) *c-fos*. Induction of *c-fos* marks stimulus-elicited brain activity (Chaudhuri 1997; Harlan and Garcia 1998), and is a marker of neuroplasticity associated with acute drug use (Nestler 2001). Up-regulation of *c-fos* levels in the mPFC occurs during context- or cue-induced reinstatement of heroin-seeking in adult rats (Bossert et al. 2011; Koya et al. 2006; Kuntz et al. 2008; Schmidt et al. 2005). We used com-

puter-guided, unbiased stereology, a highly accurate analysis technique, to estimate the overall number of Fos-ir+ and Fos-ir- neurons over the entire rostral-to-caudal extent of the prelimbic and infralimbic mPFC subregions. We hypothesized that the mPFC of adolescent-onset experimental groups would show less activation (a lower proportion of Fos-ir+ neurons) during heroin-seeking than adult-onset groups, and less activation in mPFC would correlate with attenuated heroin-seeking in adolescent-onset groups.

1.6   Summary

In this series of experiments, we have gathered data from adolescent vs. adult male rats that help to determine whether age-at-onset of opiate intake influences: 1) the acute reinforcing effects of morphine or heroin; 2) the enduring effects of morphine or heroin on relapse behavior; 3) the severity of withdrawal from heroin; 4) the intensity of heroin-induced locomotor sensitization; and 5) mPFC activation during heroin-seeking. The results described herein provide insight into the consequences of adolescent opiate use, using validated animal models of human addiction. Our data add to a growing body of literature that may provide knowledge toward developing new targets for drug abuse treatment in adolescent addicts that could ultimately offer better prognosis for teenagers seeking treatment.

Table 1.1 Outline of Chapters

2	Age-dependent morphine intake and cue-induced reinstatement, but not escalation in intake, by adolescent and adult male rats
	Tests short vs. long access self-administration conditions for 24 d, followed by15 d abstinence, 18 d extinction, and a single test of cue-induced reinstatement of morphine-seeking
3	Self-Administration of Heroin and Reinstatement of Heroin-Seeking in Adolescent vs. Adult Male Rats
	Experiment 1 tests self-administration at a fixed ratio one schedule of reinforcement for 13 d across 2 doses, followed by a single 7-hr test of extinction responding (6-hrs) and cue-induced reinstatement of heroin-seeking (1-hr) after 1 vs. 12 d abstinence. *Experiment 2* tests self-administration at fixed ratio 1-5 schedules of reinforcement for 9 d, self-administration at a progressive ratio schedule of reinforcement for 6 d across 3 doses, followed by a single test of cue-induced reinstatement of heroin-seeking after 12 d abstinence
4	Attenuated effects of experimenter-administered heroin in adolescent vs. adult male rats: physical withdrawal and locomotor sensitization
	Tests somatic and locomotor signs of spontaneous withdrawal and locomotor sensitization during, and 12 d after, a 13 d escalating dose regimen
5	Attenuated Fos immunoreactivity in prelimbic and infralimbic medial prefrontal cortex during heroin-seeking behavior in adolescent vs. adult male rats
	Tests for Fos-ir in the medial prefrontal cortex during a single test of heroin-seeking after 12 d abstinence from heroin self-administration

2 Chapter 2 - Age-dependent morphine intake and cue-induced reinstatement, but not escalation in intake, by adolescent and adult male rats

by

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2.1 Abstract

Despite increasing rates of opioid abuse by human adolescents, few laboratory experiments address adolescent vulnerability to opiates. We examined intravenous morphine self-administration after adolescent- vs. adult-onset, followed by extinction and cue-induced reinstatement. Adolescent male Sprague-Dawley rats [postnatal day (P) 35 at start] and adults (P91) acquired lever pressing maintained by 0.375 mg/kg/infusion morphine on a fixed ratio one schedule of reinforcement. Subjects were subsequently divided into short or long daily access conditions (ShAcc, 1-hr vs. LgAcc, 8-hr; 18 sessions). After extinction, cue-induced reinstatement was recorded over 1 hr. During the first six 1-hr acquisition sessions and continuing throughout ShAcc conditions, adolescent-onset rats self-administered less morphine than adults, an effect commonly interpreted as higher drug sensitivity. In contrast under LgAcc conditions, escalation of morphine intake was similar across ages. Extinction of drug-seeking was similar across ages, although rats from LgAcc conditions pressed more than ShAcc conditions. Notably, cue-induced reinstatement was less robust in rats that began morphine self-administration during adolescence vs. adulthood. Although increased sensitivity of younger rats to morphine reinforcement under ShAcc conditions might help explain opioid abuse by human adolescents, lower rates of reinstatement in younger rats might suggest that adolescent development includes some protective factors that dampen the long-term impact of early drug intake.

Keywords: narcotic; periadolescent; ontogeny; relapse; extended access; limited access; drug loading; in vivo.

2.2 Introduction

Adolescent and young adult humans aged 12-25 years old are more likely than any other age group to abuse pain relievers (SAMHSA 2009). Specifically, opioid narcotics are the third most frequently abused drugs among 12th graders (behind alcohol and marijuana), and adolescent use of two synthetic opioids (Oxycontin and Vicodin) has increased dramatically since 2002 (Johnston et al. 2010). Drug use and abuse among adolescents is particularly alarming because initiation of drug use during adolescence may increase the propensity for addiction in adulthood (Anthony and Petronis 1995; Clark et al. 1998; Kandel et al. 1992). Moreover, opioidergic pain killers are prescribed for pediatric patients, despite a lack of knowledge about potential long-term detrimental effects in young people (Carlezon and Konradi 2004; Duedahl and Hansen 2007). These human use trends call for basic research on adolescent vulnerability to drugs of abuse, particularly opioid narcotics.

Rodent models have good face validity for studying the behavioral and physiological changes associated with human adolescence (Adriani and Laviola 2004; Smith 2003; Spear 2000). Adolescence in rodents, often termed periadolescence because a precise definition is elusive, may be limited to approximately two weeks between postnatal days (P) 28 and 42 (Spear 2000; Spear and Brake 1983). In many species, including both primates and rodents, transition from youth to adulthood is characterized by robust behavioral, morphologic, metabolic, hormonal, and neurochemical changes (Spear 2000). For example, adolescents exhibit high levels of social interaction and play (Brown 1990; Panksepp 1981), high levels of risk-taking, sensation-seeking, or novelty-seeking (Adriani et al. 1998; Douglas et al. 2003; Zuckerman 1992), and perhaps elevated basal or no-

velty-stimulated motor activity (Spear and Brake 1983; Stansfield and Kirstein 2006), although not all studies confirm the latter effect (Bolanos et al. 1998; Frantz et al. 2007; Frantz and Van Hartesveldt 1999). When some of these characteristics are displayed by adult rodents, they are associated with a higher propensity to self-administer drugs of abuse (Ambrosio et al. 1995; Belin et al. 2008; Piazza et al. 1989). Coupled with high levels of drug use in humans during adolescence, these findings suggest that adolescence could be a critical period of heightened vulnerability to the reinforcing effects of drugs, perhaps including opioid narcotics (Adriani and Laviola 2004; Crews et al. 2007; Laviola et al. 1999; Spear 2000).

Indeed adolescent rats are differentially sensitive, compared with adults, to some physiological effects of the prototypical opiate morphine. For example, weanling and adolescent male rats (P21 and P28-35) become tolerant to the analgesic effects of re-peated morphine injections more quickly than older males (Ingram et al. 2007; Wang et al. 2005). With regard to motor activity, acute morphine injections stimulate more loco-motion in adolescent (P35) vs. adult male rats (Spear et al. 1982), and repeated morphine injections induce more motor sensitization among P30-32 male rats than P65-67 males (White and Holtzman 2005). Results are mixed on age differences in morphine condi-tioned place preference; P35 male rats completely failed to show a preference in one study (Bolanos et al. 1996), whereas adolescent and adult, male and female rats all showed similar levels of morphine place preference in another (Campbell et al. 2000). Together these results suggest that responsivity to opiates changes during development, and that specific tests on the reinforcing effects of opiates are necessary.

The first aim of the present study was to compare the reinforcing effects of morphine between adolescent and adult male Sprague-Dawley rats in the intravenous (i.v.) drug self-administration model. Therefore, we allowed adolescent rats (P35 at start) or adults (P91 at start) to acquire lever pressing maintained by morphine in 1-hr (ShAcc) daily sessions on a fixed ratio one (FR1) schedule of reinforcement (Kruzich et al. 2003).

The second aim of our study was to explore morphine self-administration in an escalation model that might mirror the transition from recreational drug use to compulsive addiction in humans (Ahmed et al. 2000). Thus, we compared morphine intake between conditions of short and long daily access to morphine (1-hr vs. 8-hr per day; ShAcc vs. LgAcc, respectively), in rats that acquired self-administration in adolescence or adulthood, generally following protocols from Ahmed et al. (2000) and Walker et al. (2003). In those studies, ShAcc conditions resulted in stable daily drug intake over several weeks, whereas LgAcc conditions of either 8- or 11-hrs per day produced gradual escalation to a new, higher rate of daily drug intake.

The third aim was to analyze the long-term effects of morphine intake using animal models of drug craving and relapse following abstinence (Ahmed et al. 2000; Shaham et al. 2003). Thus, extinction of drug-seeking in the absence of morphine, and cue-induced reinstatement of drug-seeking was compared across age and access (ShAcc vs. LgAcc) groups.

Based in part on the high rates of drug use during adolescence among humans (Johnston et al. 2010; SAMHSA 2009), as well as experiments suggesting rapid physiological and behavioral adaptations to morphine among adolescent rats (Ingram et al. 2007; Spear et al. 1982; Wang et al. 2005; White and Holtzman 2005), we hypothesized

that rats that begin self-administration during adolescence are more sensitive than rats that begin in adulthood to both acute and long-term effects of morphine. Thus, adolescent-onset rats should take less morphine under ShAcc conditions, but should escalate faster and to higher levels of drug intake under LgAcc conditions than adult-onset rats. Rats that acquire morphine self-administration during adolescence should also take longer than older adults to extinguish drug-seeking in the absence of morphine, and should reinstate drug-seeking in the presence of morphine-associated cues to a higher level than older adults.

2.3   Methods

Subjects

Male Sprague-Dawley rats (Zivic Miller; New Castle, PA) arrived in the laboratory at P22 (n=10) or P78 (n=16) for adolescent-onset or adult-onset age groups, respectively. Rats were housed in groups of two or three in a temperature and humidity controlled vivarium and maintained on a 12 hr light/dark cycle, with lights off at 0700 hr. All behavioral testing occurred at approximately the same time every day during the dark phase. Body weights were recorded daily to monitor health and to titrate drug doses. Food and water were freely available in home cages and self-administration chambers during long access conditions. All procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) and approved by The Institutional Animal Care and Use Committee (IACUC) of Georgia State University.

Drugs

Morphine sulfate (Mallinckrodt, Inc.; Hobart, NY), methohexital sodium (1%, Brevital Sodium, King Pharmaceuticals, Inc.; Bristol, TN), and Timentin antibiotic (GlaxoSmith-Kline; Research Triangle Park, NC), all were dissolved in sterile saline and filtered through a 25 μm syringe filter (Fisher Scientific, Inc.; Pittsburgh, PA) before i.v. administration.

Equipment

Morphine self-administration was conducted in operant chambers housed in sound-attenuating cubicles (MedAssociates, Inc.; St Albans, VT). Each chamber was equipped with two retractable levers. Only one lever was extended during morphine self-administration and reinstatement phases (see below). Pressing on the lever initiated a syringe pump with a 5 rpm motor (PVM-1000VS, Med Associates Inc.; St Albans, VT) to deliver an infusion via a stainless steel swivel and a polyethylene tube attached to the catheter portal on each subject's back. Drug delivery and data collection were controlled by a computer system using Med Associates software (Med PC IV).

Intravenous (i.v.) Catheter Implantation

Intravenous catheters were constructed as described (Caine and Koob 1993), with minor modifications (Shahbazi et al. 2008). Briefly, silastic tubing was fitted onto a guide cannula (Plastics One; Roanoke, VA) bent at a right angle and encased in dental cement anchored with a 2.5-cm circular mesh for subcutaneous, mid-scapular placement. The silastic tubing was 10 cm long for adolescent rats and 12 cm for adults.

Adolescent (P29-31) and adult (P84-86) rats were surgically catheterized in the right jugular vein, generally according to Caine et al. (1993), with minor modifications (Shahbazi et al. 2008). Briefly, rats were anesthetized with an isoflurane-oxygen vapor mixture (4-5% for initial anesthetization and 1.5-2.5% for the remainder of surgery). Catheter tubing was passed subcutaneously from the back and inserted into the right jugular vein (two cm for adolescents or four cm for adults) and tied in place with sutures. During recovery, adolescent and adult rats received 0.15 or 0.2 ml, respectively, of the antibiotic Timentin (Ticarcillin Disodium and Clavulanate Potassium; 100 mg/ml, i.v.) twice daily for two days post-surgery, then once daily for the remainder of the experiment. Catheters were also flushed daily with 0.15-0.3 ml heparinized saline (30 USP units/ml) to promote catheter patency. Catheter patency was tested one day before the start of experimentation and once per week on a day of recess from drug self-administration, by injecting 0.1-0.4 ml of a short-acting barbiturate anesthetic, Brevital, through the catheter. If muscle tone was not lost within 3 sec, the catheter was presumed defective and the subject was not included in the analysis.

Morphine Self-Administration

The drug dose was titrated daily based on individual body weight to administer 0.375 mg/kg/infusion morphine, and infusion volume was varied accordingly based on a 0.625 ml infusion over 5 sec for an adult rat weighing 350 g. This dose was mid-range+ based on pilot experiments (Doherty et al. 2006; Ogbonmwan et al. 2007). After each drug infusion, a 20-sec time out (TO) period was signaled by switching on a cue light above the lever and switching off a house light and white noise. Responses during TO were record-

ed but had no scheduled consequences. During the extinction phase only, a second lever was extended to record "non-specific" motor effects; presses on this inactive lever were recorded but had no scheduled consequences.

The four phases of behavioral testing appear in the experimental timeline (Table 2.1): pre-escalation for 6 daily sessions, escalation for 18 sessions, 15-day recess, extinction for 18 sessions and reinstatement for 1 session. Sessions were conducted six consecutive days per week, except for extinction sessions conducted five days per week. Adolescent and adult rats were counterbalanced across 14 test chambers. Following 5-7 days of post-surgical recovery, adolescents (P35 at start; n=10) and adults (P91 at start; n=16) were allowed to acquire lever pressing maintained by morphine on an FR1 schedule of reinforcement during six 1-hr sessions (pre-escalation). Subsequently, adolescent and adult rats were divided into four groups for the escalation phase, counterbalanced by body weight and rates of morphine self-administration averaged over the last three pre-escalation sessions. Two groups remained under conditions of 1-hr daily access to morphine (short access, or ShAcc; 5 adolescents and 7 adults), while two groups transitioned to extended access conditions of 8-hr per day (long access, or LgAcc; 5 adolescents and 9 adults). In two adolescent and two adult subjects in LgAcc conditions, self-mutilation was observed briefly during self-administration, but was immediately ameliorated by "chew toys" placed in the operant chambers.

Recess, Extinction and Reinstatement

After the escalation phase, all rats received a 15 day recess from testing. They were not exposed to test chambers or drugs, although they were handled and weighed periodically.

Daily catheter flushing ceased at the beginning of the recess. Extinction testing was conducted for 1-hr per day, 5 days per week over 3.5 weeks (total 18 sessions). Two levers were extended into the chamber (one previously active, one new but inactive), but presses produced no scheduled consequences (Ahmed et al. 2000). The second lever was added during extinction to record "non-specific" motor effects. Drug-associated cues were not presented, i.e. house light remained on, white noise and cue light remained off for the duration of each extinction session.

Cue-induced reinstatement of drug-seeking was tested in a single 1-hr session that began with non-contingent presentation of drug-associated cues, i.e. cue light above the active lever turned on and house light and white noise turned off for 20 sec. Subsequently, the previously active lever was extended into the chamber and each appropriate response resulted in cue presentation. Only one lever was present during reinstatement testing in order to mimic the drug-taking environment during self-administration (Ahmed et al. 2000).

Data Analysis

To assess possible effects of daily morphine intake on growth during adolescent development, body weights were analyzed during morphine self-administration, the first day of extinction testing, and one day after reinstatement. During self-administration, body weights were analyzed separately for each age group using a two-way between-within mixed measures analysis of variance (ANOVA), with access condition and sessions (repeated) as factors. Body weights before the first extinction session and the day after

reinstatement sessions were compared across access conditions using Student's t-tests for independent samples. In addition at P101, the only age at which weight was directly comparable between the younger and older age groups, body weights were analyzed using a two-way between subjects ANOVA, with age at onset and access condition as factors. Finally, as a measure of morphine dependence (Gellert and Holtzman 1978), the percent body weight lost during weekly abstinence from morphine self-administration (weekend recess of approximately 48 hrs) was analyzed using a three-way mixed measures ANOVA with age, access condition, and time (repeated measure) as factors.

For pre-escalation (sessions 1-6), the number of morphine infusions per session was compared using a two-way mixed measures ANOVA, with age and sessions (repeated) as factors. Total morphine intake (mg/kg) summed over all pre-escalation sessions was also compared across ages using an independent samples t-test. During the escalation phase (sessions 7-24), the number of infusions per session was compared within access conditions using two-way mixed measures ANOVAs, with age and sessions (repeated) as factors. A planned comparison between the first and last escalation sessions (session 7 vs. 24) was also conducted separately for all four age and access conditions using paired samples t-tests. Total morphine intake (mg/kg) summed over the entire escalation phase was compared across ages using independent samples t-tests. To compare outcomes from ShAcc vs. LgAcc conditions, the number of infusions taken during only the first 15 min "loading phase" of each session was analyzed using a three-way mixed measures ANOVA, with age, access condition, and sessions (repeated) as factors. To analyze control of behavior exerted by discriminative cues, the percentage of "inappropriate" lever presses was calculated as the sum of presses during drug infusion and TO,

divided by the total number of presses on the active lever. Percent inappropriate responding was analyzed using a three-way mixed measures ANOVA, with age and access condition as between subjects factors, and sessions as a repeated measure.

During extinction the number of lever presses per session was subjected to a four-way mixed measures ANOVA with age, access condition, lever (active vs. inactive), and sessions (repeated) as factors. For the cue-induced reinstatement test, the number of presses per session was analyzed in a two-way between subjects ANOVA with age and access condition as factors. To determine whether morphine intake during self-administration influenced lever pressing during reinstatement, a Pearson's correlation analysis was conducted on total morphine intake over 18 sessions and lever presses during reinstatement. In all cases, follow-up ANOVAs and post-hoc tests were conducted as appropriate. $P < 0.05$ was considered significant.

2.4   Results

Body Weight

Rats in both age groups gained weight throughout morphine self-administration (Fig. 2.1; panel a). However, adolescent-onset rats in the ShAcc condition gained more than adolescent-onset rats in the LgAcc condition, as suggested by a significant access X session interaction ($F_{17,136}=5.73$; $p < 0.001$), although the effect was not robust enough to reveal a significant effect of access condition on the last day of self-administration ($t_8=1.66$; $p = 0.14$). Also, there was only one age (P101) at which body weights of the adolescent- vs. adult-onset groups could be compared directly, i.e. the day of reinstatement testing for the adolescent-onset group vs. the fourth day of the escalation phase for the adult-onset group, as marked with a dagger symbol on Fig. 2.1a. At P101, a significant main effect of age at onset revealed that the adolescent-onset group weighed more than the adult-onset group ($F_{1,25}=13.32$; $p < 0.001$), regardless of access condition ($F < 1.0$; N.S.).

Loss of body weight, a classic sign of opiate withdrawal (Gellert and Holtzman 1978), was analyzed during each weekend recess period from self-administration (post-session Saturday to pre-session Monday; Fig. 2.1; panel b). Adolescent body weight gains declined across successive recess periods, while adult body weight losses increased over successive recess periods, as suggested by a trend toward a significant age X time interaction ($F_{2,44}=3.19$; $p=0.051$) in a mixed measures three-way age X access condition X time (repeated) ANOVA. A separate two-way ANOVA on body weight in only the adolescent-onset group confirmed that body weight gains declined across recess periods via a main effect of time ($F_{2,16}=18.09$; $p<0.001$), but no main effect of access condition

(F1,8=1.55; p=0.25) nor access condition X time interaction (F < 1.0; N.S.). Similarly a two-way ANOVA on body weight in only the adult-onset group confirmed that body weight losses increased across recess periods via a main effect of time (F2,28=3.76; p<0.05), but no main effect of access condition (F1,14=2.78; p=0.12) nor access condition X time interaction (F2,28=1.61; p=0.22) was recorded. Subjects in the LgAcc condition tended to either gain less (adolescents) or lose more (adults) body weight than subjects in the ShAcc condition, as revealed by a trend toward a main effect of access condition (F1,22=4.2; p = 0.052).

Pre-Escalation Phase of Morphine Self-Administration

Adolescent-onset rats took fewer infusions of morphine than adults during the pre-escalation phase (Fig. 2.2), as confirmed by a significant age X session interaction on infusions per session (F5,120=2.43; p < 0.05), and a targeted t-test on session six (t24=-2.1; p < 0.05). The main effect of age on number of infusions (F1,24=3.75; p = 0.071), as well as the age difference in total morphine intake (t24=-1.89; p = 0.071; inset) just missed statistical significance.

Short Access (ShAcc) to Morphine Self-Administration (1 hr per session)

Over 18 daily 1-hr sessions (ShAcc), adolescent-onset rats continued to take fewer morphine infusions than adults (Fig. 2.3; panel a), although both age groups increased their morphine intake over sessions. Thus, main effects of age (F1,10=10.58; p < 0.01) and session (F17,170=7.27; p < 0.001) were significant. Specifically, adolescent-onset rats tended to increase their drug intake over all 18 sessions, whereas adult-onset rats in-

creased their intake over approximately 7 sessions then reached a plateau. Paired t-tests comparing sessions 7 vs. 24 separately for each age group revealed only a trend toward increased infusions by adolescents (t_4=-2.34; $p = 0.08$), but confirmed a significant increase in morphine infusions among adults (t_6=-4.38; $p < 0.01$). Total morphine intake summed over 18 sessions was significantly lower in adolescents compared to adults (t_{10}=-3.25; $p < 0.01$; inset). The percent inappropriate responding did not differ by age group ($F_{1,10}$=2.31; p=0.16), session ($F_{17,170}$=1.38; p=0.15), or age X session interaction ($F < 1.0$; N.S.) in ShAcc conditions (Fig. 2.3; panel b).

Long Access (LgAcc) to Morphine Self-Administration (8 hr per session)

Over 18 daily 8-hr sessions (LgAcc), adolescent- and adult-onset rats escalated the number of infusions per session to similar degrees (Fig. 2.4; panel a). Rates of increase were similar across age groups. For example, the younger group reached an average of 61.2 ± 17.7 infusions by session 7 and 150.2 ± 39.6 by session 24, whereas adults reached 63.1 ± 9.9 infusions by session 7 and 214.9 ± 57.9 by session 24. There was no significant main effect nor any interactions with age on the number of infusions ($F < 1.0$; N.S.) or total morphine intake ($F < 1.0$; N.S.). Only the main effect of session was significant ($F_{17,204}$=6.06; $p < 0.001$). Post-hoc tests revealed that morphine infusions increased above the level of the first LgAcc session from the fifth session onward ($p < 0.025$), regardless of age. However, t-tests comparing session 7 vs. 24 separately for each age group revealed only a trend toward increased infusions in adolescents (t_4=-2.66; $p = 0.057$), but confirmed an increase in daily infusions among adults (t_8=-2.77; $p < 0.025$). Analysis of individual subject data revealed a subset of adults (n=3) that escalated their

morphine intake to a higher degree than all other animals in LgAcc conditions; dividing

number of infusions data into quartiles places only this subset of adults in the upper quar-

tile from session 12 onward (data not shown).

In terms of stimulus control over lever pressing, adolescent-onset rats exhibited

more inappropriate presses than adults (Fig. 2.4; panel b). The main effect of age was

significant ($F_{1,11}=4.81$; $p < 0.05$), but neither the main effect of sessions ($F_{17,187}=1.26$;

$p=0.22$), nor the age X sessions interaction ($F < 1.0$; N.S.) was significant.

Short vs. Long Access Comparisons

Comparisons across age and access conditions were conducted using the number of infu-

sions during the first 15 min "loading phase" of each session (Fig. 2.5). Adult rats in the

ShAcc condition loaded the most, compared with all other age and access groups. Thus,

there were significant main effects of age ($F_{1,22}=28.29$; $p < 0.001$), access condition

($F_{1,22}=13.31$; $p < 0.01$), and session ($F_{17,374}=5.1$; $p < 0.001$), as well as significant in-

teractions of age X access condition ($F_{1,22}=15.11$; $p < 0.01$) and session X access condi-

tion ($F_{17,374}=2.2$; $p < 0.01$). Post-hoc one-way ANOVAs comparing all groups on each

session confirmed that adults in the ShAcc condition took more morphine than any other

age or access group from the eighth session onward ($p < 0.05$). Similar outcomes were

observed when only the first hour of each session was compared across age and access

conditions, per the analysis of Ahmed and colleagues (2000; data not shown).

Extinction of Morphine-Seeking

In the absence of morphine, all subjects initially preferred the lever previously paired with morphine (active lever in Fig. 2.6; panel a) over the new (inactive; panel b) lever, but gradually decreased all lever pressing to low levels. A four-way mixed measures ANOVA [age X access condition X lever (active vs. inactive) X sessions (repeated)] produced no significant main effect of age (F < 1.0; N.S.), nor any interactions with age (F < 1.0; N.S.). However, a main effect of sessions confirmed the gradual extinction, regardless of age or access condition ($F_{17,408}=18.45$; p < 0.001). Also, a main effect of access condition ($F_{1,24}=4.2$; p < 0.05) showed that subjects in LgAcc conditions exhibited more extinction responding on the active lever than subjects in ShAcc conditions.

Cue-Induced Reinstatement

When drug-associated cues were reintroduced after extinction, rats that acquired morphine self-administration as adolescents reinstated lever pressing to a lesser degree than older adults, but access condition failed to influence reinstatement (Fig. 2.7). Thus, a two-way age X access ANOVA revealed a significant main effect of age ($F_{1,22}=9.17$; p < 0.01), but neither the main effect of access (F < 1.0; N.S.) nor the age X access interaction was significant (F < 1.0; N.S.). To determine whether morphine intake during self-administration influenced lever pressing during reinstatement, a Pearson's correlation analysis was conducted. A positive relationship was identified between total morphine intake (mg/kg) and number of lever presses during reinstatement among subjects in ShAcc, but not LgAcc conditions (ShAcc: 0.659; two-tailed p = 0.02; LgAcc: 0.188; two-tailed p = 0.52; data not shown).

2.5 Discussion

This report on morphine self-administration in adolescent vs. adult rats reveals important age differences in morphine intake and reinstatement of morphine-seeking after extinction. Under conditions of short daily access (1-hr per day), rats that acquired morphine self-administration as adolescents consistently took less morphine than adults. In contrast under long access conditions (8–hr per day), rats in both age groups escalated their morphine intake similarly. Perhaps most strikingly, cue-induced reinstatement of morphine-seeking after extinction was less robust in rats that took morphine during adolescence compared to rats that self-administered as adults, regardless of daily access conditions. Together these results only partially support our hypotheses that younger rats are more sensitive than adults to the acute and long-term reinforcing effects of morphine.

Morphine self-administration and related behaviors expressed by subjects in the present study confirm prior results from this and other laboratories. First, the assertion that morphine reinforced lever pressing in our laboratory conditions is confirmed by several factors. Stable self-administration occurred in the present experiment. Lever discrimination was observed during a two-lever choice procedure in a pilot study (Doherty et al. 2006) and during extinction in the present study (Bossert et al. 2007). A burst of extinction responding occurred when saline was substituted for morphine in our pilot study (Doherty et al. 2006; Peltier et al. 2001), and reinstatement of lever pressing after reintroduction of drug-associated cues in the present study (LaLumiere and Kalivas 2008). Second, escalation of morphine intake under long daily access conditions replicates numerous prior reports with opiate or stimulant drug reinforcers (Ahmed and Koob 1998a; Ahmed et al. 2000; Buccafusco and Bain 2007; Chen et al. 2006; Glass et al.

2005; Glass et al. 2004; Kenny et al. 2006; Kruzich et al. 2003; Lenoir and Ahmed 2007; Morgan et al. 2002; Paterson and Markou 2004; Wee et al. 2007). Third, higher rates of lever pressing during extinction by subjects from LgAcc compared with ShAcc groups replicates prior work (Ahmed et al. 2000; Ferrario et al. 2005; Lenoir and Ahmed 2007). Lastly, even the higher percentage of "inappropriate responding" exhibited by adolescent-onset rats compared to adults in the present LgAcc conditions is consistent with higher levels of impulsivity or lack of stimulus control previously noted in adolescents (Adriani and Laviola 2003; Sagvolden and Sergeant 1998; Shahbazi et al. 2008; Spear and Brake 1983).

Among the most important of the present new findings is that adolescent-onset rats consistently took less morphine than adults under ShAcc conditions. On an FR schedule of reinforcement in short daily sessions, lower rates of intake are usually elicited by higher doses per infusion (Arnold and Roberts 1997; Carroll and Lac 1997; Koob et al. 1984). Therefore, the present results suggest an adolescent hypersensitivity to the reinforcing effects of morphine, consistent with age differences in morphine-stimulated motor activity and sensitization (Spear et al. 1982; White and Holtzman 2005), as well as heightened vulnerability to opiates among human adolescents (Johnston et al. 2010; SAMHSA 2009). On the other hand, slower rates of acquisition (Perry et al. 2007) and fewer number of infusions per session (Belluzzi et al. 2005) have been interpreted to reflect hyposensitivity. Extensive research will be necessary to confirm either interpretation and describe the neural basis for either of these age differences.

Relatively little is known about adolescent development of brain reinforcement circuitry. Very few studies on the ontogeny of opioid receptors include the adolescent

phase of development, and ontological studies that do bracket adolescence do not provide a clear explanation for observed behavioral differences. For example, the density of μ-opioid receptors in the nucleus accumbens and other forebrain regions rises to adult levels already by P30 (Talbot et al. 2005) and agonist binding affinity appears similar in the forebrain of P28 and adult rats (Spain et al. 1985). However, less efficient coupling between μ-opioid receptors and G-proteins in P30 vs. adult rats fits a profile of adolescent hyposensitivity to morphine (Talbot et al. 2005). With regard to reinforcement circuits involving mesolimbic dopamine transmission, a transient overexpression of dopamine receptors in the nucleus accumbens and prefrontal cortex is observed specifically during adolescence (Andersen and Teicher 2000; Andersen et al. 2000), along with fluctuations in basal and dopamine agonist-stimulated cAMP levels (Andersen 2002). It is entirely possible that similar transient effects will be revealed in opioid receptor systems and/or that these known changes in dopamine signaling could contribute to the age differences in morphine self-administration presently reported.

In addition to neurochemical maturation, endocrine system changes could contribute to age differences in drug self-administration. Indeed corticosterone suppression decreases the locomotor-stimulating effects of morphine (Deroche et al. 1993); blocking glucocorticoids attenuates morphine-induced dopamine efflux in the nucleus accumbens (Marinelli et al. 1998), and gonadal steroid hormones increase the potency of morphine in a hot plate test (Stoffel et al. 2003). Unfortunately, comparisons across different self-administered drugs do not reveal the same age-dependent results as the present study [e.g. no age differences in cocaine self-administration (Frantz et al. 2007; Kerstetter and Kantak 2007; McQuown et al. 2007) or higher intake after adolescent onset of nicotine or

amphetamine self-administration (Belluzzi et al. 2005; Shahbazi et al. 2008; Shram et al. 2008a)]. Thus, general conclusions about adolescent sensitivity to behavioral reinforcement by drugs of abuse are not warranted.

An unexpected observation in our experiments was the gradual increase in morphine intake under ShAcc conditions, which was significant for adults and trended toward significant for adolescents (p=0.08). This increase contrasts with numerous reports of stable intake over weeks of heroin or cocaine self-administration under similar schedules and access conditions (Ahmed and Koob 1999; Ahmed et al. 2000; Bossert et al. 2007; Kenny et al. 2006), but corroborates a recent report of slightly increased heroin intake over 27 daily 1-hr sessions (Lenoir and Ahmed 2008). In a manner specific to opiates, either tolerance could drive up rates of intake (Zernig et al. 2007 for review), or increasingly aversive states of withdrawal could drive up intake through negative reinforcement (Kenny et al. 2006; Koob and Le Moal 1997; Schulteis and Koob 1996). In either case, a lack of significant increase over sessions by adolescent-onset rats compared with adults could reflect adolescent hyposensitivity to these morphine effects. Adolescent hyposensitivity to tolerance is not supported by research on morphine analgesia (Ingram et al. 2007; Wang et al. 2005). However, adolescent hyposensitivity to aversive drug withdrawal does corroborate mounting evidence from research showing adolescent hyposensitivity to acute aversive drug effects in nicotine- or amphetamine-conditioned taste aversion (Infurna and Spear 1979; Wilmouth and Spear 2004), as well as physical and affective signs of nicotine withdrawal (Infurna and Spear 1979; O'Dell et al. 2006; O'Dell et al. 2007; Shram et al. 2008b; Wilmouth and Spear 2004). Further, adolescent hyposensitivity to drug withdrawal may even be supported by the present observation that

the younger cohort exhibited less "drug-loading" than adults during the first 15 min of each session, possibly reflecting less aversive interoceptive states just prior to self-administration sessions. Finally, body weight gain among rats in the younger cohort was not affected during abstinence from morphine over three intermittent periods (weekends), whereas adult rats lost weight in a classic sign of opiate withdrawal (Gellert and Holtzman 1978). However, the interpretation of body weight change across age groups is confounded by the normal growth curve for adolescent rats during development, contrasted with the relatively flat rate of body weight gain among adults. Given that behavioral reinforcement and aversive drug withdrawal are mediated by discrete neural systems (Koob and Le Moal 2008; Schulteis and Koob 1996), it is possible that adult-like sensitivity to behavioral reinforcement is coupled with resistance to aversive drug withdrawal during ontological development, leading to lower and more stable rates of morphine self-administration by adolescent-onset compared with adult-onset rats under ShAcc conditions.

Long access self-administration conditions are thought to model the transition from recreational drug use to compulsive drug addiction (Ahmed and Koob 1998b). In contrast to the clear age differences in the present ShAcc conditions, no significant age differences in long access testing were observed, although the three highest "escalators" were all adult rats and they comprised the upper quartile in the data range from session 12 onward. The gradual escalation of drug intake exhibited by both age groups extends numerous reports on heroin, fentanyl, cocaine, and methamphetamine (Ahmed and Koob 1998b; Ahmed et al. 2000; Chen et al. 2006; Kenny et al. 2006; Kitamura et al. 2006; Lenoir and Ahmed 2007; 2008; Morgan et al. 2002; Wee et al. 2007) to include morphine

and adolescent male rats. However, insofar as a faster rate and greater degree of escalation reflect increased vulnerability to the transition from periodic drug use to compulsive drug abuse (Ahmed and Koob 1999; Ahmed et al. 2000; Chen et al. 2006; Mantsch et al. 2003; O'Brien et al. 1986; Walker et al. 2003), our data do not support the contention that adolescent onset of drug-taking heightens vulnerability to transition from periodic to compulsive drug-seeking or addiction (Anthony and Petronis 1995; Clark et al. 1998; Kandel et al. 1992; Laviola et al. 1999; SAMHSA 2009; Smith 2003).

Lastly, we considered the long-term effects of morphine self-administration using an animal model of drug craving and relapse following abstinence and extinction. Unlike prior reports (Ahmed and Koob 1998b; Ahmed et al. 2000; Lenoir and Ahmed 2007), rats that took morphine under LgAcc conditions in our study failed to exhibit more robust reinstatement of drug-seeking after extinction, relative to rats in ShAcc conditions. Two factors could explain this contradiction: 1) drug-associated cues rather than acute drug administration or stressors were used to trigger reinstatement, and 2) although escalation was greater in LgAcc conditions, rats in both access groups actually increased intake over sessions. Despite these factors, rats that acquired morphine self-administration during adolescence showed less robust cue-induced reinstatement of lever pressing than older adults. Whereas total drug intake during self-administration has correlated with lever pressing during reinstatement previously (Liu et al. 2008) as well as in the present ShAcc cohorts, no such correlation was observed among the present LgAcc cohorts, suggesting that prior drug intake does not entirely explain the age differences in reinstatement. Alternatively, the amount of cue-induced reinstatement may reflect the prior strength of the reinforcing stimulus (Kenny 2007; Shaham et al. 2003; Zhou et al. 2007), or the negative

affect triggered by cue presentation during withdrawal (Kenny and Markou 2005a; Kenny et al. 2006). In either case, our results suggest that younger rats are less sensitive than older adults to these enduring effects of drug self-administration. Similarly, rats that self-administered cocaine as adolescents showed lower rates of cue-induced reinstatement of cocaine-seeking than adults (Li and Frantz 2009). Also, rats that took cocaine as adolescents showed less long-term cognitive impairment than adults (Kerstetter and Kantak 2007); and rats given MDMA during adolescence failed to cross-sensitize to cocaine reinforcement when their adult counterparts did (Frantz and Parsons 2001). Together these results suggest that adolescence could be a period during which neuroprotective factors dampen some long-term drug effects.

In all, the present results extend research on age differences in vulnerability to both acute and long-term reinforcing effects of morphine, using the i.v. self-administration model which has strong face and predictive validity (Ator and Griffiths 2003). Although hypersensitivity of adolescent rats to the acute reinforcing effects of morphine could explain some of the present results, hyposensitivity of adolescent rats to morphine-associated tolerance, withdrawal, and/or cue-induced reinstatement may reflect developmental protection from some acute and long-term detrimental effects of morphine. If verified in future studies, including clinical investigations, these findings might suggest a better prognosis for adolescent compared with adult drug abusers in behavioral or pharmacological therapy for drug dependence.

2.6 Acknowledgements

2.7 Tables & Figures

Table 2.1

	Arrive in lab	Catheter surgery	Pre-Escal (6 sessions)	Escalation (18 sessions)	Recess (15 days)	Extinction (18 sessions)	Cue Reins-tate (1 session)
Table 2.1. Postnatal age in days (d) at start of each experimental phase.							
Adolescent-onset	22d	28-29d	35d	42d	63d	78d	101d
Adult-onset	78d	84-86d	91d	98d	119d	134d	157d

Fig. 2.1
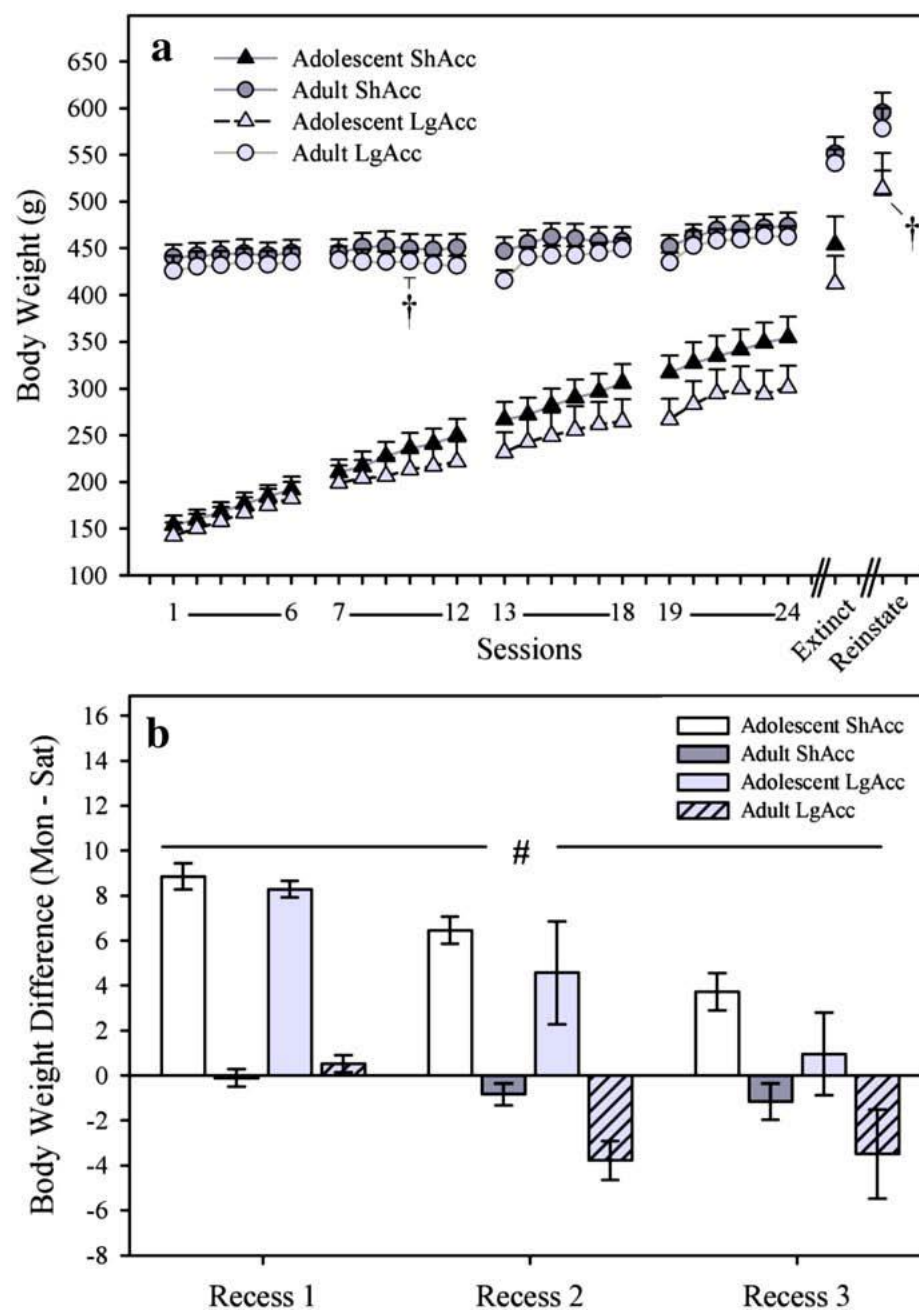
Fig. 2.1 (a) Body Weight (g) in all subjects. Rats in both age groups gained weight over days, but ShAcc adolescents gained more weight than their LgAcc counterparts. "Extinct" indicates weight before the first session of extinction. "Reinstate" indicates weight one day after the reinstatement session. Dagger symbols indicate days on which the adolescent-onset and adult-onset groups were 101 days of age; see text for body weight comparisons. (b) Percent Body Weight Change over Weekend Recess from Morphine Self-Administration (change between sessions 6-7 for Recess 1, 12-13 for Recess 2, 18-19 for Recess 3). Over each weekend recess, adolescents continued to gain body weight, while adults lost weight (main effect of age; # $p < 0.001$). Subjects in the LgAcc condition tended to lose more weight than subjects in the ShAcc condition. The change in body weight tended to decrease over time. All points or bars represent mean +/- SEM (n= 5 ShAcc adolescents; n=7 ShAcc adults; n=5 LgAcc adolescents; n=9 LgAcc adults).

Fig. 2.2
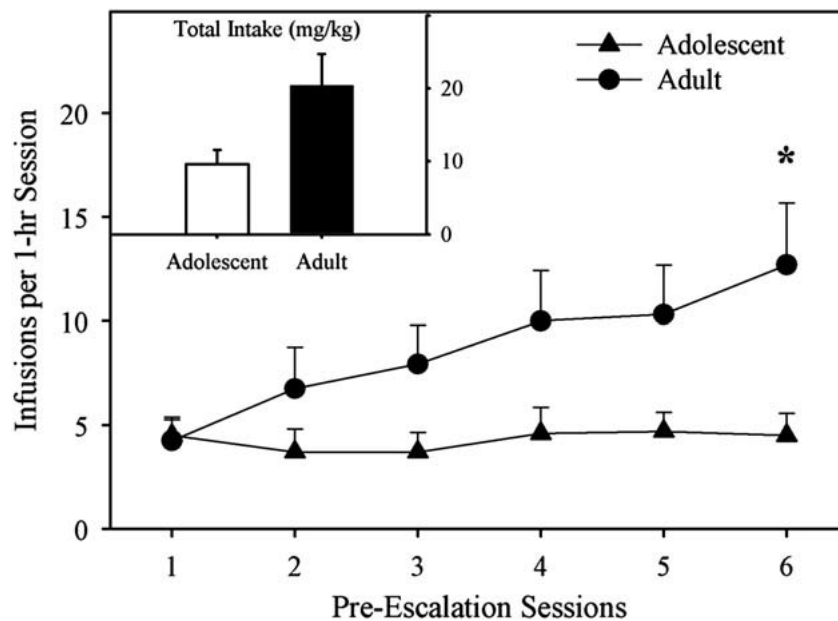
Fig. 2.2 Morphine Infusions during Pre-Escalation. Adolescents self-administered less morphine than adults. Post-hoc t-tests revealed a significant age effect on session 6 only (* $p < 0.05$). *INSET.* Total Morphine Intake during the Pre-Escalation Phase. All points or bars represent mean +/- SEM (n= 10 adolescents; n=16 adults).

Fig. 2.3

49
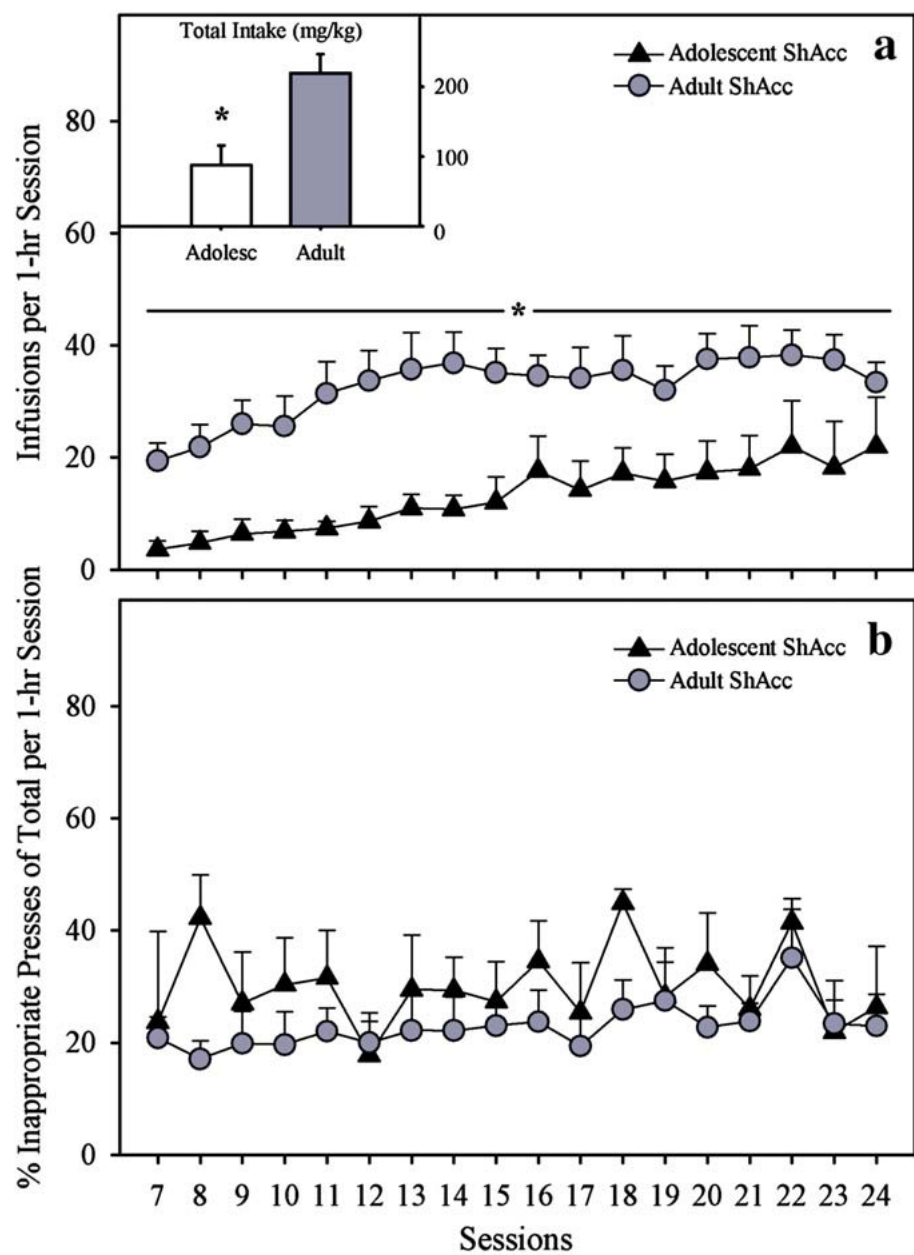
Fig. 2.3 (a) Daily Morphine Infusions Under Short Access (ShAcc) Conditions. Adolescents took less morphine than adults over 18 sessions (main effect of age; * p<0.01). *INSET.* Total Morphine Intake (mg/kg) Under ShAcc Conditions. Adolescents took less morphine than adults (main effect of age; * p < 0.01). (b) Percent Inappropriate Lever Presses During Self-Administration Under ShAcc Conditions. Adolescents and adults displayed similar percent inappropriate lever responses over sessions. All points or bars represent mean +/- SEM (n= 5 adolescents; n=7 adults).

Fig. 2.4
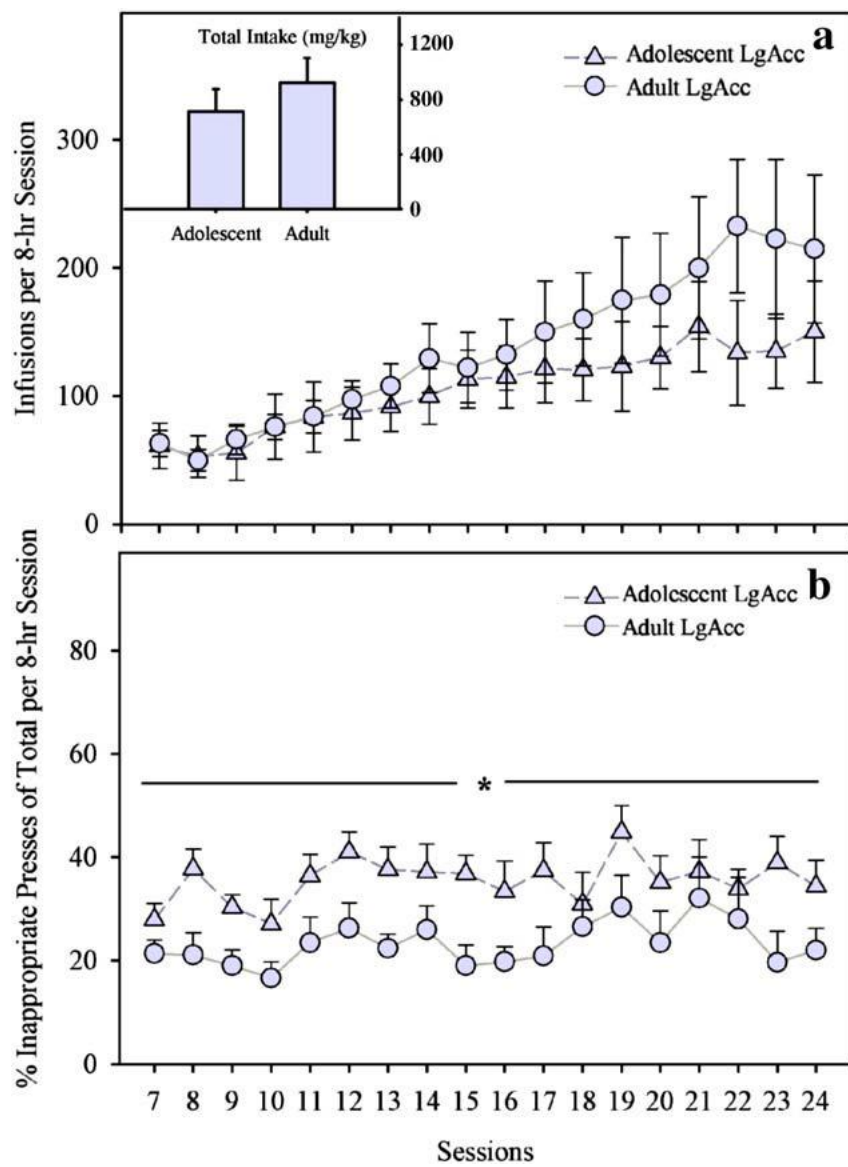
Fig. 2.4 (a) Daily Morphine Infusions Under Long Access (LgAcc) Conditions. Adolescents and adults took similar amounts of morphine. *INSET.* Total Morphine Intake (mg/kg) Under LgAcc Conditions. (b) Percent Inappropriate Lever Presses During Self-Administration Under LgAcc Conditions. Adolescents exhibited more inappropriate presses compared to adults (main effect of age; * $p < 0.05$). All points or bars represent mean +/- SEM (n= 5 adolescents; n=9 adults).

Fig. 2.5
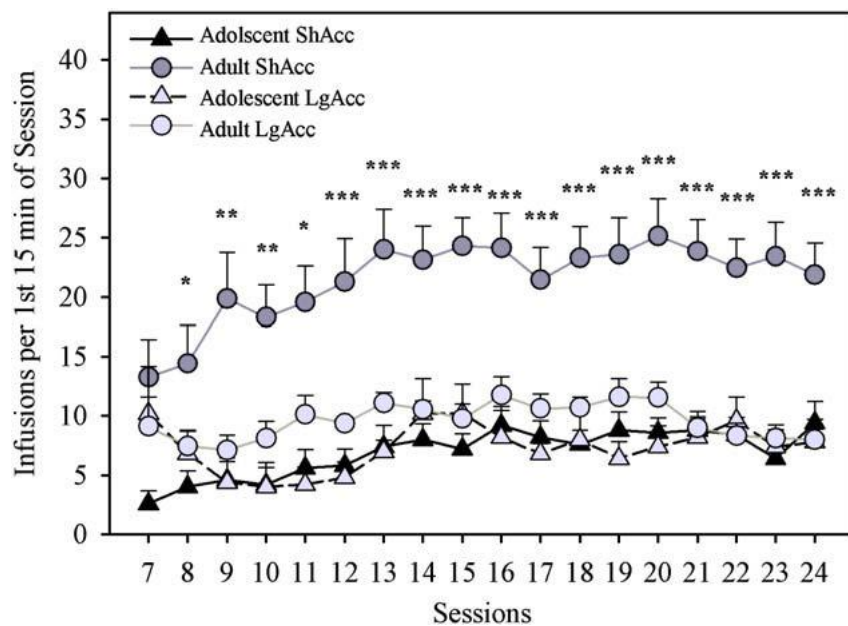
Fig. 2.5 Daily Morphine Infusions in the First 15 min per Session. Adults under ShAcc conditions took more infusions than other groups from session 8 onward (adult ShAcc different from all other groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Points represent mean +/- SEM (n= 5 ShAcc adolescents; n=7 ShAcc adults; n=5 LgAcc adolescents; n=9 LgAcc adults).

Fig. 2.6
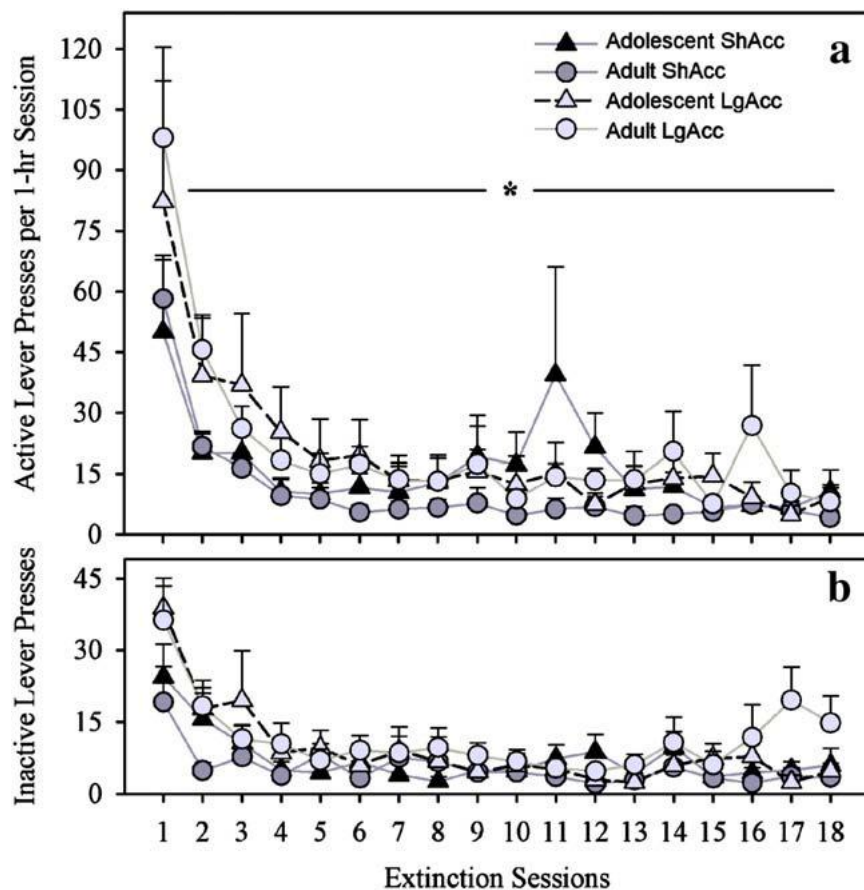
Fig. 2.6 (a) Extinction of Active Lever Pressing in 1-hr Sessions. No significant age effects were observed, but LgAcc subjects pressed more than ShAcc subjects (main effect of access condition; * $p < 0.05$). (b) No Differences in Inactive Lever Pressing During 1-hr Extinction Sessions. All points represent mean +/- SEM (n= 5 ShAcc adolescents; n=7 ShAcc adults; n=5 LgAcc adolescents; n=9 LgAcc adults).

Fig. 2.7
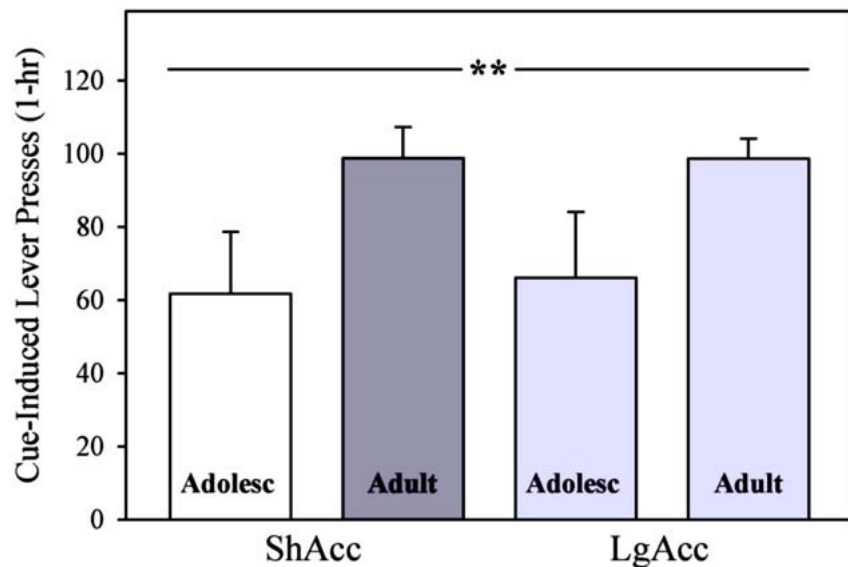
Fig. 2.7 Cue-Induced Reinstatement of Morphine-Seeking. Subjects that acquired morphine self-administration as adolescents reinstated lever pressing after reintroduction of drug-associated cues to a lesser degree than older adults, regardless of access conditions (main effect of age; ** $p < 0.01$). Bars represent mean +/- SEM (n= 5 ShAcc adolescents; n=7 ShAcc adults; n=5 LgAcc adolescents; n=9 LgAcc adults).

3   Chapter 3 - Self-Administration of Heroin and Reinstatement of Heroin-

Seeking in Adolescent vs. Adult Male Rats

By

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3.1   Abstract

Rationale Heroin abuse is prevalent among teenagers, and early onset drug use might predict long-term drug dependence. Yet adolescent sensitivity to drug reinforcement has not been explored thoroughly in animal models. *Objectives* This study aimed to compare intravenous (i.v.) self-administration of heroin, as well as extinction and reinstatement of heroin-seeking, in adolescent vs. adult male rats. *Methods* Adolescent (35 days old at start) and adult (86 days old at start) male Sprague-Dawley rats spontaneously acquired lever-pressing maintained by i.v. heroin infusions. In Experiment 1, self-administration was tested on a fixed ratio 1 schedule of reinforcement (0.05 and 0.025 mg/kg/infusion), followed by within-session extinction and reinstatement tests after 1 or 12 days of abstinence. In Experiment 2, self-administration was tested on a progressive ratio schedule (0.0125 – 0.1 mg/kg/infusion), followed 12 days later by a single cue-induced reinstatement test in the absence of extinction. *Results* In Experiment 1, adolescent rats self-administered more heroin than adults. After 1 or 12 days of abstinence, adolescents exhibited less heroin-seeking than adults, although levels of heroin-seeking increased over abstinence period for both age groups. In Experiment 2, adolescents and adults reached the same maximal number of infusions (breakpoint), although adolescents earned more infusions when response requirements were low. For cue-induced reinstatement in the absence of extinction, heroin-seeking was similar across ages. *Conclusions* Lower levels of heroin-seeking suggest that younger rats are less sensitive than adults to some residual effects of heroin intake.

3.2 Introduction

Drug abuse is prevalent in the U.S. and is typically initiated in adolescence (Johnston et al. 2010; SAMHSA 2009). For example, 250,000 high school students report using the opiate drug, heroin, at least once in their lifetime, a trend that has held steady for decades (Johnston et al. 2010; SAMHSA 2009). Early onset of drug use may also increase chances of later drug addiction (Anthony and Petronis 1995; Clark et al. 1998; Kandel et al. 1992; Palmer et al. 2009). Whether or not these phenomena can be attributed to a biological vulnerability to drugs during adolescence is unclear. Animal models provide the opportunity to test for a potential biological basis of adolescent drug abuse.

Adolescence in rodents occurs between postnatal days (P) 28 and 60 (Smith 2003; Spear 2000; Spear and Brake 1983). In many species, including primates and rodents, this transition from youth to adulthood is characterized by robust behavioral, morphologic, metabolic, hormonal, and neurochemical changes, some of which are likely to influence responses to drugs (Spear 2000). Yet adolescent exposure to opiate drugs has been explored only minimally with animal models. We know of only two comparisons of opiate intake in adolescent vs. adult subjects using the intravenous (i.v.) drug self-administration model. In our report, adolescent male rats self-administered less morphine than adult males during 1-hr daily sessions on a fixed ratio (FR) schedule of reinforcement (Doherty et al. 2009). Similarly in the other report, adolescent male mice self-administered less oxycodone than adult male mice (Zhang et al. 2009). These experiments used doses on the descending limb of a typical dose–effect function for opiate self-administration in adults (Beardsley et al. 2004; Martin et al. 1996; Smith et al. 1976),

leading to the interpretation that the drug was more potent in the adolescents than adults. Consistent with this interpretation, adolescent male mice had higher levels of oxycodone-stimulated extracellular dopamine than adults in the striatum (Zhang et al. 2009). Adolescent male rats were also more sensitive to the psychomotor stimulating or sensitizing properties of morphine compared to adults (Spear et al. 1982; White and Holtzman 2005), whereas results were mixed on age differences in morphine conditioned place preference (Bolanos et al. 1996; Campbell et al. 2000).

To extend this type of research to heroin, per se, the first aim of the present study was to test the hypothesis that adolescent male rats are more sensitive to the acute reinforcing effects of heroin than adults. In Experiment 1, we tested i.v. heroin self-administration on a FR schedule in both age groups using doses on the descending limb of a typical inverted-U dose-effect function for adult rats. We predicted that higher sensitivity in adolescents would result in lower rates of heroin self-administration, because a leftward shift in the dose-effect function would appear as a decrease in response rate. In Experiment 2, we tested for age differences in the reinforcing efficacy of heroin using a progressive ratio (PR) schedule in which lever-press requirements increased with each infusion (Hodos 1961). We predicted that higher sensitivity in adolescents would result in completion of higher response requirements, i.e. a higher break point.

Given that long-lasting vulnerability to relapse is perhaps the biggest challenge in human drug addiction (Chung and Maisto 2006; O'Brien 1997), the main focus of both of the present experiments was to test for age differences in the residual effects of heroin, as measured in extinction and reinstatement tests. Initial rates of extinction responding in the absence of the previously self-administered drug provide a measure of motivated

drug-seeking after abstinence (Conrad et al. 2008). Subsequent reinstatement of drug-seeking after extinction can be triggered by re-exposure to the drug-taking environment (context-induced reinstatement), discrete cues previously paired with the drug (cue-induced reinstatement), stress, or to the drug itself (reviewed in Shaham et al. 2003). Furthermore, context- (Shalev et al. 2001) or cue-induced (Grimm et al. 2003) reinstatement increases over time in abstinence for adult male rats, a phenomenon known as 'incubation of drug craving' (Bossert et al. 2005). We and others have begun to explore extinction and reinstatement after adolescent-onset of drug self-administration. Surprisingly, rats that self-administered morphine or cocaine as adolescents (adolescent-onset groups) exhibited attenuated cue-induced reinstatement and less robust incubation than adults, regardless of age differences in drug intake (Anker and Carroll 2010; Doherty et al. 2009; Li and Frantz 2009). To extend this work to heroin, the second aim of Experiments 1 and 2 in the present study was to compare heroin-seeking after 1 or 12 days of abstinence among adolescent- vs. adult-onset groups. Heroin-seeking was measured in three ways: 1) *extinction responding* during re-exposure to the drug-taking environment; 2) *cue-induced reinstatement* of drug-seeking after extinction, and 3) *extinction responding in the presence of cues*. We chose a maximum of 12 days in abstinence because cue-induced reinstatement of heroin-seeking is reliable at that time point in adult male rats, and peaks at just 24 days in abstinence (Shalev et al. 2001). Based on prior reports of attenuated morphine- or cocaine-seeking in adolescent-onset groups (Doherty et al. 2009; Li and Frantz 2009; Anker and Carroll 2010), we predicted that rats that self-administered heroin as adolescents would exhibit less heroin-seeking than adults.

3.3 Methods

Subjects

Male Sprague-Dawley rats (Charles River, Raleigh NC) arrived in the laboratory at post-natal days (P) 22 (n=41) or P70-74 (n=37) for adolescent or adult age groups, respectively. Rats were housed in groups of two or three in a temperature and humidity controlled vivarium (targeted at 68-72°F and 50% humidity) and maintained on a 12-hr light/dark cycle (lights off at 0700 hr). All testing occurred at approximately the same time every day in the dark phase. Body weights were recorded daily to monitor health and adjust drug doses. Food and water were available *ad libitum* in home cages. Principles of laboratory animal care were followed. All procedures complied with the NIH *Guide for Care and Use of Laboratory Animals* (7th Ed., 1998), and were approved by the Institutional Animal Care and Use Committee of Georgia State University.

Drugs

Heroin HCl (generous gift from NIDA), methohexital sodium (1% Brevital Sodium, King Pharmaceuticals, Inc.; Bristol, TN), and Timentin antibiotic (GlaxoSmithKline; Research Triangle Park, NC), were all dissolved in sterile saline and filtered through a 25 μm syringe filter (Fisher Scientific, Inc.; Pittsburgh, PA) before i.v. administration. Drug solution concentrations were 0.022, 0.0438, 0.0876, and 0.1752 mg/ml for heroin doses of 0.0125, 0.025, 0.05, and 0.1 mg/kg/infusion, respectively.

Equipment

Heroin self-administration was conducted in operant conditioning chambers housed in sound-attenuating cubicles (MedAssociates, Inc.; St Albans, VT). Each chamber was equipped with two retractable levers, both of which extended into the chamber during self-administration sessions. One lever was designated active and the other inactive. Presses on the active lever initiated a syringe pump with a 5 rpm motor (PVM-1000VS, Med Associates Inc.; St Albans, VT) to deliver a drug infusion via a stainless steel swivel and a polyethylene tube protected by a metal coil and attached to the catheter portal on each subject's back. Presses on the inactive lever were recorded but had no scheduled consequences. Drug delivery and data collection were controlled by a computer using Med Associates software (Med PC IV). I.v. catheters were made as described (Caine et al. 1993), with minor modifications including a shorter length of tubing inserted into the jugular vein for adolescents (2 cm) vs. adults (4 cm)(Shahbazi et al. 2008).

Intravenous (i.v.) Catheter Implantation

Adolescent (P28-29) and adult (P76-81) rats were surgically catheterized in the right jugular vein, generally according to Caine et al. (1993), with minor modifications (Shahbazi et al. 2008). Briefly, rats were anesthetized with an isoflurane-oxygen vapor mixture (4-5% initially; 1.5-2.5% for maintenance). Catheter tubing was passed subcutaneously from the back and inserted into the right jugular vein, sutured in place, and glued (cyanoacrylate). In recovery, adolescent and adult rats received 0.15 or 0.2 ml, respectively, of Timentin antibiotic (Ticarcillin Disodium and Clavulanate Potassium; 100 mg/ml, i.v.) twice daily for two days, then once daily for the remainder of the experiment. Catheters

were also flushed daily with 0.15-0.4 ml heparinized saline (30 USP units/ml). Catheter

patency was tested one day before, once on the recess day (see below), and one day after

heroin self-administration, by injecting 0.1-0.4 ml of a short-acting barbiturate anesthetic

(Brevital) through the catheter. If muscle tone was not lost within 3-sec, the catheter was

presumed defective and the subject was not included in the analysis.

The experimental timeline for Experiment 1 and 2 is shown in Fig. 3.1.

Experiment 1 Behavioral Testing Procedures

Self-Administration. Following 5-7 days of post-surgical recovery, adolescents (P35 at

start; body weight 139 g $\pm$ 1.89) and adults (P82-87 at start; body weight 385 g $\pm$ 4.21)

were allowed to spontaneously acquire lever pressing maintained by heroin. The drug

dose was adjusted daily based on individual body weight by changing the infusion vo-

lume and duration, using a 0.2 ml infusion over 4-sec standard for a 350 g rat. After each

drug infusion, a 20-sec time out (TO) period was signaled by a compound stimulus (2 sec

cue light above the active lever, 5-sec burst of white noise, house light off for 20 sec).

Responses during TO were recorded but had no scheduled consequences. Placement of

adolescents vs. adults was counterbalanced across 14 chambers. A FR1 schedule was

used in 3 hr daily sessions, with a change of dose after 6 days to help determine whether

the drug was maintaining the lever-pressing (0.05 mg/kg/infusion for 6 days, one recess

day, then 0.025 mg/kg/infusion for 7 days).

Extinction and Reinstatement. After 1 or 12 days of forced abstinence from heroin in the

home cage, a within-session extinction and reinstatement test was conducted, such that

six 1-hr extinction sessions were followed by a single 1-hr cue-induced reinstatement test (Grimm et al. 2003; Grimm et al. 2001; Li and Frantz 2009; 2010). During extinction and reinstatement, rats were connected to the tether but not the infusion tubing. Drug-paired cues were absent during extinction, i.e. lever presses had no schedule consequences. Between each extinction session, the two levers retracted and the house light turned off for five minutes. The subsequent cue-induced reinstatement test began with non-contingent presentation of drug-paired cues, i.e. the cue light, white noise, and house light sequence described above. During the remainder of the reinstatement test, presses on the active lever produced cue sequences identical to those during heroin self-administration, and the pump turned on, although no syringe was loaded. The only difference between self-administration and the cue-induced reinstatement test was that drug solution was not infused during reinstatement.

Experiment 2 Behavioral Testing Procedures

Self-Administration. Experiment 2 was designed to provide insight on potential age differences in heroin efficacy as a reinforcer using a PR schedule of reinforcement. To begin, adolescent (n=22) and adult (n=19) rats were allowed to spontaneously acquire lever-pressing reinforced by heroin (0.05 mg/kg/infusion) on a FR1 schedule of reinforcement for 3 days, followed by increasing lever press requirements in preparation for the PR schedule (3-d on FR2; one recess day; 3-d on FR5). The PR schedule then commenced with the same dose for 3 days (0.05 mg/kg/infusion), one recess day, then division of rats into two dose-groups to self-administer either a lower (0.0125 mg/kg/infusion) or higher dose (0.1 mg/kg/infusion) on the PR schedule for 3 more days. The progression of re-

sponse requirements on the PR schedule was calculated by the equation: response ratio = $(5 \times e^{(0.2 \times \text{infusion number})})-5$, rounded to the nearest integer (Roberts and Bennett 1993). For example over 28 reinforcers, requirements were as follows: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 603, 737, 901, 1102, and 1347. The session ended when a rat failed to achieve the next response requirement within 1-hr, and length was capped at 9-hrs.

Extinction Responding in the Presence of Cues. After 12 days of forced abstinence, rats were tested for heroin-seeking in a single 1-hr session. This session was identical to the 1-hr cue-induced reinstatement test in Experiment 1. (Extinction responding in the absence of drug-paired stimuli was not measured in Experiment 2.)

Data Analysis

Heroin Self-Administration. For heroin self-administration on FR schedules of reinforcement (Experiments 1 and 2), the number of infusions per session was analyzed using a two-way mixed-measures analysis of variance (ANOVA) with age as the between-subjects factor and session as the within-subjects repeated measure. For Experiment 1, infusions were also averaged over the six or seven sessions at each dose, and analyzed using an ANOVA with age as the between-subjects factor and dose as the repeated measure. Total heroin intake (mg/kg) was calculated as the sum of all infusions over all sessions (multiplied by dose), and compared using unpaired t-tests. Lever presses per session were analyzed using a three-way ANOVA with age, session, and lever (active vs. inactive) as factors. To compare control of behavior exerted by drug-paired cues, the percent of non-reinforced responding was calculated as the sum of non-reinforced presses

(presses on the active lever during drug infusion or TO + all presses on the inactive lever), divided by the total number of presses, then analyzed in a two-way age x sessions ANOVA. For the PR schedule (Experiment 2), results were averaged across the three sessions at each dose. To test for dose-dependent responding, both the number of infusions per session and session length (min) were compared using two-way age x dose ANOVAs, followed by paired or unpaired t-tests as appropriate. After viewing cumulative infusions graphs, we also used an age x dose ANOVA to test for differences in the number of infusions earned by 125-min into the testing sessions, as that time point was the first breakpoint for any subject, and thus the last time point at which all subjects could be analyzed in this way.

Extinction and Reinstatement. For Experiment 1, active or inactive lever presses during extinction and cue-induced reinstatement were analyzed using separate two-way mixed-measures ANOVAs at each abstinence time point (1 or 12 days), with age as the between-subjects factor and session as the within-subjects repeated measure. For cue-induced reinstatement, the number of active lever presses during the reinstatement session was also compared directly with the number of active lever presses in the last extinction session using an age x session (extinction vs. reinstatement) ANOVA. An additional analysis of extinction responding in Experiment 1 was conducted, such that the number of active lever presses in the first hr of extinction was calculated as a percentage of the number of active lever presses in the first hr over the last three self-administration sessions, then compared across age groups using separate t-tests at each abstinence period. For time-dependent changes in heroin-seeking after abstinence (i.e. incubation), the number of active or inactive lever presses during extinction sessions was analyzed using sepa-

rate three-way mixed-measures ANOVAs with age, abstinence period, and sessions as factors. Time-dependent changes in cue-induced reinstatement were also analyzed using a two-way age x abstinence period ANOVA. To compare heroin-seeking after self-administration on the PR schedule (Experiment 2), the number of active lever presses during extinction responding in the presence of cues was analyzed using unpaired t-tests. To help determine whether drug self-administration influenced reinstatement, Pearson's correlations tested for associations between total heroin intake and active lever presses during heroin-seeking. In all cases, follow-up ANOVAs and post-hoc tests were conducted as appropriate, with $\alpha = 0.05$ (n.s. = not significant).

3.4   Results

Experiment 1 – Heroin self-administration on a FR1 schedule of reinforcement, followed by extinction and reinstatement.

Self-Administration. Adolescent rats self-administered more heroin than adults on a FR1 schedule of reinforcement (Fig. 3.2a). The number of infusions per session differed according to main effects of age [$F_{(1,35)}=4.77$, $p<0.05$], session [$F_{(12,420)}=106.02$, $p<0.001$] and an age x session interaction [$F_{(12,420)}=1.80$, $p<0.05$], with a gradual increase in heroin intake over sessions and more consistent age differences in week one compared with week two. However, follow-up t-tests with Bonferroni's correction failed to reveal age differences at individual time points. Regardless of age group, infusions were dose-dependent, per a main effect of dose [$F_{(1,35)}=357.98$, $p<0.001$]. Total heroin intake (mg/kg) over all sessions was also higher for adolescents than adults [t=2.38, df=35, $p<0.05$](Fig. 3.2a inset). Rats in both age groups pressed more on the active vs. inactive

lever, according to a main effect of lever [$F_{(1,70)}=17.34$, $p<0.001$](Fig. 3.2b). A significant age x session x lever interaction was observed [$F_{(12,840)}=2.33$, $p<0.01$], but testing for age differences in active lever presses using t-tests with Bonferroni's correction at each individual session again failed to reveal any statistically significant age effects. In terms of the percent of non-reinforced responding, adolescents had higher rates than adults, according to a main effect of age [$F_{(1,35)}=10.91$, $p<0.01$ - data not shown].

Extinction and Reinstatement. Adolescent-onset groups exhibited less extinction responding and cue-induced reinstatement after abstinence than adults, although a higher rate of extinction responding and cue-induced reinstatement was observed after 12- vs. 1-day abstinence in both age groups (Fig. 3.3a and b). For extinction responding, significant age x session interactions were observed at both abstinence time points [1-d: $F_{(5,70)}=2.57$, $p<0.05$; 12-d: $F_{(5,95)}=2.8$, $p<0.025$], and follow-up analysis confirmed that adolescent-onset groups pressed less than adults during the first hr of extinction [1-d: t=2.4, df=14, $p<0.05$; 12-d: t=2.5, df=19, $p<0.025$]. Even in the first 15 min of testing, adolescent-onset groups already made fewer active lever presses than adults [1-d: t=-3.7, df=14, $p<0.01$; 12-d: t=-3.4, df=19, $p<0.01$; data not shown]. When the first hr of extinction was analyzed as a percent of responding during self-administration (Fig. 3.3c), lever-pressing among the younger groups was lower than the older groups at each abstinence time point [1-d: t=-3.7, df=14, $p<0.01$; 12-d: t=-2.3, df=19, $p<0.05$]. For both age groups at both abstinence time points, lever pressing decreased across extinction sessions, according to a main effect of session [1-d: $F_{(5,70)}=11.13$, $p<0.001$; 12-d: $F_{(5,95)}=70.28$, $p<0.001$]. Finally, regardless of age group, overall extinction responding was greater af-

ter 12-days abstinence than 1-day, according to a main effect of abstinence time point
[$F_{(1,33)}$=28.55, p<0.001].

In cue-induced reinstatement after 12 days of abstinence, re-presentation of drug-paired cues appeared to trigger less heroin-seeking in adolescents than adults, as suggested by an age x abstinence time point interaction [$F_{(1,33)}$=4.61, p<0.05]. Follow-up t-tests for age differences after 12 days abstinence just missed significance [t=-2.00, df=19, p=0.06], however. Reinstatement increased over time in abstinence (incubation) for only the adult-onset group [Adolescent-onset: n.s. p>0.05; Adult-onset: t=-3.13 df=16 p<0.01] (Fig. 3.3a vs. 3.3b).

Pressing on the inactive lever during extinction and reinstatement did not differ by age or abstinence time point. Nor did total heroin intake (mg/kg) correlate with active lever pressing during extinction for any group (Fig. 3.3d and e).

Experiment 2 – Heroin self-administration (PR schedule) and extinction responding in the presence of cues.

Self-Administration. Adolescent rats self-administered more heroin than adults during the acquisition phase of Experiment 2 (FR schedules preceding PR; 0.05 mg/kg/infusion). The number of infusions per session differed according to main effects of age [$F_{(1,39)}$=5.55, p<0.05] and session [$F_{(8,312)}$=7.04, p<0.001] (Fig. 3.4a). Total heroin intake (mg/kg) over all 9 sessions of FR testing was also higher for adolescents than adults [t=2.16, df=39, p<0.05] (Fig. 3.4a inset). Rats in both age groups pressed more on the active vs. inactive lever, according to a main effect of lever [$F_{(1,78)}$=39.08, p<0.001](Fig.

3.4b), although adolescents pressed more than adults on both levers, according to a main effect of age [$F_{(1,78)}$=7.18, p<0.01]. In contrast to Experiment 1, the percent of non-reinforced responding did not differ by age (data not shown).

Adolescent and adult rats reached the same maximal number of infusions (break-point) and session lengths on the PR schedule (Fig. 3.5a and b; 0.0125 mg/kg/infusion dose: adolescent n=10, adult n=8; 0.05 mg/kg/infusion dose: adolescent n=22, adult n=19; 0.1 mg/kg/infusion dose: adolescent n=12, adult n=11). Number of infusions and sessions lengths did not vary by age, but did vary by main effects of dose [infusions: $F_{(2,81)}$=4.15, p<0.05; session lengths: $F_{(2,81)}$=68.9, p<0.001], such that subjects earned fewer infusions of the 0.0125 vs. 0.1 mg/kg/infusion doses [t=-2.34, df=39, p<0.05], and fewer of the 0.05 vs. 0.1 mg/kg/infusion doses [t=-3.45, df=22, p<0.025], but infusions of 0.0125 vs. 0.05 mg/kg/infusion doses did not differ [n.s. p>0.05](Fig. 3.5a). Session lengths were also dose-dependent (Fig. 3.5b), comparing 0.0125 vs. 0.05 mg/kg/infusion doses [t=9.3, df=17, p<0.001], 0.0125 vs. 0.1 mg/kg/infusion doses [t=-13.2, df=39, p<0.001], and 0.05 vs. 0.1 mg/kg/infusion doses [t=-7.4, df=22, p<0.001]. Graphs from individual representative rats show that adolescents took more infusions earlier in the sessions than adults (steeper slope of cumulative infusions) at the 0.05 and 0.1 mg/kg/infusion doses (Fig. 3.6a-b,d-e,g-h). Targeted analysis driven by these observations confirmed that adolescents had earned more infusions by 125-min into the session at 0.05 [t=2.83, df=39, p<0.01] and 0.1 mg/kg/infusion [t=2.30, df=21, p<0.05](Fig. 3.6c,f,i).

Extinction Responding in the Presence of Cues. Simultaneous re-exposure to the drug-taking environment and drug-paired cues after 12 days of abstinence (extinction

responding in the presence of cues) triggered heroin-seeking behavior similarly in adolescent- and adult-onset groups, and the number of presses correlated with prior heroin intake (Fig. 3.7). Presses on the active lever did not differ across age groups [n.s. $p>0.05$, Fig. 3.7 inset], although they correlated positively with total heroin intake, according to a significant Pearson's coefficient (adolescent-onset: $r=0.43$; two-tailed $p<0.05$; adult-onset: $r=0.71$; two-tailed $p<0.001$).

3.5   Discussion

In the present study, adolescent rats self-administered more heroin than adults when re-
sponse requirements were low, i.e. during spontaneous acquisition on FR1-5 schedules of
reinforcement and early in sessions on the PR schedule. As response requirements in-
creased later in PR sessions, however, adolescents earned the same number of infusions
as adults over a 10-fold dose-range, suggesting that age differences in the acute reinforc-
ing potency and efficacy of heroin are not robust. Perhaps more importantly, extinction
responding and reinstatement of heroin-seeking after abstinence were attenuated after
adolescent-onset of heroin self-administration on a fixed ratio schedule of reinforcement,
compared with adult-onset. This study extends our work on age differences in sensitivity
to acute and residual reinforcing effects of drugs of abuse, and again surprisingly sug-
gests that younger rats may be less sensitive than adults to some enduring effects of drug
self-administration.

Heroin appeared to maintain lever-pressing in the present experiments. For ex-
ample, all rats gradually discriminated between the heroin-associated (active) lever and
the inactive lever over the first few days of behavioral reinforcement, as expected for
spontaneous acquisition procedures. In addition, decreasing the unit dose of heroin avail-
able on a FR schedule of reinforcement resulted in the expected increase in number of
infusions per session (Weeks and Collins 1964). On the PR schedule, infusions earned
through the first two hours of the sessions were clearly dose dependent and the session
length increased with dose as expected (Hodos 1961), although we did not observe robust
effects of dose on the maximal number of infusions (breakpoint). Finally, all subjects
exhibited extinction of lever-pressing in the absence of heroin, and "incubation of drug

craving" was demonstrated as heightened extinction responding in both age groups and heightened cue-induced reinstatement in adult-onset groups over longer abstinence periods (Shalev et al. 2001; Zhou et al. 2009).

We had hypothesized that adolescent rats would be more sensitive than adults to the acute reinforcing effects of heroin, and predicted that this would be reflected as lower rates of drug intake on a FR schedule of reinforcement with the doses we tested on the descending limb of a dose-effect function (Arnold and Roberts 1997; Carroll and Lac 1997; Koob et al. 1984). In other words, higher potency of heroin in adolescents would produce a leftward shift in the inverted U-shaped function. In contrast, adolescent rats took more infusions than adults. If these results are a component of a rightward shift in the dose-effect function, then they suggest lower potency of heroin in younger animals, and fail to support our hypothesis. Alternatively, higher rates of responding could reflect an upward shift in the dose-effect function, which is interpreted as higher reinforcing efficacy. In the absence of a full dose-effect function, which is difficult to generate in the short period of rodent adolescence, we attempted to use a PR schedule of reinforcement to help interpret these data. On the PR schedule, higher reinforcing efficacy of heroin should result in a higher breakpoint. The present lack of age difference in breakpoints in fact failed to clarify the results from the FR schedule, and further called into question the robustness of age differences in the acute reinforcing effects of heroin. When we conducted a more detailed analysis of responding on the PR schedule, we did confirm that adolescents again took more heroin than adults when response requirements were low, but not when behavioral demands increased. Whether this relatively modest age difference is explained by pharmacokinetic or pharmacodynamic effects of the drug, or by age

differences in motor activation or impulsivity, cannot be determined from the present data set.

With regard to the role of age-dependent motor activation in drug self-administration, we and others have indeed reported higher rates of non-reinforced responding or a lack of stimulus control among younger vs. older animals during operant conditioning tests (Doherty et al. 2009; Shahbazi et al. 2008; Adriani and Laviola 2003; Sagvolden and Sergeant 1998; Spear and Brake 1983). Also, impulsivity may be a vulnerability factor for drug use (Cardinal et al. 2001; Perry and Carroll 2008). Although the present study does not rule out a role for motor effects in the modest age differences in heroin self-administration, at least two factors detract from this explanation. First, the role of impulsivity in reward and reinforcement by opiate drugs, per se, has been questioned (McNamara et al. 2010). Second, unpublished preliminary results from our laboratory suggest that locomotion in the operant conditioning chambers does not differ across age groups.

Relapse to drug abuse is the crux of drug addiction (REFS). We tested for age differences in extinction responding and cue-induced reinstatement of heroin-seeking. In partial support of our hypothesis, extinction responding after 1 or 12 days of abstinence was lower among adolescent- vs. adult-onset groups, as was cue-induced reinstatement following extinction after 12 days of abstinence (Experiment 1). The strength of these results lies in comparison with previous reports of lower extinction and/or cue-induced reinstatement of cocaine- or morphine-seeking in adolescent-onset groups (Doherty et al. 2009; Li and Frantz 2009; Anker and Carroll 2010).

Many factors influence reinstatement of drug-seeking in adult rats, such as amount of prior drug intake, rate of extinction learning, memory of drug-cue associations, and aversive withdrawal. Amount of drug intake did correlate with heroin-seeking behavior for both age groups in the present Experiment 2, as it has before (Liu et al. 2008; Zhang et al. 2004). Yet total heroin intake failed to correlate with heroin-seeking behavior in the present Experiment 1. Previously, morphine intake correlated with cue-induced reinstatement of morphine-seeking under short daily access conditions, but not extended 8-hr daily access (Doherty et al. 2009). Thus, prior drug intake cannot entirely explain age differences in reinstatement. Other evidence rules out faster extinction learning as an explanation for age differences in extinction; similar to our prior report on morphine-seeking (Doherty et al. 2009), the present adolescent-onset groups made fewer lever-presses than adults even within the first 15-min of extinction testing, i.e. the downward slope of extinction responding was not steeper among younger cohorts, as would be expected if they learned faster than adults (Everitt et al. 2001; Taylor et al. 2009).

Alternatively, the memory of drug-cue associations could be compromised in younger rats, thereby attenuating their extinction responding or reinstatement. Especially with psychostimulant drugs, drug-cue associations in adult rats facilitate both self-administration and reinstatement of drug-seeking (Chaudhri et al. 2006). Although no evidence of compromised learning and memory among adolescent-onset groups was observed in a test of sucrose pellet self-administration and reinstatement (Li and Frantz 2010), we cannot rule out a specific memory-impairing effect of drugs in younger subjects. In fact, adolescent rats given systemic nicotine did demonstrate less nicotine-paired cue conditioning than adults (Schochet et al. 2004).

Perhaps the most likely explanation of the present age differences in extinction responding and cue-induced reinstatement, however, is lower levels of aversive drug withdrawal among adolescent compared with adult rats. In adults, drug withdrawal contributes to drug-seeking after abstinence, perhaps in an attempt to alleviate an aversive state (Kenny et al. 2006; Koob and Le Moal 2008). Mounting evidence suggests that adolescent subjects exhibit less aversive drug withdrawal than adults. For example, adolescent male rodents exhibit fewer physical and affective signs of nicotine withdrawal (Infurna and Spear 1979; O'Dell et al. 2006; O'Dell et al. 2007; Shram et al. 2008b; Wilmouth and Spear 2004; Natividad et al. 2010), or morphine withdrawal (Hodgson et al. 2009), and fewer "hangover-like" effects of ethanol (Doremus et al. 2003; Varlinskaya and Spear 2004). Preliminary results from our laboratory suggest that adolescents also exhibit fewer somatic and locomotor signs of withdrawal from chronic systemic heroin (Doherty et al. 2010). Although the present heroin self-administration conditions were unlikely to induce severe dependence per se, less aversive withdrawal in adolescents could lead to less extinction responding and reinstatement.

Of significant note, heroin-seeking measured as extinction responding in the presence of cues in the present Experiment 2 did not differ by age, similar to results for *cocaine*-seeking (Li and Frantz, unpublished observations). Perhaps the compound stimulus of re-exposure to the drug-taking environment simultaneous with re-presentation of discrete drug-paired cues drives drug-seeking more effectively in younger cohorts. Alternatively for the present study, experience with the high response requirements of the PR schedule could promote higher rates of drug-seeking in the younger animals.

Overall our data contribute to a body of literature suggesting that adolescent subjects may be less vulnerable than adults to some long-term drug effects (Shram et al. 2008a; Kerstetter and Kantak 2007; Frantz and Parsons 2001). These results counter predictions based on human survey data (Anthony and Petronis 1995; Clark et al. 1998; Kandel et al. 1992; Palmer et al. 2009), perhaps indicating that social factors and other variables not modeled in most rodent drug self-administration studies influence drug abuse by human adolescents. If verified in future studies, the present model might help reveal new targets for treatment of drug abuse by younger addicts, and may even predict better treatment outcomes for them.

3.6   Acknowledgements

3.7 Figures
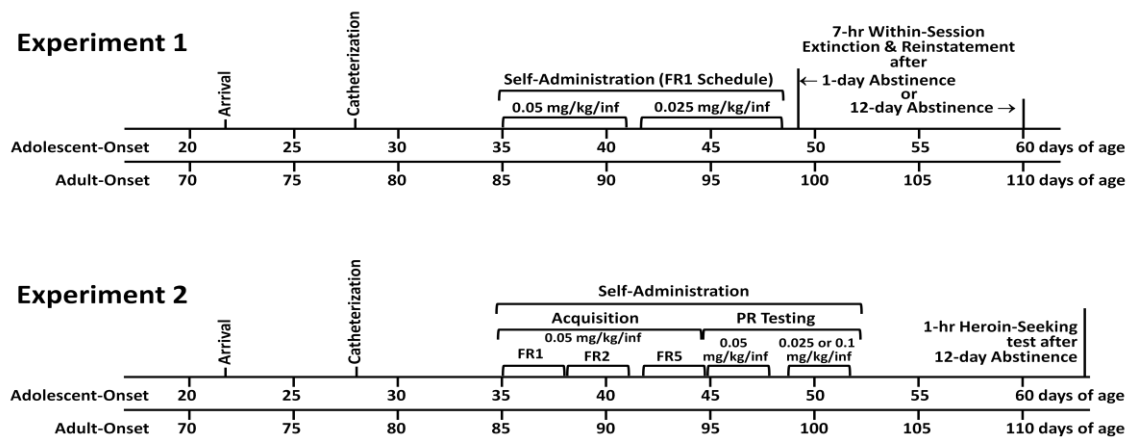
Fig. 3.1

Fig. 3.1 *Timeline of Experiments 1 and 2*

Fig. 3.2
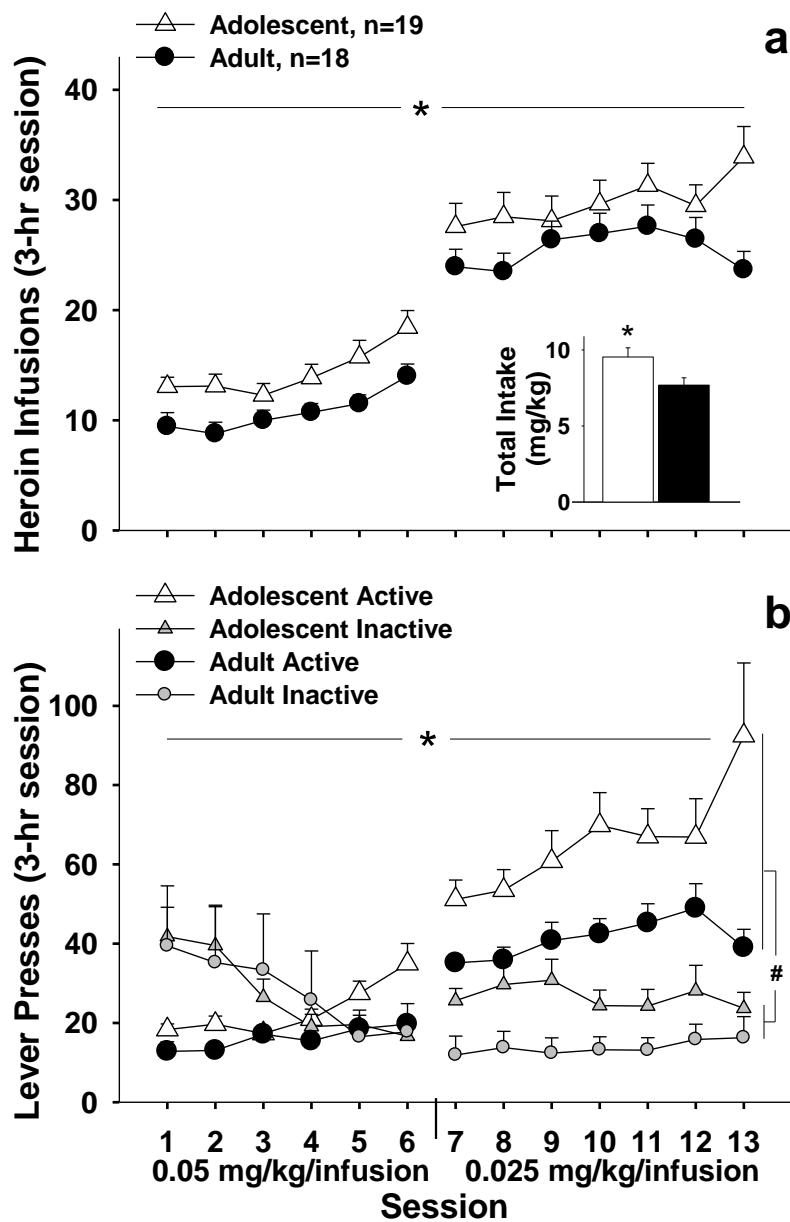
Fig. 3.2 *Experiment 1* (a) Number of infusions and total intake on a FR1 schedule of reinforcement (* main effect of age, p<0.05). *Inset* Total intake (mg/kg) (* p<0.05). (b) Active and inactive lever presses on a FR1 schedule of reinforcement (* main effect of age, p<0.01; # main effect of lever, p<0.001). All points or bars represent mean +/- SEM

Fig. 3.3
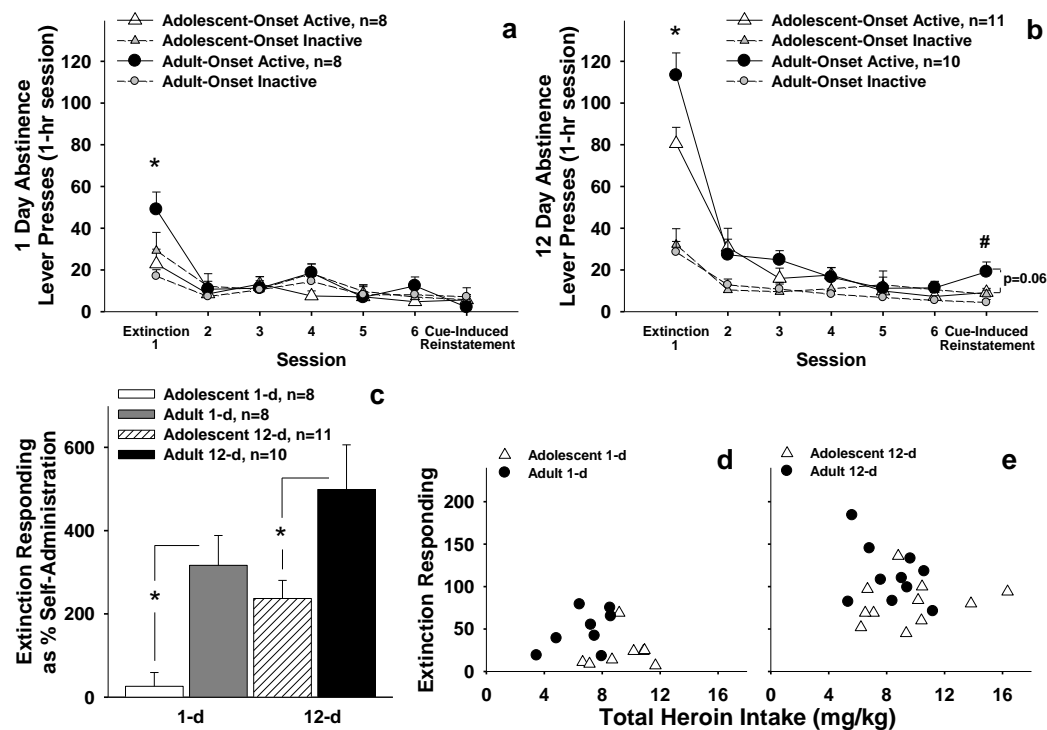
Fig. 3.3 *Experiment 1* Extinction and reinstatement of heroin-seeking after 1 (a) or 12

(b) days of abstinence. Adolescent-onset groups pressed less on the active lever during

the first hr of extinction testing than adult-onset groups (* p<0.05). Cue-induced reins-

tatement increased from 1 to 12 days of abstinence in adults (# p<0.05). Age differences

in cue-induced reinstatement just missed significance after 12 days of abstinence

(p=0.06). (c) Active lever presses during the first hr of extinction (data from *a* and *b*),

expressed as a percentage of the average number of active lever presses during the first hr

of testing in the last three self-administration sessions (effect of age * p<0.05). Total

prior heroin intake (mg/kg) did not correlate with active presses the first hour of extinc-

tion for (d) 1-d abstinence or (e) 12-d abstinence groups. All points or bars represent

mean +/- SEM

Fig. 3.4
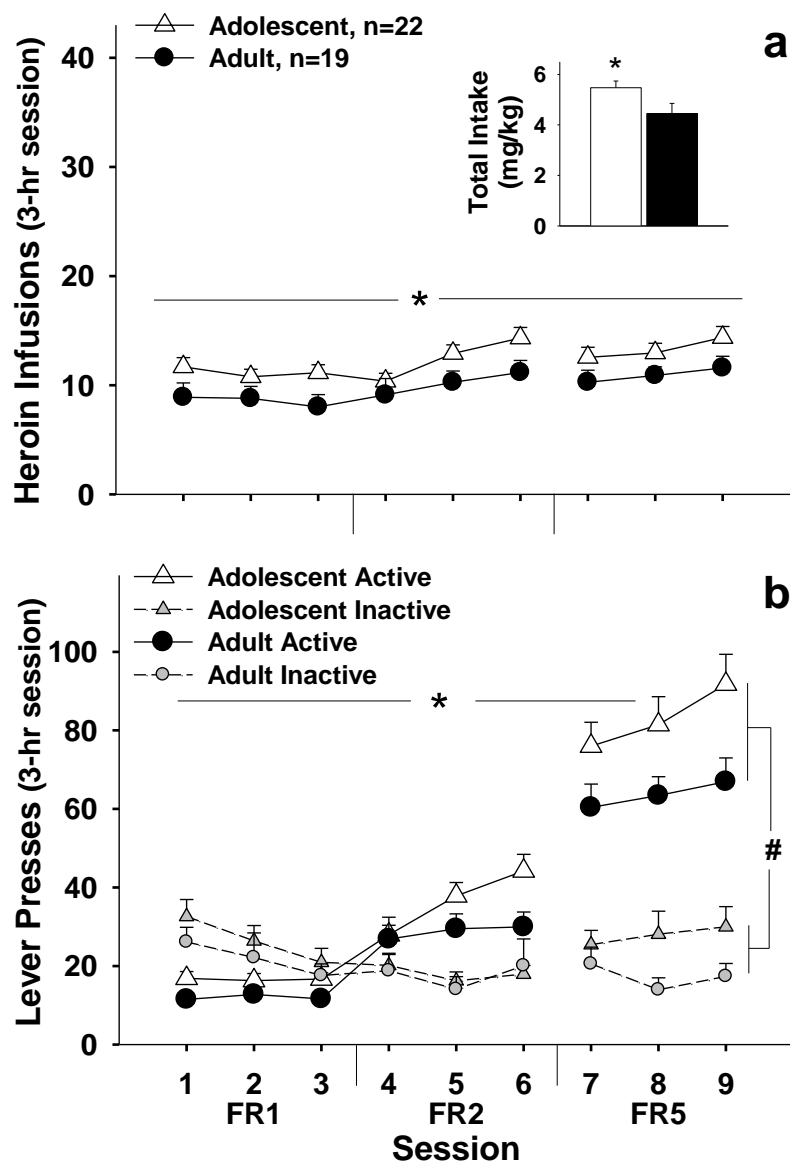
Fig. 3.4 *Experiment 2* (a) Number of infusions and total intake on FR schedules of rein-

forcement at 0.05 mg/kg/infusion (* main effect of age, p<0.05). *Inset* Total intake

(mg/kg) (* p<0.05). (b) Active and inactive lever presses on FR 1-5 schedules of rein-

forcement (* main effect of age, p<0.01; # main effect of lever, p<0.001). All points or

bars represent mean +/- SEM

Fig. 3.5
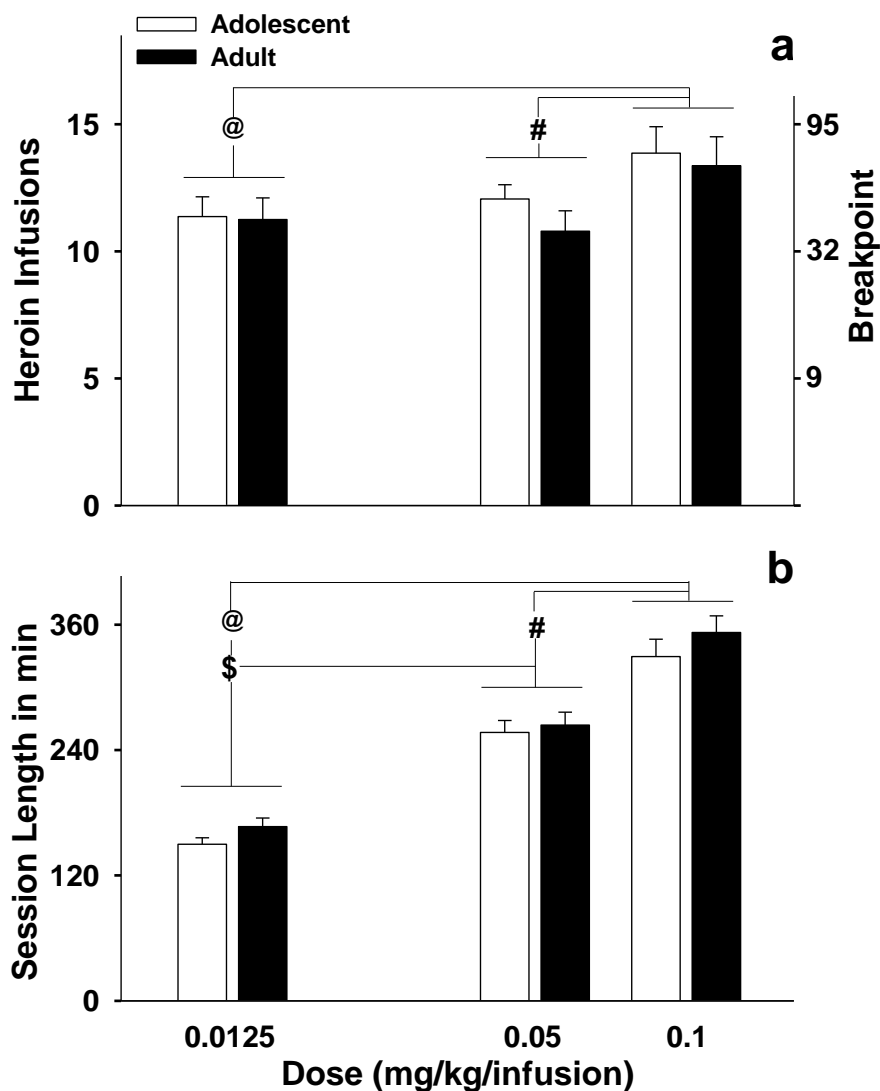
Fig. 3.5 *Experiment 2* (a) Number of infusions on a PR schedule of reinforcement, aver-
aged across three sessions at each dose comparing (effect of dose: 0.0125 vs. 0.1 doses @
p<0.05; 0.05 vs. 0.1 doses # p<0.025). (b) Session lengths on a PR schedule of rein-
forcement averaged across three sessions at each dose (effect of dose: 0.0125 vs. 0.05
doses $ p<0.001; 0.0125 vs. 0.1 doses @ p<0.001; 0.05 vs. 0.1 doses # p<0.001). All
bars represent mean +/- SEM

Fig. 3.6
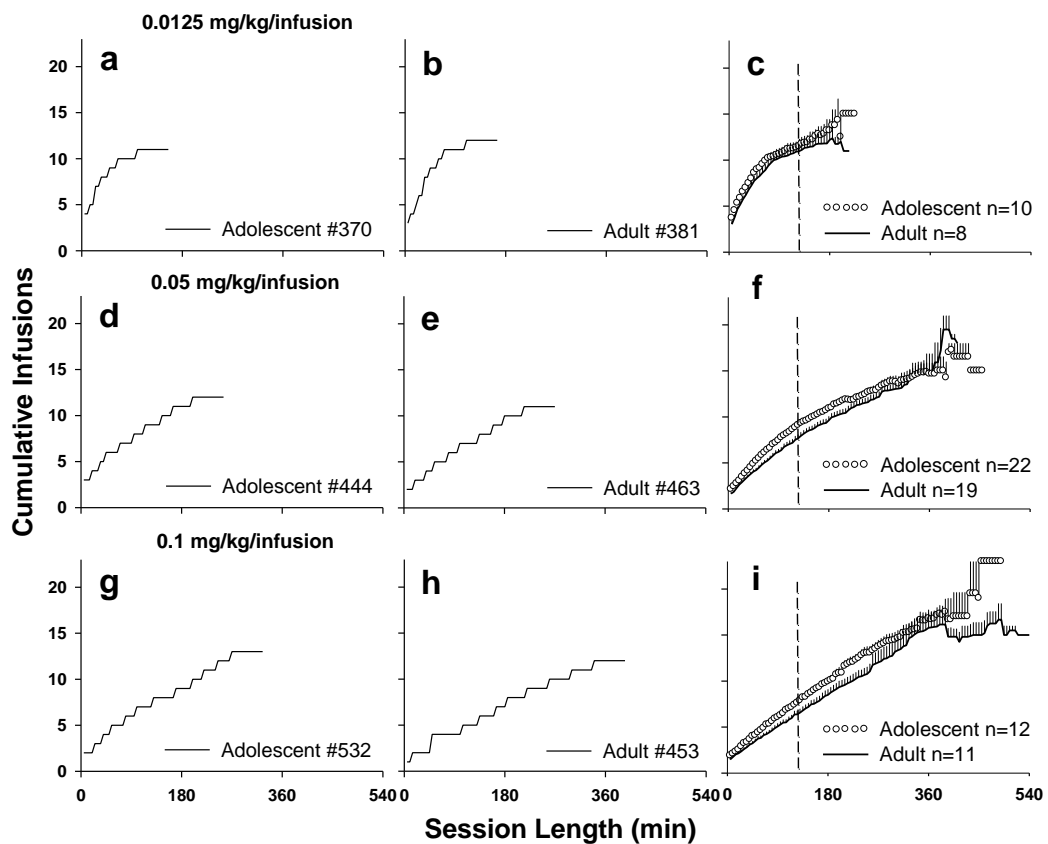
Fig. 3.6 *Experiment 2* Cumulative infusions earned for individual representative rats during a sample PR session at 0.0125 mg/kg/infusion (a-b), 0.05 mg/kg/infusion (d-e), and 0.1 mg/kg/infusion (g-h). Average total infusions earned at 5 min intervals throughout PR self-administration sessions at 0.0125 mg/kg/infusion (c), 0.05 mg/kg/infusion (f), and 0.1 mg/kg/infusion (i). Number of infusions earned by 125 min (dashed line) differed by age, p<0.01 and dose, p<0.001. All points represent mean +/- SEM

Fig. 3.7
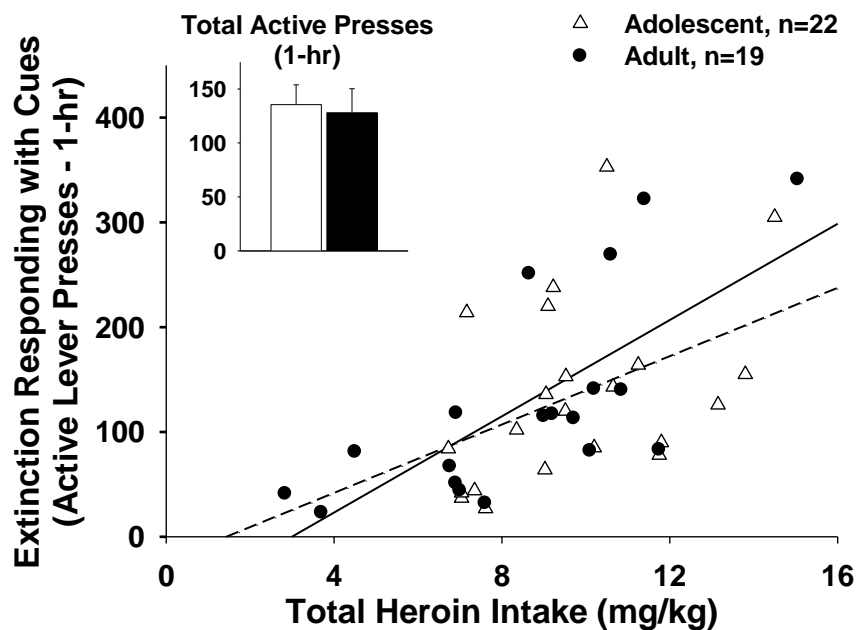
Fig. 3.7 *Experiment 2* Extinction responding in the presence of previously heroin-paired cues after 12 days of abstinence. The number of presses correlated positively with prior heroin intake (Pearson's correlation: adolescent-onset r=0.43, two-tailed, p<0.05 dashed trend line; adult-onset r=0.71, two-tailed, p<0.001 solid trend line). *Inset* Total active lever presses during the heroin-seeking test. All bars represent mean +/- SEM and points represent individual rats

4 Chapter 4 - Attenuated effects of experimenter-administered heroin in adolescent vs. adult male rats: physical withdrawal and locomotor sensitization.

By

James M. Doherty and Kyle J. Frantz

4.1 Abstract

Objectives. Heroin abuse is prevalent among human adolescents, and early-onset drug use might increase chances of later drug addiction. Prior work from our laboratory suggests, however, that adolescent male rats are actually less sensitive than adults to some reinforcing effects of heroin. In the present study, we tested two likely behavioral correlates of sensitivity to reinforcement in animal models: signs of withdrawal and locomotor sensitization after repeated drug exposure. *Methods.* Adolescent (35 days old at start) and adult (79 days old at start) male Sprague-Dawley rats were administered an escalating dose regimen of heroin (i.p.), increasing from 1.0 to 8.0 mg/kg i.p. every 12 hours, across 13 days. Somatic signs of opiate withdrawal were observed and counted starting 12 hrs after the last heroin injection and continuing over 120 hrs; locomotor activity was recorded at the same time. Heroin-induced locomotor activity was measured at four time points: immediately after the first heroin injection, after 7 and 13 days of escalating heroin dosing, and finally after 12 days of abstinence; matrix crossings were counted from videotaped 45 min sessions, always after a 1 mg/kg heroin (or saline control) challenge injection. Body weight and food intake were measured daily. *Results.* Somatic and locomotor signs of spontaneous withdrawal were less robust among adolescents than adults at the 24-48 hr time points. Heroin-induced locomotor sensitization tended to be lower among adolescents at all measured time points. Adolescents also exhibited an attenuated effect of heroin on body weight and food intake. *Conclusion.* Reduced withdrawal and sensitization is consistent with the reduced reinforcing effects of heroin among adolescent male rats observed previously, and further suggests that heroin may produce less de-

pendence in younger rats. Thus, it is possible that adolescent rats will reveal important

neuroprotective factors that could be utilized in treatment for heroin addiction.

4.2   Introduction

Drug abuse is prevalent in the U.S. and is typically initiated in adolescence (Johnston et al. 2010; Mark et al. 2001; SAMHSA 2009). In fact, roughly 250,000 high school students recently reported using heroin at least once in their lifetime, a trend that has held steady for decades, while rates of synthetic opioid abuse by adolescents are on the rise (Johnston et al. 2010; SAMHSA 2009). Numerous reports suggest that early-onset drug use increases chances of later drug addiction (Anthony and Petronis 1995; Clark et al. 1998; Kandel et al. 1992; Palmer et al. 2009). However, whether or not these phenomena can be attributed to biological vulnerability associated with the adolescent phase of development is unclear (Shram et al. 2008a). Thus, a need exists to use animal models of adolescent drug exposure to help identify the potential role of biological vulnerability during adolescence in drug use and addiction. While adolescent exposure to psychostimulants has received some recent attention, the animal experimentation on adolescent sensitivity to the effects of opiates is more sparse.

Aversive withdrawal is thought to drive drug-seeking in acute and long-term conditions through the process of negative reinforcement (Kenny and Markou 2005a; Kenny et al. 2006; Koob et al. 1992). In human opiate addicts, the withdrawal syndrome is well characterized and follows a precise time course. The initial phase (within 6-hrs) consists of dysphoria, anxiety, and irritability without overt somatic signs and is considered the motivational component of withdrawal because addicts desire to relieve this aversive state by continued drug use. Subsequently, the intensity of somatic withdrawal signs increase and last for at least 2 days (reviewed in Frenois et al. 2005; O'Connor and Fiellin 2000). The severity of withdrawal from heroin has not been tested in adolescent vs. adult

rodents, so the first aim of the present study was to test for somatic signs of withdrawal after repeated drug exposure in adolescent vs. adult male rats. We used an experimenter-administered, escalating heroin dose regimen intended to produce dependence and to reveal somatic signs of spontaneous withdrawal from heroin upon cessation of drug injections (Antonilli et al. 2005; Ventayol et al. 1997; Yang et al. 2006; Zhou and Kalivas 2008). Mounting evidence from experiments with drugs other than heroin suggest that adolescent subjects exhibit less aversive withdrawal compared to adults. For example, adolescent male rats exhibit fewer physical and affective signs of nicotine withdrawal (Infurna and Spear 1979; Natividad et al. 2010; O'Dell et al. 2006; O'Dell et al. 2007; Shram et al. 2008b; Wilmouth and Spear 2004) and fewer "hangover-like" effects of ethanol (Doremus et al. 2003; Varlinskaya and Spear 2004). The drop in extracellular levels of dopamine in the NAcc during precipitated withdrawal from nicotine is also attenuated in adolescents (Natividad et al. 2010). Adolescent mice also exhibit less affective withdrawal from morphine compared to adult mice, as measured in forced swim and locomotor tests (Hodgson et al. 2009). Although not tested in a state of withdrawal, adolescent male rats are also less sensitive to the aversive properties of nicotine, amphetamine, or ethanol as measured by conditioned taste aversion (Infurna and Spear 1979; Vetter-O'Hagen et al. 2009; Wilmouth and Spear 2004). Therefore, our hypothesis is that adolescent male rats exhibit less withdrawal from heroin compared adults, and we predict that escalating doses of heroin administered to younger rats should produce fewer somatic and locomotor signs of spontaneous withdrawal within five days of the last heroin injection.

Locomotor sensitization is often a behavioral correlate of reward and reinforcement (Robinson and Berridge 1993), and perhaps a correlate of relapse to drug-seeking as well (Vanderschuren and Pierce 2010). In adult rats, long-term sensitized locomotor responses are observed after repeated injections of morphine (reviewed in Vanderschuren and Kalivas 2000), or heroin (Paolone et al. 2007; Pontieri et al. 1997; Ranaldi et al. 2009). Although not tested with heroin, adolescent male rats exhibit heightened morphine-stimulated motor activity and greater long-term locomotor sensitization after repeated morphine injections, compared to adults (Spear et al. 1982; White et al. 2008; White and Holtzman 2005). Given that we have observed some differences in the reinforcing effects of morphine vs. heroin in adolescent vs. adult male rats (Doherty et al. 2009; Doherty and Frantz in press), this experiment tested heroin-induced locomotor sensitization, per se. We measured locomotor activity after challenge doses of heroin during, and 12 days after, the experimenter-administered escalating dose regimen mentioned above. Based on heightened acute and sensitized locomotor activation by morphine in adolescent vs. adult male rats (Spear et al. 1982; White et al. 2008; White and Holtzman 2005), our hypothesis was that adolescent male rats exhibit heightened heroin-induced locomotor sensitization compared to adult male rats.

4.3   Methods

Subjects

A total of 40 male Sprague-Dawley rats (Charles River, Raleigh NC) arrived in the laboratory at postnatal day (P) P29 or P72 for adolescent or adult age groups, respectively. Rats were housed in groups of two or three in a temperature and humidity controlled vivarium (targeted at 68-72°F and 50% humidity) and maintained on a 12-hr light/dark

cycle, with lights off at 0700 hr. All behavioral testing occurred at approximately the same time every day. Food and water were available ad libitum in home cages. Principles of laboratory animal care were followed, and all procedures complied with the NIH Guide for Care and Use of Laboratory Animals (8th Ed., 2011) and were approved by the Institutional Animal Care and Use Committee of Georgia State University.

Drugs

Heroin HCl (generous gift from NIDA) was dissolved in sterile saline and filtered through a 25 µm syringe filter (Fisher Scientific, Inc.; Pittsburgh, PA) before intraperitoneal (i.p.) administration.

Procedures

Rats received daily injections of saline or heroin and were observed for somatic signs of withdrawal and tested for heroin-induced locomotor activity: adolescent-saline (n=10), adolescent-heroin (n=10), adult-saline (n=10), adult-heroin (n=9; 1 rat died from an apparent overdose following an injection of heroin). Testing began at P35 for the adolescent groups and P79 for the adult groups (Table 4.1). All injections (i.p.; 1 ml/kg), observations of withdrawal, and locomotor activity tests occurred in the same testing room under red light illumination. Body weight and food intake were measured daily. To compare with other data from our lab, rats were group housed (2-3 per cage) and food intake is reported as an average per cage with all rats in a cage receiving the same daily treatment with saline or heroin.

Escalating dose treatment (days 1–13). Every 12-hr for 13 days (at approximately 09:00 and 21:00 hr), rats received injections of either saline or heroin (daily mg/kg increased from 2.0 to 15.0). Rats were injected and placed individually in holding cages for 10 min. This dose regimen was chosen to produce dependence on heroin as measured by observable signs of withdrawal on abrupt cessation of drug administration (Antonilli et al. 2005; Ventayol et al. 1997; Yang et al. 2006; Zhou and Kalivas 2008).

Observations of withdrawal

Two classes of withdrawal signs were measured: counted somatic signs and observed body weight loss. Signs of spontaneous opiate withdrawal were observed and counted starting 12-hr after the last heroin injection and continuing at 24-hr intervals over 5 days. Following a method modified from Glover and Davis (2008) and (Maldonado et al. 1992), experimenters blind to treatment groups observed rats for the following somatic signs of opiate withdrawal: chewing, teeth chatter, eye twitching, head shakes, genital grooming, abdominal spasms, forepaw shaking, vocalizations, abnormal posture, jumping, wet dog shakes, and piloerection. For each rat, indices of withdrawal were counted in 5-min blocks for 45 min. Each withdrawal sign was quantified by assigning a score of "1" for each aforementioned sign if it occurred at all during a 5 min block, resulting in a maximum score of "9" for each sign over the 45 min observation period. As an additional measure of withdrawal severity, body weight lost since the last observation period was included in the withdrawal score (expressed as percent change in a 12-24 hr period). As mentioned above, food intake was monitored daily throughout the entire experiment and

used as another measure of withdrawal from heroin. Locomotor activity during each

withdrawal observation was also measured (see below for details of the locomotor testing

apparatus).

Locomotor testing

After six days of acclimation to our vivarium and daily handling, rats underwent a base-

line locomotor test with an injection of saline (day BL). Treatment groups were then

formed counterbalanced by baseline activity. Heroin-induced locomotor activity (1

mg/kg i.p. heroin, or saline control) was measured at 4 time points: immediately after the

first heroin injection (day 1), on the 7th day of escalating heroin dosing [see above] (day

7), on the 13th and last day of escalating heroin dosing (day 13), and a final test of sensi-

tization after 12 days of abstinence (day 25). Rats were acclimated to the testing room

for 20 min prior to injections. Locomotor activity was tested for 45-min in clean Plexig-

las chambers (53 L x 29 W x 20 H cm; with a small amount of corncob bedding spread

on the floor). Immediately after each 45-min locomotor test with 1.0 mg/kg heroin or

saline, rats were injected with the appropriate dose of heroin or saline to continue the es-

calating dose regimen [see above]. Activity was videotaped from overhead cameras and

quantified as matrix crossings (three evenly spaced matrix zones marked on the wire top

of Plexiglas chamber with tape).

Data analysis

Withdrawal scores and locomotor activity during withdrawal were analyzed using three-

way mixed-measures analyses of variance (ANOVAs), with age and treatment as the be-

tween-subjects factors and time point as the within-subjects repeated measure. Pre-planned two-tailed t-tests comparing each class of somatic withdrawal score (observed signs, body weight change) summed across all timepoints were also used to test for specific age or treatment effects. We did not observe eye twitching or forepaw shaking in any rat regardless of age or treatment group, so data for these signs are not reported. For absolute body weight, separate two-way mixed-measures ANOVAs were conducted for each age group, with treatment as the between-subjects factor and time point as the within-subjects repeated measure. Food intake was measured for each cage of 2-3 subjects, divided by the number of subjects, and reported as percent change from baseline or total food consumed from baseline through the fifth day of withdrawal. For food intake as percent baseline, separate two-way mixed-measures ANOVAs were conducted for each age group, with treatment as the between-subjects factor and time point as the within-subjects repeated measure. Total food consumed was analyzed using a two-way ANOVA with age and treatment as the between-subjects factors. For heroin-induced locomotor activity, no group differences occurred in baseline activity (day BL) or activity during the first test of heroin-induced locomotion (day 1), so locomotor activity was expressed as percent change from day 1 and analyzed using a three-way mixed measures ANOVA with age and treatment as between-subject factors and time point as the within-subjects repeated measure. Follow-up ANOVAs, and paired or unpaired two-sided t-tests with Bonferroni's correction were used for post-hoc comparisons, as appropriate. Results were considered significant if $p < 0.05$.

4.4   Results

Somatic signs of spontaneous withdrawal

Adolescents exhibited fewer somatic signs of spontaneous withdrawal than adults after an escalating dose regimen of heroin injections (Fig. 4.1 and Table 4.2). Specifically, adolescents treated with heroin displayed fewer somatic signs of withdrawal after abrupt cessation of drug injections compared to heroin-treated adults, as suggested by an age x treatment interaction [F(1,35)=5.50, p<0.05]. Follow-up analyses using Tukey's pairwise comparisons confirmed that the adolescent-heroin group displayed fewer somatic signs of spontaneous withdrawal than the adult-heroin group (p<0.01), and that among adult groups, rats treated with heroin displayed more somatic signs of withdrawal than the saline group (p<0.05). No significant effect of treatment occurred across adolescent groups. Also, no significant interactions occurred between timepoint and age or treatment.

Analysis of each individual somatic sign of withdrawal summed across all timepoints (Table 4.2) revealed that no individual observed sign differed by treatment in the younger group. In contrast, the adult-heroin group displayed more genital grooming [t=-2.3, df=17, p<0.05], piloerection [t=-2.6, df=17, p<0.025], and percent body weight lost [t=4.4, df=17, p<0.001], compared to their saline counterparts [t=2.5, df=17, p<0.025]. Comparisons across age groups revealed that the adolescent-heroin group displayed fewer wet dog shakes [t=-3.5, df=17, p<0.01], less piloerection [t=-2.6, df=17, p<0.025], and a lower percent of body weight loss [t=4.5, df=17, p<0.001], than the adult-heroin group.

Locomotor signs of spontaneous withdrawal

Matrix crossings during testing for withdrawal was not affected in the adolescent-heroin group compared to their saline counterparts; in contrast, the adult-heroin group exhibited fewer matrix crossings than the adult-saline group (Fig. 4.2). Generally, adolescents were more active during withdrawal tests compared to adults, regardless of treatment, as suggested by a main effect of age [$F(1,35)=5.08$, $p<0.05$]. No main effect of heroin treatment on locomotion during withdrawal occurred, nor were there any interactions with age, although matrix crossings differed across time, according to a main effect of time point [$F(5,175)=5.72$, $p<0.001$]. Pre-planned t-tests at each time point (with Bonferroni's corrections) revealed that matrix crossings during withdrawal tests did not differ in adolescent-heroin vs. adolescent-saline groups at any time point tested. The adult-heroin group was less active than the adult-saline group at the 12-hr time point [$t=2.5$ $df=17$ $p<0.025$]. The adolescent-heroin group was more active than the adult-heroin group during withdrawal tests at time points of 12-hr [$t=2.5$ $df=17$ $p<0.025$], day 1 [$t=3.3$ $df=17$ $p<0.01$], day 2 [$t=2.6$ $df=17$ $p<0.025$], day 4 [$t=2.8$ $df=17$ $p<0.025$], and day 5 time points [$t=2.5$ $df=17$ $p<0.025$]. Furthermore, when activity during withdrawal tests was summed across all time points, the adolescent-heroin group displayed significantly more activity than the adult-heroin group [$t=3.5$, $df=17$, $p<0.01$]. (Fig. 4.2 inset).

The effect of heroin on body weight

Adolescents exhibited an attenuated effect of heroin on body weight compared to adults (Fig. 4.3). No difference in weight gain occurred between adolescent rats administered daily saline vs. heroin over the entire course of the experiment [n.s. treatment effect

p>0.05]. In fact, body weight in adolescents increased steadily throughout the experi-ment, according to a main effect of time point [$F_{(18,324)}=2413.54$, $p<0.001$]. A signifi-cant time point x treatment interaction [$F_{(18,324)}=3.16$, $p<0.001$] was not robust, as fol-low-up unpaired t-tests (with Bonferroni's corrections) at each time point did not reveal any significant treatment effects in this age group, including during withdrawal tests. As expected, the adult-heroin group failed to exhibit normal rates of weight gain over the entire course of the experiment, compared to their adult-saline counterparts. The greatest difference was manifest during withdrawal from the escalating heroin treatment. Thus, body weight in adults differed according to a time point x treatment interaction [$F_{(18,306)}=11.10$, $p<0.001$]. Follow-up unpaired t-tests at each time point separately (with Bonferroni's corrections) revealed strong trends for a treatment effect on days 6 through 13, suggesting reduced body weight in adult-heroin vs. adult-saline groups (e.g. p values ranging from 0.089-0.054). We expected that the greatest disruptions in body weight would occur during withdrawal, and thus performed targeted unpaired t-tests for treatment effects on body weight at each withdrawal day. The adult-heroin group had significantly lower body weight than the adult-saline group at each withdrawal timepoint [$p<0.025$]. By 11 days abstinence from heroin (ABST 11), weight differences between adult treatment groups were no longer significant.

The effect of heroin on food intake

Effects of heroin to decrease food intake were attenuated in adolescents, compared to adults (Fig. 4.4). There was no main effect of treatment, nor interactions with treatment on food intake as a percent of baseline consumption by adolescents. Food intake by ado-

lescents increased steadily throughout the experiment, though, according to a main effect of time point [$F(17,136)=53.86$, $p<0.001$]. We expected disruptions in food intake during testing for withdrawal, and thus performed targeted unpaired t-tests for treatment effects on food intake at each withdrawal day. At withdrawal day 1, the adolescent-heroin group consumed significantly less food than the adolescent-saline group [$t=2.5$, $df=17$, $p<0.01$].

As expected, the adult-heroin group consumed significantly less than their saline counterparts across the experiment (Fig. 4.4). Food intake by adults differed according to main effects of treatment [$F(1,7)=40.03$, $p<0.001$] and a treatment x time point interaction [$F(17,119)=1.93$, $p<0.05$]. Follow-up unpaired t-tests at each time point separately revealed strong trends for a treatment effect on days 1 through 13 (e.g. p values ranging from 0.035-0.005, but Bonferroni's correction reducing the alpha level to 0.004), and significantly less food intake by the adult-heroin vs. adult-saline group on days 6 and 7 [$p<0.004$]. We expected the greatest disruptions in food intake during testing for withdrawal, and thus performed targeted unpaired t-tests for treatment effects on food intake at each withdrawal day. The adult-heroin group consumed significantly less food than the adult-saline group during the first 2 days of withdrawal [$p<0.05$].

Overall, heroin treatment did not affect the total food consumed (g) in adolescent rats, while the adult-heroin group reduced their food consumption significantly (Fig. 4.4 inset). With regard to total food consumed, a two-way ANOVA revealed a significant age x treatment interaction [$F(1,18)=5.49$, $p<0.05$], and follow-up pairwise t-tests (with Bonferroni's corrections) confirmed that total food consumed was significantly reduced in the adult-heroin vs. adult-saline groups ($p<0.01$), while no difference occurred in adolescent-heroin vs. adult-heroin rats or adolescent-heroin vs. adolescent-saline rats.

Heroin-induced locomotor sensitization

Both adolescent and adult rats exhibited similar levels of heroin-induced locomotion (Fig. 4.5). Matrix crossing per test day, analyzed as percent change from day 1, did not differ according to age [n.s. $F(1,35)=1.78$, $p>0.05$], nor were there any interactions with age. Heroin did produce a sensitized locomotor response, as suggested by main effects of treatment [$F(1,35)=12.72$, $p<0.001$] and test day [$F(3,105)=3.93$, $p<0.05$] and a treatment x test day interaction [$F(3,105)=5.49$, $p<0.025$]. Follow-up pairwise t-tests (with Bonferroni's corrections) collapsed across age revealed significant heroin sensitized locomotor responses at day 7 [$t=-3.76$, $df=37$, $p<0.001$], day 13 [$t=-2.83$, $df=37$, $p<0.01$], and day 25 [$t=-2.79$, $df=37$, $p<0.01$], compared to day 1.

4.5 Discussion

Adolescents treated daily with escalating doses of heroin exhibited attenuated somatic signs of spontaneous withdrawal and less disruption in body weight and food intake, compared to adults. Adolescents and adults had similar heroin-induced locomotor sensitization.

The present display of fewer withdrawal signs after heroin in adolescent vs. adult male rats adds to a growing body of literature on attenuated somatic withdrawal in adolescent rodents. For example, less intense somatic withdrawal has been reported among adolescent rats after nicotine (O'Dell et al. 2007) or alcohol (Doremus et al. 2003; Varlinskaya and Spear 2004). Although not exactly the same, somatic signs of withdrawal are likely to mirror degree of affective withdrawal, which is modeled in animals using intracranial self-stimulation (ICSS) to measure brain reward thresholds (Kenny and Markou 2005a; Kenny et al. 2006). Affective withdrawal may be a driving force for vulnera-

bility to relapse in drug addicts (Koob and Le Moal 2008). Thus, perhaps the most convincing evidence of attenuated withdrawal among adolescents is the fact that brain reward thresholds measured after adolescent nicotine exposure were not affected in younger rats, compared to disruptions in older rats (O'Dell et al. 2006). In addition, the drop in extracellular levels of dopamine in the NAcc during precipitated withdrawal from nicotine is attenuated in adolescents vs. adults (Natividad et al. 2010). Importantly, our data set with heroin align with data from Hodgson et al (2009) in which adolescent mice displayed less affective withdrawal from morphine as measured during forced swim and locomotor tests compared to adult mice. Thus, adolescent rodents exhibit less somatic and affective withdrawal from various drugs of abuse compared to adults.

Adolescents are also less sensitive to the aversive properties of drugs. Adolescent rats display insensitivity to the conditioned place aversive properties of nicotine (Shram et al. 2008b), and delta-9-tetrahydrocannabinol (THC) or cannabinoid agonists (Pandolfo et al. 2009; Quinn et al. 2008), and THC is less anxiogenic in adolescent vs. adult rats (Schramm Sapyta et al. 2007). Results on conditioned place aversive using alcohol are mixed; adolescent rats display conditioned place preference at mid-range doses that adult rats find aversive (Philpot et al. 2003), while another study reported no age difference in conditioned place aversion to alcohol (Pautassi et al. 2009). Although not tested in a state of withdrawal, adolescent male rats are also less sensitive to the aversive properties of nicotine, amphetamine, ethanol or THC as measured by conditioned taste aversion (Infurna and Spear 1979; Schramm Sapyta et al. 2007; Vetter-O'Hagen et al. 2009; Wilmouth and Spear 2004). Thus, adolescent rodents less sensitive to the aversive properties of various drugs of abuse compared to adults.

We did not observe a strong withdrawal syndrome in either age group, an effect with at least two possible explanations. In order to align intake and subsequent spontaneous withdrawal in our previous results with heroin self-administration in adolescent and adult rats (Doherty and Frantz in press), we intentionally chose a heroin dose regimen on the lower end of the total heroin doses given in previous studies (Antonilli et al. 2005; Ventayol et al. 1997; Yang et al. 2006; Zhou and Kalivas 2008), and we chose to quantify spontaneous, rather than precipitated, withdrawal signs. Spontaneous withdrawal signs in rodents are difficult to measure due to individual variability in observable withdrawal signs, but our data align with other reports on the amount and time course of spontaneous somatic signs of withdrawal from opiates (Cicero et al. 2002; Papaleo and Contarino 2006). In addition, we wanted to avoid problems with appropriate antagonist doses across age groups. Of note, it is not uncommon to observe somatic signs of withdrawal in rats treated with saline (O'Dell et al. 2006), as all of these somatic signs can be seen as a part of the general repertoire of behavior in rodents.

We used locomotor activity during withdrawal as a measure of general malaise, i.e. less locomotion might reflect more malaise. Our locomotor activity data during withdrawal correlates well with our other measures of somatic signs withdrawal. Younger rats did not exhibit malaise during withdrawal, but older rats had significantly depressed motor activity compared to age-matched saline-treated controls.

The effects of heroin on body weight and food intake were attenuated in adolescent vs. adult rats. The direct measures of body weight and food intake support our data on the somatic and locomotor signs of spontaneous withdrawal, with adolescents exhibiting attenuated effects of heroin on all measures. These data also support our published

data on attenuated effects of self-administered morphine on body weight disruptions in adolescent vs. adult male rats (Doherty et al. 2009) and unpublished observations of body weight disruptions during self-administration of heroin (Doherty and Frantz). Of significant note, age differences in the effect of heroin on body weight was a major contributor to the calculated score of somatic withdrawal, and it is possible that the demand for growth during the developmental period of adolescence opposes any detrimental effect of heroin on the related measures of body weight and food intake.

Adolescents and adults exhibited similar heroin-induced locomotor sensitization. Similar heroin-induced locomotor sensitization during the escalating dose regimen supports our self-administration data showing similar heroin intake in adolescent and adult male rats (Doherty and Frantz in press). In addition, similar heroin-induced locomotor sensitization after 12 d abstinence suggests a possible disconnect between the neural systems controlling psychomotor sensitization to heroin and the systems controlling heroin-seeking behavior after operant conditioning. In general, we did not observe robust locomotor sensitization, which could be due to our escalating dosing regimen. Locomotor sensitization is best tested using intermittent, not daily, administration. Nevertheless, both adolescents and adults increased locomotor activity significantly above saline treated controls, suggesting that our protocol succeeded in sensitizing heroin-induced locomotion. Comparing our withdrawal and sensitization data suggests that incentive-sensitization might play a lesser role than negative reinforcement in driving heroin self-administration or even drug-seeking after abstinence. It is possible that decreased negative reinforcement in adolescents contributes to attenuated drug-seeking after abstinence in rats that self-administer heroin (Doherty and Frantz in press), morphine (Doherty et al.

2009) and cocaine (Li and Frantz 2009) as adolescents compared to rats that self-administer as adults.

As noted in the introduction, age-dependent patterns of self-administration of morphine and heroin can be different (Doherty et al. 2009; Doherty and Frantz in press). The present heroin-induced locomotor sensitization results highlight yet another behavior with a different age-dependent outcome compared to reports with repeated morphine (White et al. 2008; White and Holtzman 2005). We report here that adolescents exhibit similar heroin-induced locomotor sensitization compared to adults. In contrast, White and Holtzman (2005, 2008) report more sensitized locomotor activity in adolescent vs. adult male rats.

Overall, our results during withdrawal converge to reveal a less severe withdrawal syndrome in adolescent vs. adult male rats. Reduced withdrawal in adolescents suggests that heroin produces less dependence in younger rats. Our results of attenuated withdrawal and physical indicators of heroin use in adolescent compared to adult male rats extends and supports our published results showing attenuated enduring effects of self-administering heroin, morphine or cocaine during the adolescent period on drug-seeking after abstinence (Doherty and Frantz in press; Doherty et al. 2009; Li and Frantz 2009). The lack of age difference in heroin-induced locomotor sensitization seen here also compliments the lack of robust age differences in the amount of heroin self-administered (Doherty and Frantz in press). If less withdrawal in adolescent rats translates to human adolescent drug addicts, adolescent addicts may respond better than adults to treatments aimed alleviating the motivational component of withdrawal.

4.6   Acknowledgements

4.7   Tables & Figures

Table 4.1

	Arrive	Baseline locomotor test (Day BL)	1st Heroin locomotor test (Day 1)	Escalating dose regimen (Days 1-13)	2nd Heroin locomotor test (Day 7)	3rd Heroin locomotor test (Day 13)	Withdrawal observations (Days 13-18)	Last Heroin locomotor test (Day 25)
Table 4.1. Timeline of experimentation, indicating postnatal age in days (d) at start of each experimental phase.								
Adolescent	29 d	35 d	36 d	36-48 d	42 d	48 d	48-53 d	60 d
Adult	72 d	78 d	79 d	79-91 d	85 d	91 d	91-97 d	103 d

Table 4.2

Table 4.2. Somatic signs of withdrawal summed across all timepoints (±SEM).
* Effect of treatment, within age groups. # Effect of age, between heroin-treated rats.

	Treatment	Chewing	Teeth chatter	Head shakes	Genital grooming	Abdominal spasms	Vocalizations	Abnormal posture	Jumping	Wet dog shakes	Piloerection	Body weight lost	Total
Adolescent	Saline	0.1 ±0.1	0.8 ±0.5	0.3 ±0.2	4.8 ±1.2	0.1 ±0.1	0.6 ±0.3	0.0 ±0.0	7.7 ±2.4	7.2 ±1.7	0.0 ±0.0	0.0 ±0.0	**21.6 ±4.0**
	Heroin	0.1 ±0.1	1.9 ±0.9	0.6 ±0.3	3.3 ±0.9	0.3 ±0.2	0.5 ±0.3	0.0 ±0.0	3.5 ±1.3	5.7 ±1.0	0.0 ±0.0	-0.3 ±0.1	**16.2 ±2.5**
Adult	Saline	0.3 ±0.2	3.0 ±1.3	0.5 ±0.3	2.7 ±0.4	0.4 ±0.3	0.6 ±0.2	0.0 ±0.0	5.2 ±1.5	8.3 ±2.4	0.0 ±0.0	-0.2 ±0.2	**21.2 ±4.1**
	Heroin	0.6 ±0.2	3.8 ±1.8	1.1 ±0.5	5.6* ±1.2	0.3 ±0.2	1.1 ±0.3	0.9 ±0.6	3.0 ±1.4	12.1# ±1.5	1.5*# ±0.6	-2.3*# ±0.4	**32.2*# ±3.0**

Fig. 4.1
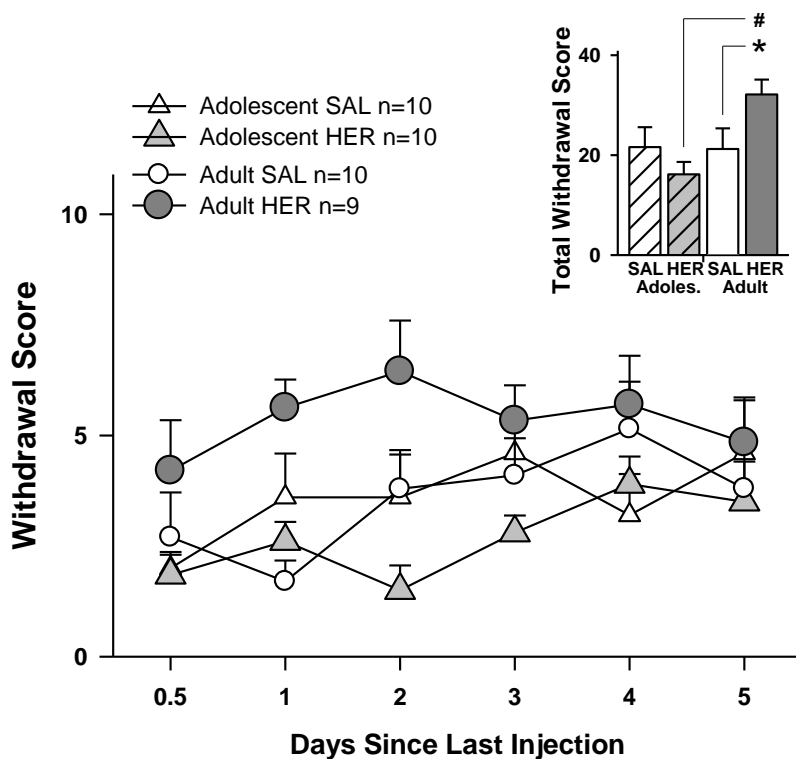
Fig. 4.1 Somatic signs of spontaneous withdrawal from repeated systemic heroin. The adult-heroin group trended toward more signs of withdrawal than the adult-saline and adolescent-heroin groups. *Inset.* Total withdrawal scores summed over all time points (days 0.5-5 since last injection). The adult-heroin group exhibited significantly more signs of withdrawal compared to the adolescent-heroin (# $p<0.05$) and adult-saline groups (* $p<0.05$). SAL=saline treatment, HER=heroin treatment. All points and bars represent mean +/- SEM

Fig. 4.2
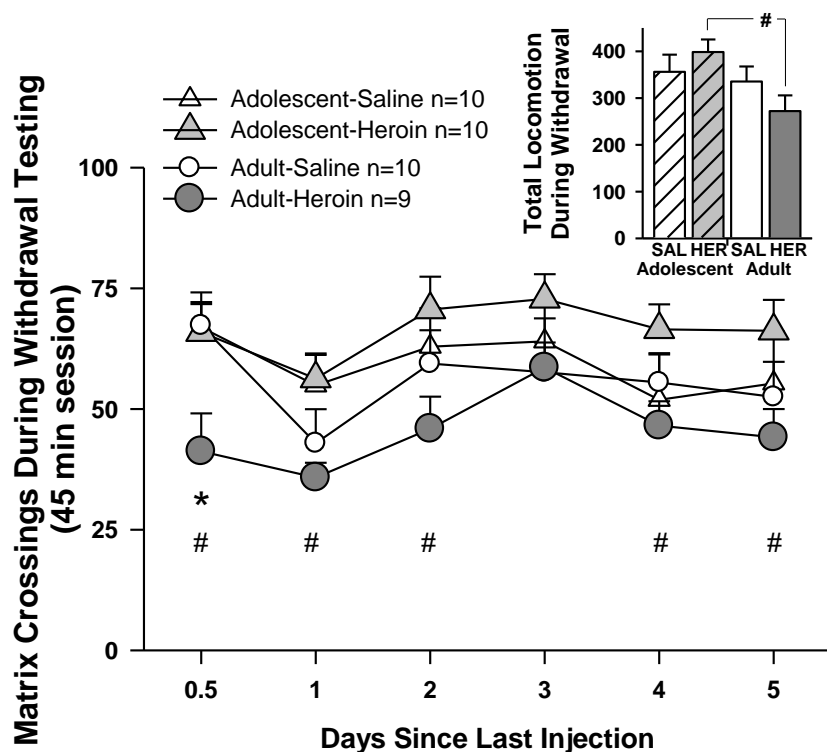
Fig. 4.2 Locomotor signs of spontaneous withdrawal from repeated systemic heroin. The adult-heroin group exhibited significantly less locomotion at only 0.5 day since the last heroin injection compared to their saline counterparts (* $p<0.05$). The adult-heroin group exhibited significantly less locomotion at 0.5-2 days, and days 4 and 5 since the last heroin injection compared to the adolescent-heroin group (# $p<0.05$). *Inset.* Total locomotion summed over all time points during withdrawal (days 0.5-5 since last injection). The adult-heroin group exhibited significantly less locomotion compared to the adolescent-heroin group (# $p<0.05$). SAL=saline treatment, HER=heroin treatment. All points and bars represent mean +/- SEM

Fig. 4.3
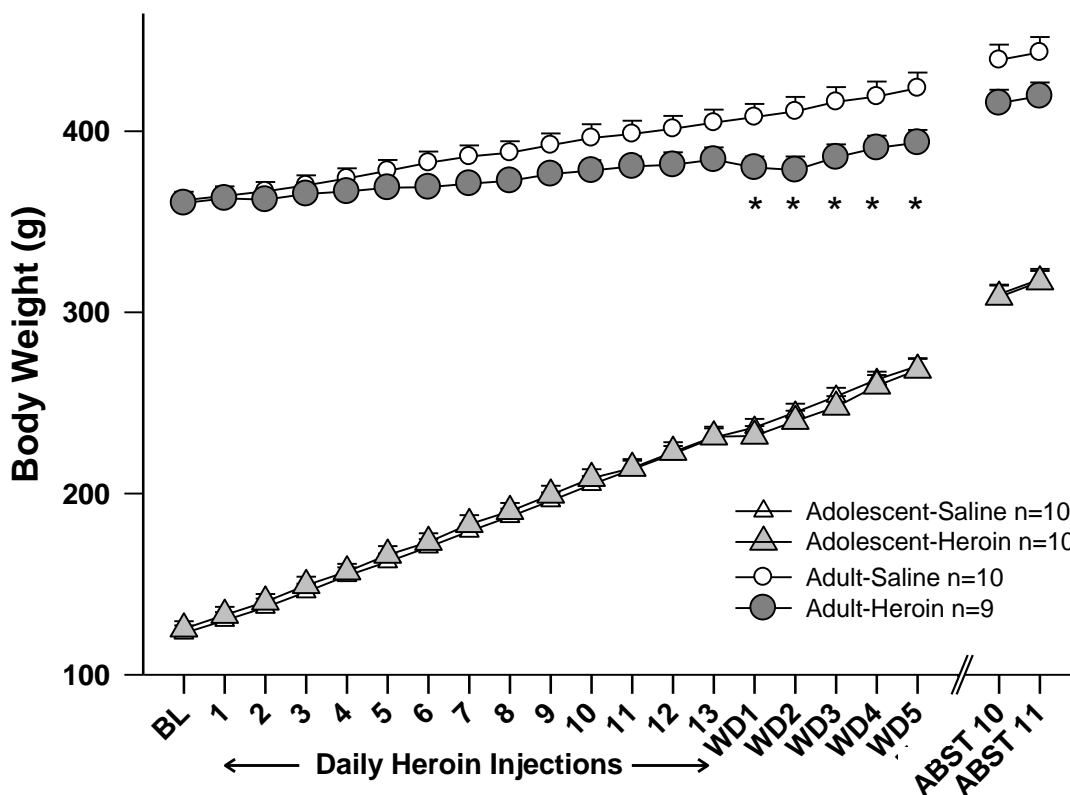
Fig. 4.3 The effects of repeated systemic heroin and withdrawal on body weight (g). The adult-heroin group did not gain as much weight as their saline counterparts, becoming significant during the first 5 days of withdrawal from heroin (* $p<0.05$). The adolescent groups did not differ from each other. BL=baseline, WD=withdrawal, ABST=abstinence. All points represent mean +/- SEM

Fig. 4.4
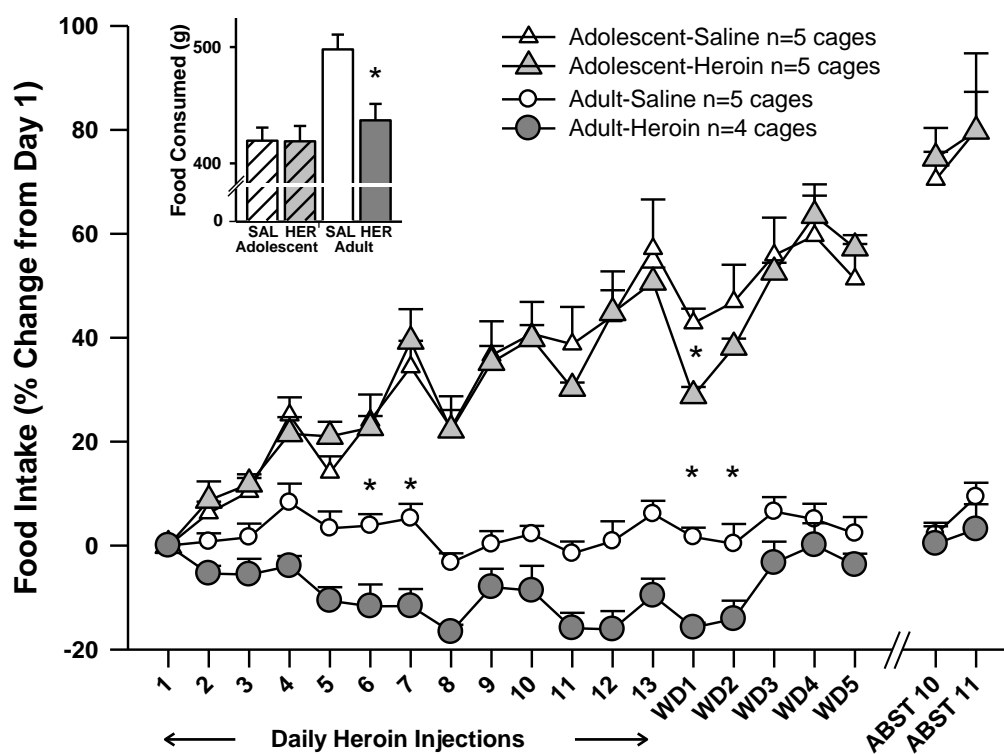
Fig. 4.4 The effects of repeated systemic heroin on food intake, expressed as percent change from day 1. The adult-heroin group trended toward eating less than their saline counterparts troughout the daily injection regimen, becoming significantly less at days 6 and 7, and days 1 and 2 of withdrawal (* $p<0.05$). The adolescent-heroin group ate significantly less than their saline counterparts at only day 1 of withdrawal (* $p<0.05$). *Inset.* Total food consumed from day 1 through the 5th day of withdrawal. The adult-heroin group ate significantly less than their saline counterparts (* $p<0.05$). Rats were group house by age and food intake is reported as an average per cage with all rats in a cage receiving the same daily treatment with saline or heroin. WD=withdrawal, ABST=abstinence, SAL=saline treatment, HER=heroin treatment. All points and bars represent mean +/- SEM

Fig. 4.5
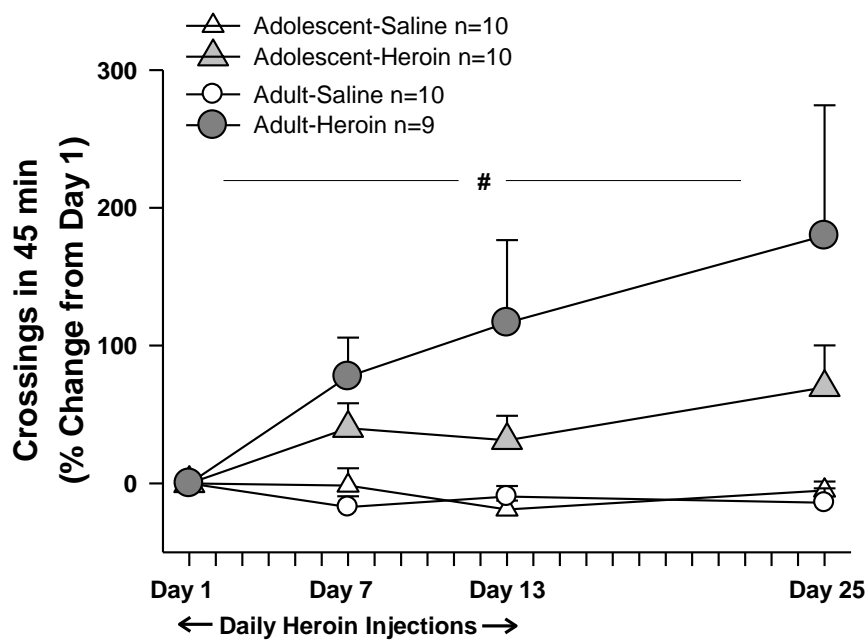
Fig. 4.5 Heroin-induced locomotor sensitization, expressed as percent change from day 1. The heroin groups exhibited significantly more locomotor activity after challenge doses of heroin (1 mg/kg, i.p.) compared to saline treated groups (main effect of treatment # $p < 0.05$). All points and bars represent mean +/- SEM

5 Chapter 5 – Attenuated Fos immunoreactivity in prelimbic and infralimbic medial prefrontal cortex during heroin-seeking behavior in adolescent vs. adult male rats

BY

James M. Doherty, Bradley M. Cooke & Kyle J. Frantz

5.1 Abstract

Rationale Heroin abuse is prevalent among teenagers. However, we have reported attenuated heroin-seeking behavior in male rats that self-administered heroin as adolescents, compared to adults. *Objectives* In order to provide insight regarding brain regions that might underlie age differences in heroin-related behavior, this study tested for a correlation between medial prefrontal cortex (mPFC) activation and attenuated heroin-seeking among adolescent male rats by measuring Fos, the protein product of the immediate early gene *c-fos*. *Methods* Adolescent (35 days old at start) and adult (86 days old at start) male Sprague-Dawley rats were allowed to spontaneously acquire lever pressing maintained by heroin (13 3-hr daily sessions; fixed ratio 1 schedule of reinforcement; 0.05 and 0.025 mg/kg/infusion). After 12 days of abstinence in the home cage, rats were tested for heroin-seeking in a 1-hr session back in the operant conditioning chamber. Immediately after the heroin-seeking test, rats were sacrificed and brain tissue was processed for Fos immunohistochemistry. Stereology was used to estimate the total number of Fos+ and Fos- neurons over the rostral-to-caudal extent of the prelimbic and infralimbic medial prefrontal cortex (mPFC). *Results* Adolescent and adult rats self-administered similar amounts of heroin, but rats that self-administered as adolescents (adolescent-onset) exhibited attenuated heroin-seeking compared to adults (adult-onset). Similarly, the ratio of Fos+ / Fos- neurons within prelimbic and infralimbic mPFC was attenuated in the adolescent-onset group, compared with the adult-onset group. *Conclusions* Lower level of neural activity within the mPFC is associated with attenuated heroin-seeking behavior in the adolescent-onset group. Thus, the mPFC could play a key role in age-dependent enduring effects of heroin self-administration in male rats.

5.2   Introduction

Drug abuse is prevalent in the U.S. and is typically initiated in adolescence (Johnston et al. 2010; Mark et al. 2001; SAMHSA 2009). In fact, more than 4 million people are currently addicted to heroin in the U.S. and majority of these addicts initiated use during adolescence (Johnston et al. 2010; SAMHSA 2009). Numerous reports suggest that early-onset drug use increases the chances of later drug addiction (Anthony and Petronis 1995; Clark et al. 1998; Kandel et al. 1992; Palmer et al. 2009). Whether or not these phenomena can be attributed to biological vulnerability associated with the adolescent phase of development, however, is unclear (Shram et al. 2008a). Animal models of adolescent drug exposure allow testing for the potential role of biological vulnerability during adolescence in drug use and addiction.

Long-lasting vulnerability to relapse to drug-taking during periods of abstinence is a major focus for the treatment of drug addiction (Chung and Maisto 2006; O'Brien 1997). In animal models of human relapse, drug-seeking after abstinence from i.v. drug self-administration in an operant conditioning chamber can be measured in several ways, including resistance to extinction of responding on a drug-associated lever and reinstatement of responding on the drug-associated lever triggered by re-exposure to the drug-taking environment (context), to discrete cues previously paired with drug infusions, to stress, or to the drug itself (reviewed in Shaham et al. 2003). These extinction and reinstatement animal models have just begun to be tested in adolescent subjects. A striking paucity of animal literature on adolescent sensitivity to opiates in general, and extinction or reinstatement of opiate-seeking in particular, makes research in this field challenging. Male rats that self-administer heroin, morphine, or cocaine as adolescents (known as ado-

lescent-onset groups) exhibit attenuated extinction responding and/or cue-induced reinstatement of drug-seeking after abstinence compared to rats that took drugs as adults (known as adult-onset groups) (Doherty and Frantz in press; Doherty et al. 2009; Li and Frantz 2009), which we interpret as lower sensitivity to some long-term motivational effects of drug-taking during adolescent development. Other studies with drugs in the psychostimulant class support our interpretation of less long-term drug effects in adolescent vs. adult male rats, although some mixed results exist. For example, adolescents exhibit faster extinction of nicotine-seeking (Shram et al. 2008a), less nicotine-paired cue conditioning (Schochet et al. 2004), faster extinction and less reinstatement of cocaine conditioned place preference (Balda et al. 2006, but see Brenhouse and Andersen 2008 for opposite results), and less severe cognitive impairment after cocaine intake in an amygdala-dependent task (Kerstetter and Kantak 2007, but see Harvey et al. 2009 for greater impairment after cocaine intake in a orbitofrontal task). We have also found that aversive withdrawal from heroin, which is thought to drive drug-seeking through negative reinforcement (Kenny and Markou 2005a; Kenny et al. 2006), is less severe in adolescent vs. adult male rats (Chapter 4; Doherty and Frantz in preparation).

To begin to explore the neural circuitry underlying the attenuated drug-seeking we observed in adolescent vs. adult rats, the present study focused on two forebrain areas known to be important for drug-seeking in adults, the prelimbic and infralimbic areas of the medial prefrontal cortex (mPFC). The mPFC plays a critical role in relapse among human drug addicts, as well as in animal models of relapse behavior (Kalivas and Volkow 2005; Volkow et al. 2003). The prelimbic and infralimbic areas are thought to play opposing roles during drug-seeking, promoting or inhibiting cocaine-seeking, respective-

ly (Peters et al. 2009). However, a recent report actually suggests heroin-seeking may be promoted by activity in either region (Bossert et al. 2011). Moreover, the PFC and its related circuitry continue to mature through adolescent development (Adriani and Laviola 2004; Andersen et al. 2000; Crews et al. 2007; Kalsbeek et al. 1988; O'Donnell 2010b; Spear 2000), suggesting that it might either be more vulnerable to long-term detrimental effects of early drug exposure, or its maturational trajectory could facilitate "recovery" from early drug insult. Therefore the aim of the present study was to determine the proportion of Fos immunoreactive (Fos-ir) neurons activated during a one hour heroin-seeking test conducted 12 days after the last of 13 heroin self-administration sessions in adolescent- vs. adult-onset male age groups, along with comparison to age-matched saline control groups. We stained brain tissue for Fos, which is the protein product of the immediate early gene (IEG), *c-fos*. Induction of *c-fos* marks stimulus-elicited brain activity (Chaudhuri 1997; Harlan and Garcia 1998), and is a marker of neuroplasticity associated with acute drug use (Nestler 2001). Up-regulation of *c-fos* levels in mPFC occurs after context- or cue-induced reinstatement of heroin-seeking in adult rats (Bossert et al. 2011; Koya et al. 2006; Kuntz et al. 2008; Schmidt et al. 2005). We used computer-guided unbiased stereology to estimate the overall number of Fos-ir positive (+) and Fos-ir negative (-) neurons over the rostral-to-caudal extent of the prelimbic and infralimbic mPFC (neurons were identified by counterstaining with cresyl violet). We hypothesized that the mPFC of the adolescent-onset male group would show less neuronal activation in the mPFC (lower proportion of Fos+ neurons) during heroin-seeking than the adult-onset male group, and that levels of activation in mPFC would correlate with heroin-seeking.

5.3 Methods

Subjects

Male Sprague-Dawley rats (Charles River, Raleigh NC) arrived in the laboratory at post-natal days (P) 22 (n=41) or P70-74 (n=37) for adolescent or adult age groups, respectively. Rats were housed in groups of two or three in a temperature and humidity controlled vivarium (targeted at 68-72°F and 50% humidity) and maintained on a 12-hr light/dark cycle, with lights off at 0700 hr. All behavioral testing occurred at approximately the same time every day during the dark phase. Body weights were recorded daily to monitor health and to adjust drug doses. Food and water were available ad libitum in home cages. Principles of laboratory animal care were followed, and all procedures complied with the NIH Guide for Care and Use of Laboratory Animals (8th Ed., 2011) and were approved by the Institutional Animal Care and Use Committee of Georgia State University.

Drugs

Heroin HCl (generous gift from NIDA), methohexital sodium (1% Brevital Sodium, King Pharmaceuticals, Inc.; Bristol, TN), and Timentin antibiotic (GlaxoSmithKline; Research Triangle Park, NC), were all dissolved in sterile saline and filtered through a 25 μm syringe filter (Fisher Scientific, Inc.; Pittsburgh, PA) before i.v. administration.

Self-administration and Reinstatement Procedures

Equipment. Heroin self-administration was conducted in operant conditioning chambers housed in sound-attenuating cubicles (MedAssociates, Inc.; St Albans, VT). Each cham-

ber was equipped with two retractable levers. Presses on the active lever initiated a syringe pump with a 5 rpm motor (PVM-1000VS, Med Associates Inc.; St Albans, VT) to deliver an infusion via a stainless steel swivel and a polyethylene tube protected by a metal coil and attached to the catheter portal on each subject's back. Presses on the inactive lever were recorded but had no scheduled consequences. Drug delivery and data collection were controlled by a computer system using Med Associates software (Med PC IV). The i.v. catheters for drug self-administration were made as previously described (Caine et al. 1993), with minor modifications including a shorter length of tubing inserted into the jugular vein for adolescents (2 cm) compared with adults (4 cm)(Shahbazi et al. 2008).

Intravenous (i.v.) catheter implantation. Adolescent (P28-29) and adult (P76-81) rats were surgically catheterized in the right jugular vein, generally according to Caine et al. (1993), with minor modifications (Shahbazi et al. 2008). Briefly, rats were anesthetized with an isoflurane-oxygen vapor mixture (4-5% for initial anesthetization and 1.5-2.5% for the remainder of surgery). Catheter tubing was passed subcutaneously from the back and inserted into the right jugular vein, tied in place with sutures, and glued with cyanoacrylate. During recovery, adolescent and adult rats received 0.15 or 0.2 ml, respectively, of the antibiotic Timentin (Ticarcillin Disodium and Clavulanate Potassium; 100 mg/ml, i.v.) twice daily for two days post-surgery, then once daily for the remainder of the experiment. Catheters were also flushed daily with 0.15-0.4 ml heparinized saline (30 USP units/ml) to promote catheter patency. Catheter patency was tested one day before, once on the recess day, and one day after heroin self-administration, by injecting 0.1-0.4 ml of

a short-acting barbiturate anesthetic (Brevital) through the catheter. If muscle tone was not lost within 3-sec, the catheter was presumed defective and the subject was not included in the analysis.

Self-administration. Following 5-7 days of post-surgical recovery, adolescents were P35 at start (body weight 143 g ± 1.7) and adults were P82-87 (body weight 388 g ± 5.2). We tested four treatment groups: adolescent-onset saline n=6, adolescent-onset heroin n=6, adult-onset saline n=5, and adult-onset heroin n=5. All groups were allowed to spontaneously acquire lever pressing maintained by heroin (or saline) on a fixed ratio one (FR1) schedule of reinforcement over 14 days, with a change of dose after 6 days to help test whether the drug was maintaining the lever-pressing (0.05 mg/kg/infusion for 6 days, one recess day, then 0.025 mg/kg/infusion for 7 days; 3-hr daily sessions). The drug dose was adjusted daily based on individual body weight by changing the infusion volume and duration, using a 0.2 ml infusion over 4-sec standard for a 350 g rat. After each drug infusion, a 20-sec time out (TO) period was signaled by a compound stimulus that included turning on the cue light above the active lever, a 5-sec burst of white noise, and switching off the house light. Responses during TO were recorded but had no scheduled consequences. Placement of age and treatment groups were counterbalanced across 14 test chambers.

Heroin-seeking. After 12 days of forced abstinence in the home cage, we tested heroin-seeking for 1-hr under extinction conditions. Heroin-seeking is defined as responding on the lever previously associated with heroin infusions (active lever). Rats were connected

to the metal coil tether but not the infusion tubing, and the house light remained on. Neither the compound stimulus of the cue light and white noise, nor TO signals were presented after presses on either the active or inactive lever.

Immunohistochemistry Procedures

Tissue collection. Immediately following the end of the one hour heroin-seeking test, rats were removed from the operant chambers, placed individually in clean holding cages, and left undisturbed in a quiet, dark, adjacent room for 30 min. At 90 min after the start of the extinction session, rats were injected with Sleepaway (1.0 ml i.p.) and intracardially perfused with 100 ml of phosphate buffer (0.1M, pH 7.3), followed by 200 ml 4% paraformaldehyde in phosphate buffer (0.1M, pH 7.3) with the descending aorta clamped closed. Brains were removed, post-fixed in 4% paraformaldehyde in phosphate buffer (0.1M, pH 7.3) overnight at 4°C, and allowed to submerge in 30% phosphate-buffered sucrose at 4°C before serial sectioning on a freezing microtome (four series of 40 μm-thick sections). Brain sections were collected in ethylene glycol-based cryoprotectant and stored at -20° C until immunohistological processing.

Fos-ir detection. Brain sections from animals in each treatment group were processed simultaneously in the same multi-well tissue rack to ensure that each group received equal procedural processing. All rinses and incubations were performed at room temperature unless otherwise specified. Immunolabeling for Fos, the protein product of *c-fos*, was carried out on every eighth section through the rostral-to-caudal extent of the prelimbic and infralimbic mPFC, using a primary polyclonal antibody (rabbit anti-Fos Santa

Cruz Biotechnology, Santa Cruz, CA). Sections were first incubated in primary anti-Fos antibody (diluted 1:500 in 0.5 M tris-buffered saline (TBS) containing 1% normal goat serum (NGS), 2% bovine serum albumin (BSA), 0.3% Triton-X 100 (TX100)) for 48 hrs at 4°C. Sections were next incubated in biotinylated secondary goat anti-rabbit antibody for 1.5 hr (1:600 dilution in 0.5 M TBS-1% NGS, 2% BSA, 0.3% TX100, Vector Laboratories, Burlingame, CA), followed by incubation in avidin–biotin complex for 1.5 hr (Vector Elite, Vector Laboratories, Burlingame, CA). Finally, sections were reacted with nickel-enhanced diaminobenzidine. The first or second antibodies were excluded from several sections from subjects in each age and treatment groups in each round of processing to determine background non-specific staining, which was uniformly low. Sections were mounted on gelatin-coated slides, dehydrated, counter-stained with cresyl violet for distinguishing cell bodies, cleared with xylene and coverslipped with Permount.

Stereological Analysis of Fos-ir Neurons

Under brightfield illumination, unbiased total number of Fos-ir+ and Fos-ir- neurons were estimated from coded slides using the optical fractionator probe in Stereo Investigator software (MBF Bioscience, Williston, VT, USA) by a researcher blind to experimental groups. An outline of the prelimbic and infralimbic area was first traced on one hemisphere at 2.5X magnification on sections at bregma intervals separated by 0.5 mm before subsequent counting of neurons at 100X. Counts from the prelimbic area were obtained from six sections per brain, corresponding to planes 4.7, 4.2, 3.7, 3.2, 2.7, and 2.2 mm anterior to bregma (Fig. 5.4a; Paxinos and Watson 1998). Counts from the infralimbic area were obtained from three sections per brain: corresponding to planes 3.2, 2.7,

and 2.2 mm anterior to bregma (Fig. 5.4a). Neurons were counted using a Zeiss M1 AxioImager microscope (Carl Zeiss, Oberkochen, Germany) with a Plan Apochromat 100 X oil-immersion objective (1.4 NA) and a systematic random selection sampling scheme that yielded an average of 228 ±3 prelimbic and 65 ±2 infralimbic counting sites per rat. The counting frame size was 25 μm^2, and the virtual x–y sampling grid size was 155 X 184 μm. The counting frame height was 8 μm, with 2 μm guard zones, resulting in a 5000 μm^3 counting volume per counting site. The mean section thickness after dehydration was 14.5 μm. Stereo Investigator moves the motorized microscope stage from one counting site to the next. At each counting site, the researcher manually focuses through each counting frame volume, clicking on Fos-ir+ or Fos-ir- neurons as they come into focus within the inclusion zone. Fos-ir+ neurons possessed dark brown cytoplasm and black nuclei, while Fos-ir – neurons possessed light violet-clear cytoplasm and dark violet nuclei (Fig. 5.4b). The software allows counting only within the three-dimensional inclusion volume defined by the exclusion lines and the counting frame height. Following standard procedures, a neuron was counted if and only if its plasma membrane was contained within or was touching the inclusion line of the counting frame without touching the exclusion line (Mouton 2002). The average coefficient of error for all rats was 0.07 for the prelimbic area and 0.14 for the infralimbic area.

Data Analysis.

Heroin self-administration. For heroin self-administration, the number of heroin infusions per session was analyzed using a three-way mixed-measures analysis of variance (ANOVA) with age (adolescent vs. adult) and treatment (heroin vs. saline self-

administration) as between-subject factors, and session as the within-subjects repeated measure. The number of infusions was also averaged per dose and analyzed using a three-way mixed-measures ANOVA with age and treatment as between-subject factors and dose (0.05 vs. 0.025 mg/kg/infusion) as the repeated measure. Total intake was summed across both doses (all 13 self-administration sessions) and compared using independent samples t-tests.

Heroin-seeking. For heroin-seeking after 12 days of forced abstinence from self-administration, lever presses during the 1-hr heroin-seeking test were analyzed using a three-way ANOVA with age and treatment as between-subject factors and lever (active vs. inactive) as the within-subject factor. Additionally, responding on the active lever in rats that self-administered heroin was expressed as fold change from responding on the active lever in counterpart rats that self-administered saline (labeled "relative respond-ing"), then compared across age groups using an independent sample t-test.

Fos-ir. In our stereological analysis of Fos-ir, systematic random selection of the count-ing frames guarantees that each and every neuron in a defined volume has an equal prob-ability of being sampled. Additionally, the counting frame rules guarantee that neurons cannot be counted more than once. Therefore, neuron counts are unbiased using this me-thod, and it is valid to estimate total neurons per brain region from the fraction that was sampled. The optical fractionator estimates the total number of neurons in the prelimbic or infralimbic volume sampled. Estimated total number (Fos-ir+ plus Fos-ir- neurons), and the ratio of Fos-ir+ to total neurons in the prelimbic or infralimbic cortex were ana-

lyzed separately using two-way ANOVAs with age and treatment as between-subjects factors. Fos-ir differences across the rostral-to-caudal extent of the prelimbic or infralimbic were analyzed by calculating the ratio of Fos-ir+ to total neurons from rats that self-administered heroin, expressing those values relative to age-matched controls, and comparing them across age and bregma coordinate using two-way ANOVAs with age as the between-subject factor and bregma coordinate as the repeated measures within subject factor. To determine if heroin-seeking correlated with Fos-ir+ expression, a Pearson's correlation tested the association between active lever presses during heroin-seeking and the ratio of Fos-ir+ to total neurons in the prelimbic and infralimbic areas of the mPFC in age and treatment groups separately. Data were analyzed using SPSS (SPSS Inc., Chicago, IL, USA). Data are expressed as the means +/- SEM. In all cases, follow-up ANOVAs and post-hoc tests were conducted as appropriate, with $p < 0.05$ considered significant.

5.4 Results

Heroin Self-Administration. Adolescent and adult rats self-administered similar amounts of heroin, with similar patterns of acquisition (Fig. 5.1). A three-way ANOVA revealed that number of infusions per session differed according to main effects of treatment [$F(1,18)=24.7$, $p<0.001$] and session [$F(12,216)=6.4$, $p<0.001$] and a treatment x session interaction [$F(12,216)=14.8$, $p<0.001$], but no main effects or interactions with age. Rats self-administering heroin took more infusions then those receiving saline vehicle infusions, especially in the second week of testing. Heroin intake increased gradually across sessions. Regardless of age group, infusions per session increased as dose per infusion decreased, as confirmed by a significant main effect of dose [$F(1,18)=17.72$, $p<0.01$].

Total heroin intake (mg/kg) did not differ across age groups [t=-0.19, df=7, p>0.05](Fig. 5.1 inset).

Heroin-Seeking. Rats that self-administered heroin as adolescents (adolescent-onset group) exhibited less heroin-seeking after abstinence than rats that self-administered heroin in adulthood (adult-onset group), and rats that self-administered heroin exhibited more responding than saline vehicle groups (Fig. 5.2). A three-way ANOVA revealed that lever pressing during heroin-seeking differed according to significant main effects of treatment [F(1,18)=39.02, p<0.001], lever [F(1,18)=21.83, p<0.001], and interactions between age and treatment [F(1,18)=5.09, p<0.05], age and lever [F(1,18)=7.47, p<0.05], and treatment and lever [F(1,18)=30.05, p<0.001]. Follow-up analysis on active lever presses using Tukey's pairwise comparisons confirmed that among those rats that self-administered heroin, the adolescent-onset group pressed fewer times on the active lever than the adult-onset group (p<0.025), and heroin experienced groups pressed significantly more than saline groups (adolescent-onset: p<0.01; adult-onset: p<0.001). Follow-up analysis on inactive lever presses using Tukey's pairwise comparisons confirmed that adolescent-onset groups pressed more on the inactive lever than adult-onset groups (p<0.05), and the adolescent-onset saline group pressed more times on the inactive lever than the adult-onset saline group (p<0.05). When expressed relative to active lever pressing among saline controls, relative responding in heroin experienced rats was attenuated in adolescent- compared to adult-onset groups [t=-4.95, df=9, p<0.001](Fig. 5.3).

Fos-ir. The expression of Fos-ir+ neurons was attenuated in the prelimbic (Figs. 5.6, 5.7, 5.8) and infralimbic (Figs. 5.10, 5.11, 5.12) areas of the mPFC of adolescent- vs. adult-onset rats during heroin-seeking. *Prelimbic Fos-ir.* With regard to total estimated number of neurons (Fos-ir+ plus Fos-ir-), no differences across age or treatment groups were recorded (Fig. 5.5). When comparing the ratio of Fos-ir+ to total neurons, heroin-seeking induced a significantly higher proportion of Fos-ir+ neurons in the prelimbic area of the adult-onset heroin group, compared to their saline counterparts, an effect not observed in the adolescent-onset heroin group (Fig. 5.6). Thus, a two-way ANOVA on the ratio of Fos-ir+ to total neurons in the prelimbic area resulted in a significant interaction of age X treatment [$F(1,18)=7.1$, $p<0.025$], and follow-up post-hoc Tukey's pairwise comparisons confirmed significant differences of treatment within adult-onset groups (*$p<0.01$) and age within rats that self-administered heroin (#$p<0.025$). When the ratio of Fos-ir+ neurons to total was expressed relative to saline counterparts, analysis differed by bregma coordinate, with the adult-onset heroin group exhibiting more robust Fos-ir+ expression toward the caudal pole of prelimbic area, compared to the adolescent-onset heroin group (Fig. 5.7). A two-way ANOVA revealed a significant interaction of age X bregma coordinate [$F(5,45)=3.13$, $p<0.05$], with follow-up independent samples t-tests (with Bonferroni's correction) at each bregma coordinate confirming that the adult-onset group had significantly more Fos-ir+ neurons ($p<0.05$) at bregma coordinate +2.7. Active lever presses during the heroin-seeking test were not significantly associated with the ratio of Fos-ir+ to total neurons in the prelimbic area for any age or treatment group (Fig. 5.8).

Infralimbic Fos-ir. Regardless of drug treatment, total estimated number of neurons (Fos-ir+ plus Fos-ir-) in the infralimbic area of the mPFC was higher for adolescent-onset compared to adult-onset groups, according to a significant main effect of age [$F(1,18)=16.56$, $p<0.001$] (Fig. 5.9). When comparing the ratio of Fos-ir+ to total neurons, heroin-seeking induced significant Fos-ir+ neurons in the infralimbic area of the adult-onset heroin group compared to saline counterparts, an effect not observed in the adolescent-onset heroin group (Fig. 5.10). Thus, a two-way ANOVA on ratio of Fos-ir+ to total neurons in the infralimbic area resulted in a significant interaction of age X treatment [$F(1,18)=9.3$, $p<0.01$], and follow-up post-hoc Tukey's pairwise comparisons confirmed significant differences of treatment within adult-onset groups (*$p<0.01$) and age within rats that self-administered heroin (#$p<0.01$). When the ratio of Fos-ir+ neurons to total were expressed as relative to saline counterparts, analysis differed by bregma coordinate, with the dult-onset heroin group exhibiting more robust Fos-ir+ expression in the infralimbic area of the mPFC, compared to the adolescent-onset heroin group (Fig. 5.11). A two-way ANOVA revealed a significant interaction of age X bregma coordinate [$F(2,18)=30.58$, $p<0.001$], with follow-up independent samples t-tests (with Bonferroni's correction) each bregma coordinate confirming that the adult-onset group had significantly more Fos-ir+ neurons ($p<0.05$) at bregma coordinates +3.2-2.2. Active lever presses during heroin-seeking were not significantly associated with the ratio of Fos-ir+ to total neurons in the infralimbic area for any age or treatment group (Fig. 5.12).

5.5   Discussion

Despite similar levels of heroin self-administration by adolescent and adult male rats, those that self-administered heroin as adolescents exhibited attenuated heroin-seeking

behavior after 12 days of abstinence, compared to adults. Furthermore, rats in this adolescent-onset group failed to show evidence of neural activation in the prelimbic and infralimbic mPFC during the heroin-seeking test, compared to the adult-onset heroin self-administration group. The proportion of Fos-ir+ neurons in the mPFC in the adolescent-onset heroin group did not differ from saline counterparts, whereas within the adult-onset heroin group a 2 to 6 fold increase in Fos-ir+ neurons relative to saline counterparts were observed.

The present heroin self-administration results contradict some of our results regarding age differences in heroin self-administration using fixed ratio schedules of reinforcement (Chapter 3; Doherty and Frantz in press), i.e. we recorded no age difference in the self-administration of heroin in the present results, whereas adolescents self-administered significantly more heroin than adults previously. In the previous study, however, we noted lack of confidence in the age effect, because effect size was small on the fixed ratio schedule of reinforcement, and absent on the progressive ratio schedule of reinforcement. We also observed weak effects of age on heroin-induced locomotor sensitization (no significant overall age difference; Chapter 4; Doherty and Frantz in preparation), further supporting our present conclusion that there is no reliable age difference in the acute reinforcing effects of heroin in adolescent vs. adult male rats.

More consistently across our studies, adolescent rats exhibit reduced enduring effects of drug use compared to adults. The present age-dependent levels of heroin-seeking behavior replicate our previous results showing attenuated heroin-seeking after 12 days of abstinence in rats that self-administered heroin as adolescents, compared with adults (present results; Chapter 3; Doherty and Frantz in press). We have also observed similar

attenuated drug-seeking in rats that self-administered morphine (Doherty et al. 2009) or cocaine (Li and Frantz 2009) as adolescents, compared to adults. In further support of reduced enduring effects of heroin in adolescent rats, somatic and locomotor signs of spontaneous withdrawal from repeated systemic heroin are attenuated, as are the effects of heroin on body weight and food intake, compared to adult rats (Chapter 4; Doherty and Frantz in preparation).

We identified a lower total number of neuron number in adolescent-onset vs. adult-onset groups, especially in the infralimbic area. Our age-dependent results on total neuron number are supported by human and rat data that also show a gradual loss, or pruning, of neurons in the transition from adolescence into adulthood (Andersen et al. 2000; Gogtay et al. 2004; Markham et al. 2007; Sowell et al. 1999). The second major finding is that rats that self-administered heroin as adolescents exhibited fewer Fos-ir+ neurons in the prelimbic and infralimbic areas of the mPFC during heroin-seeking, com-pared to adults. This pattern of neural activation, low in the adolescent-onset heroin group and high in the adult-onset heroin group, correlates with attenuated drug-seeking in adolescent- vs. adult-onset rats we have recently reported for heroin (present results; Chapter 3; Doherty and Frantz in press), morphine (Chapter 2; Doherty et al. 2009) and cocaine (Li and Frantz 2009). Low Fos-ir induced by drug-seeking among adolescents vs. adults contrasts acute nicotine or cocaine effects on *c-fos* expression in mPFC (Cao et al. 2007; Caster and Kuhn 2009; Schochet et al. 2005; Shram et al. 2007), but numerous differences result from self-administered vs. experimenter-administered drugs (e.g. Ro-binson et al. 2002; Hemby et al. 2007). Fos-ir data are only correlational and therefore do not indicate whether increased neuronal activity is a cause or a consequence of heroin-

seeking behavior, but the fact that adolescent and adult rats that self-administered saline did not differ from adolescent rats that self-administered heroin is a strong indication that age dependent neuroplasticity associated with heroin use exists. Lastly, less neural activation in the mPFC during heroin-seeking in adolescent-onset rats supports our hypothesis of less enduring effects of heroin in adolescent-onset rats.

Data from the present adult-onset groups support a recent report suggesting that heroin-seeking may actually be promoted more by activity in the infralimbic, than activity in the prelimbic cortex (Bossert et al. 2011). Our adult-onset heroin group had a higher relative increase in Fos-ir+ neurons in the infralimbic vs. prelimbic subregions, especially toward the caudal pole centered around bregma +2.7. We also report a relatively small proportion of Fos-ir+ neurons during heroin-seeking, similar to the 5-7% difference reported by Bossert et al. (2011). These investigators continued on to show that selective pharmacogenetic inactivation of these sparsely distributed, activated, neurons was a likely mechanism controlling context-induced heroin-seeking behavior in adult rats.

Thus combined with the above results, our heroin-seeking and Fos data might suggest that the mPFC of the adolescent-onset heroin group is not retrieving, processing, or communicating goal representations during the heroin-seeking test to the same degree as the adult-onset heroin group. The mPFC is implicated in drug reinforcement and relapse in humans and animal models. The PFC is critical for normal representation of goals (Hyman 2005; Miller and Cohen 2001; Perry et al. 2011), and relapse behavior (Goldstein and Volkow 2002; Kalivas and Volkow 2005; Volkow et al. 2003). In addition, processing of drug-cue associations within the PFC can be regulated by dopaminergic modulation of glutamatergic output to subcortical systems (Feltenstein and See 2008;

LaLumiere and Kalivas 2008; Piazza et al. 1991). Important for our studies, the PFC of adolescents is immature compared to adult PFC (Adriani and Laviola 2004; Lenroot and Giedd 2006; Spear 2000). Adolescents have immature dopamine modulation of inhibitory circuitry in the PFC (O'Donnell 2010b; Tseng et al. 2007; Tseng and O'Donnell 2007), and immature inhibitory modulation of mesolimbic dopamine levels (Natividad et al. 2010). Another possible reason for attenuated heroin-seeking in the adolescent-onset heroin group is that during adult drug-seeking behavior, cross-talk between the prelimbic and infralimbic is needed to seek drugs normally (activation of prelimbic vs. infralimbic neurons is thought to promote or inhibit cocaine-seeking behavior, respectively (Peters et al. 2009), and maybe the adolescent-onset group seeking heroin has deficits in communication between these mPFC subregions.

Overall, attenuated neuronal activity within the prelimbic and infralimbic areas of the mPFC during heroin-seeking in the adolescent-onset group extends and supports our published results showing attenuated enduring effects of self-administering heroin, morphine or cocaine during the adolescent period on drug-seeking after abstinence (Doherty and Frantz in press; Doherty et al. 2009; Li and Frantz 2009). Fos activation is only correlative, not mechanistic, but our results reveal a region of interest in which neuronal activation correlates to reduced relapse behavior in rats that self-administer drugs as adolescents compared to adults. Although application of results from rodent studies to the human condition is tenuous, our adolescent rats may provide a natural model to identify ontological neuroprotective factors against long-term drug effects in humans. It is conceivable that adolescents have an inherent mechanism, perhaps due to an immature prefrontal cortex, that protects from drug insult and/or faster recovery during abstinence. If re-

vealed in future research, this mechanism might provide new targets for drug abuse treatment in adolescent addicts, and ultimately offer better prognosis for teenagers seeking treatment.

5.6 Acknowledgements

5.7   Figures
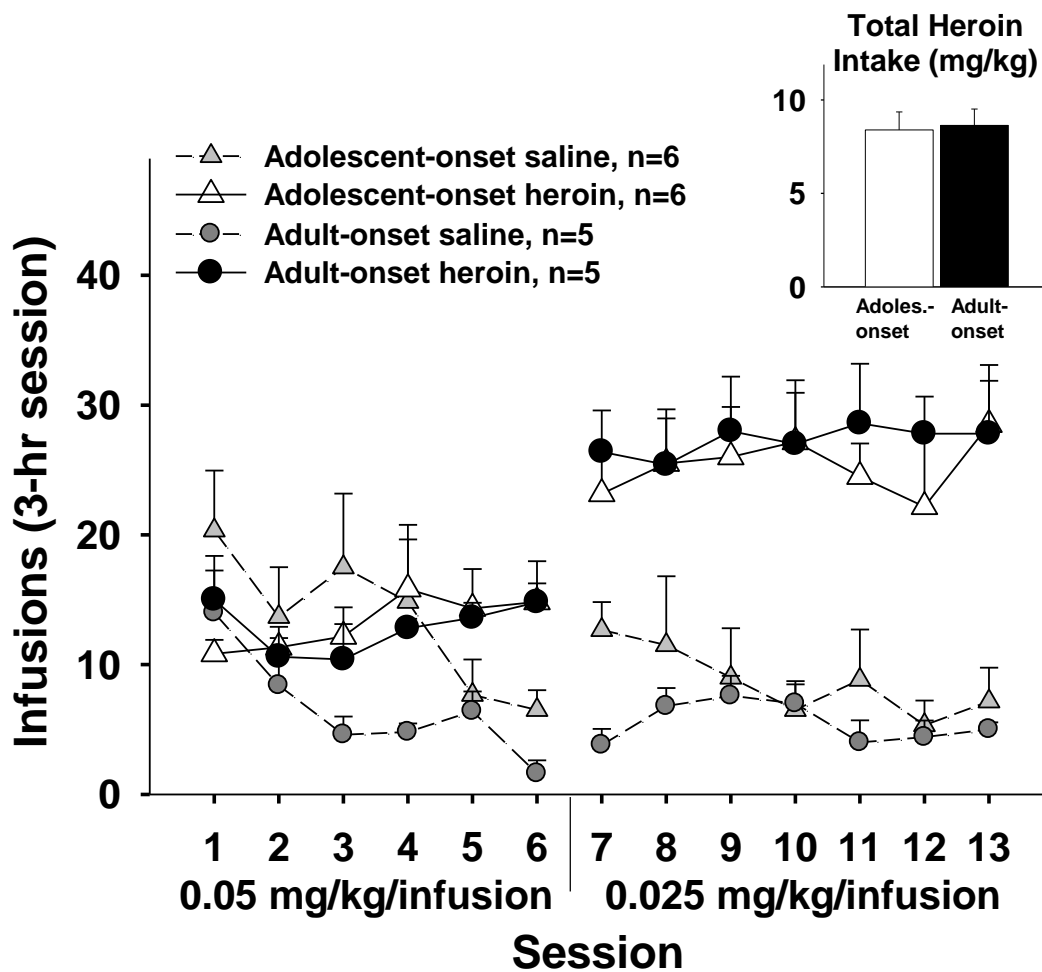
Fig. 5.1

Fig. 5.1 Number of infusions of heroin or saline and total intake on a FR1 schedule of reinforcement. Rats self-administering heroin increased infusions in response to reducing the dose of heroin, while rats self-administering saline gradually decreased infusions over sessions. Inset Total intake (mg/kg). All points or bars represent mean +/- SEM

Fig. 5.2
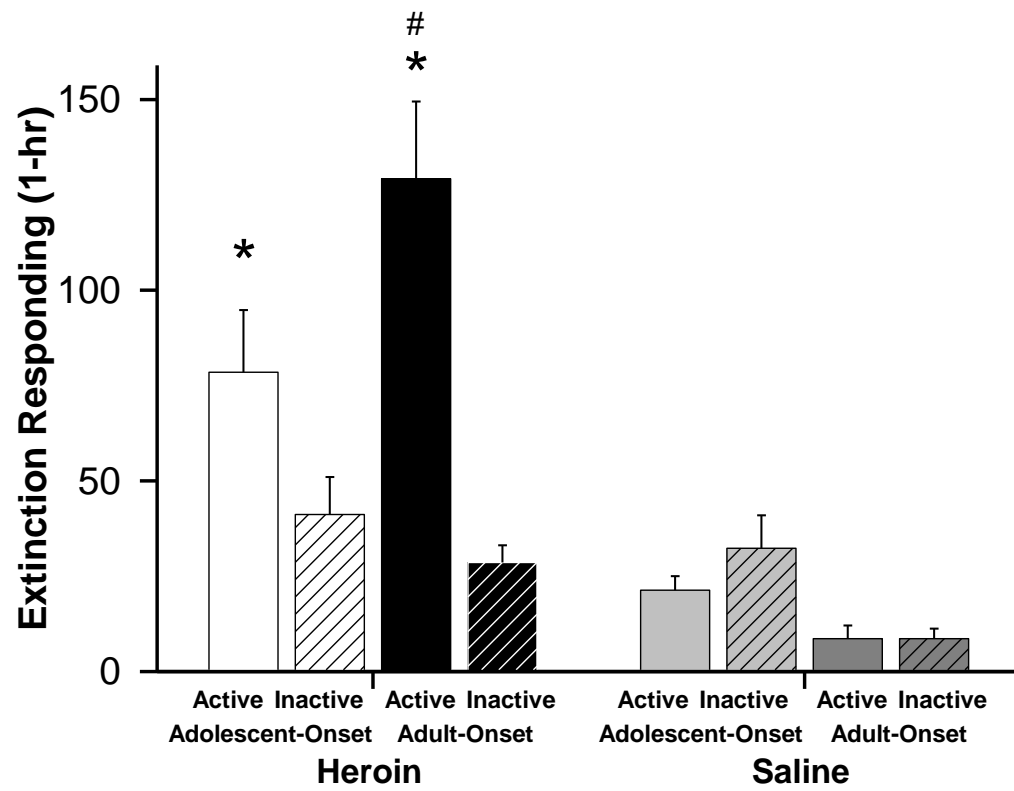
Fig. 5.2 Active and inactive lever presses during the 1-hr heroin-seeking test after 12 days of abstinence. The adolescent-onset heroin group pressed less on the active lever than the adult-onset heroin group (# $p<0.05$). Heroin groups of both ages exhibited heroin-seeking behavior by pressing more on the active lever than the inactive lever (* $p<0.05$), and by pressing more on the active lever than saline counterpart groups. All bars represent mean +/- SEM

Fig. 5.3
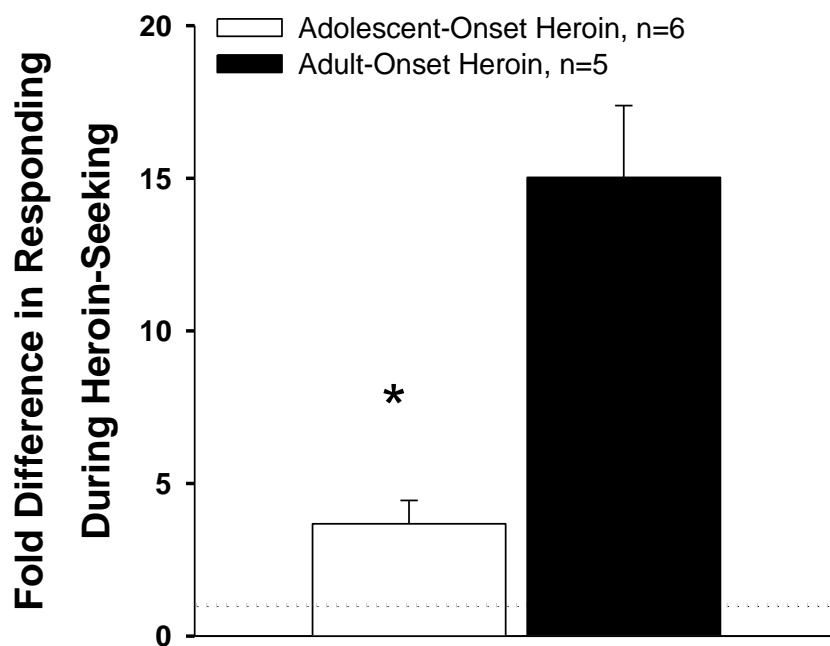
Fig. 5.3 Fold difference in responding on the active lever during the heroin-seeking test

after 12 days abstinence from heroin self-administration, expressed as change from saline

counterparts (dotted line represents data from saline counterparts). Adolescent-onset he-

roin group exhibited less relative responding than the adult-onset heroin group (*

$p<0.05$). All bars represent mean +/- SEM

Fig. 5.4a

Bregma +4.7   +4.2   +3.7   +3.2   +2.7   +2.2
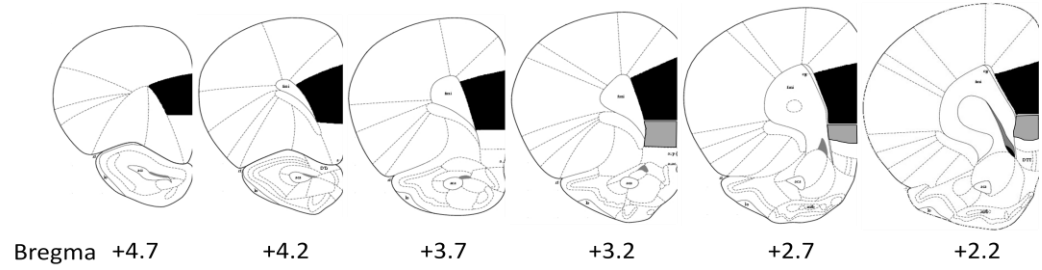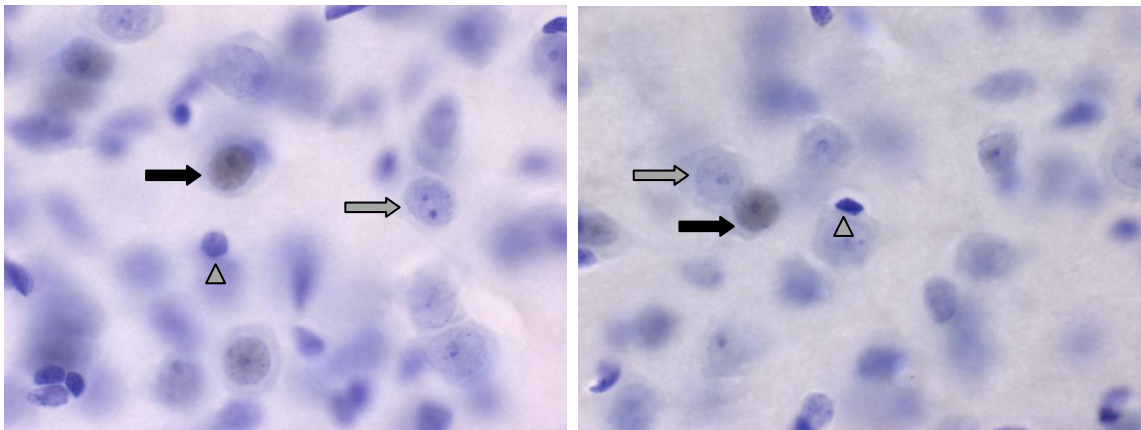
Fig. 5.4b

Fig. 5.4 (a) Prelimbic (black) and infralimbic (gray) regions of the mPFC (+4.7-2.2 from

bregma; Paxinos and Watson 1998). (b) Representative photomicrograph at 100X of pre-

limbic (left) and infralimbic (right) neurons, with a presumptive Fos-ir+ neuron (black

arrow), Fos-ir – neuron (gray arrow), and glial cell (gray triangle) indicated.

Fig. 5.5

Fig. 5.5 Total estimated neurons in the prelimbic area of the mPFC. All bars represent mean +/- SEM

Fig. 5.6
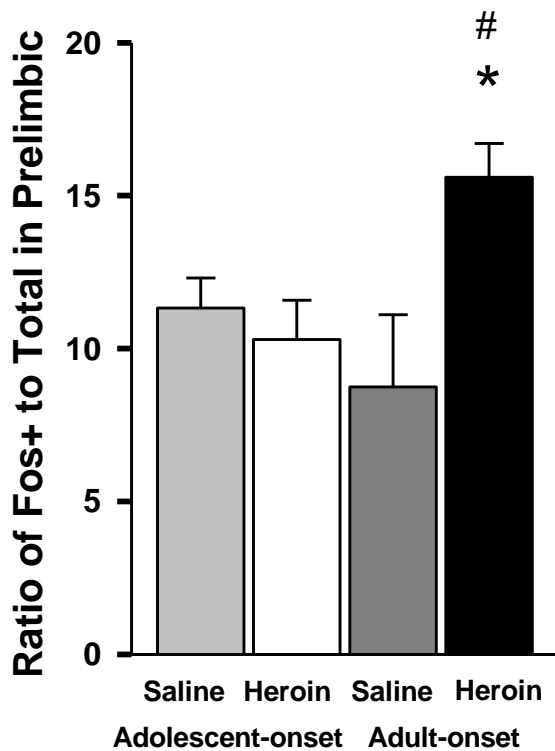
Fig. 5.6 Ratio of Fos-ir+ to total neurons in the prelimbic area of the mPFC during the heroin-seeking test after 12 days of abstinence. The adolescent-onset heroin group had fewer Fos-ir+ neurons than the adult-onset heroin group (# $p < 0.05$). The adult-onset heroin group had more Fos-ir+ neurons than the adult-onset saline group (* $p < 0.05$). All bars represent mean +/- SEM

Fig. 5.7
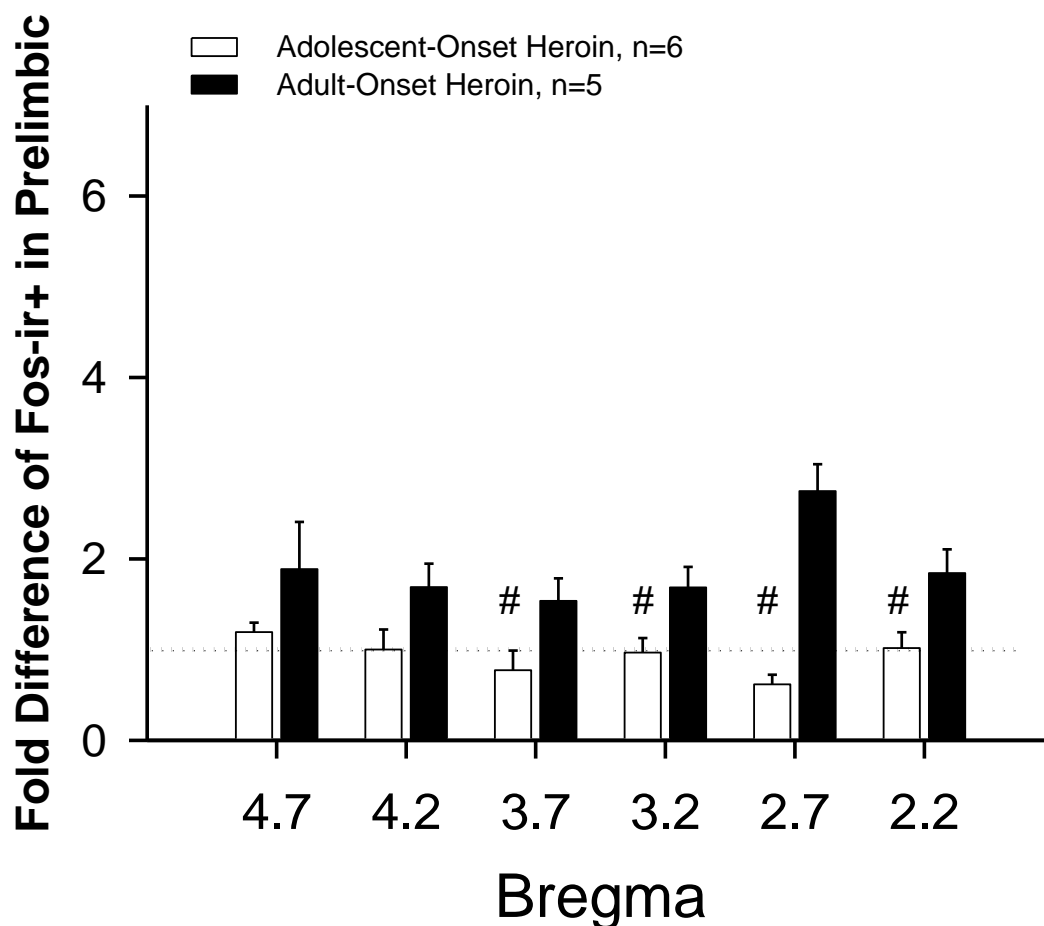
Fig. 5.7 Fold difference in Fos-ir+ neurons over the rostral-to-caudal prelimbic area of the mPFC during the heroin-seeking test after 12 days abstinence from heroin self-administration, expressed as change from saline counterparts (dotted line represents data from saline counterparts).   The adolescent-onset heroin group had fewer Fos-ir+ neurons than the adult-onset heroin group at bregma levels +3.7 to 2.2 (# $p<0.05$). All bars represent mean +/- SEM

Fig. 5.8

Fig. 5.8 Correlation of Fos-ir+ neurons in the prelimbic area of the mPFC vs. active lever presses during the heroin-seeking test after 12 days abstinence from heroin self-administration. All points represent individual rats

Fig. 5.9
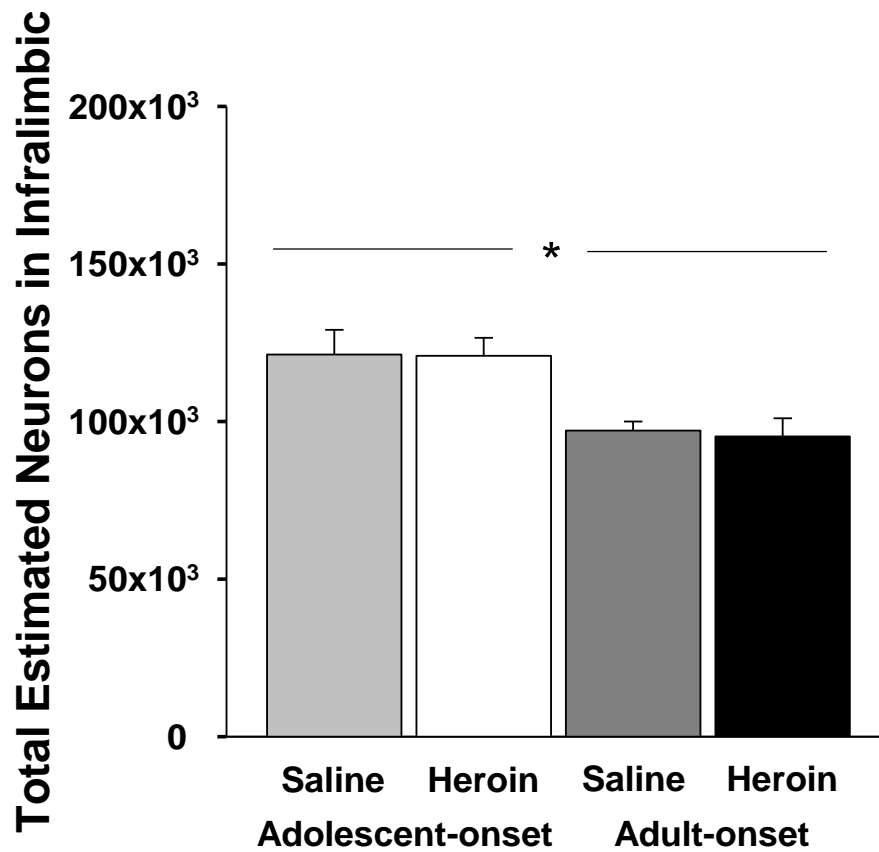
Fig. 5.9. Total estimated neurons in the infralimbic area of the mPFC. Adolescent-onset rats had more neurons than adult-onset rats (main effect of age * $p<0.05$). All bars represent mean +/- SEM

Fig. 5.10
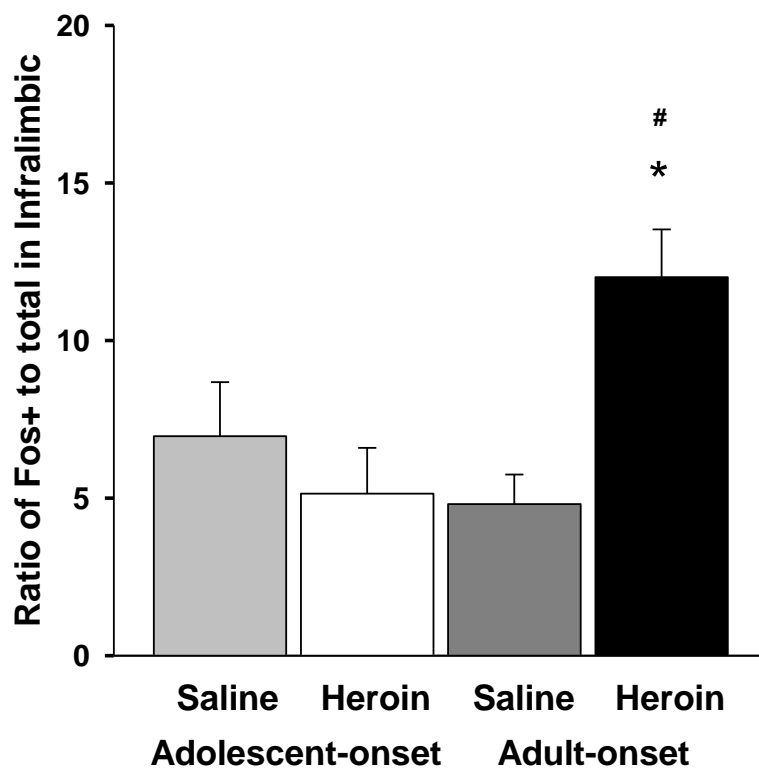
Fig. 5.10 Ratio of Fos-ir+ to total neurons in the infralimbic area of the mPFC during the heroin-seeking test after 12 days of abstinence. The adolescent-onset heroin group had fewer Fos-ir+ neurons than the adult-onset heroin group (# $p < 0.05$). The adult-onset heroin group had more Fos-ir+ neurons than the adult-onset saline group (* $p < 0.05$). All bars represent mean +/- SEM

Fig. 5.11
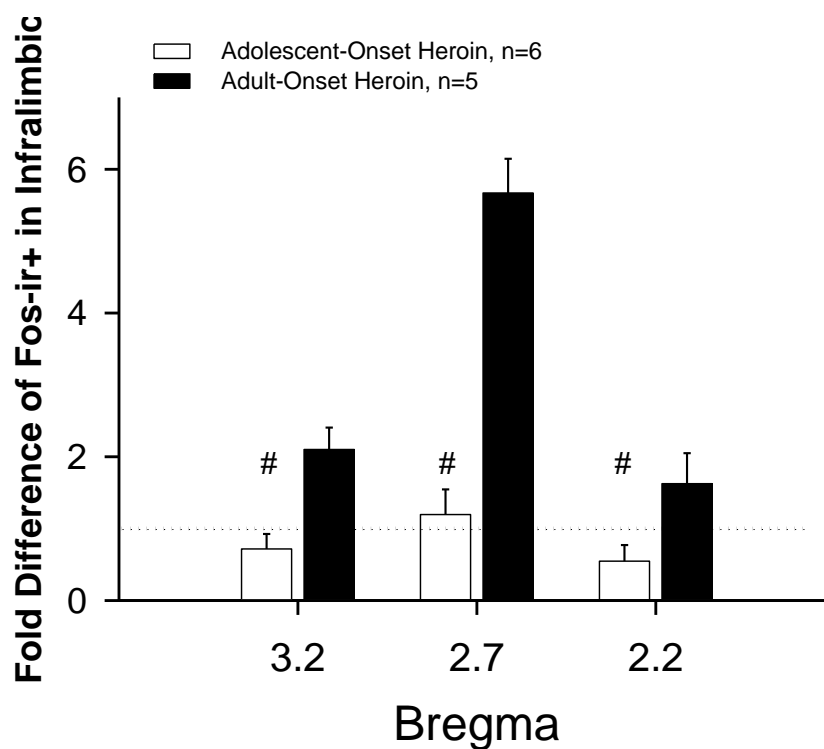
Fig. 5.11 Fold difference in Fos-ir+ neurons over the rostral-to-caudal infralimbic area of the mPFC during the heroin-seeking test after 12 days abstinence from heroin self-administration, expressed as change from saline counterparts (dotted line represents data from saline counterparts). The adolescent-onset heroin group had fewer Fos-ir+ neurons than the adult-onset heroin group at bregma levels +3.2 to 2.2 (# $p<0.05$). All bars represent mean +/- SEM

Fig. 5.12
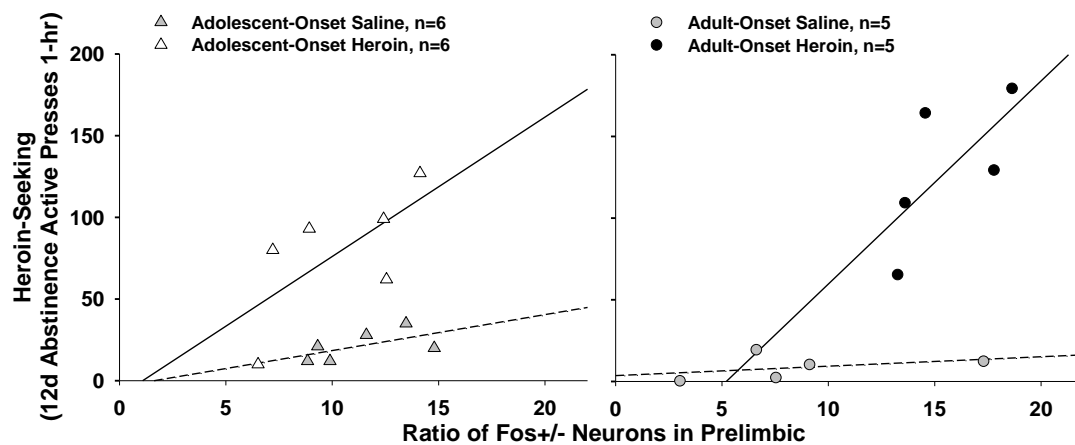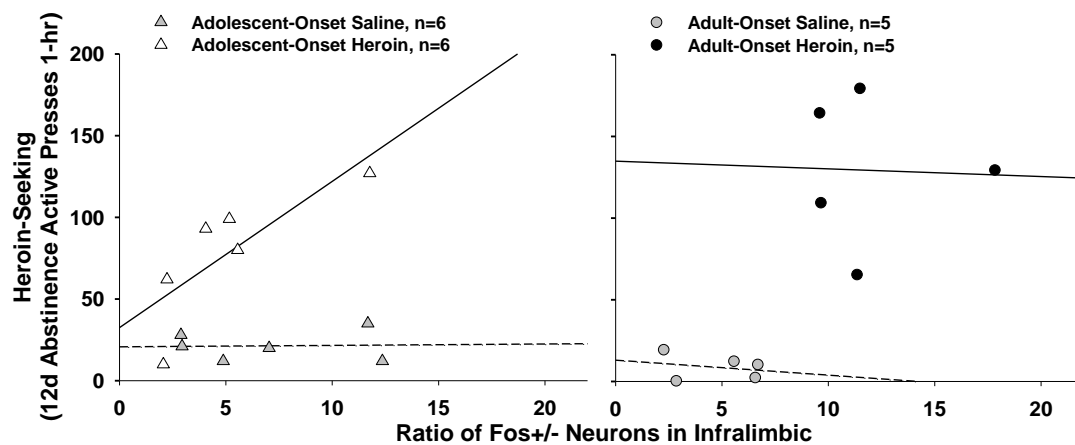

Fig. 5.12 Correlation of Fos-ir+ neurons in the infralimbic area of the mPFC vs. active lever presses during the heroin-seeking test after 12 days abstinence from heroin self-administration. All points represent individual rats

6   Introduction to general discussion

The final Chapter of this dissertation summarizes the major findings from these series of experiments testing opiate reinforcement and relapse in adolescent vs. adult male rats. We discuss some possible explanations for differential age effects across opiate or opioid reinforcers, with emphasis on pharmacokinetics and pharmacodynamics, procedural, vendor and species considerations, and inconsistent age differences in reward and reinforcement behavior and neurocircuity. We discuss our reliable finding of attenuated drug-seeking in adolescent-onset groups within the context of relevant drug-seeking literature. We attempt to rule out alternative hypotheses for attenuated drug-seeking in adolescent-onset groups. We go on to discuss how the mPFC controls drug-seeking in adolescent- vs. adult-onset rats, and we provide a review of the relevant developmental neurocircuitry of the PFC, and its major efferent target, the NAcc, that may influence age-dependent reinforcement and drug-seeking behavior. We acknowledge that our data goes against the current dogma of increased adolescent vulnerability to drug addiction. We discuss how endocrine function and sex steroids cannot entirely explain our age differences in drug effects. We then offer some limitations of using adolescent rodent models for complex human disorders. Finally, we introduce some possible future directions based on our results.

6.1   Summary

The research from this dissertation explored different stages of the addiction cycle using validated animal models. To explore the binge/intoxication stage we used the i.v. self-administration model and showed that adolescent male rats are more sensitive to the reinforcing effects morphine, but adolescents have similar sensitivity to heroin, compared to

adults. We explored the binge/intoxication stage further using a behavioral correlate of reinforcement, locomotor sensitization, and found no age difference in heroin-induced locomotor sensitization between adolescent and adult male rats. We explored the withdrawal/negative affect stage by measuring the severity of somatic and locomotor signs of spontaneous withdrawal and showed that adolescents exhibit less withdrawal from repeated heroin, as well as less effects of heroin on body weight and food intake, compared to adults. To explore the preoccupation/anticipation stage we tested for responding on the drug-associated lever during extinction and cue-induced reinstatement of opiate-seeking and showed that rats that self-administered morphine or heroin as adolescents exhibit attenuated opiate-seeking compared to adults. We then tested for a neural correlate of opiate-seeking, Fos-ir, and showed that fewer Fos-ir+ neurons in the prelimbic and infralimbic areas of the mPFC during heroin-seeking in the adolescent-onset group correlated with attenuated heroin-seeking, compared to the adult-onset group.

6.2   Explanations for differential age effects across opiate or opioid reinforcers

To our knowledge, our data are the first to test for the reinforcing effects of morphine or heroin in adolescent vs. adult male rats using the i.v. drug self-administration model. Adolescent male rats self-administer less morphine during short access conditions (1-hr/day) compared to adults (Chapter 2; Doherty et al. 2009), a pattern supported by data on the self-administration of oxycodone in adolescent vs. adult male mice (Zhang et al. 2009). In contrast, we recorded no robust effect of age on heroin intake in adolescent vs. adult male rats (Chapter 3; Doherty and Frantz in press). It is surprising that the age-dependent pattern of self-administration differs across opiates (morphine or oxycodone vs. heroin), given that reinforcing effects of opiates and synthetic opioids alike are pre-

sumed to be produced predominately by mu opioid receptor activation (Bozarth and Wise 1983; Koob et al. 1984).

6.2.1   Pharmacokinetics and pharmacodynamics

We should consider age-dependent pharmacokinetics or pharmacodynamics as factors in our results, but the unfortunate paucity of data on age differences in opiate pharmacokinetics or pharmacodynamics makes strong conclusions elusive. It is possible age-dependent absorption rates, active metabolites, or receptor activation may mediate our observed behavioral differences between the self-administration of heroin vs. morphine and oxycodone, and heroin- vs. morphine-induced locomotion. However, we did not observe robust age differences in the reinforcing effects of heroin across a wide dose range during progressive ratio testing (Chapter 3), or robust age differences in acute or sensitized locomotor effects from heroin (Chapter 4). Therefore it is unlikely that age differences in pharmacokinetics or pharmacodynamics during exposure to the drug influenced our results during testing for drug-seeking in a drug-free state.

As a class of drugs, opiates and synthetic opioids can be absorbed in the body and brain at different rates, can affect behavior with active metabolites, and can act on different receptor subtypes. Yet to our knowledge, there is no data comparing adolescent vs. adult subjects on these factors. In adult rodents tested with different opiates and synthetic opioids, differential absorption rates in the body and brain have been observed (Andersen et al. 2009; Strandberg et al. 2006). Heroin is rapidly metabolized into 6-monoacetylmorphine (6-MAM) and morphine, and morphine is metabolized into morphine-3-glucuronide and morphine-6-glucuronide (M6G). 6-MAM and M6G are active metabolites that can be reinforcing or analgesic as agonists at the mu-opioid receptor or

receptor subtype variants (reviewed in Andersen et al. 2009; Antonilli et al. 2005; Bolan et al. 2002). Opioid receptors are referred to as MOP, DOP, KOP, and NOP receptors (MOP-R, DOP-R, KOP-R, or NOP-R) for the mu (μ), delta (δ), kappa (κ), and nociceptin receptors, respectively (reviewed in Waldhoer et al. 2004). Besides these four cloned opioid receptors, pharmacological evidence exists for additional opioid receptor pheno-types (Pasternack 2001). A line of research by G. Pasternack and others has alluded to a possible mechanism by which similar opiates (i.e. morphine and heroin) or synthetic opioid compounds, or their active metabolites, can exert differential effects due to diverse splice variants of the mu-opioid receptor subtype (reviewed in Pasternak 2001).

Very few studies on the ontogeny of opioid receptors include the adolescent phase of development, and ontological studies that do bracket adolescence do not provide a clear explanation for observed behavioral differences. For example, the density of mu-opioid receptors in the NAcc and other forebrain regions rises to adult levels already by P30 (Talbot et al. 2005), and agonist binding affinity appears similar in the forebrain of P28 and adult rats (Spain et al. 1985). However, adolescents do have less efficient coupl-ing between mu-opioid receptors and G-proteins vs. adult rats (Talbot et al. 2005), and perhaps less efficient coupling in adolescents could blunt the effects of mu-opioid agoinsts, such as morphine and heroin.

For further consideration of age differences in pharmacokinetics or pharmacody-namics, we turn to extant data on adolescent vs. adult psychostimulant and alcohol phar-macokinetics. Adolescent rats must be administered 1.5 times higher amounts of nicotine than adults to achieve similar blood levels of nicotine and the metabolite of nicotine, co-tinine (O'Dell et al. 2006), and adolescent rats had lower levels of NAcc cocaine levels

after i.v. injections of high doses of cocaine compared to adults (Frantz et al. 2007). In contrast, no age differences occurred between adolescent vs. adult rats in basal or cocaine-stimulated dopamine levels in the NAcc (Frantz et al. 2007), cocaine concentration in brain homogenate after i.v. administration (Schramm Sapyta et al. 2007), or in blood alcohol levels between adolescent and adult male mice administered ethanol (Hefner and Holmes 2007). Although general rules about age differences in metabolism of exogenous psychoactive substances are unlikely, enough evidence exists to warrant direct comparisons before concrete conclusions regarding the contributions of pharmacokinetics or pharmacodynamics to opiate or synthetic opioid effects in adolescents vs. adults can be made.

6.2.2   Procedural considerations

Procedural differences might account for inconsistent age-dependent patterns of opiate or opioid self-administration. First, the daily access period was different between morphine and heroin tests, which is important because varying access to the drug can dramatically influence intake, and therefore the effects of the drug (Vendruscolo et al.; Zernig et al. 2007). During the morphine experiment, we specifically tested for age differences using dramatically different access periods to the drug (1- vs. 8-hr/day; Chapter 2). The most robust age effects during morphine self-administration were in rats tested under short access conditions, with adults self-administering significantly more morphine than adolescents. In fact, robust age differences occurred even within the first 15 min loading phase of each experiment. Also, the only other published report testing self-administration with a opiate-like compound in adolescent vs. adult rodents (Zhang et al. 2009) supports our results with morphine. Zhang et al. (2009) used 2-hr daily sessions

and adolescent male mice self-administrated less oxycodone than adult male mice. However, during our heroin self-administration experiment we did not see any robust age differences in intake and the access condition was intermediate between what was tested in the morphine experiment (heroin: 3-hr/day; Chapter3). We also did not observe age differences in heroin intake during the loading phase, a pattern similar to 8-hr morphine groups. Furthermore, when allowed to self-administer heroin up to 9-hrs per day during progressive ratio testing, there were no overall age differences in the amount of heroin self-administered. Therefore, it is possible that longer (>2-hr) daily access periods self-administering opiates reduces any robust age differences in the reinforcing effects of opiates.

6.2.3 Vendor and species considerations

We cannot rule out vendor or species differences as a possible factor contributing to different patterns of self-administration between our studies of morphine or heroin self-administration in rats and self-administration of oxycodone in mice (Zhang et al. 2009). After completion of our morphine experiment, our vendor (Zivic Miller; New Castle, PA) stopped selling Sprague-Dawley rats. Therefore, the vendor for our heroin experiments was different (Charles River, Raleigh, NC). Also, although our results with morphine support results with oxycodone in mice (Zhang et al. 2009), heroin self-administration has not been tested in mice. Evidence exists for strain or species phenotypic differences in numerous behaviors, including the reinforcing and/or analgesic properties of opiates in rats (Martn et al. 1999; Ray and Barrett 1975) and mice (Uhl et al. 1999), even in genetically similar strains.

6.2.4 Inconsistent age differences in reward and reinforcement behavior

Although tempting to try to draw general conclusions about sensitivity to reward and reinforcement during adolescence, inconsistencies in available data sets make this impossible to date. For example, in comparison to adults, adolescent rats exhibit the following characteristics: *increased* acute and sensitized morphine-stimulated locomotor activity (Spear et al. 1982; White et al. 2008; White and Holtzman 2005); *lack of or similar* morphine conditioned place preference (Bolanos et al. 1996; Campbell et al. 2000); *more or similar* sensitivity to nicotine-, cocaine-, amphetamine-, or methylphenidate-stimulated motor activity or conditioned place preference (Badanich et al. 2008; Belluzzi et al. 2004; Caster et al. 2005; Niculescu et al. 2005; Torrella et al. 2004); *more or similar* cocaine self-administration (Anker and Carroll 2010; Frantz et al. 2007; Kerstetter and Kantak 2007; Li and Frantz 2009); *more or less* nicotine self-administration (Belluzzi et al. 2005; Levin et al. 2007; Levin et al. 2003; Shram et al. 2007; Shram et al. 2008a); and *more* amphetamine self-administration (Shahbazi et al. 2008). Moreover, results vary when testing adolescent vs. adult rats self-administering natural or artificial sweeteners, with adolescents taking *less* sucrose pellets (Li and Frantz 2010) but *similar* amounts of saccharin compared to adults (Shram et al. 2008a). Lastly, brain thresholds measured by intracranial self-stimulation were *similar* at baseline in adolescents vs. adults, revealing similar basal activity in brain reinforcement circuitry (O'Dell et al. 2006). Thus, effects of drugs of abuse, as well as natural rewards vary across rewarding or reinforcing stimulus and subject populations, and require further investigation.

The locomotor-stimulating properties of morphine and heroin also differ in adolescent vs. adult rats and may influence self-administration. Our data suggest no age differ-

ence in acute or sensitized locomotor response to heroin, although adolescents did tend to exhibit less or delayed sensitization compared to adults (Chapter 4). Of note, we did not observe robust locomotor sensitization, probably due to our escalating dosing regimen (locomotor sensitization is best tested using intermittent, not daily, administration). In contrast, with acute or sensitized morphine-stimulated locomotion adolescents exhibit more sensitivity to the psychomotor stimulating or sensitizing properties of morphine compared to adults (Spear et al. 1982; White et al. 2008; White and Holtzman 2005).

6.2.5 Reward and reinforcement neurocircuity similarities between adolescent and adult rats

A number of neurocircuitry similarities between adolescent and adult rats cloud explanations of age-dependent reinforcement or drug-seeking behavior. For example, the density of mu opioid receptors in the NAcc and other forebrain regions rises to adult levels already by P30 (Talbot et al. 2005) and mu opioid receptor agonist binding affinity appears similar in the forebrain of P28 and adult rats (Spain et al. 1985). Also, levels of acute cocaine-induced *c-fos* mRNA expression and dopamine transporter binding were similar across ages in some brain areas (NAcc, dorsal caudate putamen, and lateral bed nucleus of the stria terminalis; Cao et al. 2007). Furthermore, adolescent and adult male rats have similar basal (Frantz et al. 2007; Natividad et al. 2010; but see Badanich et al. 2006) and cocaine-stimulated dopamine in the NAcc (Badanich et al. 2008; Frantz et al. 2007), and basal levels of noradrenaline in the NAcc shell and PFC (Carboni et al.; Silvagni et al. 2008). Taken together, clearly some important reward and reinforcement neurocircuitry that have been heavily implicated in the effects of drugs in adult subjects are similar across development.

6.3   Consistent attenuation of drug-seeking in adolescent-onset groups

Despite the major inconsistencies in acute reward and reinforcement across age groups, data to date on drug-seeking after abstinence are independent of age differences in drug intake, reproducible, and similar across drug class. The data from Chapters 2, 3, and 5 showed that rats that self-administered morphine or heroin as adolescents exhibited attenuated drug-seeking after a period of abstinence and/or extinction compared to rats that self-administered as adults. Similar age differences occurred in drug-seeking behavior regardless of procedural differences between morphine and heroin experiments (see 6.2.2).

6.3.1   Summary of relevant drug-seeking data in adolescent- vs. adult-onset groups

Our results with opiates are also supported by recent reports suggesting adolescent subjects are less vulnerable than adults to other enduring drug effects. We have recently shown that rats that self-administered cocaine as adolescents exhibited attenuated time dependent cocaine-seeking compared to adults (Li and Frantz 2009). Also, a recent report (Anker and Carroll 2010) replicated the attenuated cue-induced reinstatement of cocaine-seeking in adolescent- vs. adult-onset male rats, although, cocaine- and yohimbine-induced reinstatement of drug-seeking was higher in the adolescent-onset group compared to adults. Also for cocaine, compared to adult rats, adolescent rats exhibited faster extinction and less "reinstatement" of cocaine conditioned place preference (Balda et al. 2006; but see Brenhouse and Andersen 2008 for opposite results); a lack of cross-sensitization to cocaine self-administration following systemic MDMA exposure during adolescence (Frantz and Parsons 2001), and less severe cognitive impairment in an amygdala-dependent task after cocaine intake during adolescence (Kerstetter and Kantak

2007; but see Harvey et al. 2009 for greater impairment after cocaine intake in a orbito-frontal task). For nicotine, rats that self-administered nicotine as adolescents extin-guished their drug-seeking faster than those that took the drug as adults (Shram et al. 2008a). Also for nicotine, adolescent rats did not exhibit signs of nicotine-cue condition-ing, unlike adults, and adolescents displayed less robust nicotine-induced locomotor sen-sitization than adults (Schochet et al. 2004). Thus, at least in rodents, adolescence may be a developmental period associated with less severe long-term consequences of drug use, compared to initiating drug use in adulthood.

6.3.2 Alternative hypotheses for attenuated drug-seeking in adolescent-onset groups

A number of alternative hypotheses and possible explanations are plausible for why rats that self-administer drugs during adolescence exhibit attenuated drug-seeking. Many fac-tors influence drug-seeking behavior in adult rats, such as amount of prior drug intake, indiscriminate motor activation, impulsive behavior, rate of extinction learning, sensitivi-ty to drug-seeking stimuli, inadequate memory or recall of drug-cue associations, and aversive withdrawal; each of these can be considered for its role in age-dependent drug-seeking.

6.3.2.1 Prior drug intake

Prior drug intake cannot entirely explain age differences in drug-seeking. Adolescents as a group took similar amounts of heroin (Chapter 3) and cocaine (Li and Frantz 2009), but less morphine (Chapter 2) than adults. Yet all adolescent-onset groups reinstated drug-seeking to a lower level than adults. Thus, no matter what the pattern of drug-taking, or procedural differences during self-administration testing, adolescent-onset groups exhibit

attenuated drug-seeking. On the other hand, the amount of prior heroin intake did corre-late with cue-induced reinstatement in the absence of extinction for both age groups in rats that were tested using a progressive ratio (PR) schedule of reinforcement (Chapter 3), as it has before (Liu et al. 2008; Zhang et al. 2004). It is unclear, though, if conditions of the progressive ratio testing influenced age-dependent heroin-seeking behavior, i.e. dif-ferences in the amount of training (the schedule demands a high degree lever pressing) or large differences in intake due to testing across a wide dose range.

6.3.2.2 *Indiscriminate motor activation*

With regard to indiscriminate motor activation underlying age differences in self-administration and drug-seeking, preliminary results from our laboratory suggest no age differences in locomotion in the operant conditioning chambers, measured as beam breaks during self-administration and reinstatement of drug-seeking sessions (Fig. 6.1). Generally, rats of both ages exhibited increased motor activation during each self-administration session, compared to saline counterparts (data not shown), but activity was surprisingly stable across sessions and there was no affect of age. Low levels of respond-ing on the inactive lever might also weaken support for that explanation. Furthermore, although tenuous to directly compare behavior in operant conditioning chambers to open field behavior, we also do not see robust age differences in baseline or heroin-induced locomotor activity (Chapter 4).

6.3.2.3 *Impulsive behavior*

Impulsivity has been cited as a vulnerability factor for drug use (Cardinal et al. 2001; Per-ry et al. 2007). Our adolescent rats exhibit more impulsive lever pressing behavior or lack of stimulus control, in the form of non-reinforced responding during self-

administration (Fig. 6.2; Doherty et al. 2009; Shahbazi et al. 2008). Numerous other reports also show high impulsive behavior in adolescent vs. adult rodents (Adriani and Laviola 2003; Sagvolden and Sergeant 1998; Spear and Brake 1983). However, attenuated drug-seeking in adolescent-onset groups is opposite to what would be predicted from high impulsive behavior in adolescents. Notably, the role of impulsivity in reward and reinforcement by opiates, per se, has been questioned (McNamara et al. 2010). Future research is required to clarify the influence of such factors in adolescent drug self-administration and drug-seeking.

6.3.2.4   *Rate of extinction learning*

Another attractive explanation for lower levels of drug-seeking among adolescent-onset groups is that younger rats learn more quickly during extinction that presses on the drug-associated lever are no longer drug-associated. A steeper downward slope would be expected if the adolescent-onset groups were learning about the new extinction conditions faster than adult-onset groups (Everitt et al. 2001; Taylor et al. 2009). However, the downward slope of extinction responding was not steeper among younger cohorts reinstating heroin-seeking at either 1 or 12 days abstinence (Fig. 6.3). We have also observed a similar slope of extinction rate during cocaine-seeking behavior (Li and Frantz 2009). Furthermore, the ability of adolescent and adult rats to extinguish sucrose pellet-seeking (Li and Frantz 2010) or responding for a chocolate solution (Andrzejewski et al. 2011) was not different across ages. Thus, it is unlikely that adolescents have a general advantage in learning during extinction. Of note, when adolescents rats are tested for extinction behavior during adolescence (as opposed to most of our conditions), some evidence does exist that adolescent rats are actually impaired in their extinction learning compared

to adults; adolescents exhibit more lever pressing after repeated extinction sessions when tested for extinction of lever pressing for a chocolate solution (Andrzejewski et al. 2011), and adolescents exhibit impaired retention of extinction of a conditioned fear response (McCallum et al. 2010).

6.3.2.5   *Sensitivity to drug-seeking stimuli*

The stimulus reinstating drug-seeking behavior (i.e. drug-taking context, discrete drug-paired cues, context+cue, stress, or the systemic administration of the drug itself) appears to be an important factor in age-dependent drug-seeking behavior. Research on nicotine-cue associations in adult rats shows a facilitatory and synergistic role of cue associations on both drug self-administration and reinstatement of drug-seeking behavior (Chaudhri et al. 2006), and in one study adolescent rats given systemic nicotine did demonstrate less nicotine-paired cue conditioning than adults (Schochet et al. 2004). Similarly, when we tested rats for context+cue-induced reinstatement of heroin-seeking without any prior extinction sessions (Chapter 3 Fig.3.6 ; Fig. 6.4 below), no overall age difference occurred in drug-seeking. Furthermore, the portion of the drug-seeking test that we see the most robust age difference is in the initial 15 min of testing, and testing context+cue-induced reinstatement abolishes that initial age difference. A similar result occurs when testing cocaine-experienced rats for context+cue -induced reinstatement of cocaine-seeking (Li and Frantz unpublished observations). Also, when adolescent vs. adult male rats were tested for extinction of food reinforcers, age differences depended on the presence of discrete cues and on the motivational state of the rat (i.e. food deprivation; Sturman et al. 2010). We have also tested another type of drug-seeking stimuli, systemic injection of small amounts of the test drug just prior to returning the rat to the operant conditioning

chamber. When we tested adolescent- vs. adult-onset groups for morphine-induced reinstatement of drug-seeking no age differences were observed (Fig. 6.5; morphine-induced reinstatement tested 1 day after cue-induced reinstatement; Ogbomnwan Y., Doherty JM. and Frantz KJ unpublished observations). Similarly, no age difference in cocaine-seeking occurred when systemic cocaine was tested (Li and Frantz 2009). Of note, a recent study found adolescent-onset groups exhibited higher levels of cocaine-induced reinstatement of cocaine-seeking in adolescent- vs. adult-onset male rats (Anker and Carroll 2010). Thus, it seems drug-seeking stimuli vary in their ability to drive age-dependent drug-seeking behavior.

6.3.2.6   *Inadequate memory or recall of drug-cue associations*

It is possible, instead, that adolescent-onset groups are impaired in the ability to retain, or remember, reinforcer-cue associations during abstinence. However, data from our laboratory provide an important control for this possibility. Although adolescents self-administer fewer sucrose pellets than adults, no age differences in the reinstatement of sucrose-seeking behavior over time in "abstinence" were observed (Li and Frantz 2010). This lack of age differences in sucrose-seeking behavior suggests that the age-dependent drug-seeking is specific for drugs of abuse, such as opiates and psychostimulants. Of note, we cannot yet rule out that it is the drug itself that impairs learning and/or memory, thus resulting in lower levels of extinction responding and/or cue-induced reinstatement of drug-seeking specifically in younger cohorts of male rats.

6.3.2.7   *Aversive withdrawal*

Perhaps the most likely explanation for age differences in drug-seeking is lower levels of aversive drug withdrawal among adolescent compared with adult rats. In humans and

adult rats, negative reinforcement during drug withdrawal may drive drug-seeking beha-vior, perhaps in an attempt to alleviate an aversive state (Frenois et al. 2005; Kenny and Markou 2005a; Kenny et al. 2006; O'Connor and Fiellin 2000). We show that, indeed, adolescents exhibit attenuated somatic and locomotor signs of spontaneous withdrawal from repeated systemic heroin, and attenuated effects of heroin on body weight and food intake (Chapter 4). Thus, attenuated withdrawal in adolescents provides a possible beha-vioral correlate to attenuated drug-seeking in adolescent- vs. adult-onset rats. Further-more, numerous studies from other labs also observe less withdrawal or less sensitivity to aversive properties of drugs of abuse in adolescent vs. adult rodents (Doremus et al. 2003; Hodgson et al. 2009; Infurna and Spear 1979; Natividad et al. 2010; O'Dell et al. 2006; O'Dell et al. 2007; Shram et al. 2008b; Varlinskaya and Spear 2004; Vetter-O'Hagen et al. 2009; Wilmouth and Spear 2004). Note, however, that most of the self-administration conditions we employ (excluding escalation conditions) are not likely to induce severe dependence and withdrawal (Koob et al. 1984). Future studies should test for age differences during withdrawal from self-administration conditions that produce dependence, and test explicitly whether or not less aversive withdrawal among adoles-cents also directly contributes to acute drug self-administration and attenuated drug-seeking behavior.

6.3.3 Activation (Fos-ir) of medial prefrontal cortex controls drug-seeking in ado-lescent- vs. adult-onset groups

A paucity of data exists testing neural mechanisms of drug-seeking in adolescent- vs. adult-onset groups with a history of drug self-administration. We report attenuated neur-al activation (Fos-ir+ neurons) in the prelimbic and infralimbic areas of the mPFC during

a test for heroin-seeking behavior in rats that self-administered heroin as adolescents, compared to adults (Chapter 5). The attenuated Fos-ir data in the adolescent-onset groups is consistent with attenuated heroin- (Chapter 3; Doherty and Frantz in press; Chapter 5), morphine- (Chapter 2; Doherty et al. 2009), and cocaine-seeking (Li and Frantz 2009) in adolescent- vs. adult-onset groups. Low Fos-ir induced by drug-seeking among adolescents vs. adults contrasts acute nicotine or cocaine effects on *c-fos* expression in mPFC (Cao et al. 2007; Caster and Kuhn 2009; Schochet et al. 2005; Shram et al. 2007), but numerous differences result from self-administered vs. experimenter-administered drugs (e.g. Robinson et al. 2002; Jacobs et al. 2005). Fos-ir data are only correlational and therefore do not indicate whether increased neuronal activity is a cause or a consequence of heroin-seeking behavior, but the fact that adolescent and adult rats that self-administered saline did not differ from adolescent rats that self-administered heroin is a strong indication that age dependent neuroplasticity associated with heroin use exists. Lastly, less neural activation in the mPFC during heroin-seeking in adolescent-onset rats supports our hypothesis of less enduring effects of heroin in adolescent-onset rats.

We identified a lower total number of neuron number in adolescent-onset vs. adult-onset groups, especially in the infralimbic area (Chapter 5). Our age-dependent results on total neuron number are supported by human and rat data that also show a gradual loss, or pruning, of neurons in the transition from adolescence into adulthood (Andersen et al. 2000; Gogtay et al. 2004; Markham et al. 2007; Sowell et al. 1999). It is possible that neurons within the infralimbic area involved in modulating drug-seeking

behavior get pruned in adolescent rats, leading to attenuated drug-seeking behavior, compared adults.

Our heroin-seeking and Fos data might suggest that the mPFC of the adolescent-onset heroin group is not retrieving, processing, or communicating goal representations during the heroin-seeking test to the same degree as the adult-onset heroin group. The mPFC is implicated in drug reinforcement and relapse in humans and animal models. The PFC is critical for normal representation of goals (Hyman 2005; Miller and Cohen 2001; Perry et al. 2011), and relapse behavior (Goldstein and Volkow 2002; Kalivas and Volkow 2005; Volkow et al. 2003). Also, processing of drug-cue associations within the PFC can be regulated by dopaminergic modulation of glutamatergic output to subcortical systems (Feltenstein and See 2008; LaLumiere and Kalivas 2008; Piazza et al. 1991). Important for our studies, the PFC of adolescents is immature compared to adult PFC (Adriani and Laviola 2004; Lenroot and Giedd 2006; Spear 2000). Adolescents have immature dopamine modulation of inhibitory circuitry in the PFC (O'Donnell 2010b; Tseng et al. 2007; Tseng and O'Donnell 2007), and immature inhibitory modulation of mesolimbic dopamine levels (Natividad et al. 2010). Another possible reason for atte-nuated heroin-seeking in the adolescent-onset heroin group is that during adult drug-seeking behavior, cross-talk between the prelimbic and infralimbic is needed to seek drugs normally (activation of prelimbic vs. infralimbic neurons is thought to promote or inhibit cocaine-seeking behavior, respectively; Peters et al. 2009), and maybe the adoles-cent-onset group seeking heroin has deficits in communication between these mPFC sub-regions. Of note, we report no age difference the reinstatement of sucrose-seeking be-tween adolescent-onset and adult-onset male rats (Li and Frantz 2010), which suggests

any age-dependent deficits in neural processing with the mPFC that contribute to seeking behavior is specific for drugs of abuse, such as opiates and psychostimulants.

6.3.4 Developmental circuitry of the PFC, and its major efferent target, the NAcc, may influence age-dependent reinforcement and drug-seeking behavior

We know that dopamine, glutamate, and GABA circuits in the PFC, NAcc, and amygdala, among others, play important roles in extinction responding and cue-induced reinstatement in adult male rats (Bossert et al. 2005; Shaham et al. 2003). Without direct tests of these systems during drug-seeking behavior in adolescent-onset treatment groups, we can only speculate that developmental changes in any of these systems [we also know that these systems undergo dramatic developmental changes during adolescence (as reviewed in Adriani and Laviola 2004; Crews et al. 2007; Spear 2000)] could dampen the long-term effects of drugs of abuse in adolescent-onset groups.

Perhaps the most important consideration with regard to developmental changes in reward and reinforcement circuitry is the target of morphine and heroin itself: the mu opioid receptor. The density of mu-opioid receptors in the NAcc and other forebrain regions rises to adult levels already by P30 (Talbot et al. 2005) and agonist binding affinity appears similar in the forebrain of P28 and adult rats (Spain et al. 1985). However, adolescents do have less efficient coupling between mu-opioid receptors and G-proteins in vs. adult rats (Talbot et al. 2005), and perhaps less efficient coupling in adolescents could blunt the effects of mu-opioid agonists, such as morphine and heroin.

With regard to mesolimbic dopamine circuitry, a recent study in rats showed an increased risk preference after a history of adolescent alcohol exposure that was associated with altered dopamine signaling to the risk, but not altered dopamine signaling to

reward valuation (Nasrallah et al. 2011). Also, a transient overexpression of dopamine receptors and increases in associated second messenger activity in the PFC and NAcc is observed specifically during adolescence (Andersen and Teicher 2000; Andersen et al. 2000; Brenhouse et al. 2008), along with evidence of blunted second messenger system function in neurons that possess dopamine receptors (Andersen 2002). Neurons that possess dopamine D1 receptors and project from PFC to NAcc are thought to drive drug-seeking behavior (Brenhouse et al. 2008; LaLumiere and Kalivas 2008). These same neurons undergo pruning during the developmental period spanning the end of heroin or cocaine self-administration (P48-50) and the test for heroin- or cocaine-seeking behavior (P60 +) (Andersen et al. 2000), and so it is possible that elimination of these neurons driving drug-seeking during abstinence contributes to less drug-seeking behavior in adolescent-onset rats. Along those lines, extinction of drug-cue associations in tests for cocaine conditioned place preference was facilitated in adolescents by elevating dopamine and norepinephrine in the PFC during extinction training with systemic atomoxetine, and by microinjection of the D1 receptor agonist SKF38393 directly into the PFC (Brenhouse et al. 2010). Also, blunted second messenger system activation in adolescent-onset rats during the drug-seeking test could contribute to less activation within those neurons driving drug-seeking behavior. Dopamine D2 receptors located on GABA neurons are thought to allow flexibility in learning (Ernst et al. 2009; Tseng and O'Donnell 2007), and adolescent rats possess a greater number of dopamine D2 receptors within the PFC (Brenhouse et al. 2008), although it is yet unclear which neuron type these overexpressed D2 receptors occur on. Therefore, it is also possible that during abstinence adolescent-onset rats have greater flexibility in learning, allowing adolescents to place a greater val-

ue on other non-drug rewards (i.e. social companionship, food, etc.), which might contribute to less drug-seeking compared to adults when re-entering the drug-taking environment.

6.4   Acknowledging the current dogma of increased adolescent vulnerability to drug addiction

Numerous studies contradict our surprising results by describing greater/more long-term effects of adolescent exposure to drugs of abuse. For example, systemic D-9-tetrahydrocannabinol (THC) exposure during adolescence increases the sensitivity to heroin self-administration in adulthood, increases striatal preproenkephalin mRNA expression in the NAcc shell, and potentiates mu opioid receptor GTP-coupling in mesolimbic and nigrostriatal brainstem regions (Ellgren et al. 2007). Rats that initiate nicotine self-administration during adolescence display higher nicotine intake as adults relative to rats that initiate self-administration as adults (Adriani and Laviola 2003). Using the conditioned place preference model, Brenhouse and Andersen (2008, 2010) report that adolescent vs. adult male rats exhibit delayed extinction and more reinstatement of a conditioned place preference for the cocaine-paired compartment, but results were dose- and cue- specific. Rats that self-administered cocaine as adolescents also exhibit more drug-primed and stress-induced reinstatement of cocaine-seeking compared to adults (Anker and Carroll 2010). Also, rats that self-administered cocaine during adolescence exhibit more severe cognitive impairment in an orbitofrontal -dependent task when tested in adulthood, compared to rats that self-administered as adults (Harvey et al. 2009). This incomplete summary of evidence of increased adolescent sensitivity to drug use is oppo-

site the trajectory of our results, thus more research is needed to clarify adolescent rodent sensitivity to drugs.

6.5 Endocrine function and sex steroids cannot entirely explain age differences in drug effects

Endocrine system changes could contribute to age-dependent acute and long-term drug effects, since dramatic changes occur in sex and stress hormone levels during adolescence. Indeed, adult females will work harder for and self-administer more morphine or heroin than adult males (Cicero et al. 2003), and adult female rats reach acquisition criteria for i.v. heroin or cocaine faster than adult male rats (Lynch et al. 1998; but see Stewart et al. 1996 for no sex difference in heroin self-administration in rats). Numerous sex differences occur in analgesic, reinforcing, discriminative, and motoric effects of opioids, as well as opioid receptor density and dopaminergic response to exogenous opiate administration (reviewed in Craft 2008). Also, conflicting data exists suggesting some sex-dependent effects of adolescent cannabis or a synthetic cannabinoid agonist (CP 55,940) exposure on adult heroin or morphine self-administration behavior, endogenous peptide levels, and receptor efficiency (Biscaia et al. 2008; Ellgren et al. 2007). Furthermore in adult rodents, corticosterone suppression decreases the locomotor-stimulating effects of morphine (Deroche et al. 1993); blocking glucocorticoids attenuates morphine-induced dopamine efflux in the NAcc (Marinelli et al. 1998), and gonadal steroid hormones increase the potency of morphine in a hot plate test (Stoffel et al. 2003). Unfortunately, comparisons across different drugs do not reveal the same age-dependent results as presented here [e.g. no age differences in cocaine self-administration (Frantz et al. 2007; Kerstetter and Kantak 2007; McQuown et al. 2007) or higher intake after adolescent on-

set of nicotine or amphetamine self-administration (Belluzzi et al. 2005; Shahbazi et al. 2008; Shram et al. 2008a)]. Thus, general conclusions about endocrine and sex hormone-related adolescent-specific sensitivity to behavioral reinforcement by drugs of abuse are not warranted.

6.6   Limitations of using adolescent rodent models for complex human disorders

We have only tested a couple possible factors known to be involved in addiction, but certainly other factors are involved. Moreover, adolescent human drug vulnerability may end up not being biologically based. Other factors known to be important for human drug abuse are social factors such as peer-pressure, cognitive factors such inhibition and impulsivity, environmental factors such as access to drugs, and internal factors such as response to stress. Also, we have initially focused on only one way of reinstating drug-seeking behavior, context or discrete cues, but stress- and the drug itself are also used to induce reinstatement of drug-seeking behavior. And maybe continued drug intake is important, as our adolescent rats only take drugs during the short period of adolescence, and not continued into adulthood.

Some characteristics of rodent adolescence may limit its use as a model for human adolescence. Adolescence in rodents is quiet short, spanning only approximately 20 days (Spear 2000 for review). Thus, experiments intended to take place during the adolescent period in rodents must be modified to fit in this short time span and may therefore not be as thorough, possibly leading to inconclusive interpretations. For example, full dose effect functions during self-administration are needed for accurate conclusions regarding the reinforcing potency and efficacy of the test drug, but rats usually take approximately five days to learn to acquire the association of the lever and the infusion of the

drug, and acquisition must come before any test of dose effects. It is possible to pre-train rats to lever press for food pellets to speed up acquisition, but then food deprivation is used to motivate the rats, which adds possible confounding motivational states. It is also possible to speed up testing phases by running two self-administration sessions per rat per day, but that would also reduce the number of rats tested at one time and drug effects from the first test of the day might carry over to the second test of the day. Another technical drawback is the need to modify the i.v. catheters for the initial small size of adolescent rats, but also allow for the rapid growth spurt. Our lab can only reliably catheterize adolescent rats starting as early as P28, which means worthwhile young adolescent days are spent recovering from surgery. Also, great care must be taken to maintain i.v. catheters for long periods, yet despite caution most labs using i.v. catheters consistently lose approximately 10% of rats to catheter malfunction.

6.7   Future Directions

Guided by the framework of current literature and the present results, a number of future directions are worth pursuing based on factors known to be important for reinforcement and reinstatement of drug-seeking in adult subjects and development of the nervous system in adolescents. First, there seems to be a dichotomy in adolescent vs. adult reinstatement of cocaine-seeking (Anker and Carroll 2010), in that adolescent- vs. adult-onset male rats exhibit less context/cue-induced, but more cocaine- and yohimbine-induced reinstatement of drug-seeking. It would be worthwhile to test if this same pattern of reinstatement behavior was exhibited in adolescent- vs. adult-onset rats with a history of heroin or morphine self-administration. Adolescents may be exceptionally vulnerable to stress (Spear 2009), and in adult rats modulation of the central nervous stress system im-

pacts heroin-seeking behavior (Banna et al. 2010; Shaham et al. 2000; Zhou and Kalivas 2008). Also of interest, in adult male rats the context in which heroin vs. cocaine or amphetamine are self-administered (home cage vs. separate chamber and room) seems to important for amount of drug intake and Fos mRNA levels in the brain (Celentano et al. 2009), but it is not known if this environmental factor also influences age-dependent drug-seeking behavior. As mentioned above, maybe drug use beginning during adolescence and continuing into adulthood would be a more influential drug history pattern for adolescent vulnerability and would be a better model of adolescent-onset human drug addicts, compared to our current short two week self-administration conditions. Indeed, long-term self-administration (3 months) seems be important for the appearance of human addiction-like behaviors in rats (Deroche-Gamonet et al. 2004).

Our lab has yet to test which neurotransmitter systems are regulating age-dependent drug-seeking (i.e. opioidergic, dopaminergic and glutamatergic systems within reward and reinforcement areas in the brain). Obvious candidates exist known to control drug-seeking in adult rodents that also go through dramatic changes during the adolescent developmental period. In regards to the opioid system [maintenance pharmacotherapy with opioid ligands methadone, naltrexone, or buprenorphine are currently the gold standard treatment for human opiate addicts], mu opioid receptor efficiency is blunted during early adolescence compared to adulthood in rats (Talbot et al. 2005), and in adult rats methadone maintenance reduces heroin- and cocaine-induced reinstatement of drug-seeking (Leri et al. 2004). In regards to the dopamine system, dopamine D1 receptors are over-expressed and associated second messenger system activity is blunted during adolescence (Andersen 2002; Andersen and Teicher 2000; Brenhouse et al. 2008), and in adult rats

injection of the dopamine D1-family receptor antagonist (SCH 23390) into the dorsola-

teral, but not dorsomedial, striatum attenuates heroin-seeking behavior (Bossert et al.

2009).

In regards to the glutamate system, adolescence and puberty are intimately linked

to proper glutamate system function (Parent et al. 2005). Electrophysiological data sug-

gest greater AMPA receptor contribution to excitatory postsynaptic currents (EPSCs) in

the NAcc among adolescents vs. adults (Kasanetz and Manzoni 2009), but in PFC in spe-

cific populations of interneurons, NMDA- but not AMPA-mediated current might contri-

bute more in adolescents vs. adults (Wang and Gao 2009). In the PFC, as well, increased

basal expression of specific isoforms of AMPA receptor subunits GluR1/3 in adolescent

vs. adult rats was observed (Stine et al. 2001), whereas another study suggested possible

more GluR1 but less GluR2-4 subunit expression in younger rats (although this later

comparison was made in younger P21, vs. P110 adults; Talos et al. 2006). Also, AMPA

receptors exhibit age-, dose-, and brain area-dependent changes following adolescent vs.

adult administration of nicotine (Adriani et al. 2004), suggesting that age-at-onset of drug

intake is important for some drug-related long-term glutamate system plasticity. In adult

rats systemic and intra-VTA injections of the glutamate group II metabotropic receptor

agonist (LY379268) reduces glutamate release and attenuates heroin-seeking in adult rats

(Bossert et al. 2004). Also, glutamate AMPA receptor subunit ratio/activity within

mPFC and NAcc are critical for drug-seeking behavior in adult rats; GluR2 and GluR3

subunit downregulation were associated with cue-induced heroin-seeking and blocking

GluR2 endocytosis in infralimbic, but not prelimbic, mPFC attenuates cue-induced he-

roin-seeking (Van den Oever et al. 2008); and blockade of GluR2-lacking receptors in

NAcc attenuates "incubation" of cocaine-seeking (Conrad et al. 2008). Furthermore in adult rats, treatment with N-Acetylcystine, which restores cystine–glutamate tone after repeated drug use, reduces extinction responding and cue- and heroin-induced (Zhou and Kalivas 2008) and cocaine-seeking behavior (Moussawi et al. 2011).

Lastly, the cellular and molecular mechanisms of neuroplasticity have emerged as targets for drug addiction research, but little is known about neuroplasticity in rats that self-administer drugs as adolescents. Our lab has begun to explore expression of plasticity genes, such as BDNF, Arc and Erk, during abstinence and reinstatement of drug-seeking in rats that self-administered cocaine as adolescents (Li and Frantz in preparation). It would be interesting to find out if the effects of cocaine self-administration in adolescent rats on neuroplasticity genes can generalize to rats with a history of adolescent morphine or heroin self-administration, especially during protracted withdrawal.

6.8   Overall Summary & Conclusion

In summary, these combined findings have provided important information regarding the neurobehavioral consequences of adolescent opiate use. Our results offer evidence using validated animal models of addiction that adolescent rats may provide a natural rodent model for protection against some acute and long-term effects of opiates. Although the patterns of morphine vs. heroin self-administration can be different (e.g. adolescents self-administer less morphine, but similar amounts of heroin, than adults), rats that self-administered either opiate during adolescence showed attenuated reinstatement of drug-seeking compared to adults. Adolescents also exhibited less severe somatic and locomotor signs of withdrawal, and less heroin-induced sensitization compared to adults. Lastly, less neural activation in the mPFC of adolescent-onset rats was associated with attenuated

heroin-seeking behavior, compared to adult-onset rats (measured as Fos, the protein product of the immediate early gene, *c-fos,* in the prelimbic and infralimbic areas of the mPFC). It is possible that adolescent's lack of negative effects would promote heightened recovery during abstinence. Also, this adolescent model might help reveal new targets for drug abuse treatment in human adolescent addicts and ultimately offer better prognosis for teenagers seeking treatment. Our results contradict the current dogma regarding adolescent sensitivity to the long-term effects of drugs. Since drug use peaks during human adolescence and early onset drug use is thought to make an individual more likely to be an addict as an adult, one would expect higher levels of adult addicts to match the high levels of adolescent-onset drug use. Our adolescent male rats might actually be a model of the majority of the human population who experiment with drugs during adolescence but whom the drugs do not impart increased vulnerability to become full blown addicts in adulthood.

6.9 Supplementary figures

Fig. 6.1
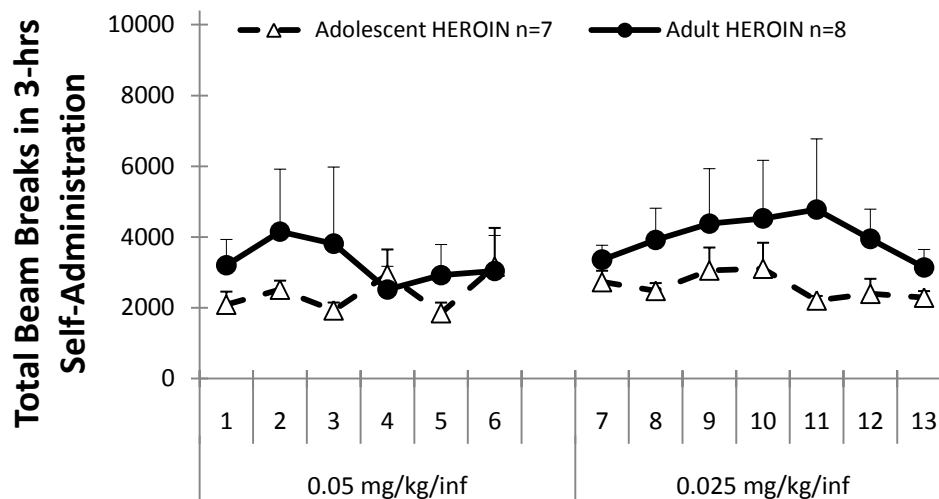
Fig. 6.1. Total beam breaks during heroin self-administration sessions within the operant chambers. Age groups did not differ in activity within the operant chambers. All points represent mean +/- SEM

Fig. 6.2
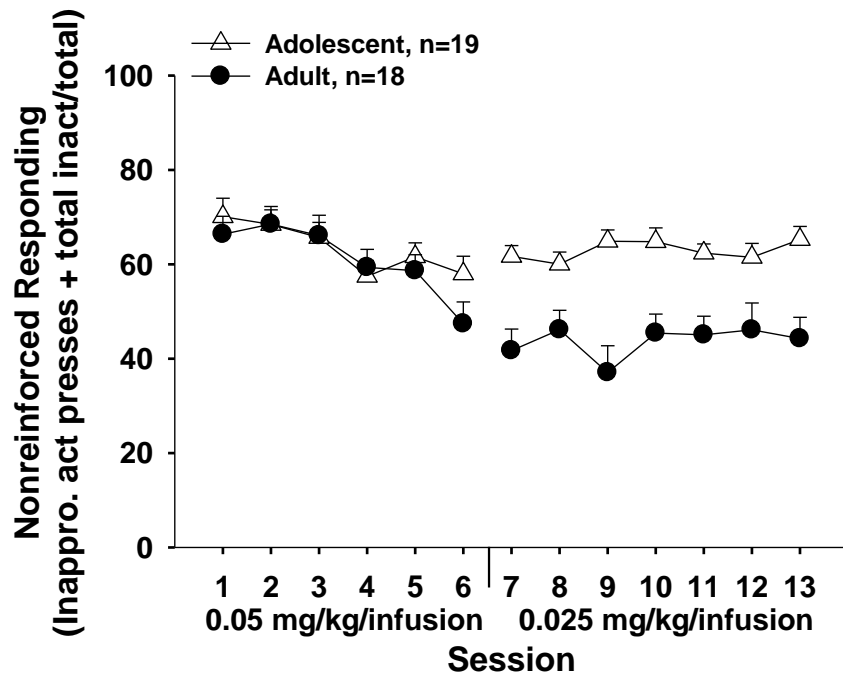
Fig. 6.2. Nonreinforced responding during heroin self-administration sessions. Adolescent male rats self-administering heroin exhibit more nonreinforced responding compared to adults. All points represent mean +/- SEM

Fig. 6.3
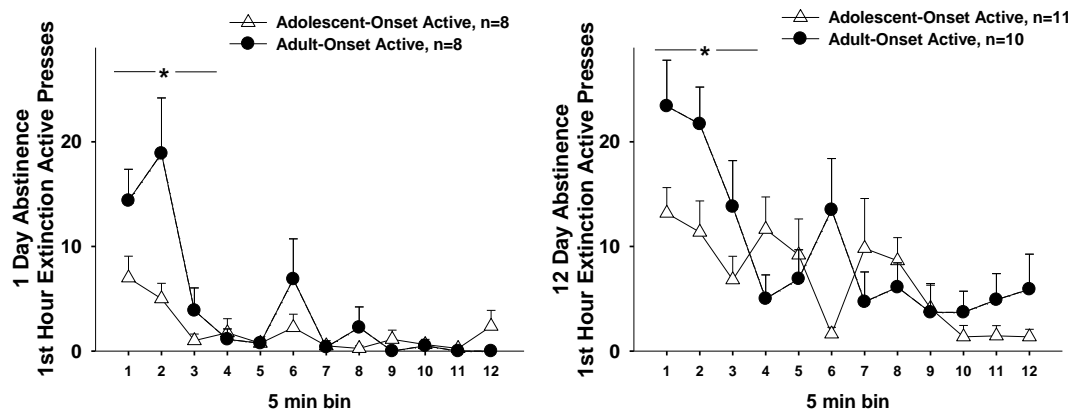
Fig. 6.3. Active lever presses per 5 min bin during the initial hour of extinction testing

after 1 (left) or 12 (right) days of abstinence from heroin self-administration. Rats that

self-administered heroin as adolescents exhibit less active lever presses during the initial

15 min of the heroin-seeking test compared to rats that self-administered heroin as adults,

regardless of abstinence period (* p<0.01). All points represent mean +/- SEM

Fig. 6.4
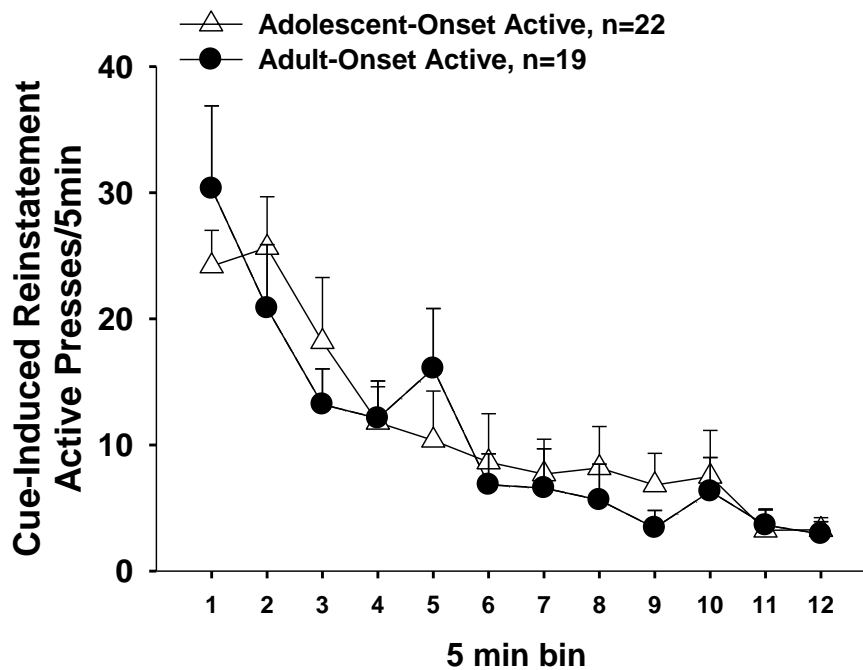
Fig. 6.4. Cue-induced reinstatement without extinction after 12 days of abstinence from

heroin self-administration on a progressive ratio schedule of reinforcement. Age groups

did not differ in active lever presses per 5 min bin. All points represent mean +/- SEM

Fig. 6.5
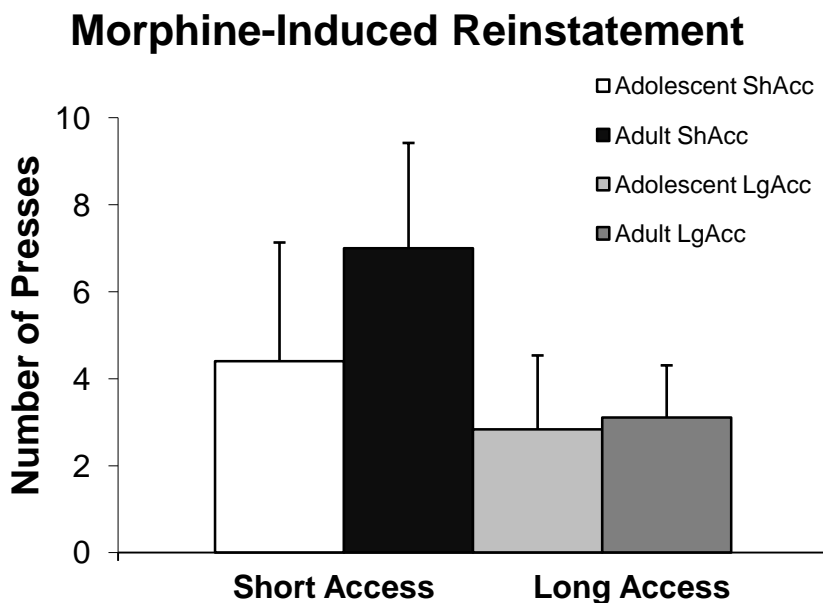
Fig. 6.5. Morphine-induced reinstatement of active lever presses one day after cue-induced reinstatement. Age and access groups did not differ in the ability of morphine (10 mg/kg i.p.) to reinstate active lever pressing during the one hour test. Access conditions during morphine self-administration: ShAcc=short access (1-hr/d) adolescent n=5, adult n=7; LgAcc=long access (8-hr/d) adolescent n=5, adult n=9. All points represent mean +/- SEM

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