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Attenuated Cocaine Seeking After Adolescent-Onset of Cocaine Self-Administration in Male Rats: Behavior, Environment, and Genes

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ATTENUATED COCAINE SEEKING AFTER ADOLESCENT-ONSET OF COCAINE SELF-ADMINISTRATION IN MALE RATS: BEHAVIOR, ENVIRONMENT, AND GENES

by

CHEN LI

Under the direction of Kyle J. Frantz

ABSTRACT

Recreational drug use peaks in the developmental stage of adolescence. Nevertheless, the potential association between early onset of drug use and higher rates of addiction in adulthood has not been thoroughly studied. In this series of experiments, we used a rodent model of adolescence and behavioral assessments of intravenous (i.v.) cocaine self-administration and reinstatement of cocaine-seeking to explore age differences in these cocaine-related behaviors, and then tested for the influence of environmental enrichment and for correlations between behavior and expression of plasticity genes. Male rats were trained to self-administer cocaine during either adolescence (adolescent-onset groups) or adulthood (adult-onset groups), and reinstatement of drug-seeking induced by cocaine or cocaine-associated cues was assessed after various durations of abstinence from cocaine intake. First, although taking similar amounts of

cocaine during self-administration, adolescent-onset groups surprisingly exhibited attenuated time-dependent increases in cue-induced reinstatement compared with adults. On the other hand, time-dependent increases in cue-induced reinstatement of sucrose-seeking were similar across age groups, suggesting that age differences in reinstatement of cocaine seeking depend on specific effects of cocaine, not a compromised ability among younger rats to associate cues with rewards. Then we asked whether the attenuated reinstatement may be due to rapid developmental re-organization of reinforcement circuits (high plasticity) in adolescent-onset groups. To stimulate or inhibit neuroplasticity, subjects experienced environmental enrichment and impoverishment during abstinence. Environmental manipulations had no effect in adolescent-onset groups, whereas the enriched environment attenuated cue-induced reinstatement in adult-onset groups compared with their impoverished counterparts. Last we tested age differences in basal or cocaine-related expression of two neuroplasticity-related genes, activity-regulated cytoskeletal-associated gene (*arc*) and brain-derived neurotrophic factor (*bdnf*). Age differences in gene expression after self-administration or cue-induced reinstatement were brain region specific. Overall, *arc* expression in the nucleus accumbens (NAc) and *bdnf* expression in the medial prefrontal cortex (mPFC) was higher in adolescent-onset than in adult groups. Together our data suggest that adolescence may be a period of relative biological resistance to some long-term drug effects.

INDEX WORDS: Ontogeny, Addiction, Relapse, Neuroplasticity, Enrichment, Gene

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1. GENERAL INTRODUCTION

This general introduction gives background on drug addiction in humans, especially with regard to the peak of drug use in adolescents. Rodent models for studying drug use in adolescent are described. Next, neuroplasticity induced by drug exposure and developmental organization in brain motivational circuitry is summarized, and effects of modifying neuroplasticity by environmental manipulations on drug addiction are introduced. Finally, the roles of neuroplasticity-related genes in drug addiction are discussed.

1.1 Vulnerability of Adolescents in drug addiction: human study

1.1.1 Cocaine addiction and relapse

Cocaine, an alkaloid obtained from the coca plant, is a powerful stimulant of the central nervous system. It is abused mostly likely because it produces intense feelings of euphoria that last for 15 minutes to 1 hour. According to an evidence-based assessment of the harms of addictive drugs, cocaine is ranked both the second-most addictive and the second-most harmful of 20 popular recreational drugs (Nutt et al., 2007). In 2009, 4.8 million Americans age 12 and older abused cocaine (SAMHSA, 2009), making cocaine abuse a severe social and medical problem. Although it is agreed that cocaine addiction is a brain disease, there are still no effective pharmacological treatments for cocaine addiction.

For its acute effects, cocaine blocks the dopamine transporter, a membrane-bound protein for reuptake of dopamine from the synaptic cleft into the neuronal terminals. Thus cocaine increases synaptic dopamine levels (Volkow et al., 1999). Dopamine then binds to more postsynaptic dopamine receptors, which are G-protein coupled receptors, and activates signaling pathways that enhance neuronal activity and promote short-term neuroplasticity. Dopamine is

one of the most important neurotransmitters in brain reward pathways. Mesolimbic and mesocortical dopamine pathways, arising from the ventral tegmentum and projecting to limbic system (including the nucleus accumbens, amygdala, hippocampus, and septal area) and cortex respectively, function in the initiation and maintenance of goal-directed and reward-mediated behaviors. Under normal conditions, these systems activate and release dopamine during both procurement and consumption of natural rewards, such as food, water and sex, and reinforce behaviors required for survival. Chronic cocaine intake induces profound, long-lasting neuroadaptations in brain reward pathways leading to addiction (Dackis and O'Brien, 2001, Kalivas and O'Brien, 2008). (See section 1.3.1 for more details).

Relapse, referring to reinitiation of cocaine seeking and cocaine taking behavior after abstinence from cocaine use, is one of the primary obstacles in treatment of cocaine addiction. Relapse can happen over days, months or even years after stopping the daily use of drugs like cocaine. Three main factors can trigger relapse: emotional stress, drugs, and drug-associated environmental cues (Dackis and O'Brien, 2001, Drummond, 2001). At early stages of relapse, addicts report that emotional disturbance, e.g. stress, anger and isolation, drive the motivation to eliminate these feelings by using drugs (Koob and Le Moal, 1997). Re-exposure to the abused drugs or even other addictive agents appears to drive relapse due to the fact that releasing dopamine in the nucleus accumbens is a common mechanism for addictive agents. Human studies show that cocaine itself causes a rebound of cocaine craving shortly after its administration (Jaffe et al., 1989). Finally, environmental cues, over many pairings with cocaine-taking, can produce intense craving in addicted individuals, presumably through classical conditioning (O'Brien et al., 1992). Positron emission tomography studies of human addicts show that cocaine-associated environmental cues activate brain reward pathways mediating the

craving for drugs (Childress et al., 1999). Since chronic cocaine exposure causes dramatic changes in neural circuitry, cognitive intervention and behavior therapy are usually insufficient to control relapse behavior. Thus, developing pharmacological intervention is urgent and necessary, but requires further understanding of the biological basis of the drug addiction phenomenon.

1.1.2 Drug use in adolescents

Adolescence in humans is suggested to be a vulnerable developmental stage to drug addiction. The vulnerability of adolescents to illicit drug abuse and addiction is supported by data from the National Survey on Drug Use & Health (NSDUH), which is a survey on the prevalence, patterns, and consequences of drug use and abuse in the general U.S. civilian population aged 12 years and older (SAMHSA, 2009). This survey shows that an estimated 21.8 million Americans aged 12 or older are current (past month) illicit drug users, and among them, an estimated 3.1 million used an illicit drug for the first time within the past 12 months. More than half are younger than age 18 when they first used.

The early onset of drug use may cause susceptibility to develop dependence on or abuse of illicit drugs (Laviola et al., 1999, O'Malley and Johnston, 2007, SAMHSA, 2009). NSDUH shows that for marijuana, among those whose onset of use is 14 or younger, 12.6 percent are classified with illicit drug dependence or abuse; however, among those who had first used marijuana at age 18 or older, only 2.1 percent are classified as dependent. For alcohol, among those whose onset of use was 14 or younger, 17.5 percent are classified with alcohol dependence or abuse among; however, among those who have first used alcohol at age 18 or older, only 3.7 percent are classified as dependent. However, whether or not these phenomena can be attributed to a biological vulnerability of adolescent development is unclear, especially given that a

manifestation of vulnerability to drugs among humans might be adolescent onset of drug use; if so, few vulnerable individuals would be included in any survey group of adult-onset drug users (Shram et al., 2007). Animal models provide a platform to address these questions, since the onset age of drug use in animals can be easily manipulated and controlled (Schramm-Sapyta et al., 2009).

1.1.3 Characteristics of adolescents

Adolescence is the period of physical and psychological development from the onset of puberty to maturity, and it usually starts at age 13 years old and ends around age 19. Under the influence of a surge of gonadal hormones, adolescents undergo a number of physical changes, including secondary sex characteristics, and become sexually mature (Teicher, 1956). Besides physical changes, adolescents also undergo dramatic psychological changes, including cognitive, emotional and attitudinal maturation (Teicher, 1956). Adolescence is a period in which individuals seek independence but are still not equipped to cope with new challenges. Under the drive of sexual maturation, independence, and aggression to achieve and dominate, adolescents exhibit unique characteristics, e.g. novelty-seeking, risk-taking and impulsive behavior, often making their behavior frustrating and frightening to adults, and even to themselves (Teicher, 1956). It is now believed that those "bizarre" behaviors are due to the developing prefrontal cortex, which is one of the last brain regions to reach maturation (Teicher et al., 1998, Spear, 2000b). The prefrontal cortex performs so-called "executive functions", including defining a goal, forming strategies and planning, focusing attention, impulse control and delaying gratification, modulation of intense emotions and inhibiting inappropriate behavior and initiating appropriate behavior. With an immature prefrontal cortex, it is difficult for adolescents not to engage in certain activities; even though they realize that the harmful consequence may be

caused by such risky behavior. These psychological characteristics may make adolescence a vulnerable stage for illicit drug abuse and addiction.

1.2 Introduction to chapters 2 and 3

To answer the question whether adolescent onset of cocaine use leads to long-term vulnerability to addiction, we used an adolescent model in rodents. To test for adolescent-specific patterns of drug-taking and drug-seeking behavior, the intravenous drug self-administration, extinction, and reinstatement models were used.

1.2.1 Adolescence in rodents

In rodents, adolescence can be defined as a transitional period sometime between postnatal days (PND) 28 and 60 (Spear and Brake, 1983, Spear, 2000a, Spear, 2000b, Smith, 2003). In addition to sexual maturation, rodents during this developmental stage also exhibit similar characteristic behaviors to primates, including increases in peer-directed social interactions and elevations in novelty-seeking and risk-taking behaviors (Spear, 2000b, Crews et al., 2007).

Similar structural and functional changes in brain are observed in adolescent humans and rodents as they mature to adults. For example, for the maturation of neocortex, a massive loss of synapses in cortical brains regions occurs in both humans and rodents, and forebrain dopamine receptors levers also prune (Teicher et al., 1995, Andersen et al., 2002b). Moreover, rates of oxygen consumption and glucose utilization in neocortex and forebrain are greater in human adolescents (Chugani et al., 1987), suggesting a higher level of activity in these brain regions during development. In addition, other limbic regions such as amygdala and hippocampus mature dramatically in both humans and rodents (Kellogg et al., 1998, Yurgelun-Todd, 2007).

Therefore, adolescent rodents can be used as a model to investigate drug addiction in human adolescents. In our experiments, adolescent-onset groups self-administered cocaine

between postnatal days 35-50, and their drug intake and later cue-induced reinstatement behavior were compared with adult-onset groups that started taking cocaine as young adults (postnatal day ~ 80-~95).

1.2.2 Self-administration, extinction and reinstatement model

Self-administration and reinstatement models in rodents are widely used to mimic drug intake followed by drug-, cue- or stress-induced relapse of drug-seeking in humans (Shaham et al., 2003). The model usually consists of several phases. First, rats acquire lever-pressing maintained by intravenous (i.v.) infusions of cocaine (or other drugs or natural rewards) in operant conditioning chambers and establish associations between their behavior, environmental cues and the drug. Then they undergo a variable period of abstinence in their home cages. Finally, with or without extinction phases that extinguish lever pressing under the condition when drug is not available, rats are re-exposed to self-administration chambers and/or the conditioned stimuli, drug or stress to reinitiate lever-pressing behavior. On the contrary, saline and saline associated cues have no such reinforcing effects. The validity of the reinstatement model in particular is supported by the evidence that drug or drug-associated environmental cues activate similar brain circuits in human and rodents (Kalivas and McFarland, 2003, Bossert et al., 2005, See, 2005). Moreover, somewhat analogous to persistent relapse in humans, cue-induced reinstatement in rodents also increases over time after self-administration in a phenomenon known as “incubation of drug craving” (Grimm et al., 2001, Lu et al., 2004c). Thus, we used the self-administration and cue-induced reinstatement procedure to compared cocaine taking and seeking in adolescent- vs. adult-onset groups of male rats (chapter 2). Based on previous studies showing that adolescent rats take similar amount of cocaine as adults (Frantz et al., 2007) and the human literature, we hypothesized that there would be no age differences in cocaine self-administration,

but taking cocaine during adolescence would cause higher levels of later reinstatement in adolescent-onset groups compared with adults.

Cue-induced reinstatement of sucrose seeking has been used as a control procedure for studying cue-induced reinstatement of drug-seeking (Bossert et al., 2005). Sucrose seeking can be reinitiated after abstinence and/or extinction by exposure to cues associated with the presentation of rewards, and cue-induced reinstatement of sucrose seeking also increases over abstinence periods, but to a lesser extent than cocaine seeking (Grimm et al., 2003, Lu et al., 2004c, Grimm et al., 2005). Thus, to exclude the possibility that the attenuated cue-induced reinstatement of cocaine seeking in adolescent rats is due to relatively poor association between cues and rewards compared with adults (and not due to a specific effect of cocaine), and also to investigate age differences in food-related reward and reinforcement, we study sucrose self-administration and cue-induced reinstatement in adolescent- vs. adult-onset groups (chapter 3) We hypothesized that both age groups would demonstrate similar robust time-dependent increases in cue-induced reinstatement of sucrose seeking.

1.3 Introduction to chapter 4

1.3.1 Cocaine induces neuroplasticity changes in brain motivational circuitry

The possible neural mechanisms mediating the age differences in cocaine seeking were further explored. The phenomenon that persistent vulnerability to relapse to cocaine seeking could last for many years after abstinence indicates that there must be long-lasting changes in brain structures and functions that mediate the pathological behavior. It is hypothesized that cocaine and other addictive agents, “hijack” the mesolimbic and mesocortical pathways that guide goal-directed behavior and ensure survival needs under normal conditions. Three stages of addiction have been defined to include acute drug effects, transition to addiction, and end-stage addiction

(Kalivas and Volkow, 2005). Through this process, recreational drug use becomes compulsive, and develops into long lasting vulnerability to relapse (Kalivas and Volkow, 2005). At the stage of acute drug effects, cocaine-induced increases in synaptic dopamine activate cAMP-dependent protein kinase (PKA), which in turn phosphorylates transcriptional regulators such as cAMP response element binding protein (CREB). Phosphorylation of CREB induces immediate early gene expression, such as c-fos (Konradi et al., 1994), thus promoting short-term neuroplastic changes and facilitating drug-associated learning. At the stage of transitioning to addiction, repeated administration of cocaine induces accumulated neuroplastic changes lasting over days or weeks, among which ΔFosB is the most studied molecular adaptation. ΔFosB modulates the synthesis of AMPA glutamate receptor subunits and increases the number of dendritic branches and spines (Kelz et al., 1999). Moreover, accumulation of ΔFosB correlates with the development of behavioral sensitization to repeated cocaine injections (Kelz et al., 1999, Nestler et al., 2001), suggesting its role in the transition to addiction. At the last stage of addiction, permanent changes at functional and structural levels can last for years; although counterintuitive, some changes appear to increase during drug withdrawal rather than decreasing (Shalev et al., 2002, Grimm et al., 2003). The most significant changes may be in the glutamatergic projection from prefrontal cortex to nucleus accumbens, a final common pathway for initiating drug-seeking behavior (Figure 1.1) (Kalivas et al., 2005).

The changes in the pathway of the prefrontal cortex to the nucleus accumbens have been shown on several aspects. Neuroimaging studies in human subjects suggest a reduced baseline activity in the prefrontal cortex in addicts, compared with controls (Jentsch and Taylor, 1999). Drug-associated cues also induce strong activity in the prefrontal cortex, and the magnitude of changes in metabolic activity correlates with self-reported drug craving (Wexler et al., 2001).

Morphological studies on medium spiny neurons in the nucleus accumbens, which are the principle neurons in the nucleus accumbens and receive glutamatergic projections from the prefrontal cortex, report increases in dendritic length and dendritic spine density, indicating increases in the number of excitatory synapses (Robinson and Kolb, 1999, Robinson et al., 2001, Shen et al., 2009). Microdialysis studies on extracellular glutamate in prefrontal-nucleus accumbens synapses show a decreased baseline level, but an enhanced cocaine- or cocaine-associated cue-induced release of glutamate (Baker et al., 2003). This hyperactivity of excitatory synapse is also supported by a growth in the number of postsynaptic AMPA receptors (Boudreau et al., 2007), and changes in the subunit of AMPA receptors, such as more frequent insertion of GluR2-lacking AMPA receptors, which are more permeable to calcium than other AMPA receptor subtypes (Conrad et al., 2008b). Moreover, modification of gene expression (e.g. Homer1 PSD-95, *bdnf* and *arc*) is also involved in cocaine addiction (Yao et al., 2004, Ghasemzadeh et al., 2009).

1.3.2 Developmental organization

During adolescence, the mesolimbic and mesocortical pathways, including prefrontal cortex, nucleus accumbens, amygdala, etc, are undergoing robust developmental organization (Smith, 2003, Crews et al., 2007). Cortical regions in adolescents show hypermetabolism suggesting higher levels of neuronal activity. The absolute volume of cortex reduces during adolescence due to the loss of substantial synapses, especially the excitatory glutamatergic connections (van Eden et al., 1990). A variety of different neurotransmitter systems are under specific developmental changes. For instance, dopamine input to the prefrontal cortex is at the highest level during adolescence; dopamine receptor numbers peak during adolescence, then prune to adult level in the prefrontal cortex (Brenhouse et al., 2008). Electrophysiological studies also show the

maturation of dopamine-dependent prefrontal cortical functions during adolescence (Tseng and O'Donnell, 2007). Similarly, dendrites and synapses in the nucleus accumbens, amygdala, hippocampus and hypothalamus are also undergoing pruning and reorganization (Choi et al., 1997, Tarazi et al., 1999, Zehr et al., 2006, Zehr et al., 2008). Thus, it is possible that this wide array of developmental changes in the mesolimbic and mesocortical pathways during adolescence actually reduce cue-induced reinstatement of cocaine seeking in younger animals by preventing or reversing the pathological neuroplasticity induced by cocaine, per se. To promote or constrain neuroplasticity during abstinence and test whether adolescent rats are more or less sensitive than adults to the influence of plasticity on reinstatement of cocaine seeking after abstinence, we introduced environmental manipulations during abstinence in our model.

1.3.3. Environmental enrichment vs. impoverishment

Classic manipulations that stimulate or inhibit brain plasticity include environmental enrichment or environmental impoverishment, respectively (van Praag et al., 2000, Lewis, 2004, Laviola et al., 2008). An enriched environment can be defined as "a combination of complex inanimate and social stimulation" and animals are usually housed in a big group, in a large cage, and opportunity to explore periodically changed "toys". Enriched environments produce experience-dependent neuroplasticity, improve learning and memory, and attenuate the addictive effects of psychostimulant drugs (Laviola et al., 2008). On cellular and molecular levels, enriched environments increase experience-dependent neuroplasticity including transcription of various genes, synaptogenesis and adult neurogenesis (van Praag et al., 2000). On the other hand, environmental impoverishment in which animals are housed in a limited space and socially isolated limits such neuroplasticity and increases effects of psychostimulant drugs (Stairs et al., 2006). Based on the experimental timeline of exposure to environmental manipulations, the

effects of environmental enrichment on drug addiction have been categorized as preventive or curative.

Preventive effects

For the preventive effects, animals are exposed to environmental enrichment prior to any contact with drugs, usually from weaning and through the entire experimental procedure, since it is agreed that peri-adolescent development is a sensitive period for long-lasting influence of environmental manipulations. Results obtained from a variety of behavioral models suggest that environmental enrichment may have opposite effects on specific behaviors induced by cocaine (Solinas et al., 2010). For example, locomotor effects of cocaine are greater in rats housed in enriched condition (Bowling and Bardo, 1994, Bardo et al., 1995a, Green et al., 2010). Rewarding effects of cocaine are also enhanced by enrichment, as a lower dose of cocaine establishes a conditioned place preference (CPP) in enriched rats compared with controls in standard housing (Bardo et al., 1995b, Green et al., 2010). On the other hand, environmental enrichment reduces the reinforcing effects of psychostimulants and the long-term neuroadaptations produced by repeated administration of psychostimulants (Bardo et al., 2001, Green et al., 2002, Stairs et al., 2006). For example, using self-administration models, rats housed in enriched conditions take less cocaine, and show decreased motivation for the drug measured by a progressive schedule of reinforcement, in which more and more effort will be needed to receive the next drug infusion (Bardo et al., 2001, Green et al., 2002). Meanwhile, development of sensitization, the resistance to extinction, and the propensity to relapse are also decreased in rats reared in enriched conditions (Stairs et al., 2006). Since the self-administration procedure better represents drug-seeking and taking behavior in human, it is generally accepted that environment enrichment has a preventive effects on drug addiction. However, for those

studies, animals are usually housed in different conditions upon weaning and throughout the development stage and experimental procedure. The goal for treatment of drug addiction is to extinguish and prevent drug-seeking and taking behavior after addiction has developed. Thus it is important to know whether environmental enrichment provided after drug exposure can "treat" addiction.

Curative effects

Using CPP and the self-administration model, it is clear that environmental enrichment provided only during the abstinence period can decrease the ability of drug-associated cues or stress to reinstate drug-seeking behavior (Solinas et al., 2008, Chauvet et al., 2009, Thiel et al., 2009, Thiel et al., 2010). However, these two models provide discrepant results in the ability of cocaine priming injection to reinstate drug-seeking, i.e. environmental enrichment reduces cocaine-induced reinstatement of CPP (Solinas et al., 2008), but has no effects on drug-induced reinstatement of self-administration (Chauvet et al., 2009). This discrepancy may be explained by the fact that CPP and self-administration measure different aspects of drug addiction (Sanchis-Segura and Spanagel, 2006). Moreover, the curative effects are supported by the evidence that environmental enrichment reduces cocaine-induced brain activity, as measured by c-Fos expression, in mesolimbic and mesocortical regions (Solinas et al., 2009).

Environments may have more profound effects on younger animals than on older animals. Adolescent rats are more affected by environmental stress than adults (Smith 2003). Also, adolescents exhibit more novelty-seeking behavior in an inescapable novel environment and novel object exploration than do adults (Philpot and Wecker 2008). To test whether adolescent rats are more or less sensitive than adults to the influence of promoting or constraining plasticity on reinstatement of cocaine seeking after abstinence, we introduced environmental

manipulations during abstinence in our model (chapter 4). We hypothesized that (1) environmental enrichment during 60 days of abstinence from i.v. cocaine self-administration would attenuate cue-induced reinstatement more among rats that self-administer cocaine as adolescents, compared with adults; (2) environmental isolation during 60 days of abstinence from i.v. cocaine self-administration would elevate cue-induced reinstatement more among rats that self-administer cocaine as adolescents than adults.

1.4 Introduction to chapter 5

On the molecular level, neuroplasticity is mediated in part by changes in gene expression (Loebrich and Nedivi, 2009). Two molecules that are associated with reinstatement of cocaine seeking and play known roles in neuroplasticity in adult rats are activity-regulated cytoskeleton-associated protein (Arc) and brain-derived neurotrophic factor (BDNF). The study reported in chapter 5 tests for correlation between Arc and/or BDNF gene expression and cocaine-related behaviors across age groups.

1.4.1 Arc in cocaine addiction

Arc is an immediate early gene expressed in response to cellular activity that plays an important role in learning and memory, at least in rodents. Induced by a variety of cellular signals, such as BDNF and glutamate bound at NMDA receptors, *arc* mRNA migrates to dendrites and accumulates at sites of synaptic activity where it is translated into protein and contributes to formation, strengthening, and stabilization of synapses (Bramham et al., 2008, Bramham et al., 2010, Shepherd and Bear, 2011). For a specific example, Arc increases endocytosis of AMPA receptors, eventually leading to LTD following some patterns of neuronal activity (Chowdhury et al., 2006). Also during the early phase of LTP, Arc is necessary for the expression of LTP; whereas during late phase of LTP, Arc regulates F-actin polymerization and stabilization, which

leads to expansion of postsynaptic structures and maintenance of LTP (Messaoudi et al., 2007). Behavioral studies also show Arc's role in learning and memory. For example, inhibition of Arc expression in hippocampus impairs consolidation of long-term memory for a spatial water maze task without affecting acquisition or short term memory tasks (Guzowski et al., 2000). *Arc* knockout mice are deficient in numerous behaviors, such as spatial learning, fear conditioning, conditioned taste aversion memory, and long-term object-recognition memory (Plath et al., 2006).

Effects of cocaine depend on long-term synaptic changes in the rewarding and reinforcement pathway, and Arc appears to be important in cocaine-related behaviors in rats. For example, a single injection of cocaine is sufficient to up-regulate *arc* expression in the mesocortical system, although it declines to basal level after 24 hours (Fumagalli et al., 2006), After the last treatment in a chronic cocaine exposure in the same study, *arc* expression increased for a longer period (days) (Fumagalli et al., 2006), suggesting a correlation between duration of cocaine exposure and duration of Arc effects. Re-exposure to cocaine-associated environmental contexts or cues also up-regulates *arc* expression, suggesting Arc involvement in conditioned learning for incentive motivational effects of cocaine-related cues (Hearing et al., 2008, Zavala et al., 2008, Ziolkowska et al., 2011). Finally, experimental suppression of *arc* expression in the caudate putamen slows down the process of 'extinction learning' in the absence of a previously self-administered drug (cocaine), thereby confirming a role for Arc in behavioral changes related to cocaine self-administration (Hearing et al., 2010). Since cocaine induces synaptic plasticity in the mesolimbic and mesocortical circuitry, and *arc* expression plays an important role in expression and maintenance of synaptic plasticity, arc expression in these regions, e.g. mPFC and NAc, should be involved in cocaine-induced long-term changes (Figure 1.1).

1.4.2 BDNF in cocaine addiction

BDNF is a member of the "neurotrophin" family of growth factors and its major functions include supporting the survival of existing neurons as well as promoting the growth and differentiation of new neurons and synapses. Neuronal activity triggers releases of BDNF, which binds to and actives tropomyosin sensitive receptor receptors kinase B (TrkB). Phosphorylation in the intracellular domains of TrkB then triggers the downstream signal cascades, such as mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and phospholipase Cɣ (PLCɣ), which regulate gene transcription, protein translation, and trafficking (Kuczewski et al., 2009, Yoshii and Constantine-Paton, 2010). BDNF thus contributes to synaptic plasticity through both functional and structural changes. For example, BDNF modulates LTP through promoting presynaptic release of neural transmitter (Xu et al., 2000), as well as increasing the density of dendritic spines (Shimada et al., 1998).

Various studies have shown possible links between BDNF and central nervous system disorders (Lipsky and Marini, 2007). For example, a role for BDNF in memory is supported by human studies and conditional knockout mice studies showing that mutant impairs both structure and function of hippocampus (e.g. contextual fear conditioning) (Egan et al., 2003, Monteggia et al., 2004). A disruption of BDNF-dependent neuronal plasticity is associated with development of depression, and antidepressant drugs increase BDNF levers in hippocampus (Castren and Rantamaki, 2010). Moreover, individuals carrying a BDNF variant show more chance to have anxiety disorders, suggesting a role of BDNF in anxiety-related behavior (Jiang et al., 2005). Finally, the linkage between BDNF and drug dependence and addiction has been shown in both human and animal studies (Ghitza et al., 2010).

BDNF is involved in the relapse of cocaine seeking modeled in experimental animals (Figure 1.1). Although oversimplified, Figure 1.1 shows that BDNF is synthesized in the PFC and VTA, transported to the nucleus accumbens, secreted from presynaptic component, then it binds to TrkB receptors there. It is up-regulated by cocaine and accumulates in the prefrontal cortex and nucleus accumbens of adult male rats during drug abstinence (Grimm et al., 2003). Its specific role in reinstatement appears to vary by brain region, however. Injection of BDNF into the ventral tegmental area (VTA) or the nucleus accumbens (NAc) immediately after a final cocaine self-administration session promotes subsequent reinstatement of drug seeking (Lu et al., 2004a, Graham et al., 2007). On the other hand, injection of BDNF into the prefrontal cortex (PFC) immediately after a final cocaine self-administration session attenuates reinstatement of drug-seeking (Berglind et al., 2007). Notably, none of these treatments is effective if given immediately prior to reinstatement testing, suggesting that the functions of BDNF to influence cue-induced reinstatement occur concurrently or in temporal proximity to cocaine effects. The attenuating effects of BDNF in the medial prefrontal cortex (mPFC) on cocaine seeking depend on Trk receptor-mediated activation of extracellular signal-regulated kinase (ERK) signaling in the nucleus accumbens (Whitfield et al., 2011).

1.4.3 Age-dependent expression in Arc and BDNF

Although developmental assays of Arc and BDNF levels and activity are scarce, their expression appears to be age-dependent, leading us to predict that they could be involved in age differences in cocaine-related behavior. For example, basal expression levels of *arc* are 1.5-3-fold higher in the cortex of adolescent male rats than adults (Schochet et al., 2005). BDNF levels gradually decrease throughout the lifespan in male rats (Silhol et al., 2005). Moreover, amphetamine induced less expression of *arc* and *bdnf* mRNA in hippocampal and cortical brain

regions of juvenile than adult rats (Banerjee et al., 2009). Based on the fact that suppression of *arc* expression in striatum impairs extinction, we hypothesized that cocaine and cocaine associated cues would induce higher expression of *arc* in the prefrontal cortex and nucleus accumbens of younger animals than adults. Also, based on the report that BDNF in the prefrontal cortex attenuates, whereas BDNF in the nucleus accumbens increases the cue-induced reinstatement in adult rats, we hypothesized that cocaine and cocaine-associated cues would induce higher expression of BDNF mRNA in the prefrontal cortex but lower expression in the nucleus accumbens of younger animals than in adults.

1.5 Summary

In summary, our specific aims include the following: 1) compare age differences in cocaine self-administration and reinstatement behavior in male rats; 2) test effects of environmental manipulations on reinstatement behavior in adolescent- and adult-onset groups; 3) test for correlations between expression of neuroplasticity-related genes in reinforcement-related brain regions and the behaviors of cocaine taking and/or seeking in adolescent- and adult-onset groups. Ultimately these studies provide insight regarding adolescent sensitivity to drugs and can contribute to the development of targeted drug treatment protocols for adolescent addicts.

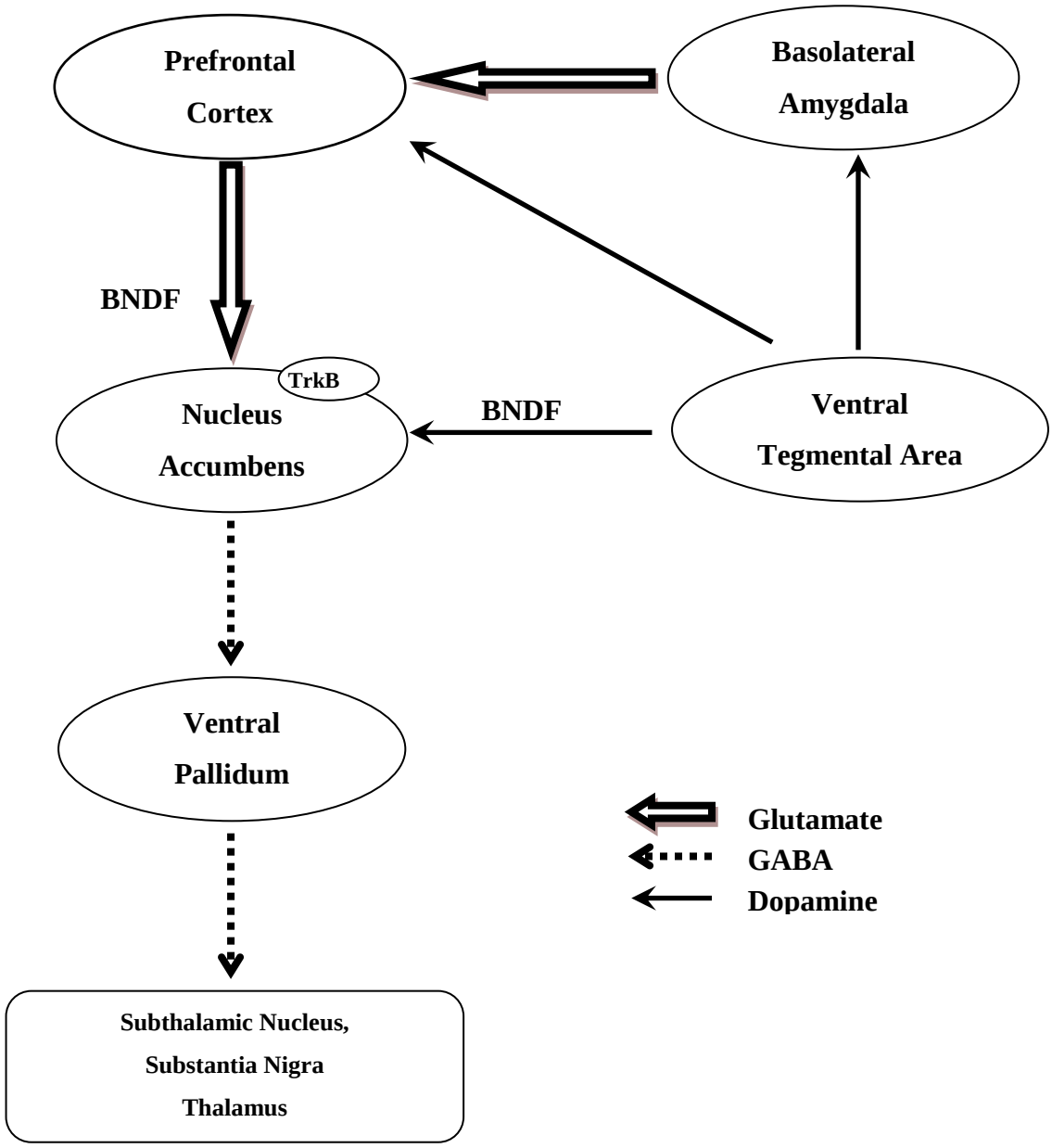

Figure 1.1 Simplified diagram of neural circuitry mediating reinstatement of cocaine seeking. Cocaine induces neuroplastic changes in brain motivational circuitry that mediate the long lasting behavior of addiction, including prefrontal cortex, nucleus accumbens, amygdala, etc. This circuitry is also undergoing robust developmental organization. We hypothesize that the developmental neuroplasticity may prevent or reverse cocaine-induced neuroadaptations.

2. CHAPTER 2: ATTENUATED INCUBATION OF COCAINE SEEKING IN MALE RATS TRAINED TO SELF-ADMINISTER COCAINE DURING PERIADOLESCENCE

By

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2.1 Abstract

Rationale and objectives: Although onset of drug use during adolescence appears to increase long-term vulnerability to drug dependence in humans, relatively little is known about extinction and reinstatement of drug-seeking after periadolescent onset of drug self-administration in laboratory animals. Furthermore, although cue-induced reinstatement of cocaine-seeking increases progressively during abstinence from cocaine self-administration in adult subjects, this "incubation of cocaine craving" remains unexplored after adolescent drug intake in animal models.

Methods: We allowed periadolescent (postnatal day 35; PND35 at start) and adult (PND83-95 at start) male Wistar rats to self-administer cocaine (0.36 mg/kg/infusion) in 2-h daily sessions on a fixed ratio 1 (FR1) schedule of reinforcement over 14 days. Then we compared extinction and cue- or cocaine priming-induced reinstatement (10 mg/kg cocaine, i.p.) of cocaine-seeking in both age groups after 30 days of abstinence in home cages. In separate cohorts, we tested for time-dependent increases in cue-induced reinstatement over approximately 1, 14, 30 or 60 days of abstinence in both age groups.

Results: Adolescent and adult rats self-administered similar amounts of cocaine. Subsequent cue-induced reinstatement was lower in the adolescent-onset group after a 30-day abstinence period, but cocaine priming-induced reinstatement did not differ across ages. Also extinction responding and time-dependent increases in cue-induced reinstatement (incubation) were less pronounced in rats that took cocaine as adolescents, compared with adults.

Conclusions: Surprisingly, these results may reflect resistance among adolescent subjects to some enduring effects of drug self-administration, such as reward learning.

2.2 Introduction

Adolescence among humans is a developmental stage associated with vulnerability to drug use and abuse (Laviola et al., 1999, O'Malley and Johnston, 2007). People aged 18 to 20 years report the highest rates of illicit drug use compared to other age groups, and individuals who initiate drug-taking at age 14 or younger are almost five times more likely to classify themselves as drug dependent in adulthood than those who first tried drugs after age 18 (SAMHSA, 2006). However, whether or not these phenomena can be attributed to a biological vulnerability of adolescent development is unclear, especially given that a manifestation of vulnerability to drugs among humans might be adolescent onset of drug use; if so, few vulnerable individuals would be included in any survey group of adult-onset drug users (Shram et al., 2007). Animal models of adolescent drug intake may help to identify the potential role of biological vulnerability in drug abuse.

In rodents, adolescence can be defined as a transitional period sometime between postnatal days (PND) 28 and 60 (Spear and Brake, 1983, Spear, 2000a, Smith, 2003) and shares several key characteristics with primate adolescence (Laviola et al., 1999, Spear, 2000a, Smith, 2003, Crews et al., 2007). As a gold standard measure of drug abuse liability (Schuster and Thompson, 1969, Meisch, 1982), drug self-administration has been used to study adolescent vulnerability to drug reward and reinforcement. Intravenous (i.v.) drug self-administration by adolescent rodents varies across drugs and subject populations (Levin et al., 2003, Belluzzi et al., 2005, Frantz et al., 2007, Kantak et al., 2007, Levin et al., 2007, Shram et al., 2007, Shahbazi et al., 2008, Doherty et al., 2009), and thus requires further investigation. The first aim of the present study was to replicate and extend our previous results that no age differences exist in cocaine self-administration. We allowed periadolescent (PND35 at start) and adult (PND83-95 at start) male

Wistar rats to acquire lever-pressing maintained by i.v. infusions of cocaine. We also compared outcomes using two different drug dosing methods, changing either volume or concentration of the i.v. drug infusions to account for daily changes in body weight.

Long-lasting vulnerability to relapse to drug-seeking, or drug-taking during periods of abstinence, is one of the major challenges for treatment of drug addiction (Hunt et al., 1971, Sayette et al., 2000, Mezinskis et al., 2001, Chung and Maisto, 2006). Yet relapse or reinstatement of drug-seeking after adolescent drug use remains almost entirely unexplored in animal models. To the best of our knowledge, only one study explicitly analyzed extinction and reinstatement in animals that acquired drug self-administration as adolescents (Shram et al., 2007); nicotine-induced reinstatement of nicotine-seeking did not differ across age groups, but Wistar rats that took nicotine as adolescents extinguished their drug-seeking faster than those that took the drug as adults. The second aim of the present study was to compare patterns of extinction and cue- or drug priming-induced reinstatement of cocaine-seeking after a 30-day abstinence period (Grimm et al., 2001, Shaham et al., 2003) in rats that self-administered cocaine during adolescence (adolescent-onset groups) vs. adulthood (adult-onset groups).

Finally, incubation of drug craving refers to time-dependent increases in cue-induced reinstatement of drug-seeking after abstinence from drug self-administration in rodents (Grimm et al., 2001, Lu et al., 2004c). Although incubation may contribute to the persistent vulnerability to relapse observed in human drug addicts (Chung and Maisto, 2006), it has not been examined after adolescent-onset of drug self-administration in rats. Therefore, the third aim of the present study was to compare time-dependent changes in cue-induced reinstatement after 1, 14, 30 or 60 days of abstinence from cocaine self-administration during adolescence vs. adulthood.

Based on previous data, we predicted that neither age nor titration method would affect cocaine self-administration (Frantz et al., 2007, Crombag et al., 2008). However, based on reports of heightened vulnerability to drug dependence after adolescent onset of drug use among humans, we predicted that rats that acquired cocaine self-administration as periadolescents would show higher levels of reinstatement and more robust time-dependent increases in cue-induced reinstatement of cocaine-seeking (incubation of cocaine craving), compared with rats that acquired self-administration as adults.

2.3 Methods

Subjects

Male Wistar rats (Charles River Laboratories, Inc., Wilmington, MA) arrived in the laboratory at either postnatal day (PND) 22 or 70-82 and were housed in groups of 2-3 in a humidity (50%) and temperature controlled (22 ℃) vivarium on a 12/12 h light/dark cycle (reverse cycle, with lights on at 1900 h). Rats acclimated to these conditions for 6-8 days prior to the start of experiments. Food and water were available *ad libitum* except during self-administration sessions. All subjects were observed and/or weighed daily to assess general health and responsiveness to drug exposure. All procedures were conducted in strict adherence to the "Principles of Laboratory Animal Care" and the *National Institute of Health Guide for the Care and Use of Laboratory Animals* (NRC, 2003).

Drugs

Cocaine hydrogen chloride was obtained from Mallinckrodt Inc. (Hazelwood, MO). For rats receiving variable concentrations to account for differences in body weight, the concentration of cocaine stock solution in sterile saline was 2.5 mg/ml and was diluted for individual subjects. For the variable volume group, the concentration of stock solution was 1.25

mg/ml. (See below for details on dosing methods.) Methohexital sodium was obtained from Eli Lilly (Indianapolis, IN).

Surgery

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). Rats were anesthetized with an isoflurane/oxygen vapor mixture (4-5% for initial anesthetization and 1.5-3% during surgery), and catheter tubing was passed subcutaneously from the animal's back to the right jugular vein, inserted into the vein previously punctured with a 25 gauge needle, and tied gently with suture thread. During recovery, rats received approximately 0.2 ml Timentin (Ticarcillin Disodium and Clavulanate Potassium; 100 mg/ml, i.v.) twice daily on the first two days post-surgery, then once daily throughout the experiment. Catheters were also flushed daily with approximately 0.4 ml heparinized saline (100 USP units/1 ml). Catheter patency was confirmed in all subjects by full loss of muscle tone within 5 s of i.v. infusion of the short acting anesthetic agent, 1% Methohexital sodium, one day before the first and after the last self-administration session. Subjects that failed either patency test were eliminated from the study.

Equipment

Behavioral tests were conducted in operant conditioning chambers enclosed in sound-attenuating, ventilated environmental cubicles (Med Associates, Inc., St. Albans, VT). To start each session, a house light and white noise turned on and two levers extended into the chamber. Lever presses on the inactive lever were recorded but had no scheduled consequences. Presses on the active lever triggered a syringe pump (Med Associates, Inc., St. Albans, VT) to deliver drug solution via a stainless steel swivel (Instech Laboratories, Inc., Plymouth Meeting, PA) and

polyethylene tubing attached to a catheter portal on each animal's back. Each reinforced response lit a cue light above the lever which stayed on for the duration of the infusion. The cue light, house light, and white noise were not present during a 20 s time-out (TO20) after each infusion. Drug delivery and data collection were controlled by Med Associates software (Med PC IV).

Self-Administration

Following a 6-7-day post-surgical recovery, spontaneous acquisition of cocaine self-administration began (PND35 or 83-95), with daily 2 h sessions over 14 days conducted during the dark phase of the light-dark cycle. Non-contingent drug injections were not administered in this phase of experimentation. Lever-pressing on the active lever was reinforced by i.v. infusion of cocaine (0.36 mg/kg/infusion) under a Fixed-Ratio 1 (FR1) TO20 schedule of reinforcement.

Infusion Conditions

Two different methods to account for differences in body weight across age groups were compared, in order to verify that a switch from a previous method of adjusting concentration (Frantz et al. 2007) to a preferred current method of adjusting infusion volume would not alter behavioral outcomes. Thus, we tested the influence of varying concentration vs. varying volume of drug infusions on self-administration. Adolescent and adult subjects were assigned to either variable concentration or variable volume conditions. For variable concentration groups, the concentration of cocaine solution for each rat was titrated according to body weight, but the volume was fixed such that all subjects received 0.1 ml cocaine solution over 4 s per infusion (approximately 0.12 ml/kg/s for adolescent, and 0.06 ml/kg/s for adults). For the variable volume groups, the volume of cocaine solution for each rat was titrated according to body weight (0.07 ml/kg/s), but the concentration was fixed such that all subjects received same concentration of

cocaine solution. The volume and duration were based on a standard 0.1 ml/4 s per infusion for a 350 g rat.

Abstinence Period

After 14 days of self-administration, separate groups of rats remained in their home cages for 20-24 h, 14-15, 30-31 or 60-61 days, under normal housing conditions. Rats were handled twice per week during this abstinence period. Cue-induced reinstatement was tested at each abstinence period, but drug-induced reinstatement was tested only after 30-31 days of abstinence, in different experimental groups.

Extinction and Reinstatement

After the abstinence from cocaine self-administration, a within-session extinction and reinstatement test was conducted (Grimm et al., 2001, Grimm et al., 2003). Six 1-h extinction sessions were followed by a 1-h cue- or drug-induced reinstatement test. During extinction, rats were connected to the metal coil tether but not the infusion tubing, white noise remained off, and the house light remained on. Neither cue-lights nor TO signals were presented after presses on either lever. Five-min breaks occurred between each successive session, during which the two levers retracted and the house light turned off.

For cue-induced reinstatement tested after various abstinence periods, rats in the adolescent-onset groups were PND49-50, 63-64, 79-80, or 109-110, whereas adult-onset groups were PND97-110, 111-124, 127-140 or 157-171. Cue-induced reinstatement tests began with the onset of the house light, white noise, and a 5 s cue-light, followed by a 20 s TO during which the house light, white noise, and cue light were turned off. During the remainder of cue-induced reinstatement sessions, presses on the active lever produced cue sequences identical to those presented during cocaine self-administration, and the pump went on, although no syringe was

loaded. The only difference between self-administration and cue-induced reinstatement sessions was that drug solution was not infused during reinstatement.

For drug priming-induced reinstatement tested 30-31-days after drug self-administration, rats in the adolescent-onset group were PND79-80, whereas the adult-onset group was PND127-140. Rats were taken out of the chambers after the last extinction session, injected with 10 mg/kg cocaine (i.p.), and placed back into the chamber immediately. This dose was chosen for two reasons. First, 10 mg/kg cocaine (i.p.) is commonly used dose for cocaine priming-induced reinstatement (Schenk and Partridge, 1999, Soria et al., 2008). Second, we tested 10, followed by either 3 or 30 mg/kg cocaine priming-induced reinstatement on successive days in the same animals, and only 10 mg/kg cocaine (i.p.) induced reliable reinstatement. While 3 mg/kg did not induce reinstatement in either age group, 30 mg/kg caused stereotyped behaviors in subjects from each age group. (These preliminary data are confounded by the previous day's test and are not shown.) Parameters for drug priming-induced reinstatement sessions were identical to extinction sessions. No control injections of saline were administered before the drug priming-induced reinstatement test.

Data Analysis

For drug self-administration sessions, the number of drug infusions per session was analyzed using a three-way mixed measures analysis of variance (ANOVA) with age and infusion condition (variable concentration vs. variable volume) as between-subjects factors, and day as a within-subjects factor. Total drug intake (mg/kg) summed over the entire 14 days of cocaine self-administration was also compared using a two-way ANOVA, with age and infusion condition as between-subjects factors. The number of lever presses per session was also analyzed using a three-way ANOVA with age as a between-subjects factor, and day and lever (active vs.

inactive) as within-subjects factors. (Data were collapsed across infusion condition for the active vs. inactive levers analysis, because no differences were observed on other measures; see results.)

For extinction sessions, the number of active or inactive lever presses per session was analyzed using a two-way mixed measures ANOVA, with age as a between subjects factor, and session as a within subjects factor. To test for cue- or drug-induced reinstatement of lever-pressing, the number of active lever presses in the last extinction session was compared directly with active lever presses during the reinstatement session, using a two-way age x session ANOVA, with age as a between subjects factor and session (extinction vs. reinstatement) as a within subjects factor. To test our hypothesis regarding age differences in reinstatement directly, the number of active lever presses was also compared across age groups using unpaired t-tests.

For analysis of time-dependent changes in cocaine-seeking, total extinction responses on the active lever summed over all six 1-h extinction sessions were analyzed using a two-way between subjects age x abstinence period ANOVA. Time-dependent changes in the number of active lever presses per reinstatement session were also analyzed using a two-way age x abstinence period ANOVA. Per the analytical methods of Grimm et al. (2001, 2003), one-way ANOVAs were also conducted on each age group separately to test specifically for time-dependent increases in reinstatement. Unpaired 2-sided t-tests with Bonferroni's correction were used for post-hoc comparisons, as appropriate. Results were considered significant if $p<0.05$.

2.4 Results

Influence of age and infusion condition on cocaine self-administration

All age and titration groups showed similar patterns of reliable cocaine self-administration, which increased gradually in the first week and stabilized during the second week of testing (n=7-9/group). A three-way age x infusion condition x days ANOVA revealed that

only the main effect of days was significant [$F_{(13, 364)}$=11.12, p<0.001], but not other main effects nor interactions. Total drug intake (mg/kg) over 14-days of self-administration did not differ across age or infusion condition either (adolescent variable concentration group: 612±50 mg/kg, adult variable concentration group: 704±67 mg/kg, adolescent variable volume group: 666±72 mg/kg, and adult variable volume group: 613±33 mg/kg). Additional groups were added for subsequent extinction and reinstatement tests (n=9-16/group), and all showed similar initial patterns of cocaine self-administration. Thus, we collapsed data across infusion conditions and subsequent abstinence period groups to show similar lever presses and total intake in all adolescent vs. adult rats (Figure. 2.1a). Moreover, despite relatively high rates of non-reinforced responding on the active lever, rats clearly discriminated between active and inactive levers (Figure. 2.1b) suggesting reliable self-administration. However, no age difference was observed in either total active lever presses or inactive lever presses. A three-way age x days x lever ANOVA revealed the main effects of day [$F_{(13, 1638)}$=3.24, p<0.001], lever [$F_{(1, 126)}$=271.07, p<0.001] and day x lever interaction [$F_{(13, 1638)}$=4.22 , p<0.001], but no other main effects nor interactions.

Extinction and reinstatement after a 30-day abstinence period

No age difference was observed in cocaine self-administration (Figure. 2.1a) or extinction sessions (see below). However, after a 30-day abstinence period, rats in the adolescent-onset group showed lower levels of cue-induced reinstatement than the adult-onset group (Figure. 2.2, n=11 or 12/group), in contrast to similar levels of drug-induced reinstatement (Figure. 2.3, n=6 or 10/group).

During extinction tests before cue-induced reinstatement, both age groups exhibited more active lever pressing than inactive lever pressing in the first 1-h session (Figure. 2.2). Lever-

pressing gradually declined in both age groups by the sixth 1-h extinction session. A two-way age x session ANOVA on extinction responding revealed a significant main effect of session [$F_{(5, 105)}$=21.704, p<0.001], but neither the main effect of age, nor the age x session interaction was significant. During the cue-induced reinstatement test, re-exposure to drug-associated cues triggered lever-pressing behavior in both age groups. A two-way age x session ANOVA on active lever presses in the last extinction session vs. the reinstatement session revealed main effects of session [$F_{(1, 21)}$=23.257, p<0.001] and age [$F_{(1, 21)}$=50.840, p=0.03], but no interaction. Moreover, the adolescent-onset group exhibited less lever pressing than adults during the single cue-induced reinstatement session, confirmed by a targeted t-test [$t_{(21)}$=2.139, p=0.044]. In contrast, inactive lever presses did not differ between the last extinction session and reinstatement.

During extinction tests before drug-induced instatement, subjects exhibited similar rates of lever-pressing and extinction (Figure. 2.3). Subsequent i.p. injections of cocaine triggered similar levels of reinstatement in both age groups. A two-way age x session ANOVA comparing active lever presses in the last extinction session with the reinstatement session revealed only a main effect of session [$F_{(1, 14)}$=6.164, p=0.026], but no main effect of age, nor a significant age x session interaction. Inactive lever presses did not differ across age or session.

Time-dependent increases in cue-induced reinstatement

During extinction tests at approximately 1-, 14-, 30- or 60-day abstinence, adolescent-onset groups showed an overall lower level of responding compared with adult-onset groups (Figure. 2.4; n=9-16/group). A two-way ANOVA on the total number of active lever-presses summed over all 6 1-h extinction sessions revealed a main effect of age [$F_{(1,86)}$=10.30, p=0.002] and abstinence period [$F_{(3, 86)}$=5.77, p=0.001] but no age x abstinence period interaction.

With regard to cue-induced reinstatement after a 1-day abstinence, both age groups showed similarly low levels of cue-induced lever-pressing (Figure. 2.5). Subsequently, the adult-onset groups demonstrated more robust increases in drug-seeking from 1 to 60 days of abstinence, compared with the adolescent-onset groups. A two-way ANOVA on active lever-presses showed main effects of age [$F_{(1, 86)}$=17.29, p<0.001], abstinence period [$F_{(3, 86)}$=13.82, p<0.001], and an age x abstinence period interaction [$F_{(3, 86)}$=2.87, p=0.041]. Post-hoc t-tests showed that adolescent-onset groups responded at lower levels than adults after both 30 [$F_{(1, 21)}$=4.57, p=0.044] and 60 [$F_{(1, 26)}$=15.61 , p=0.001] days of abstinence. Separate one-way ANOVAs on each age group revealed a significant effect of abstinence period among adults [$F_{(3, 40)}$=10.802, p<0.001] and adolescent-onset groups [$F_{(3, 46)}$=3.195, p=0.032]. Post-hoc tests on the adult-onset groups confirmed that reinstatement was higher at 60 days than at 1 or 14 days of abstinence (1 vs. 60: p<0.001, 14 vs. 60: p=0.008). However, post-hoc tests on the adolescent-onset groups did not reveal specific differences, suggesting a weak overall effect.

2.5 Discussion

Results from the present experiment are consistent with previous studies from our group and others demonstrating that periadolescent and adult male rats acquire cocaine self-administration similarly (Frantz et al., 2007, Kantak et al., 2007). On the other hand, striking age differences emerged in the test of time-dependent increases in cocaine-seeking during drug abstinence, such that no age differences were observed in reinstatement after a 1-day abstinence, but cue-induced reinstatement remained low in adolescent-onset rats while increasing significantly in adult-onset rats through a 60-day abstinence period. Drug priming-induced reinstatement after a 30-day abstinence period did not differ across ages, but only one cocaine dose was tested.

During 14 daily 2-h self-administration sessions, adolescent and adult rats took similar amounts of cocaine. These results confirm several reports and our own preliminary data that no age differences exist in cocaine self-administration behavior (Frantz and Parsons, 2000, Belluzzi et al., 2005, Frantz et al., 2007, Kantak et al., 2007, Kerstetter and Kantak, 2007). Moreover, small variations in the infusion parameters (variable volume vs. variable concentration methods) had no effect on cocaine self-administration, as shown previously in adult rats (Crombag et al., 2008), despite the fact that rapid infusion enhances the reinforcing effects of drugs in a self-administration model with non-human primates (Kato et al., 1987, Panlilio et al., 1998). Overall, we conclude that cocaine has similar acute reinforcing effects in adolescent and adult male rats.

Despite similar rates of cocaine self-administration across age groups, extinction responding was lower in adolescent-onset rats than adult-onset rats during extinction tests after 1-, 14-, 30-, and 60-day abstinence periods. These age differences fail to support our hypothesis of adolescent vulnerability, but do corroborate results from Wistar rats in another test of extinction and reinstatement after adolescent drug self-administration (Shram et al., 2007). Given that lower rates of extinction responding have been interpreted as lower motivation to obtain drugs or lower conditioned incentive motivational effects of a drug-associated environment (Bossert et al., 2004, Fuchs et al., 2005, Fuchs et al., 2008), these results lead to the unexpected conclusion that adolescent-onset of drug self-administration does not heighten vulnerability to long-term motivation toward drug intake.

A major emphasis of the present study was comparing incubation of drug craving after cocaine self-administration during adolescence vs. adulthood, measured as time-dependent increases in cue-induced reinstatement of drug-seeking after abstinence from drug self-administration. Similar to extinction responding, the present results are surprising in light of

evidence supporting the hypothesis that adolescent-onset of drug intake heightens vulnerability to drug addiction (Laviola et al., 1999, SAMHSA, 2006). Whereas both age groups showed similarly low levels of cue-induced reinstatement of cocaine-seeking one day after cocaine self-administration, adolescent rats showed only weak increases in cue-induced reinstatement of drug-seeking from 1- to 60-days in abstinence, while adults demonstrated significant increases over the same time period. In other words, adolescent subjects failed to demonstrate the robust incubation of cocaine craving that has been observed repeatedly in adult subjects (Grimm et al., 2001, Lu et al., 2004b, Lu et al., 2004c).

The present results could reflect resistance among adolescent subjects to some enduring effects of drug self-administration. As such, they are consistent with other recent reports. For example, stimulus-reward learning remains intact after cocaine self-administration during adolescence, but is impaired after cocaine self-administration in adulthood (Kerstetter and Kantak, 2007). Also both somatic and affective signs of abstinence from nicotine appear less intense in adolescent compared with adult rats (O'Dell et al., 2004, O'Dell et al., 2007, Shram et al., 2008). Finally, after morphine self-administration by adolescent or adult rats, cue-induced reinstatement is attenuated in the younger cohort (Doherty et al., 2009). On the other hand, adolescent resistance to long-term drug effects clearly contradicts prior reports and hypotheses on adolescent vulnerability to addiction (Spear, 2000b, Laviola et al., 2003, Smith, 2003). Particularly with regard to cocaine, adolescent vulnerability has included specific neurocognitive deficits such as impaired spatial memory, altered inhibitory avoidance, and attentional challenges, as well as premature death (Santucci et al., 2004, Black et al., 2006, Santucci, 2008).

An alternative explanation for the present age differences in reinstatement is that subjects taking cocaine during adolescence experience a generalized reward devaluation later in

adulthood, as observed after juvenile/pre-adolescent methylphenidate exposure (Andersen et al., 2002a, Bolanos et al., 2003, Mague et al., 2005). However, not only is stimulus-reward learning intact after adolescent cocaine self-administration (Kerstetter and Kantak, 2007), but also cocaine-induced devaluation of natural rewards is less affected by cocaine treatment in adolescence than adulthood (Schramm-Sapyta et al., 2006). Additionally, sucrose-stimulated dopamine efflux in the nucleus accumbens remains intact after cocaine treatment in adolescence but not adulthood (Catlow and Kirstein, 2007). Thus, evidence available at this time does not support a general diminution in reward-related processing after adolescent cocaine self-administration.

Drug-induced reinstatement after a 30-day abstinence period did not differ across age groups, consistent with the other test of drug-induced reinstatement after adolescent drug intake (Shram et al., 2007). Possibly drug injection (but not cue presentation) is such a strong trigger of drug-seeking that it fails to reveal differential sensitivity across development, as postulated previously with regard to the reinforcing effects of cocaine (Frantz et al., 2007). A potential limitation of the present study, however, is that only one, highly effective, cocaine dose was tested. A full dose-effect analysis will be required to explore drug priming-induced reinstatement thoroughly and reach justifiable conclusions on the topic.

If confirmed in future studies, age differences in cue-induced but not drug-induced reinstatement might suggest a developmental dissociation between neural pathways mediating cue- vs. drug-induced reinstatement. For example, the amygdala has been identified as a brain region critical for both cue-induced reinstatement and its time-dependent increase during drug abstinence, but less involved in drug-induced reinstatement of drug-seeking (Quirk and Gehlert, 2003, Lu et al., 2005, See, 2005, for review, Lu et al., 2007). Moreover, glutamatergic

projections from the prefrontal cortex to the nucleus accumbens are involved in reinstatement to drug-seeking (Kalivas et al., 2005, Conrad et al., 2008b, Kalivas and O'Brien, 2008, Koya et al., 2008), as are local changes in the nucleus accumbens (Grimm et al., 2003, Hollander and Carelli, 2005, Hollander and Carelli, 2007, Kalivas and O'Brien, 2008). Thus, periadolescent anatomical and functional remodeling in such areas draws attention for future research (Alexander and Goldman, 1978, Teicher et al., 1991, Teicher et al., 1998, Tseng and O'Donnell, 2007, Brenhouse et al., 2008).

In summary, our data suggest that adolescence might be a developmental stage associated with resistance to the long-term motivation to seek cocaine or to the salience of drug-associated cues. These results counter predictions based on human survey data and rodent models other than self-administration showing that early onset of drug intake increases chances of later addiction. Thus, our results may support the idea that social factors and other variables not modeled in most rodent drug self-administration studies influence drug use and abuse by human adolescents. As a note of caution, the validity of extinction, reinstatement, and incubation of drug-seeking behavior in rodents as models of human drug-related behavior has been questioned (Katz and Higgins, 2003). Overall, if future studies from multiple lines of research converge on the conclusion that adolescence is a period of relative biological resistance to some long-term drug effects, then intervention programs targeting adolescent drug abusers may have high success rates.

2.6 Acknowledgements

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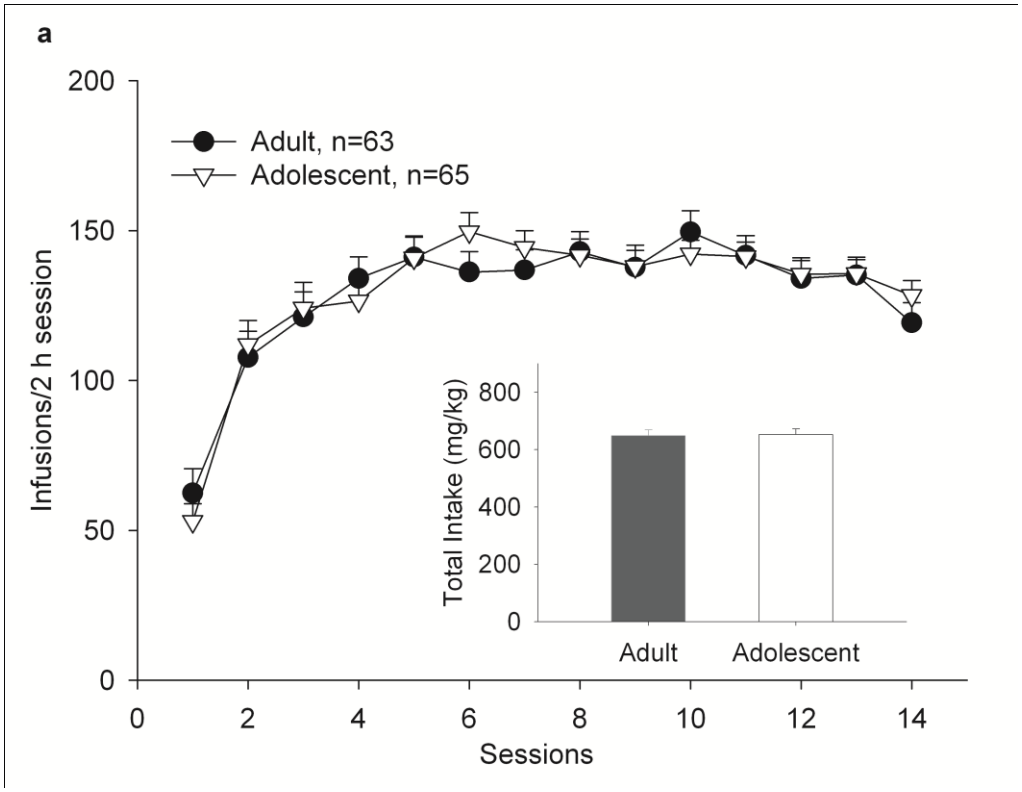
2.7 Figures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Adolescent, n=65
Infusions/2 h session
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Total Intake (mg/kg)
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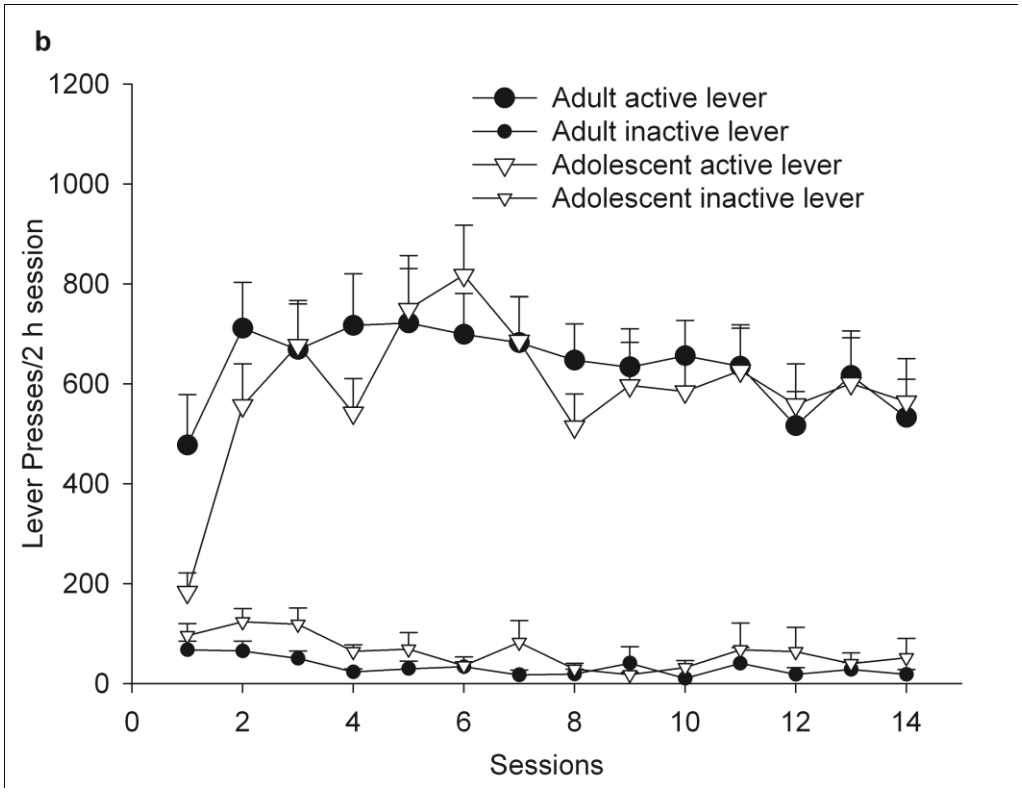
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Adult active lever
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Figure. 2.1. Fourteen consecutive daily 2 h cocaine self-administration sessions in adolescent vs. adult male rats. (a) Number of infusions during 14 daily self-administration sessions. Points represent mean ± SEM (n=63 or 65/group). Inset shows total cocaine intake summed across the entire 14 days of self-administration. Bars represent mean ± SEM. (b) Number of active vs. inactive lever presses during self-administration. Points represent mean ± SEM (n=63 or 65 /group).

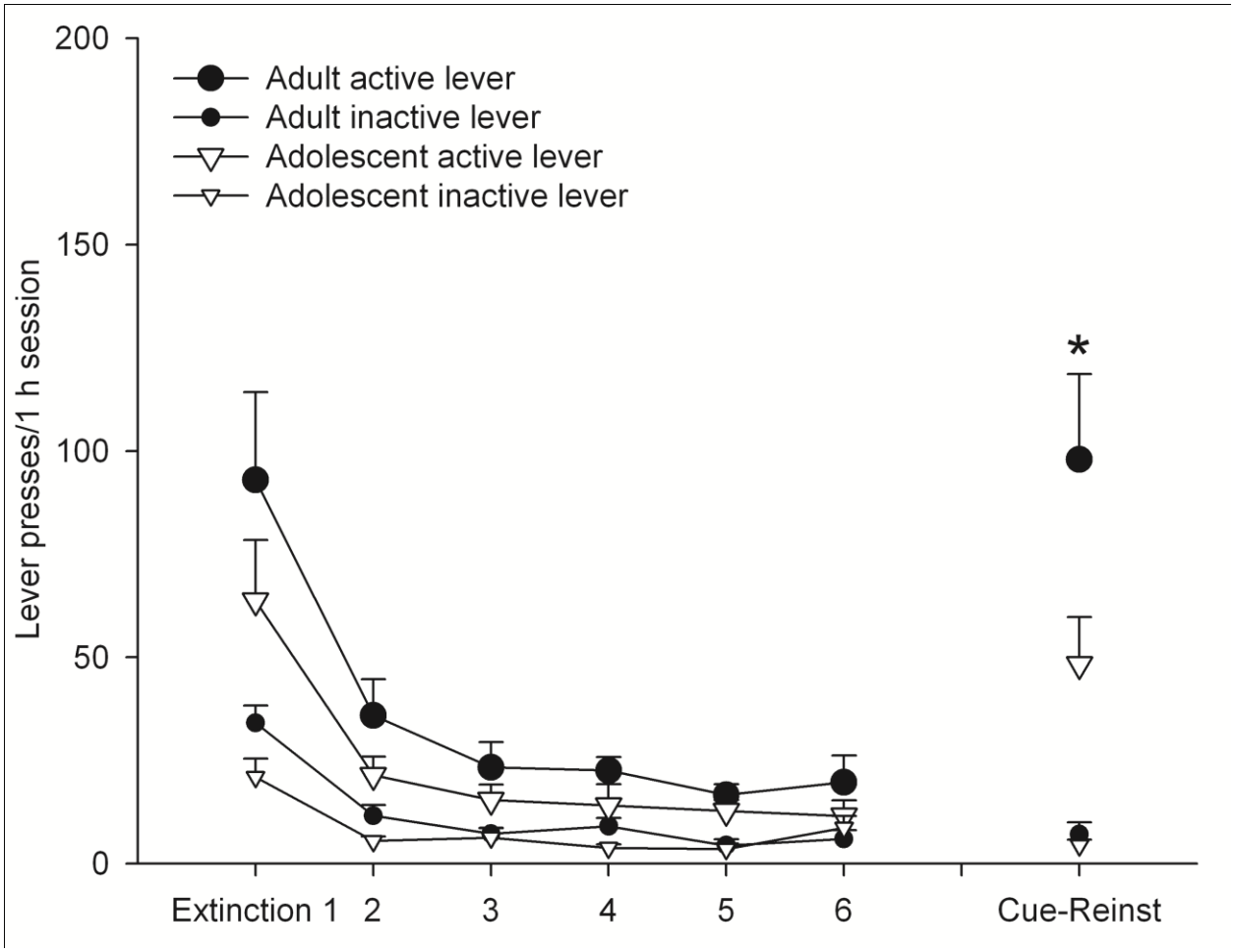


Figure. 2.2. Lever pressing in extinction and cue-induced reinstatement after 30 days of abstinence in adolescent- vs. adult-onset age groups. Points represent mean ± SEM lever presses on the drug-associated (active) lever or the inactive lever (n=11 or 12/group). Adolescents showed a lower level of cue-induced reinstatement than adults (* indicates $p<0.05$ on targeted t-test).

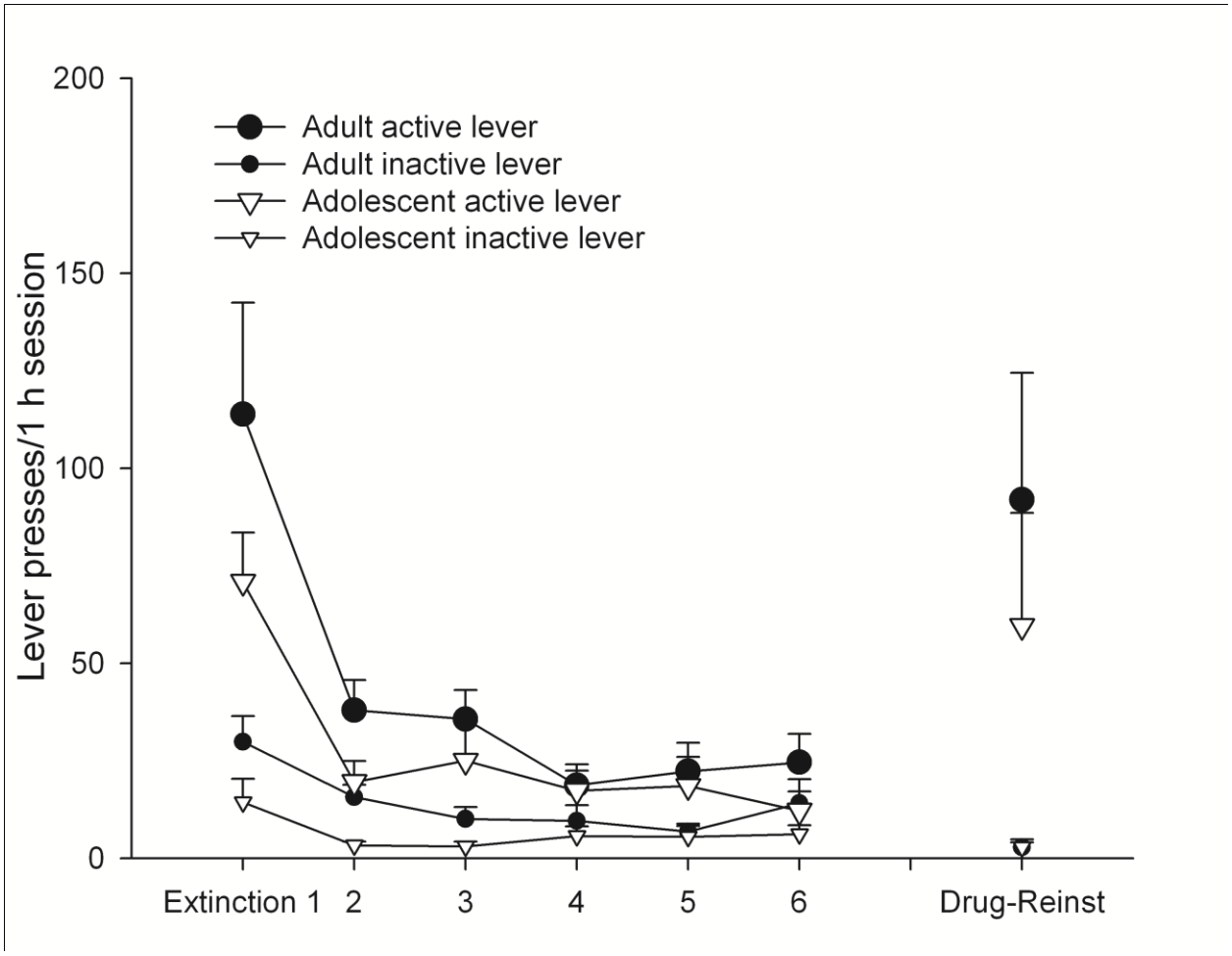


Figure. 2.3. Lever pressing in extinction and drug-induced reinstatement after 30 days of abstinence in adolescent- vs. adult-onset age groups. Points represent mean ± SEM (n= 6 or 10/group). No age differences were observed.

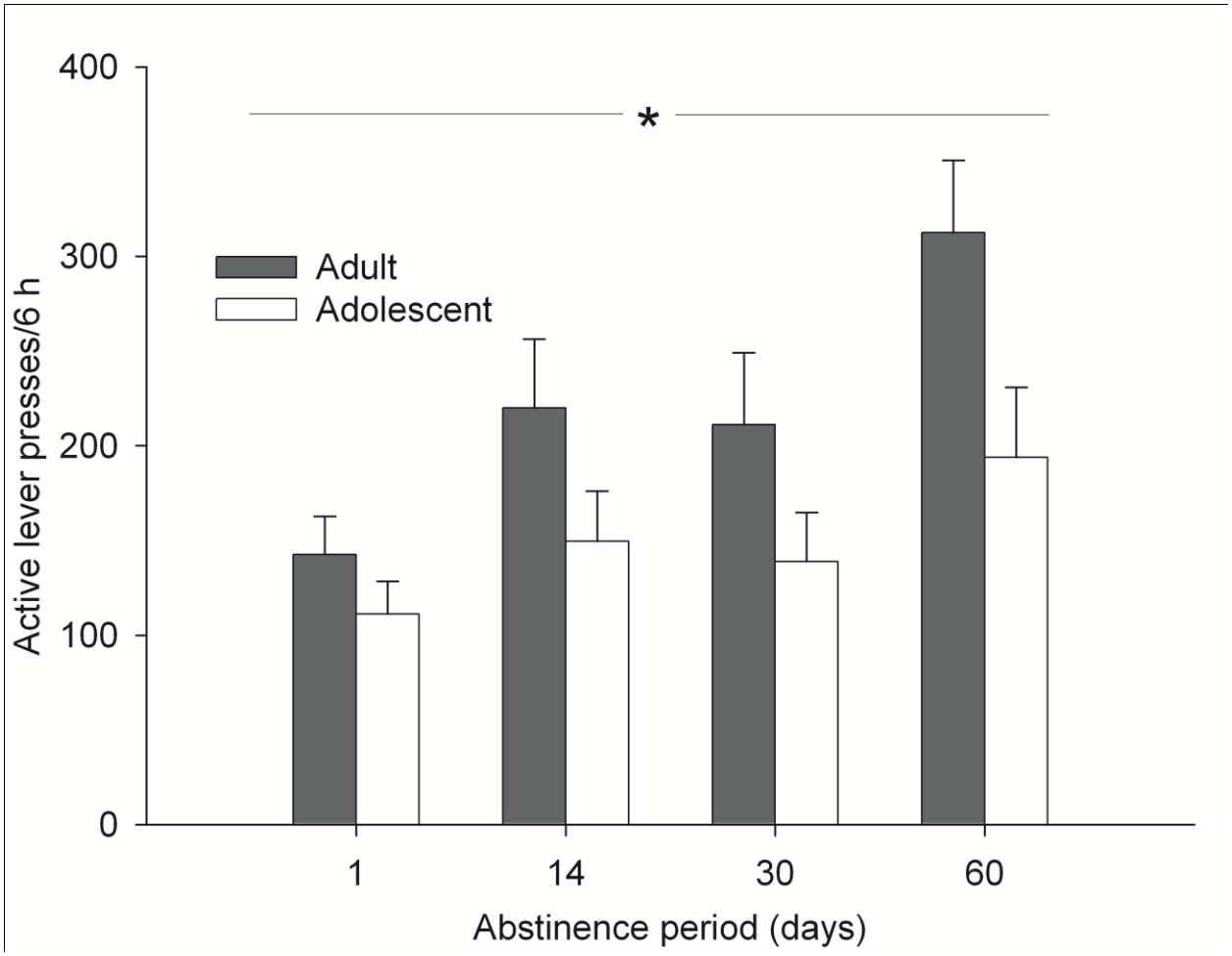


Figure. 2.4. Total extinction responses summed over 6 1-h sessions conducted after 1, 14, 30, or 60 days of abstinence from cocaine. Bars represent mean ± SEM (n=9-16/group). Adolescent-onset groups showed lower levels of extinction responding compared with adults (* indicates overall main effect of age, $p<0.05$).

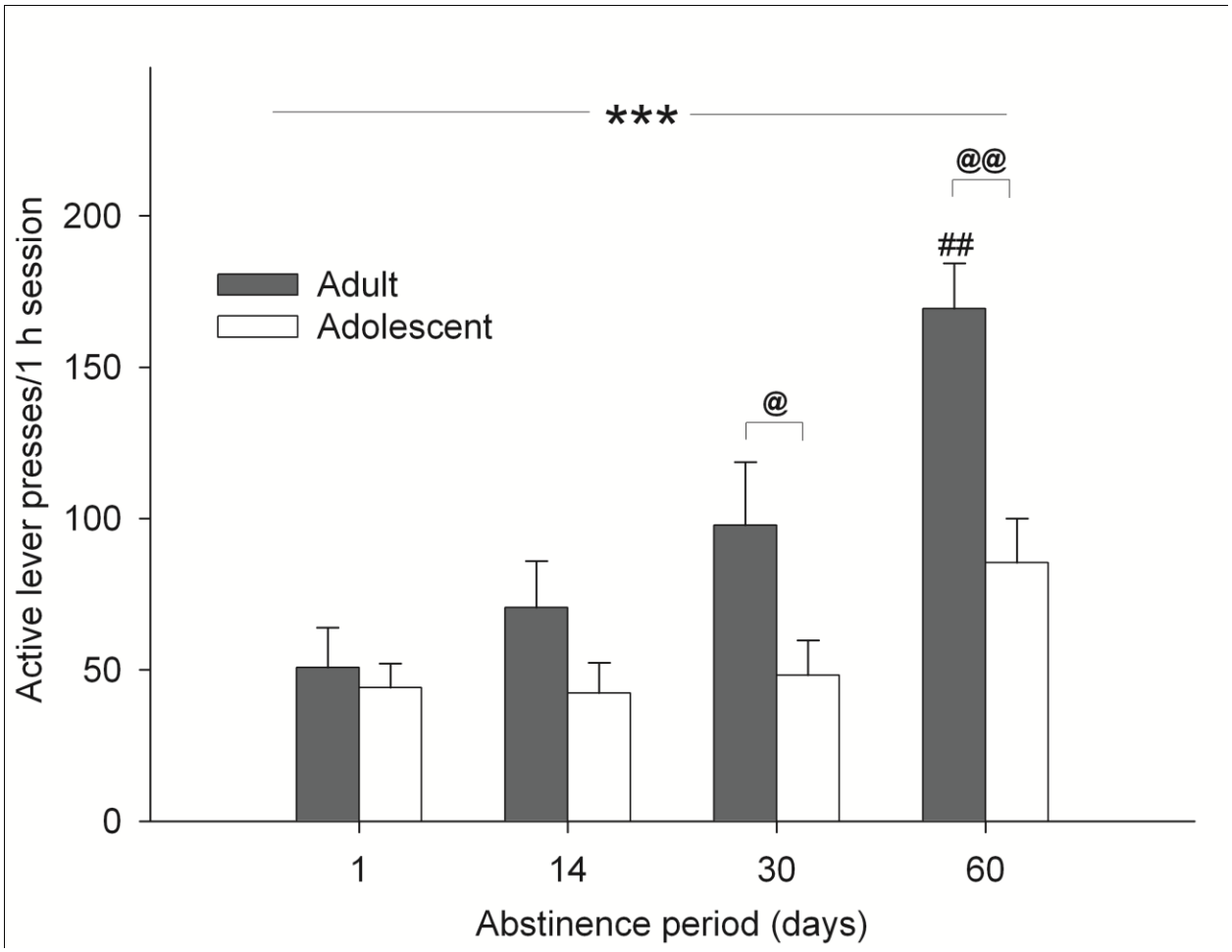


Figure 2.5. Time-dependent increases in cue-induced reinstatement after adult- but not periadolescent-onset of cocaine self-administration. Bars represent mean ± SEM (n=9-16/group). Age groups showed similar levels of cue-induced reinstatement at 1-day abstinence, but adolescent-onset groups showed overall lower levels of reinstatement (*** indicates an overall main effect of age, $p<0.001$). Only adult groups showed time-dependent increases in reinstatement ($^{\#\#}$ higher cue-induced reinstatement after 60-day compared with 1- or 14-day abstinence). Adolescents showed less cue-induced reinstatement than adults after 30- and 60-day abstinence periods ($^{@}$ $p<0.05$, $^{@@}$ $p<0.01$ on targeted t-tests).

3. CHAPTER 3: TIME-DEPENDENT INCREASES IN CUE-INDUCED REINSTATEMENT OF SUCROSE SEEKING AFTER SUCROSE SELF-ADMINISTRATION IN ADOLESCENCE

by

Chen Li & Kyle J. Frantz

Behav Brain Res 213: 109-12

3.1 Abstract

Previously we reported that time-dependent increases in cue-induced reinstatement of cocaine seeking were attenuated in rats self-administering cocaine as adolescents, compared with adults (Li and Frantz, 2009). Now using sucrose self-administration, we report time-dependent increases in cue-induced reinstatement of sucrose seeking that are similar across age groups, suggesting that age differences in reinstatement of cocaine seeking depend on specific effects of cocaine, not a compromised ability among younger rats to associate cues with rewards.

3.2 Introduction

The developmental stage of adolescence in humans is suggested to be a vulnerable period for development of drug abuse and addiction (Laviola et al., 1999, O'Malley and Johnston, 2007). Among the most challenging aspects of the addiction cycle is relapse to drug-seeking and drug-taking after abstinence. In humans and experimental animal models alike, one of the factors that can trigger relapse is drug-associated environmental cues, which acquire reinforcing properties when repeatedly paired with availability of drugs (See, 2005). If adolescence is a period of heightened vulnerability to addiction, then higher rates of cue-induced reinstatement of drug-seeking should be observed in animals self-administering drugs during adolescence compared with adults. Our previous study testing age differences in intravenous (i.v.) cocaine self-administration and cue-induced reinstatement in male rats, however, showed that after self-administration of similar amounts of cocaine, time-dependent increases in cue-induced reinstatement of cocaine seeking (known as incubation; (Grimm et al., 2001)) were surprisingly attenuated in rats that took cocaine as adolescents (adolescent-onset groups), compared with adults (adult-onset) (Li and Frantz, 2009). Whether these age differences were drug-specific or

due to a compromised ability among younger rats to associate rewards with related cues was not tested.

Cue-induced reinstatement of sucrose seeking has been used as a control procedure for studying cue-induced reinstatement of drug-seeking (Bossert et al., 2005). Moreover, sucrose self-administration and reinstatement of sucrose seeking are models that may provide insight to food-related reward, reinforcement, and addiction in humans (Lenoir et al., 2007, Diergaarde et al., 2009, Gosnell et al., 2010). Both drug-seeking and sucrose seeking can be reinitiated after abstinence and/or extinction by exposure to cues associated with the presentation of rewards, and cue-induced reinstatement of both sucrose seeking and cocaine seeking increase over abstinence periods, i.e. they show incubation (Grimm et al., 2003, Lu et al., 2004c, Grimm et al., 2005). Time-dependent increases in cue-induced reinstatement of sucrose seeking, however, are not as robust and do not last as long as increases in cocaine seeking. For example, cue-induced reinstatement of cocaine seeking peaks at approximately 90 days of abstinence, while sucrose seeking peaks at approximately 30 days (Lu et al., 2004c). Such differences between drug and natural reinforcers in behavioral phenomena are likely to reflect differences in underlying neural mechanisms. For example, antagonists of metabotropic glutamate receptor 5s (mGluR5s) attenuate cue-induced reinstatement of cocaine seeking but not sucrose seeking (Kumaresan et al., 2009). Also, brain-derived neurotrophic factor (BDNF) protein levels within the mesolimbic dopamine system progressively increase after cocaine, but not sucrose, self-administration (Grimm et al., 2003).

Considering that sucrose can be used as a control for the specific effects of cocaine in studies of behavioral reinforcement, the present experiment tested time-dependent increases in cue-induced reinstatement of sucrose seeking in adolescent vs. adult rats, mainly for comparison with

prior age differences in cue-induced reinstatement of cocaine seeking (Li and Frantz, 2009). We hypothesized that age differences in cue-induced reinstatement of cocaine seeking are due to specific effects of cocaine, not a compromised ability among younger rats to associate cues with rewards, and therefore that both age groups would demonstrate similar time-dependent increases in cue-induced reinstatement of sucrose seeking.

3.3 Methods

Male Wistar rats (Charles River Laboratories, Inc., Wilmington, MA) arrived in the laboratory at postnatal day (PND) 22 (adolescent-onset) or 70-82 (adult-onset) and were housed in groups of 2-3 in a humidity (~50%) and temperature controlled (22 ℃) vivarium on a 12/12 h light/dark cycle (reverse cycle, with lights on at 1900 h). Rats acclimated to these conditions for 13 days prior to the start of experiments. Food and water were available ad libitum except during self-administration sessions. All subjects were observed and/or weighed daily to assess general health. All procedures were conducted in strict adherence to the "Principles of Laboratory Animal Care" and the National Institute of Health Guide for the Care and Use of Laboratory Animals (NRC, 2003).

Behavioral tests were conducted in operant conditioning chambers enclosed in sound-attenuating, ventilated environmental cubicles (Med Associates, Inc., St. Albans, VT). To start each session, a house light and white noise turned on and two levers extended into the chamber. Presses on the inactive lever were recorded but had no scheduled consequences. Presses on the active lever triggered a pellet dispenser to deliver a sucrose pellet (Bio-Serv, Frenchtown, NJ) into a receptacle for oral consumption (Med Associates, Inc., St. Albans, VT). Each reinforced response lit a cue light above the lever which stayed on for 2 s. The cue light, house light, and white noise were not present during a 20 s time-out (TO20) after each pellet delivery. Sucrose

delivery and data collection were controlled by Med Associates software (Med PC IV). Sucrose pellets left unconsumed were counted at the end of each session.

Spontaneous acquisition of sucrose self-administration began at PND35 or 83-95, with 14 daily sessions during the dark phase of the circadian cycle. Sessions lasted 30-min or until rats earned a maximum of 82 pellets. Three non-contingent sucrose pellets were provided at the beginning of each session to rats that earned fewer than approximately 10 pellets per session during the first week of testing. Lever-pressing on the active lever was reinforced by a sucrose pellet presented on a Fixed-Ratio 1 (FR1) TO20 schedule of reinforcement.

After 14 days of self-administration, separate groups of rats remained in their home cages for 20-24 h, 14, 30 or 60 days, under normal housing conditions. Rats were handled and weighed twice per week during the abstinence period.

After abstinence, a within-session extinction and reinstatement test was conducted (Grimm et al., 2001, Grimm et al., 2003). Rats in the adolescent-onset groups were PND49, 63, 79, or 109, whereas adult-onset groups were PND97-110, 111-124, 127-140 or 157-171. Six 30-min extinction sessions were followed by a 30-min cue-induced reinstatement test. During extinction, white noise remained off, and the house light remained on. Neither cue-lights nor TO signals were presented after presses on either lever. Five-min breaks occurred between each successive session, during which the two levers retracted and the house light turned off. Cue-induced reinstatement tests were initiated by the house light, white noise, and a 5 s cue-light, followed by a 20 s TO during which the house light, white noise, and cue light were turned off. During the remainder of the cue-induced reinstatement sessions, presses on the active lever produced cue sequences identical to those presented during sucrose self-administration, but no

sucrose pellets were delivered. A table of average body mass and age at each experimental time point is provided (Table 3.1).

For analysis of behavior during sucrose self-administration sessions, the number of sucrose pellets acquired or consumed per session was analyzed using a two-way mixed measures analysis of variance (ANOVA) with age as a between-subjects factor, and day as a within-subjects factor. Total number of pellets earned or consumed over the entire 14 days of sucrose self-administration was also compared using an unpaired t-test. For 5 adults and 2 adolescents that reached the maximum number of pellets allowed per session (82 pellets), data were included in all analyses. A correlation between body mass and the number of sucrose pellets consumed on the last self-administration session was examined.

For extinction sessions, the number of active or inactive lever presses per session was analyzed using a two-way mixed measures ANOVA, with age as a between subjects factor, and session as a within subjects factor.

For analysis of time-dependent changes in reinstatement of sucrose-seeking, the number of active lever presses per reinstatement session was analyzed using a two-way between-subjects age x abstinence period ANOVA. In order to rule out the possibility that total number of pellets consumed influenced age differences in cue-induced reinstatement of sucrose seeking, total intake was tested as a covariate in the age x abstinence period ANOVA. Follow-up one-way ANOVAs and Tukey tests were used for post-hoc comparisons with equal sample sizes, as appropriate. All results were considered significant if $p<0.05$.

3.4 Results

As shown in Figure. 3.1, both age groups gradually increased sucrose self-administration over 14 days. However, adolescents consumed fewer sucrose pellets than adults. Thus, main

effects of age ($F_{(1, 62)}$=10.17, p=0.002) and session ($F_{(13, 806)}$=210.42, p<0.001) were significant, as was the interaction of age x session ($F_{(13, 806)}$=3.23, p<0.001). The unpaired t-test showed that total number of pellets consumed over 14-days of self-administration was also lower in adolescents than adults ($t_{(62)}$=3.19, p=0.02). Age differences in number of pellets earned were similar to number of pellets consumed, with adolescents earning fewer sucrose pellets than adults (data not shown). Pearson's test for correlation showed that the number of sucrose pellets consumed positively correlated with the body mass of the animals (r^2=0.355, p=0.004), suggesting that the lower consumption in adolescents is due to their lower body mass.

No age differences were observed in extinction (data not shown) or cue-induced reinstatement of sucrose seeking after any of the tested abstinence periods (Figure. 3.2). The main effect of age was not significant ($F_{(1,56)}$=0.25, p=0.618). Sucrose seeking increased over abstinence periods, a trend supported by the main effect of abstinence period in the two-way ANOVA ($F_{(3, 56)}$=2.72, p=0.053). Thus, lever presses during reinstatement were combined across age groups to confirm the time-dependent changes in cue-induced reinstatement using a statistical test with higher degrees of freedom. A one-way ANOVA revealed a main effect of abstinence period ($F_{(3, 60)}$=2.83, p=0.046), and follow-up testing revealed that cue-induced reinstatement after 30 days of abstinence was higher than after 1 day (p=0.041). The test using total number of pellets consumed as a covariate did not reveal significant influence of sucrose intake on cue-induced reinstatement ($F_{(1, 55)}$<0.001, p=0.987).

3.5 Discussion

Results from the present experiment yield several points of discussion. With regard to sucrose pellet self-administration, adolescent rats earned and consumed fewer sucrose pellets than adults. Moreover, 5 out of 32 adults reached the maximum number of sucrose pellets allowed during a

session whereas only 2 of 32 adolescents reached the maximum. In terms of age differences in sensitivity to sucrose reinforcers, these data reflecting behavior reinforced on a simple FR1 schedule are difficult to interpret. A classic conclusion from lower response rates on a FR schedule is that the reinforcing stimulus has higher potency, but in the present case, the interpretation is confounded by differences in body mass. Lower rates of sucrose pellet self-administration among adolescents may be explained by the lower body mass of adolescents, at approximately half the mass of adults. (See Table 1 for body mass data.)

Despite age differences in self-administration, both age groups exhibited similar time-dependent increases in cue-induced reinstatement of sucrose seeking after abstinence. Consistent with other findings (Lu et al., 2004c), time-dependent increases in cue-induced reinstatement of sucrose seeking were not as robust and did not last as long as the increases in cocaine seeking we had reported previously (Li and Frantz, 2009), e.g. cue-induced reinstatement of sucrose seeking may have peaked after approximately 30 days of abstinence in the present study. These data might suggest that adolescents are just as sensitive as adults to associations established between sucrose rewards and environmental cues, a conclusion with implications for equal vulnerability to food addiction among human adolescents and adults.

Together with our previous study on cue-induced reinstatement of cocaine seeking (Li and Frantz, 2009), the present results provide evidence that younger animals can associate cues with natural rewards to the same degree as older animals, and thus support our hypothesis that previously reported age differences in cue-induced reinstatement of cocaine seeking are due to specific effects of cocaine (Li and Frantz, 2009), not a compromised ability among younger rats to associate cues with rewards. Generally these experiments suggest that adolescence may be a developmental stage associated with protection against certain long-term pathological effects of

cocaine. Although surprising, this conclusion is supported by other recent reports. For example, stimulus-reward learning remains intact after cocaine self-administration during adolescence, but is impaired after cocaine self-administration in adulthood (Kerstetter and Kantak, 2007). Also adolescent rats show less intense somatic and affective signs of withdrawal from nicotine compared with adults (O'Dell et al., 2004, O'Dell et al., 2007, Shram et al., 2008). Finally, cue-induced reinstatement of morphine seeking is also attenuated in younger subjects after abstinence from morphine self-administration by adolescent or adult rats (Doherty et al., 2009). Nonetheless, our conclusion counters predictions based on human survey data indicating that adolescence among humans is a developmental stage associated with vulnerability to drug use and abuse (O'Malley and Johnston, 2007). These contradictions may reveal that social factors, cognitive immaturity, and other variables not modeled in most rodent drug self-administration studies influence drug use and abuse by humans.

3.6 Tables and Figures

Table 3.1. Body mass (mean ±SEM, g) and age (PND) at key experimental time points

Age Group	**Start of Self-administration (n=32/group)**	**1-day reinstatement (n=8/group)**	**14-day reinstatement (n=8/group)**	**30-day reinstatement (n=8/group)**	**60-day reinstatement (n=8/group)**
Adolescent-onset	138.75 ±1.11	231.30 ±4.11	357.03 ±8.98	407.02 ±10.12	491.55 ±7.53
PND	35	49	63	79	109
Adult-onset	386.79 ±2.49	428.36 ±6.34	463.77 ±9.51	478.46 ±16.05	532.60 ±9.63
PND	83-95	97-110	111-124	127-140	151-170

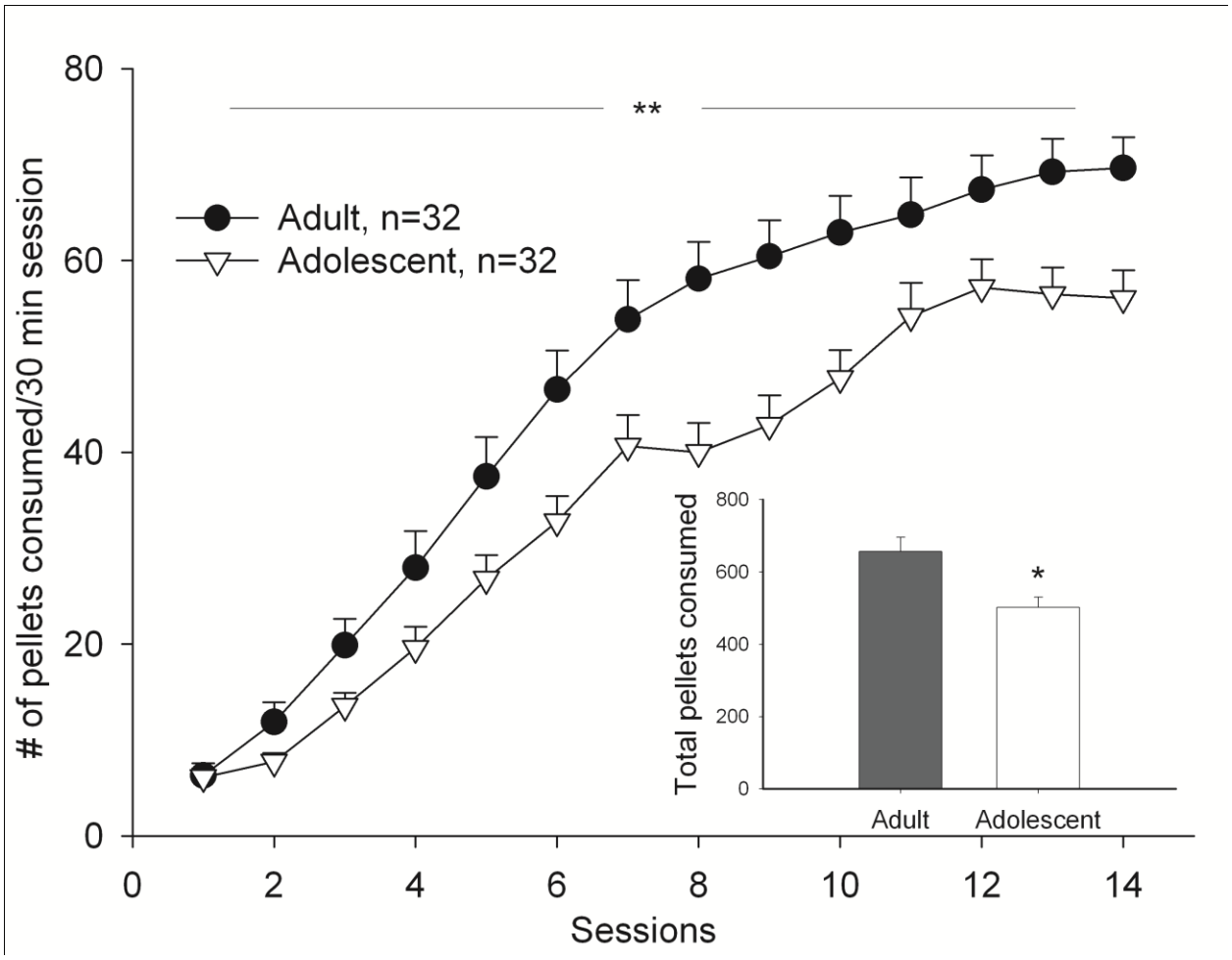


Figure 3.1. Fourteen consecutive daily 30 min sucrose self-administration sessions in adolescent vs. adult male rats. Points represent mean ± SEM number of sucrose pellets consumed (** indicates a main effect of age, $p<0.01$). Inset shows total consumption summed across the entire 14 days of self-administration. Bars represent mean ± SEM (* indicates a significant age difference, $p<0.05$).

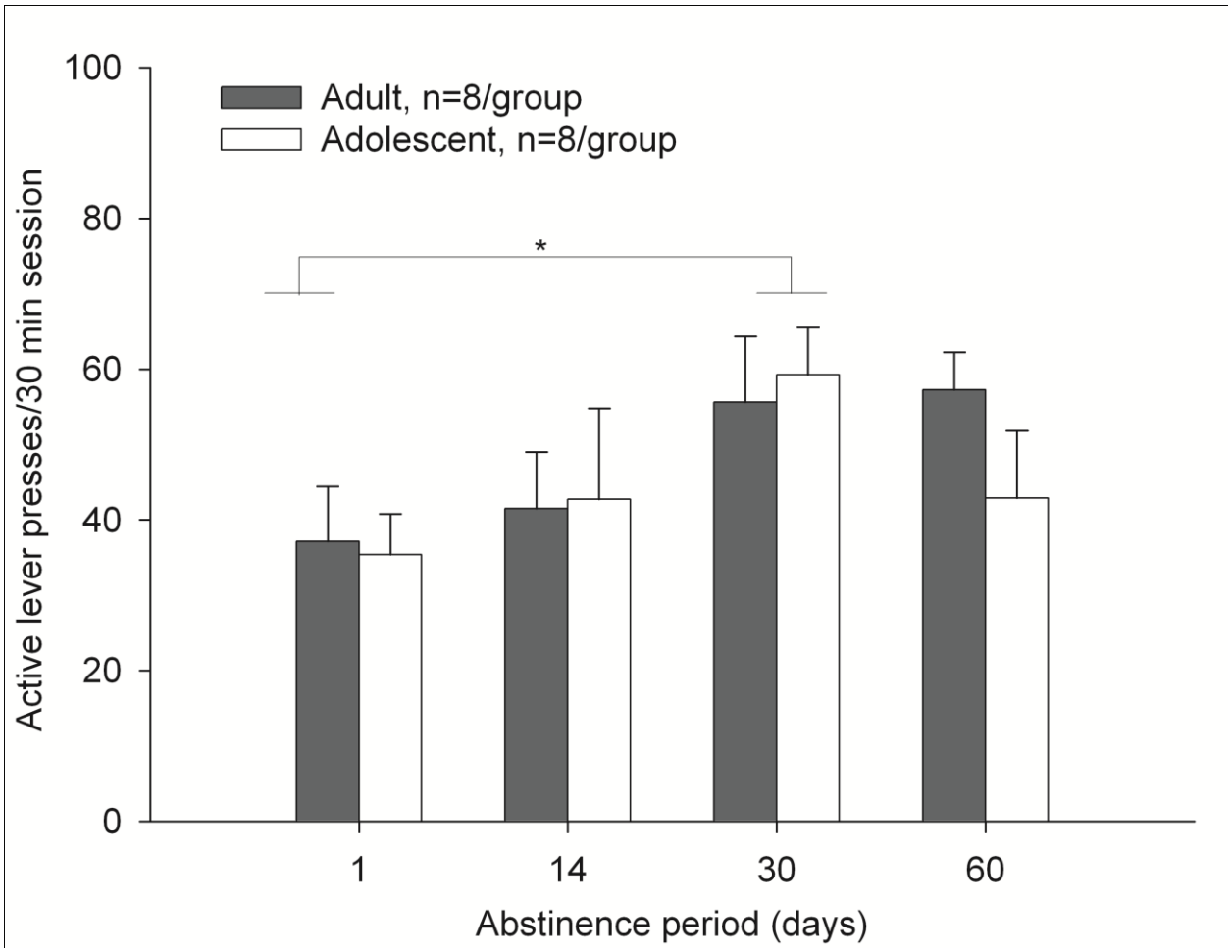


Figure 3.2. Time-dependent increases in cue-induced reinstatement of sucrose seeking. Bars represent mean ± SEM presses on the active lever. No age differences were observed (* indicates that cue-induced reinstatement after 30 days of abstinence was higher than after 1 day, $p<0.05$).

4. CHAPTER 4: AGE DIFFERENCES IN THE EFFECTS OF ENVIRONMENTAL ENRICHMENT ON CUE-INDUCED REINSTATEMENT OF COCAINE SEEKING IN MALE RATS

By

Chen Li & Kyle J. Frantz

4.1 Abstract

The time-dependent increase in cue-induced reinstatement of cocaine seeking, known as "incubation", is attenuated in male rats that self-administered cocaine during adolescence, compared with adults. We hypothesized that attenuated reinstatement may be explained by rapid developmental re-organization in brain reinforcement circuits during abstinence from cocaine in adolescent-onset groups, i.e. ongoing ontological plasticity could weaken associations between drug reward and environmental cues established during self-administration in adolescence. Classic manipulations that stimulate or inhibit brain plasticity include environmental enrichment or impoverishment, respectively, each of which influences drug taking and drug seeking. We therefore predicted that environmental enrichment or impoverishment imposed during an abstinence period between self-administration and extinction/reinstatement testing would exert greater effects on adolescent-onset vs. adult-onset groups to attenuate or enhance cocaine seeking, respectively, Thus adolescent and adult male Wistar rats were allowed to self-administer cocaine intravenously for 12 days (fixed ratio 1 schedule of reinforcement; 0.36 mg/kg/infusion; 2 hr daily sessions). Subsequently, rats were housed in one of three housing conditions: enriched (grouped, with large cages and toys), standard (paired) or impoverished (isolated). After 2 months, extinction and cue-induced reinstatement of cocaine seeking were tested, followed by tests of drug-induced reinstatement using escalating cocaine doses (0, 5, 10 mg/kg, i.p.). Consistent with previous results, extinction and cue-induced reinstatement were attenuated in the adolescent-onset group compared with adults, and this trend extended to lower levels of drug-induced reinstatement as well. Also consistent with previous results, environment enrichment decreased cue-induced reinstatement in adults, but had no effect on drug-induced reinstatement. On the other hand, environmental manipulations had no effect on reinstatement in the

adolescent-onset groups. These data confirm that adolescent subjects are resistant to some enduring effects of cocaine, but suggest that their resistance is not sensitive to environmental manipulations.

4.2 Introduction

The adolescent stage of development in humans is associated with high rates of experimentation with drugs of abuse (Laviola et al., 1999, O'Malley and Johnston, 2007, SAMHSA, 2009, Johnston et al., 2010). Whether or not this behavioral trend can be attributed to a biological vulnerability to drugs during adolescence remains unclear. Moreover, individuals who initiated drug use at an early age are more likely to classify themselves as addicts in adulthood (SAMHSA, 2009), raising the importance of studying the long-term vulnerability to relapse after adolescent onset of drug use.

Using the rodent models of intravenous (i.v.) drug self-administration, extinction, and cue-induced reinstatement (relapse) to test for biological underpinnings of adolescent drug vulnerability, our previous work indicated surprisingly that time-dependent increases in cue-induced reinstatement of cocaine seeking (i.e. incubation of drug craving; (Grimm et al., 2001)) were attenuated in male rats self-administering cocaine during adolescence (adolescent-onset groups) compared with rats taking cocaine during adulthood (adult-onset groups) (Li and Frantz, 2009). Thus, although both age groups self-administered similar amounts of cocaine, and exhibited similar levels of cue-induced reinstatement after just one day of abstinence, only the adult-onset groups and not the adolescent-onset groups showed increases in reinstatement over abstinence periods ranging from 14 days to 3 months.

One possible explanation for attenuated reinstatement among younger cohorts could be rapid developmental re-organization in brain reinforcement circuitry even during abstinence from cocaine. In other words, ongoing ontological plasticity and/or salient experiences could weaken associations between drug reward and environmental cues established during self-administration in adolescence. For example, cortical regions show hypermetabolism during

adolescence suggesting higher levels of neuronal activity (Kim et al., 2009). Many excitatory glutamatergic connections are lost during development into adulthood (van Eden et al., 1990). Dopamine input to the prefrontal cortex is at its highest level during adolescence, and dopamine receptor numbers peak during adolescence, then prune to adult levels in the prefrontal cortex (Teicher et al., 1991, Andersen et al., 2002b, Brenhouse et al., 2008). Similarly, dendrites and synapses in the nucleus accumbens, amygdala, hippocampus and hypothalamus are also undergoing pruning and reorganization during transition to adulthood (Choi et al., 1997, Tarazi et al., 1999, Zehr et al., 2006, 2008). Therefore, any cocaine-induced neuroplasticity may be overshadowed or perhaps normalized by this developmental organization in adolescent brains. In our previous study, all subjects were pair-housed (Li and Frantz, 2009); if social interactions increase rates of change in brain connectivity (i.e. induce plasticity), then adolescent-onset groups with higher basal rates of change than adults may be more likely to "replace" drug-cue associations with other relevant associations from their social experiences. This plasticity could be manifested as lower levels of reinstatement of drug seeking after abstinence in adolescents than adults. Classic manipulations that stimulate or inhibit brain plasticity include environmental enrichment or impoverishment, respectively, each of which also influences drug taking and drug seeking behavior. Environmental enrichment often consists of a combination of social and inanimate stimulation, with animals housed in groups in large cages containing periodically changed "toys" (Wurbel, 2001, Laviola et al., 2008). It contrasts with social or standard housing conditions in which small groups or pairs of same-sex and same-age animals live in approximately 10.5" x 19" x 8" Plexiglas shoebox cages with wire lids, shredded bedding, but no toys, and with impoverished conditions of social isolation in smaller wire cages without bedding or toys (Wurbel, 2001, Laviola et al., 2008). Environmental enrichment generally promotes

experience-dependent plasticity and learning, delays onset of neural degeneration, and reduces drug reinforcement (reviewed in (Nithianantharajah and Hannan, 2006, Laviola et al., 2008, Stairs and Bardo, 2009)). On the other hand, impoverished environments are associated with increased risks for psychiatric disorders, early onset of neurodegenerative diseases, and poor performance in cognition and learning tasks (Fone and Porkess, 2008, Laviola et al., 2008). Specifically in operant conditioning tasks, environmental enrichment decreases both the reinforcing potency and reinforcing efficacy of drugs of abuse (Bowling and Bardo, 1994, Bardo et al., 2001, Green et al., 2002, Solinas et al., 2008). After drug self-administration, environmental enrichment provided only during abstinence attenuates cue- or stress-induced reinstatement of drug-seeking, but surprisingly not drug-primed reinstatement (Chauvet et al., 2009, Thiel et al., 2009). Thus environmental enrichment (and possibly the associated neural plasticity) has both "preventive" and "curative" effects with regard to decreasing drug reinforcement (Stairs and Bardo, 2009, Solinas et al., 2010).

We hypothesized that if adolescence is associated with higher rates of brain plasticity than adulthood, then environmental manipulations during abstinence from cocaine should exert greater effects on brain plasticity in adolescent-onset groups of rats than adults, measured behaviorally as more robust decreases or increases in levels of reinstatement of drug seeking after environmental enrichment or impoverishment, respectively. Adolescent male rats were allowed to acquire lever-pressing maintained by i.v. cocaine, self-administered the drug in 12 short daily sessions over 14 days, and then were placed into one of three housing conditions during a 60-day abstinence period: enriched, standard (pair-housing), or impoverished. Within-session extinction responding and cue-induced reinstatement were then tested. To confirm that environmental manipulations have no effects on drug-induced reinstatement, drug-induced

reinstatement after the 60-day abstinence was also tested in the present subjects. Adult male rats were subjected to the same procedures, and they served as positive controls for the influence of environmental manipulations on cue-induced reinstatement, and thus for the specificity of environmental manipulations on cue-induced but not drug-induced reinstatement (Chauvet et al., 2009, Thiel et al., 2009).

4.3 Methods

Subjects

Male Wistar rats (Charles River Laboratories, Inc., Wilmington, MA) arrived in the laboratory at either postnatal day (PND) 22 or 70-82 and were housed in groups of two until the beginning of abstinence, in a humidity (50%) and temperature controlled (22 ℃) vivarium on a 12/12 h light/dark cycle (reverse cycle, with lights on at 1900 h). Rats acclimated to these conditions for 6-8 days prior to the start of experiments. Food and water were available *ad libitum* except during self-administration sessions. All subjects were observed and/or weighed daily to assess general health and responsiveness to drug exposure. All procedures were conducted in strict adherence to the "Principles of Laboratory Animal Care" and the *National Institute of Health Guide for the Care and Use of Laboratory Animals* (NRC, 2003).

Drugs

Cocaine hydrogen chloride was obtained from Mallinckrodt Inc. (Hazelwood, MO). The concentration of stock solution was 1.25 mg/ml, and the volume of cocaine solution for each rat was adjusted according to body weight (0.07 ml/kg/s). Thus a dose of 0.36 mg/kg/infusion was provided for each rat with an infusion duration of approximately 4.0 s for adults and 1.5 s for adolescents on the first day. Methohexital sodium (1% solution) was obtained from Eli Lilly (Indianapolis, IN).

Surgery

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 to adults (4 cm) (Shahbazi et al., 2008). Rats were anesthetized with an isoflurane/oxygen vapor mixture (4-5% for initial anesthetization and 1.5-3% during surgery), and catheter tubing was passed subcutaneously from the animal's back to the right jugular vein, inserted into the vein previously punctured with a 25 gauge needle, gently tied with suture thread, and fixed with superglue. During recovery, rats received approximately 0.2 ml Timentin (Ticarcillin Disodium and Clavulanate Potassium; 100 mg/ml, i.v.) twice daily on the first two days post-surgery, then once daily throughout the experiment. Catheters were also flushed daily with approximately 0.4 ml heparinized saline (100 USP units/1 ml). Catheter patency was confirmed in all subjects by full loss of muscle tone within 5 s of i.v. infusion (over ~2 s) of the short acting anesthetic agent, 1% Methohexital sodium, over one day before the first and after the last self-administration session. Subjects that failed either patency test were eliminated from the study.

Equipment

Behavioral tests were conducted in operant conditioning chambers enclosed in sound-attenuating, ventilated environmental cubicles (Med Associates, Inc., St. Albans, VT). To start each session, a house light and white noise were turned on and two levers, one active and one inactive, extended into the chamber. Lever presses on the inactive lever were recorded but had no scheduled consequences. Presses on the active lever triggered a syringe pump (Med Associates, Inc., St. Albans, VT) to deliver drug solution via a stainless steel swivel (Instech Laboratories, Inc., Plymouth Meeting, PA) and polyethylene tubing attached to a catheter portal on each

animal's back. Each reinforced response lit a cue light above the lever which stayed on for the duration of the infusion. The cue light, house light, and white noise were not present during a 20 s time-out (TO20) after each infusion. Drug delivery and data collection were controlled by Med Associates software (Med PC IV).

Self-Administration

Following a 6-7-day post-surgical recovery, spontaneous acquisition of cocaine self-administration began (PND35 or 83-95), with daily 2 h sessions over 12 days conducted during the dark phase of the light-dark cycle. Non-contingent drug injections were not administered in this phase of experimentation. Lever-pressing on the active lever was reinforced by i.v. infusion of cocaine (0.36 mg/kg/infusion) under a Fixed-Ratio 1 (FR1) TO20 schedule of reinforcement.

Abstinence and Environmental Conditions

After 12 days of self-administration, the animals were divided into three housing conditions: enriched, standard, or impoverished, for 60-62 days of abstinence. Assignment to housing groups was balanced based on cocaine intake over the last 3 days of self-administration.

Enriched Conditions

In enriched environmental conditions, 4-5 rats were group-housed in a "super cage", constructed from 4 standard Plexiglas cages (10.5" x 19" x 8") with wire lids connected via three 6" length x 6" diameter PVC pipes. The enriched conditions also included 10 plastic objects (toys) per super-cage, half of which were changed daily. Animals were also briefly handled and weighed twice per week.

Standard Conditions

In the standard environmental conditions, pairs of age-matched rats were maintained in standard housing conditions throughout self-administration, abstinence, and extinction/reinstatement

testing. Standard conditions were the same as conditions for all subjects after catheterization and during the self-administration phase of the experiment, and consisted of pair-housing in standard Plexiglas cages (10.5" x 19" x 8") with wire lids, as well as twice-weekly handling and weighing.

Impoverished Conditions

In the impoverished environmental condtions, subjects were individually housed in hanging wire cages (wire mesh floor and front panel, solid metal sides, back, and top; approximately 17 x 24 x 20 cm). Rats in impoverished environmental conditions were not handled nor weighed during abstinence.

Extinction and Reinstatement

After abstinence from cocaine, a within-session extinction and reinstatement test was conducted (Grimm et al., 2001, Grimm et al., 2003, Li and Frantz, 2009). Six 1-h extinction sessions were followed by one 1-h cue-induced reinstatement, and additional three 1-h drug-induced reinstatement tests. During extinction, rats were connected to the metal coil tether but not the infusion tubing, white noise remained off, and the house light remained on. Neither cue-lights nor TO signals were presented after presses on either lever. Five-min breaks occurred between each successive session, during which the two levers retracted and the house light turned off.

Cue-induced reinstatement tests began with the onset of the house light, white noise, and a 5 s cue-light, followed by a 20 s TO during which the house light, white noise, and cue light were turned off. During the remainder of the cue-induced reinstatement session, presses on the active lever produced cue sequences identical to those presented during cocaine self-administration, and the pump went on, although no syringe was loaded. The only difference between self-administration and cue-induced reinstatement was that drug solution was not infused during reinstatement.

For three drug-induced reinstatement sessions, each rat was taken out of the chamber before the start of each session, and injected with 0, 5, or 10 mg/kg cocaine (intraperitoneal) in an ascending order to minimize potential effects of preceding injections on subsequent treatments and to escalate cocaine doses gradually. They were placed immediately back into the chamber for a 1-hr test after each injection. Parameters for drug priming-induced reinstatement sessions were identical to extinction sessions, i.e. no drug-paired cues were presented.

Data Analysis

For cocaine self-administration sessions, the number of infusions per session was analyzed using a three-way mixed measures analysis of variance (ANOVA) with age and housing conditions (enriched, standard and impoverished) as between-subjects factors, and session as a within-subjects factor. The number of total active vs inactive presses per was analyzed using a four-way mixed measures analysis of variance (ANOVA) with age, housing conditions, and lever (active vs. inactive) as between-subjects factors, and session as a within-subjects factor. Similarly for extinction sessions, the number of active lever presses per session was analyzed using a three-way mixed measures ANOVA, with age and housing condition as a between-subjects factors, and session as a within-subjects factor. To test for cue-induced reinstatement of lever-pressing, the number of active lever presses in the last extinction session was compared directly with active lever presses during the reinstatement session, using a three-way mixed measures ANOVA, with age and housing condition as between-subjects factors and session (extinction vs. reinstatement) as a within-subjects factor. Specific effects of housing condition on reinstatement of lever-pressing were also analyzed using a two-way age x housing condition ANOVA. To confirm previous findings that environmental enrichment attenuates cue-induced reinstatement in adult-onset groups (Solinas et al., 2008, Chauvet et al., 2009) and to test our hypothesis that

adolescent-onset groups respond differently to environmental manipulations, separate one-way ANOVAs with housing condition as a between-subjects factor were conducted for each age group. Unpaired 2-tailed t-tests with Bonferroni's correction were used for post-hoc comparisons, as appropriate. To test for drug-induced reinstatement of lever-pressing, the number of active lever presses per session were analyzed using a three-way mixed measures ANOVA, with age and housing conditions as a between-subjects factors, dose as a within-subjects factor, and follow-up t-tests with Bonferroni's correction as above. Data were subsequently collapsed across housing condition for further analysis of drug-induced reinstatement, because neither the main effect nor any interactions with housing condition were significant; see Results. Outliers (three animals per age group) were removed when values fell greater than two standard deviations above or below the mean, as noted in Results. In all cases, results were considered significant if $p<0.05$.

4.4 Results

Consistent with previous results from our laboratory, no age differences in cocaine self-administration were observed (Figure 4.1). Given that assignment of subjects to housing conditions was balanced by mean number of infusions on the last three days of self-administration, housing condition also did not influence self-administration (Figure 4.1). Thus, a three-way age x housing condition x sessions ANOVA revealed that only the main effect of sessions was significant [$F_{(11, 57)}=14.653$, $p<0.001$], but the other main effects and interactions were not. On the first day of self-administration, adolescent rats in the enriched housing group self-administered fewer infusions than other groups, but this difference is most likely random and thus not meaningful for the conclusions of the study. A four-way age x housing condition x lever x sessions ANOVA revealed that only the main effect of lever was significant [$F_{(1,}$

$_{108)}$=163.19, p<0.001], but not other main effects nor interactions, indicating the discrimination between active vs. inactive lever during self-administration.

With regard to extinction responding after 60 days of abstinence, both age groups exhibited an expected decline of extinction responding over sessions in the absence of discrete drug paired cues (Figure 4.2), but the adolescent-onset groups made fewer lever-presses overall compared to adult-onset groups (Figure 4.3a). Thus, lever-pressing gradually declined in both age groups by the sixth 1-h extinction session such that a three-way age x housing condition x session ANOVA revealed a significant main effect of session [$F_{(5,54)}$=46.054, p<0.001]. Extinction responding also differed according to main effects of age [$F_{(1,54)}$=5.617, p=0.021] and an age x session interaction [$F_{(5,54)}$=4.580, p=0.001]. Given that housing condition did not influence extinction responding, follow-up unpaired t-tests for age differences in specific extinction sessions were conducted with data collapsed across housing groups; they confirmed the age difference in the first two but not subsequent extinction sessions (t=2.535, p=0.014; t=3.019, p=0.003 respectively).

With regard to cue-induced reinstatement of cocaine seeking, both age groups exhibited an expected increase in lever-pressing after re-presentation of discrete cocaine-paired cues (Figure 4.2). A three-way age x housing condition x session ANOVA on active lever presses in the last extinction session vs. the reinstatement session revealed main effects of session [$F_{(1,54)}$=239.12, p<0.001] and an interaction of session x housing condition [$F_{(2,54)}$=5.935, p=0.005], but no other main effects nor interactions.

Environmental manipulations altered cue-induced reinstatement in adults only, and not in the adolescent-onset groups (Figure 4.3b). The two-way age x housing condition ANOVA on active lever presses in the cue-induced reinstatement session alone revealed a main effect of

housing condition [$F_{(2,54)}$=3.962, p=0.025], a main effect of age that barely missed significance [$F_{(1,54)}$=3.690, p=0.06], but no age x housing interaction. Post hoc tests confirmed that rats in the enriched groups exhibited fewer responses than impoverished groups, regardless of age group (p=0.027). Given that we planned to test age differences in effects of environmental manipulations on cue-induced reinstatement, separate one-way ANOVAs by housing condition were conducted for each age group and revealed a main effect of housing condition in only the adult-onset group [$F_{(2,26)}$=3.627, p=0.041], and post hoc analysis once again confirmed lower levels of reinstatement in enriched condition compared with the impoverished condition (p=0.044) (Figure 4.3b).

For all subjects, cue-induced reinstatement was followed by tests of drug-induced reinstatement in which escalating doses of cocaine produced increasing numbers of lever-presses per session that differed by age but not housing condition (Figure 4.4). Outliers with extremely high rates of lever pressing that fell greater than two standard deviations above the mean were removed from the analysis: two from the adolescent enriched group; one from adolescent standard; one from adult enriched; one from adult standard; and one outlier from the adult impoverished group, with most of them noted by experimenters as exhibiting perseverative behavior or motor stereotypies near the active lever during the reinstatement testing. Given that the same number of adolescents and adults were removed, our overall interpretations are unlikely to be affected. The three-way age x housing condition x dose ANOVA revealed main effects of age [$F_{(1, 49)}$=22.071, p<0.001] and dose [$F_{(2, 49)}$=32.106, p<0.001], and interactions between age and dose [$F_{(2, 49)}$=4.774, p=0.011]. In the absence of any effects of housing condition, data were collapsed across housing groups to examine the age differences in drug-induced reinstatement. Adolescent-onset groups exhibited less lever-pressing after each dose, including the saline

vehicle treatment (0 mg/kg cocaine), than adult-onset groups. Unpaired t-tests showed age differences after saline ($t=3.881$, $p<0.001$), 5 mg/kg cocaine ($t=3.433$, $p=0.001$), and 10 mg/kg cocaine ($t=3.273$, $p=0.002$).

4.5 Discussion

The present experiment tested whether environmental manipulations during abstinence from cocaine influenced reinstatement of cocaine seeking more robustly in groups that self-administered cocaine as adolescents (adolescent-onset groups) vs. adults (adult-onset groups). As expected (Li and Frantz, 2009), no age differences in initial cocaine self-administration were observed, adolescent-onset groups made fewer lever-presses during extinction, and they tended to make fewer lever-presses during cue-induced reinstatement ($p=0.06$) than adults. With regard to effects of environmental manipulations on cue-induced reinstatement of cocaine seeking, the present study replicates prior reports on adult subjects in which enrichment was associated with lower levels of cue-induced reinstatement than impoverishment during abstinence (Solinas et al., 2008, Chauvet et al., 2009, Thiel et al., 2009). Surprisingly among the present adolescent-onset groups, however, environmental manipulations failed to change levels of reinstatement. Neither did environmental changes alter drug-induced reinstatement, although this was expected for adult subjects (Chauvet et al., 2009, Thiel et al., 2009). Finally, all adolescent-onset groups exhibited less drug-induced reinstatement than adults.

Several elements of the present study confirm that the methods were sound. For cocaine self-administration, rats in both age groups gradually acquired lever-pressing reinforced by i.v. cocaine infusions, discriminated between active vs. inactive levers, and extinguished their lever-pressing behavior in the absence of drug reinforcement during extinction tests. For environmental enrichment, adult groups serving as positive controls based on prior literature

(Chauvet et al., 2009, Thiel et al., 2009) reinstated their cocaine seeking to a lower level after abstinence in an enriched environment compared to an impoverished environment. Thus, the environmental conditions in our laboratory, although not identical to other reports, were sufficient to alter reinstatement in predictable ways after abstinence and extinction in adults. Confirming previous reports on the specificity of environmental effects, neither enrichment nor impoverishment altered drug-induced reinstatement in adults.

The lack of environmental influence on reinstatement of cocaine seeking among the present adolescent-onset groups may be explained by factors related to experimental procedures. For example, our subjects experienced enrichment only during abstinence (approximately 60 days from PND50-110), and not throughout all experimentation and thus a longer period of development (e.g. PND22-110). Moreover, our adolescent-onset groups in the different housing conditions self-administered similar amounts of cocaine, whereas rats housed in enriched conditions throughout the duration of experimentation took less cocaine than their standard-housed or impoverished counterparts (Smith et al., 2009). Age differences in the salience of environmental cues may contribute to the lack of environmental influence as well, i.e. in some cases younger subjects may be less responsive than adults to inanimate or social aspects of the environment. For example, social isolation only affects novelty seeking behavior in adults but not in adolescents (Meaney and Stewart, 1981, Douglas et al., 2004, Varlinskaya and Spear, 2008). In addition, adolescent rats appear to ignore environmental stimuli in a runway (Spear and Brake, 1983). Another possible explanation is a floor effect: in other words, standard pair housing may provide enough enrichment for younger animals that other forms of enrichment could not drive reinstatement any lower. However, the finding that environmental impoverishment did not change cue-induced reinstatement in younger animals does not support

this possibility. Finally, ongoing ontological change in the brain could create insensitivity to some external influences during adolescence. For example, excitatory glutamate projections from various cortical regions are lost during adolescent development (van Eden et al., 1990) and dopamine inputs as well as dopamine receptors are pruned (Kalsbeek et al., 1988, Teicher et al., 1995, Andersen et al., 2002b, Brenhouse et al., 2008). These developmental changes and others may counteract the well-documented cocaine-induced plasticity thought to underlie addiction (Kalivas and Volkow, 2005), and restore the structure and function of brain reward and reinforcement circuits to normal conditions during drug abstinence. More work on which specific mechanisms could underlie these possible effects remains to be done.

In fact, numerous avenues of research result from this study. This replication of prior reports with adults (Chauvet et al., 2009, Thiel et al., 2009) indicates that the ameliorative effects of environmental enrichment are specific to cue-induced reinstatement and do not generalize to drug-induced reinstatement. Such specificity may guide future studies to examine differences in effects of enrichment on plasticity in neural substrates that mediate cue- vs. drug- reinstatement separately, e.g. amygdala and ventral tegmental area (Shaham et al., 2003). Similarly, the present results confirm that enrichment fails to alter extinction responding, also directing future work on enrichment away from neural substrates for context-induced reinstatement (Hearing et al., 2008) in the absence of alternate environment exposure during abstinence (Crombag et al., 2002).

Of final note, the present data set extend our findings of lower levels of reinstatement among adolescent-onset vs. adult-onset self-administration groups from cue-induced reinstatement of cocaine, morphine, and heroin seeking (Doherty et al., 2009, Li and Frantz, 2009), to include cocaine-primed reinstatement as well. This phenomenon appears to be drug-specific, as it does not generalize to reinstatement of sucrose seeking (Li and Frantz, 2010).

Although consistent across studies from our laboratory, this phenomenon contrasts results in which drug-primed reinstatement as well as yohimbine stress-induced reinstatement were more robust in adolescent rats than adults (Anker and Carroll, 2010), again possibly explained by procedural differences including especially the fact that self-administration, daily extinction sessions, and reinstatement all took place during the short adolescent phase in Anker and Carroll's experiment. In other words, when tested with cue-induced reinstatement, rats in Anker and Carroll's experiment are still late adolescents while in present study, our rats were adults. Generally, studies from our laboratory suggest that ongoing development during adolescence protects younger animals from some of the long-term effects of early drug intake. When applied to the human condition, our data suggest that adolescence may be a period of relative biological resistance to at least some long-term effects of drugs of abuse; and also that adults may be more responsive than teenagers to environmental interventions for the treatment of drug addiction.

4.6 Figures

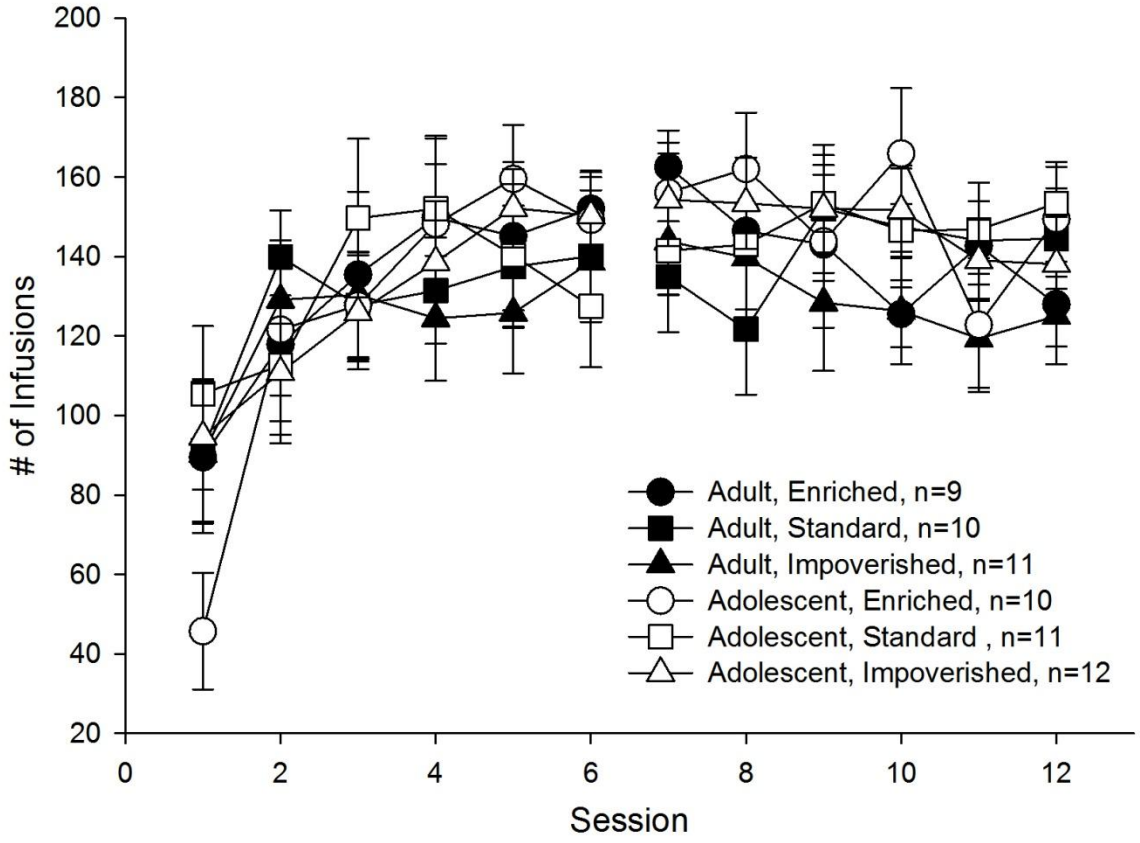
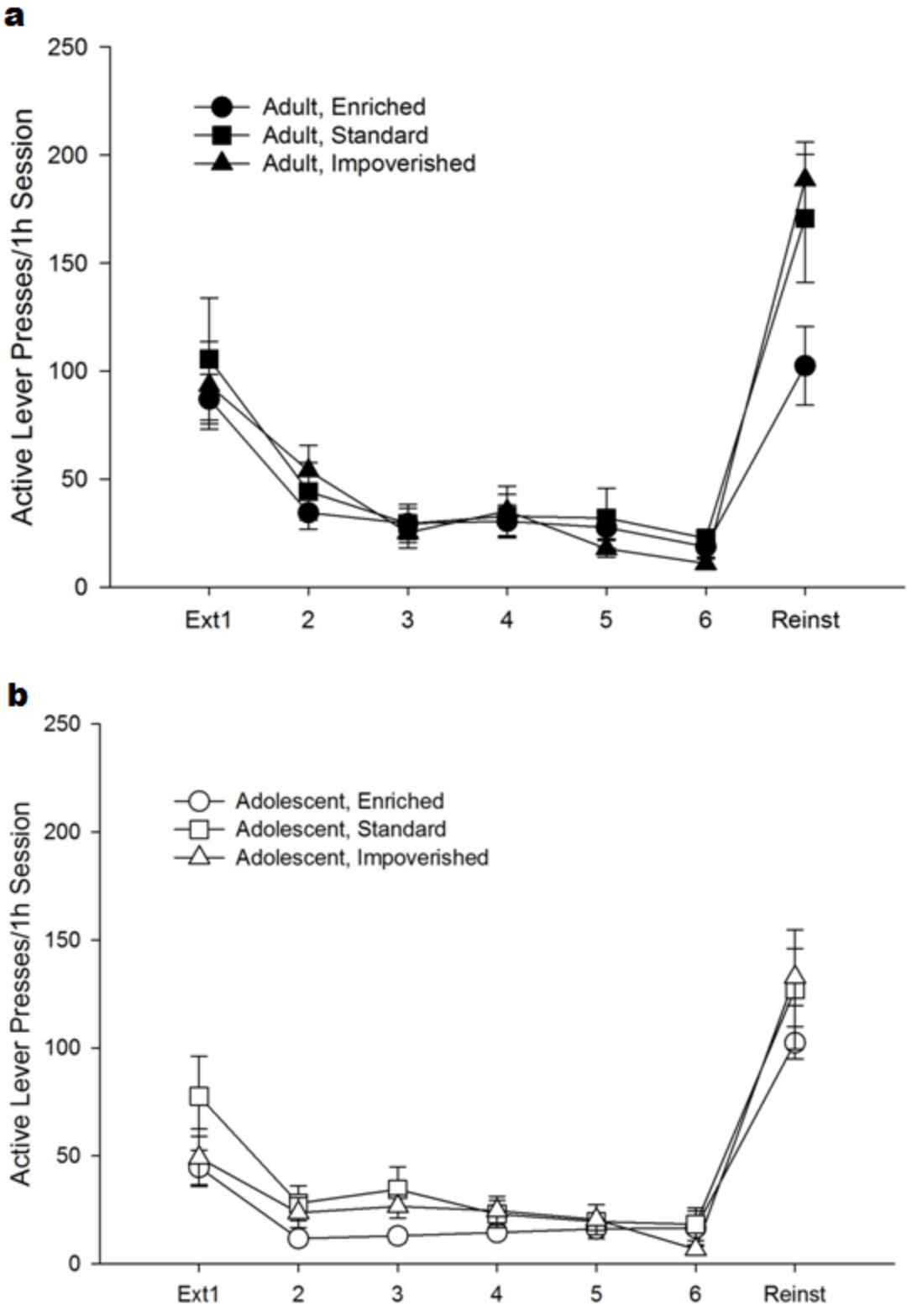


Figure 4.1. Twelve 2 h daily cocaine self-administration sessions in adolescent vs. adult male rats. Points represent mean ± SEM. Assignment of subjects to housing conditions was balanced by mean number of infusions on the last three days of self-administration. No differences among housing conditions.


a
Active Lever Presses/1h Session
250
200
150
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50
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Adult, Enriched
Adult, Standard
Adult, Impoverished
Ext1
2
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4
5
6
Reinst
b
Active Lever Presses/1h Session
250
200
150
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Adolescent, Enriched
Adolescent, Standard
Adolescent, Impoverished
Ext1
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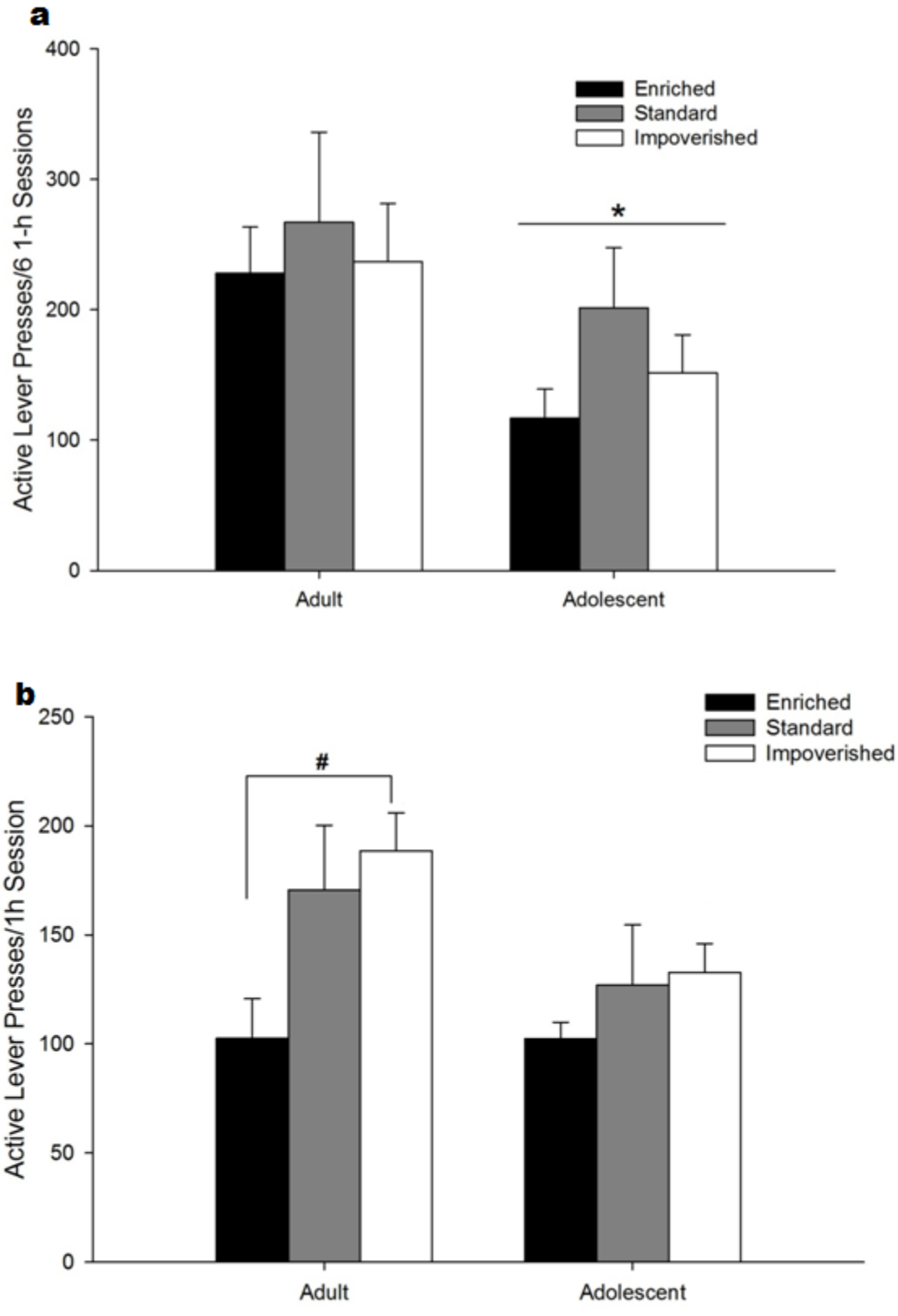
6
Reinst

Figure 4.2. Lever pressing in extinction and cue-induced reinstatement after 60 days of abstinence in adult-(a) vs. adolescent-onset (b) age groups in three housing conditions. Points represent mean ± SEM lever presses on the drug-associated active lever. All groups showed reliable extinction and cue-induced reinstatement.


a
Active Lever Presses/6 1-h Sessions
400
300
200
100
0
Enriched
Standard
Impoverished
*
Adult
Adolescent
b
Active Lever Presses/1h Session
250
200
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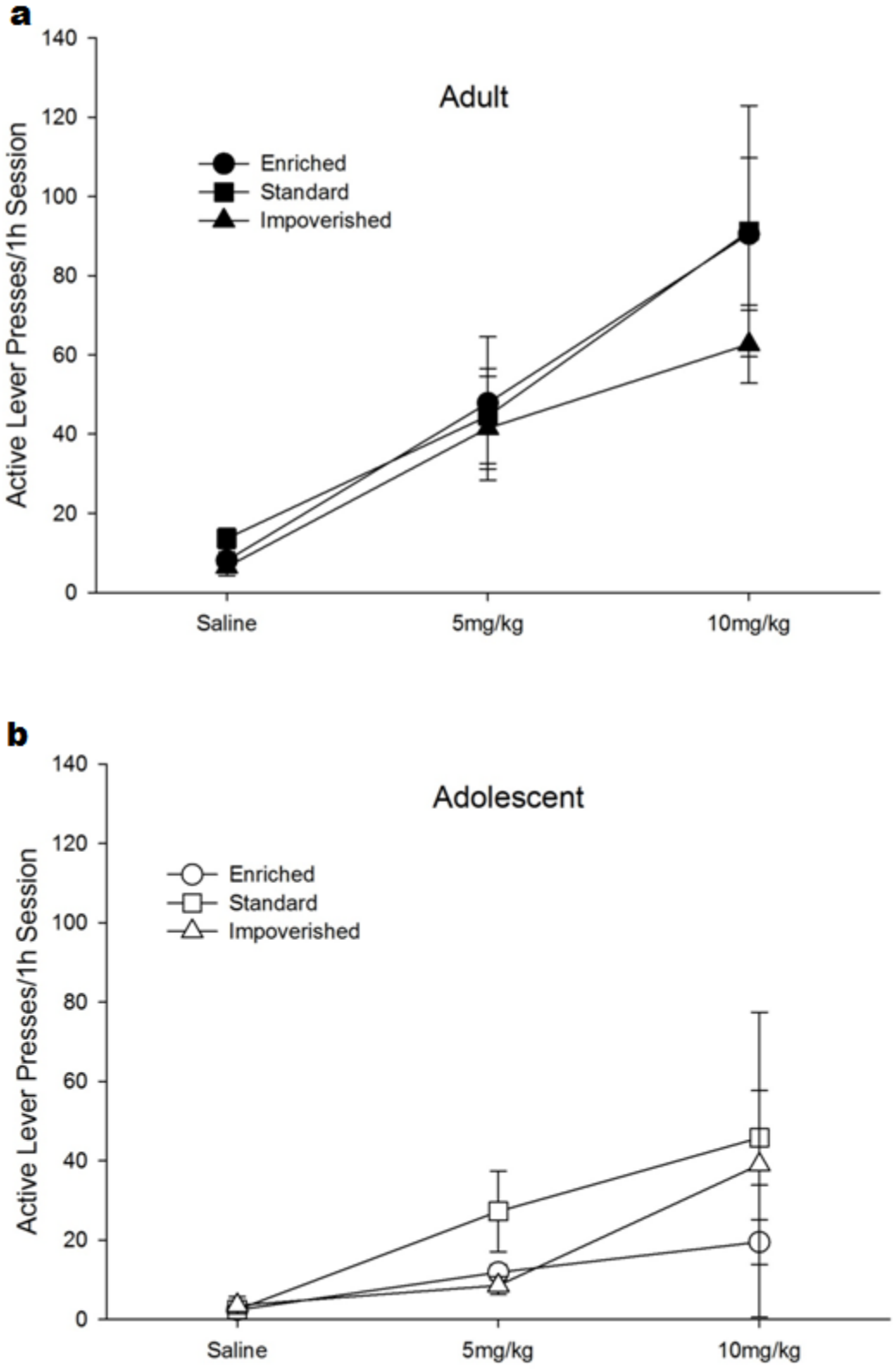
100
50
0
Enriched
Standard
Impoverished
#
Adult
Adolescent

Figure 4.3. Total extinction responses summed over 6 1-h sessions (a) and cue-induced reinstatement (b). Bars represent mean ± SEM. (a) Adolescent-onset groups showed lower levels of extinction responding compared with adults. (b) Environmental enrichment attenuated cue-induced reinstatement compared with impoverished condition only in the adult group. (* indicates differences between age groups, $p<0.05$; # indicates differences between housing conditions, $p<0.05$).


a
Adult
Enriched
Standard
Impoverished
Active Lever Presses/1h Session
140
120
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80
60
40
20
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Saline
5mg/kg
10mg/kg
b
Adolescent
Enriched
Standard
Impoverished
Active Lever Presses/1h Session
140
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Saline
5mg/kg
10mg/kg

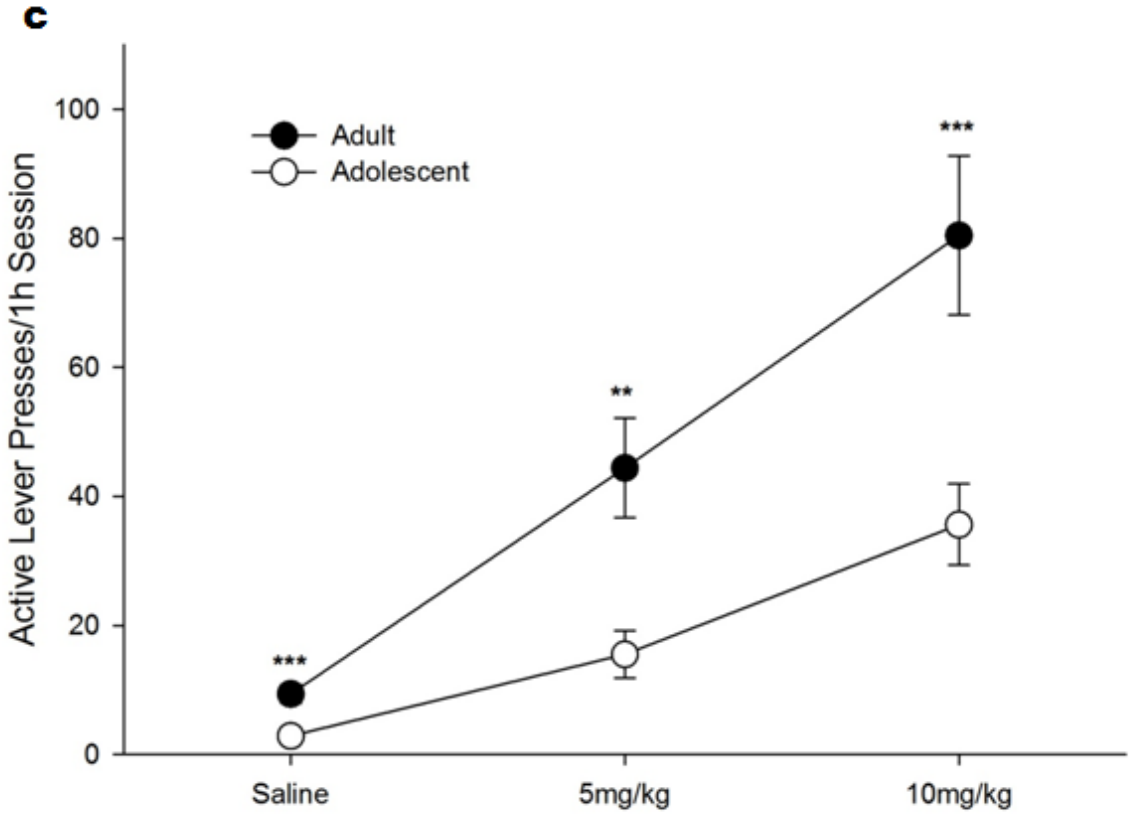

Figure 4.4. Drug-induced reinstatement among adults (a) and the adolescent-onset group (b), in which escalating doses of cocaine produced increasing numbers of lever-presses per session that differed by age but not housing condition (c). Data were collapsed among housing conditions, adolescent-onset groups showed lower levels of lever pressing than adults at all three doses (Differences between age groups, **p<0.01, ***p<0.001).

5. CHAPTER 5: AGE DIFFERENCES IN EXPRESSION OF NEUROPLASTICITY-RELATED GENES AFTER COCAINE SELF-ADMINISTRATION OR CUE-INDUCED REINSTATEMENT OF COCAINE SEEKING IN MALE RATS

by

Chen Li, Jacqueline F. McGinty & Kyle J. Frantz

5.1 Abstract

Recreational drug use peaks in the developmental stage of adolescence. Our research with adolescent vs. adult male rats, however, showed surprisingly that rats taking cocaine as adolescents were actually less vulnerable to later cue-induced reinstatement of drug-seeking than adults. Several genes related to neuroplasticity, such as activity-regulated cytoskeletal-associated gene (*arc*) and brain-derived neurotrophic factor (*bdnf*), influence cocaine self-administration and cue-induced reinstatement. Moreover, their expression is age-dependent. We hypothesized that cocaine-seeking in adolescent vs. adult rats correlates with differential expression of plasticity-related genes, in ways dependent on brain-region. Adolescent and adult male rats were allowed to acquire lever-pressing maintained by i.v. infusions of cocaine in daily two-hour sessions over 13 days. A subset of yoked controls received saline infusions. At one of three experimental time points (immediately after the last self-administration session, or after extinction and reinstatement tests at 1 day or at 60 days of abstinence), rats were sacrificed and tissue was collected to analyze *arc* and *bdnf* mRNA levels by in situ hybridization. The entire medial prefrontal cortex (mPFC) and entire nucleus accumbens (NAc) were analyzed at rostro-caudal extents where drug-related neuroplasticity occurs in adult rats. While behavioral outcomes generally replicated our previous results, age differences in gene expression were brain region specific. Whereas *arc* expression in the mPFC varied with drug treatment and time point, it was similar across age groups and trended toward negative correlation with cocaine-seeking. *Arc* mRNA in the NAc was stable across treatments and time points, but slightly higher in younger animals than adults in all conditions. *Bdnf* expression in the mPFC, but not the NAc was higher in younger animals than adults. These data support the possibility that higher levels of

neuroplasticity in reinforcement-related brain regions in younger animals than adults could contribute to age-dependent long-term effects of cocaine and cocaine-associated cues.

5.2 Introduction

Recreational drug use peaks in the developmental stage of adolescence (Laviola et al., 1999, O'Malley and Johnston, 2007, SAMHSA, 2008, Johnston et al., 2010). Individuals aged 12-24 years report the highest rates of illicit drug use (SAMHSA, 2009). Whether or not this behavioral trend can be attributed to a biological vulnerability to drugs during adolescence remains unclear. Moreover, early onset of drug use increases the likelihood of addiction in adulthood (SAMHSA, 2009), raising the importance of studying long-term vulnerability to relapse after adolescent onset of drug use.

Using the rodent models of intravenous (i.v.) drug self-administration, extinction, and cue-induced reinstatement (relapse) to investigate both acute and long-term adolescent drug vulnerability, our previous work indicated surprisingly that adolescent and adult rats took similar amounts of cocaine**,** nevertheless time-dependent increases in cue-induced reinstatement of cocaine seeking (incubation of drug craving; (Grimm et al., 2001)) were attenuated in male rats self-administering cocaine during adolescence (adolescent-onset groups), compared with rats taking cocaine during adulthood (adult-onset groups) (Li and Frantz, 2009). Moreover, research conducted in our laboratory on environmental manipulations during abstinence failed to affect cue-induced reinstatement of cocaine seeking in adolescent-onset groups, whereas in adults, environmental enrichment (vs. impoverishment) during abstinence replicated prior reports (Chauvet et al., 2009, Thiel et al., 2009) by decreasing cue-induced reinstatement (see Chapter 4). A possible explanation may involve internal factors in younger animals, such as ongoing neuroplasticity related to developmental growth and maturation, e.g. pruning of glutamatergic or

dopaminergic synapses (van Eden et al., 1990, Teicher et al., 1991, Tarazi et al., 1999, Spear, 2000b, Andersen et al., 2002b, Brenhouse et al., 2008), could eliminate neuroadaptations induced by cocaine, minimize cue-induced reinstatement to a nadir, and also limit the influence of environmental manipulations. Thus in the present experiment, we explored two of many neuroplasticity-related genes that are possible internal factors contributing to age differences in reinstatement of drug seeking. We focused on their expression in two brain regions involved in addiction generally and reinstatement of drug seeking specifically (Kalivas et al., 2005), the medial prefrontal cortex (mPFC) and nucleus accumbens (NAc).

Cocaine induces expression of plasticity-related genes, which then trigger functional and structural changes at synapses, among other cellular locations. For example, cocaine turns on immediate early genes (IEGs) such as the transcription factors, *c-fos, c-jun* and *zif/268* (Daunais and McGinty, 1994, Kosofsky et al., 1995), and activates effector genes such as activity-regulated cytoskeleton-associated gene (*arc*) and brain-derived neurotrophic factor (*bdnf*) (Le Foll et al., 2005, Fumagalli et al., 2009). Effector genes *arc* and *bdnf* have received special attention because their protein products are important in helping to form, strengthen, and stabilize synapses during learning and memory, promoting survival and differentiation of neurons (Tzingounis and Nicoll, 2006). Their expression patterns are also more precisely aligned with neuroplasticity than is *c-fos* activation because c-fos is an indicator of activation and not plasticity (Ons et al., 2004, 2010).

Arc protein in particular is necessary for long-term synaptic plasticity (Bramham et al., 2010), and several lines of research point to its role in long-term behavioral effects associated with drugs of abuse. Either cocaine itself or cocaine-associated cues up-regulate *arc* mRNA expression in reinforcement-related brain regions among adult male rats (Fumagalli et al., 2006,

Hearing et al., 2008, McGinty et al., 2010), and altered *arc* expression lasts long into abstinence from cocaine (Fumagalli et al., 2006, Freeman et al., 2008). Moreover, suppression of *arc* expression in the caudate putamen inhibits extinction learning in the absence of cocaine after self-administration (Hearing et al., 2010). Age-dependent expression of *arc* mRNA also leads us to predict that it may play a role in age differences in cocaine-related behavior (Crews et al., 2007). For example, basal *arc* expression levels are 1.5-3-fold higher in the cortex of adolescent than adult rats (Schochet et al., 2005). Also another psychomotor stimulant, nicotine, increases *arc* expression in the prefrontal cortex to a greater extent in adolescents than in adults (Schochet et al., 2005), although amphetamine induces more variable changes in *arc* among younger than older rats (Banerjee et al., 2009). Thus, the clear significance of *arc* in plasticity in general and drug-related neuroplasticity in particular, along with some potentially meaningful age differences in *arc* expression prompted us to explore its correlation with age-dependent cocaine-related behaviors in the present experiment. Together these data may suggest that high levels of *arc* expression attenuate some of the acute and long-term effects of cocaine (Hearing et al., 2010). We further hypothesized that higher *arc* mRNA expression in younger than older subjects reveals greater ongoing neural plasticity that may facilitate recovery from cocaine intake and thus minimize its long-term effects.

Similar to Arc, BDNF is another important factor involved in modulating activity-dependent synaptic plasticity. BDNF is released into target synapses during periods of high activity and stimulates morphological and functional modification of pre- and postsynaptic elements (Kuczewski et al., 2009). The involvement of BDNF in mediating cocaine effects is well-supported (McGinty et al., 2010). Cocaine or cocaine-associated cues up-regulate *bdnf* expression (Fumagalli et al., 2006, Hearing et al., 2008, McGinty et al., 2010), and BDNF

protein levels in the ventral tegmental area (VTA), nucleus accumbens, and amygdala progressively increase during cocaine abstinence. Furthermore, injection of BDNF into the VTA or NAc promotes reinstatement of cocaine-seeking, whereas BDNF administered to the mPFC attenuates reinstatement (Lu et al., 2004a, Berglind et al., 2007, Graham et al., 2007). Finally, BDNF activity can turn on *arc* transcription (Bramham et al., 2010). In terms of age differences in BDNF expression and activity across adolescent development, data are scarce. Gradual decreases in BDNF in the hippocampus and hypothalamus occur throughout the lifespan in rats (Silhol et al., 2005), but amphetamine-induced *bdnf* in the hippocampus and cortex is attenuated in juvenile compared with adult rats (Banerjee et al., 2009). Although specific predictions were complicated by the brain region- and drug-specific activity of BDNF, we also explored whether expression of *bdnf* mRNA in the mPFC and/or NAc correlated with age-dependent cocaine-related behaviors in the present experiment.

Generally, adolescent and adult male Wistar rats were allowed to acquire lever-pressing maintained by i.v. cocaine infusions, then sacrificed for post-mortem analysis of *arc* and *bdnf* mRNA levels in the mPFC or NAc by in situ hybridization. Expression levels were tested at three different experimental time points in separate groups of rats: immediately after the last self-administration session, or after within-session extinction and reinstatement testing at 1- or 60-days abstinence periods. Yoked saline controls provided comparisons of gene expression across age groups in drug-naïve rats.

5.3 Methods

Subjects

Male Wistar rats (Charles River Laboratories, Inc., Wilmington, MA) arrived in the laboratory at either postnatal day (PND) 22 or 70-82 and were housed in groups of two in a humidity (50%)

and temperature controlled (22 ℃) vivarium on a 12/12 h light/dark cycle (reverse cycle, with lights on at 1900 h). Rats acclimated to these conditions for 6-8 days prior to the start of experiments. Food and water were available *ad libitum* except during self-administration sessions. All subjects were observed and/or weighed daily to assess general health and responsiveness to drug exposure. All procedures were conducted in strict adherence to the "Principles of Laboratory Animal Care" and the *National Institute of Health Guide for the Care and Use of Laboratory Animals* (NRC, 2003), and the Georgia State University the Institutional Animal Care and Use Committee.

Drugs

Cocaine hydrogen chloride was obtained from Mallinckrodt Inc. (Hazelwood, MO). The concentration of stock solution was 1.25 mg/ml, and the volume of cocaine solution for each rat was adjusted according to body weight (0.07 ml/kg/s). Thus a dose of 0.36 mg/kg/infusion was provided for each rat with an infusion duration of approximately 4.0 s for adults and 1.5 s for adolescents on first day. Methohexital sodium (1% solution) was obtained from Eli Lilly (Indianapolis, IN). Variable duration of infusion (as opposed to variable concentration of drug solution) as a means to adjust drug dose does not alter cocaine self-administration in adolescent or adult rats (Li and Frantz, 2009).

Surgery

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). Rats were anesthetized with an isoflurane/oxygen vapor mixture (4-5% for initial anesthetization and 1.5-3% during surgery), and catheter tubing was passed subcutaneously from the animal's back to the

right jugular vein, inserted into the vein previously punctured with a 25 gauge needle, tied gently with suture thread, and fixed with superglue. During recovery, rats received approximately 0.2 ml Timentin (Ticarcillin Disodium and Clavulanate Potassium; 100 mg/ml, i.v.) twice daily on the first two days post-surgery, then once daily throughout the experiment. Catheters were also flushed daily with approximately 0.4 ml heparinized saline (100 USP units/1 ml). Catheter patency was confirmed in all subjects by full loss of muscle tone within 5 s of i.v. infusion of the short acting anesthetic agent, 1% Methohexital sodium, one day before the first and after the last self-administration session. Subjects that failed either patency test were eliminated from the study.

Equipment

Behavioral tests were conducted in operant conditioning chambers enclosed in sound-attenuating, ventilated environmental cubicles (Med Associates, Inc., St. Albans, VT). To start each session, a house light and white noise turned on and two levers extended into the chamber. Lever presses on the inactive lever were recorded but had no scheduled consequences. Presses on the active lever triggered a syringe pump (Med Associates, Inc., St. Albans, VT) to deliver drug solution via a stainless steel swivel (Instech Laboratories, Inc., Plymouth Meeting, PA) and polyethylene tubing attached to a catheter portal on each animal's back. Each reinforced response lit a cue light above the lever which stayed on for the duration of the infusion. The cue light, house light, and white noise were not present during a 20 s time-out (TO20) after each infusion. Drug delivery and data collection were controlled by Med Associates software (Med PC IV).

Self-Administration

Following a 6-7-day post-surgical recovery, spontaneous acquisition of cocaine self-administration began (PND35 or 83-95), with daily 2 h sessions over 13 days conducted during

the dark phase of the light-dark cycle. Non-contingent drug injections were not administered in this phase of experimentation. Lever-pressing on the active lever was reinforced by i.v. infusion of cocaine (0.36 mg/kg/infusion) under a Fixed-Ratio 1 (FR1) TO20 schedule of reinforcement.

Abstinence

After 12 sessions of self-administration, separate groups of rats remained in their home cages for 20-24 hours or 60-61 days, under standard housing conditions. Rats were handled twice per week during the abstinence period.

Extinction and Reinstatement

After abstinence from cocaine, a within-session extinction and reinstatement test was conducted (Grimm et al., 2001, Grimm et al., 2003, Li and Frantz, 2009). Six 1-h extinction sessions were followed by one 1-h cue-induced reinstatement test. During extinction, rats were connected to the metal coil tether but not the infusion tubing, white noise remained off, and the house light remained on. Neither cue-lights nor TO signals were presented after presses on either lever. Five-min breaks occurred between each successive session, during which the two levers retracted and the house light turned off.

Cue-induced reinstatement tests began with the onset of the house light, white noise, and a 5 s cue-light, followed by a 20 s TO during which the house light, white noise, and cue light were turned off. During the remainder of the cue-induced reinstatement session, presses on the active lever produced cue sequences identical to those presented during cocaine self-administration, and the pump went on, although no syringe was loaded. The only difference between self-administration and cue-induced reinstatement was that drug solution was not infused during reinstatement.

Yoked saline controls

To compare the baseline age differences in *arc* and *bdnf* expression, a subset of controls received yoked saline infusions. Lever presses by these subjects did not produce any scheduled consequences in any session. However, during self-administration, they received an infusion of saline accompanied by cues and time-out sequences whenever their age-matched self-administering counterparts earned a cocaine infusion. During the cue-induced reinstatement session, they were exposed to cue and time-out sequences at the same times as their cocaine-counterparts.

Tissue collection

At three experimental time points, immediately after the last self-administration session, after cue-induced reinstatement at 1-day abstinence, or after cue-induced reinstatement at 60-days abstinence, rats were sacrificed and tissue was collected for *in situ* hybridization (n=5 per group). Rats were anesthetized by 5% isoflurane for 3-4 minutes, decapitated, and brains removed in less than 4 min. Brains were fast-frozen in cold isopentane and stored at -80 ℃ until analysis.

***In situ* hybridization**

In situ hybridization was performed using established methods [J. McGinty at the Medical University of South Carolina (MUSC)] (e.g. Hearing et al., 2008) to test the expression of *arc* or *bdnf* mRNA in tissue sections containing the mPFC and NAc. The sequence for oligodeoxynucleotide probes complementary to rat *bdnf* (5'GCA TTG CGA GTT CCA GTG CCT TTT GTC TAT GCC CCT GCA GCC TTC CTT3') or *arc* (5'GGC AGC TTC AAG AGA GGA GGG GAC GGT GCT GGT GCT GGG GTG GTA3') mRNA were obtained from the McGinty Lab, and probes were synthesized commercially [Integrated DNA Technologies (IDT), Coralville, IN]. Using alpha-[^{33}P]-dATP (Perkin Elmer, Waltham, MA) and terminal

deoxynucleotidyl transferase (Roche Diagnostics, Indianapolis, IN), probes were labeled at the 3' end. Brain regions containing the mPFC or NAc were cut on a cryostat into 12-μm sections and thaw-mounted onto charged slides. To reduce nonspecific binding, brain sections were pretreated with 4% paraformaldehyde and 0.25% acetic anhydride in sterile 0.1 M triethanolamine/0.9% NaCl pH 8.0 followed by an ascending alcohol concentration series, chloroform, then a descending alcohol concentration series. Pretreated sections were then hybridized with labeled 1×10^6 counts per minute (cpm) of one of the probes in hybridization buffer at 37 ℃ for 20-24 hours. After post washes (4 X SSPE/DTT, 2 X SSPE/DTT at 55 ℃, 2 X SSPE, H_2O and alcohol, the sections were dried and laid on Biomax MR film (Kodak, Rochester, NY) along with ^{14}C standards (American Radiolabeled Chemicals, St Louis, MO) in an X-ray film cassette. Films were developed after 7 days for *arc*, or 14 days for *bdnf* to achieve an optimal signal/noise ratio.

Several controls were included to ensure reliability of the protocol. To test the efficacy of the protocol, mPFC sections, which are shown to have *arc* and *bdnf* mRNAs (Hearing et al., 2008) were used as positive control tissue. To test the specificity of probes binding only to RNA, the mPFC sections were digested with RNase (Roche Diagnostics, Indianapolis, IN) at 37 ℃ for 1 h prior to hybridization with the probes. As a control for this RNase digestion procedure, a subset of slides went through additional steps for digestion but without the RNase (Figure 5.1c).

Film autoradiograms were quantified with the NIH Image program. The measurements of ^{14}C standards were plotted against known dpm/mg, and a calibration curve was generated. The mean density was measured in selected mPFC regions including cingulate cortex, prelimibic cortex and infralimbic cortex, as well as the NAc including both core and shell (Figure 5.1a, b), in four adjacent sections per brain, and the average of the four measurements was taken for each sample.

Data analysis

For analysis of behavior during cocaine self-administration sessions, the total number of active lever presses over 12 sessions was analyzed using a three-way mixed measures analysis of variance (ANOVA), with age and drug (cocaine vs. saline) as between subjects factors, and session as a within subjects factor. For subjects in the cocaine treatment group, the number of infusions over 12 sessions was also analyzed using a three-way mixed measures ANOVA, with age and time point group (self-administration, 1-day or 60-days abstinence) as between subjects factors, and session as a within subjects factor. For extinction sessions, the number of active lever presses per session was analyzed using a four-way mixed measures ANOVA, with age, drug, and abstinence period as between subjects factors, and session as a within subjects factor. For analysis of time-dependent changes in reinstatement of cocaine-seeking, the number of active lever presses per reinstatement session was analyzed using a three-way between-subjects age x drug x abstinence period ANOVA. For analysis of *arc* or *bdnf* expression in the mPFC or the NAc, the measurements of the hybridization signals were analyzed using three-way between-subjects age x drug x time point ANOVAs. Correlation coefficients between the number of cocaine infusions and gene expression in cocaine animals after last self-administration were analyzed by a Pearson correlation test. Correlation coefficients between the number of total active lever presses in cue-induced reinstatement and gene expression in the cocaine animals were also analyzed at 1-day or 60-days abstinence. Follow-up ANOVAs, unpaired 2-tailed t-tests and Tukey post hoc tests were used as post hoc analyses as appropriate, and $p<0.05$ was considered significant.

5.4 Results

Cocaine-Related Behavior

During self-administration sessions, the total number of active lever presses in the cocaine treatment groups was higher than in yoked-saline controls (Figure 5.2a). An age x drug x session ANOVA revealed a three-way interaction [$F_{(1, 56)}$=2.133, p=0.017]. A follow-up two-way drug x session ANOVA revealed a main effect of drug [$F_{(1, 58)}$=68.702, p<0.001] that did not vary across sessions (no significant interaction). As a result, saline controls were excluded from subsequent analyses. A separate follow-up two-way age x session ANOVA conducted on cocaine self-administration groups revealed an unexpected age x session interaction [$F_{(11,308)}$=2.151, p=0.017], but follow-up t-tests with Bonferroni's correction (adjusted for unequal variance in active lever presses during sessions 1, 2, and 3, and 5) were not significant.

More importantly, no age differences were observed in the number of infusions earned by subjects in the cocaine self-administration groups (Figure 5.2b). The age x time point x session ANOVA revealed a significant overall three-way interaction [$F_{(22, 264)}$=1.88, p=0.011]. Dividing the data by age, follow-up two-way time point x session mixed measures ANOVAs showed main effects of session for both adolescents [$F_{(11, 132)}$=7.921, p<0.001] and adults [$F_{(11, 132)}$=4.206, p<0.001], confirming gradual increases in infusions per session. Separating data by abstinence time point groups, follow-up two-way age x session mixed measures ANOVAs showed only main effects of session in the group to be sacrificed immediately after the last self-administration session [$F_{(11, 88)}$=3.383, p=0.001] as well as the group sacrificed after testing at 1-day abstinence [$F_{(11, 88)}$=4.331, p<0.001]. For the 60-days abstinence group, an age x session interaction was significant [$F_{(11, 88)}$=2.195, p=0.02], but follow-up unpaired t-tests comparing two age groups at each session did not reveal any significant differences. Thus, the data were collapsed across the

time points to show no differences in the number of infusions per session across age groups (Figure 5.2b).

Extinction responding after just 1-day abstinence did not differ across age, drug, or session, but was lower than after 60-days abstinence (Figure 5.3a, b). A four-way age x drug x abstinence period x session ANOVA on active lever presses revealed several significant two-way interactions, including age x drug [$F_{(1, 32)}$=5.157, p=0.03], drug x session [$F_{(5, 32)}$=3.024, p=0.012], and abstinence period x session [$F_{(5, 32)}$=2.773, p=0.02], but no four-way or three-way interactions. Data were separated by time point for further analysis. A three-way ANOVA at 1-day abstinence revealed no main effects and no interactions. At 60-days abstinence, an age x drug x session ANOVA revealed main effects of session [$F_{(5,80)}$=4.595, p=0.001] (confirming a gradual decline in responding) and drug [$F_{(1,16)}$=7.784, p=0.013] (confirming more responding among cocaine groups than saline controls), and a two-way age x drug interaction that just missed significance [$F_{(1,16)}$=4.388, p=0.052] would have led to testing on the lack of drug effect in adolescent-onset groups (i.e. adolescent-onset of cocaine self-administration did not lead to significant cue-induced reinstatement of cocaine-seeking).

For cue-induced reinstatement tests, adolescent-onset groups responded at a lower level than adults, although cue-induced reinstatement increased over the abstinence period in both age groups and the number of active lever presses in cocaine groups was higher than in yoked-saline controls (Figure 5.3a, b). A three-way age x drug x abstinence period ANOVA revealed an age x drug interaction [$F_{(1, 32)}$=7.330, p=0.011] and a drug x abstinence period interaction [$F_{(1, 32)}$=11.034, p=0.002], but no significant three-way interaction. Data were separated by drug for further analysis. In cocaine self-administration groups, a two-way age x abstinence period ANOVA revealed main effects of age [$F_{(1, 16)}$=8.411, p=0.01] and abstinence period [$F_{(1,}$

$_{16)}$=5.157, p=0.03], but no interactions, thus confirming that adolescent-onset groups responded at a lower level than adults at both abstinence periods, and that animals in both age groups responded at higher levels after 60 days of abstinence than after 1 day of abstinence.

Gene expression

Arc expression in the mPFC

Arc expression in the mPFC did not differ by age, but was higher in cocaine groups than saline groups after the last self-administration session and testing at 1-day abstinence, but not at 60-days abstinence (Figure 5.4a). In cocaine self-administration groups, *arc* expression fell from its peak after the last self-administration session to lower levels after cue-induced reinstatement at 1-day or 60-days abstinence. On the other hand in saline controls, *arc* expression varied by time point, such that lower levels after the last self-administration session and testing at 1-day abstinence contrasted with higher levels after testing at 60-days abstinence. Statistical analysis began with a three-way age x drug x time point ANOVA and revealed only a drug x time point interaction [$F_{(1, 48)}$=5.732, p=0.006]. Data were collapsed across age groups for further analysis. Follow-up unpaired t-tests comparing drug treatment groups showed that *arc* expression was higher in cocaine groups than saline controls after the last self-administration session [t_{18}=-3.775, p=0.001] and after testing at 1-day abstinence [t_{18}=3.263, p=0.004], but not 60-days abstinence. Analyzing effects of time point separately in each drug treatment group using one-way ANOVAs revealed main effects of time in both cocaine [$F_{(2, 27)}$=5.348, p=0.011] and saline groups [$F_{(2, 27)}$=8.614, p=0.001]. In cocaine groups, *arc* expression after last self-administration session was higher than after testing at 1-day or 60-days abstinence, according to Tukey's post-hoc tests (p=0.01). In saline animals, *arc* expression was similar at the first two time points, but rose

significantly after testing at 60-days abstinence above the first (p=0.028) and second time points (p=0.001).

Arc expression in the NAc

Overall expression of *arc* in the NAc was higher in adolescent-onset groups compared with adults (Figure 5.4b). A three-way between-subjects age x drug x time point ANOVA revealed a significant main effect of age [F (1, 48)=4.891, p=0.032], but no other significant main effects nor interactions.

Bdnf expression in the mPFC

In the mPFC, *bdnf* expression was higher among adolescent-onset groups than in adults; Cocaine groups also showed overall higher expression than saline controls, and *bdnf* expression was higher after testing at 60-days abstinence than at previous time points (Figure 5.5a). A three-way age x drug x time point ANOVA revealed main effects of age [$F_{(1, 48)}$=11.217, p=0.002], drug [$F_{(1, 48)}$=8.557, p=0.005] and time point [$F_{(1, 48)}$=18.895, p<0.001], but no interactions. Tukey's post-hoc tests confirmed the higher *bdnf* expression after testing at 60-days abstinence than the previous time points (p<0.001).

Bdnf expression in the NAc

No differences were observed in *bdnf* expression in the NAc across age, drug or time point (Figure 5.5b). Generally low levels of *bdnf* expression were observed (e.g. close to background density)

Behavioral correlations with gene expression

The number of cocaine infusions earned during self-administration did not correlate with any measure of gene expression. Levels of cue-induced reinstatement at the 60-days abstinence time

point, however, trended toward a negative correlation with *arc* expression in the mPFC across both adolescent-onset and adult cocaine groups [$r_{(8)}$=-0.630, p=0.051] (Figure 5.6).

5.5 Discussion

In the present study, we confirmed our previous findings that cue-induced reinstatement of cocaine seeking was attenuated in male rats that self-administered cocaine as adolescents, compared with adults, despite similar rates of cocaine intake. Generally, age differences in the expression of *arc* and *bdnf* mRNA were brain region specific, and more profound changes were observed in the mPFC than the NAc in both age groups. Specifically, whereas *arc* expression in the mPFC varied significantly over drug treatment and time point in similar ways for both age groups, *arc* in the NAc and *bdnf* in the mPFC were higher in adolescent-onset than adult-onset groups at all time points. *Bdnf* mRNA in the NAc was low for both age groups at all time points (i.e. close to background density). Taken together, these behavioral and molecular findings provide partial support for our hypothesis that greater plasticity in younger subjects may contribute to their attenuated responsivity to cocaine or cocaine-associated cues.

Several aspects of the behavioral results confirm the validity of the self-administration model in our laboratory. First, cocaine self-administration groups of both ages gradually acquired lever-pressing over 12 2-hr sessions, and gradually discriminated between the active vs. the inactive lever (data not shown), as expected (Li and Frantz, 2009). Slight variations from our prior report were noted in which adults in the cocaine group appeared to press more on the active lever than adolescents during the first few days of testing, but the differences were not significant and are likely explained by a few rats exhibiting unusually high rates of pressing during those sessions (but none were statistical outliers). Second, the present data replicate our previous finding of no age differences in the number of cocaine infusions earned under these self-

administration parameters. Third, the addition of yoked saline controls that failed to acquire lever-pressing in the absence of cocaine infusions lends confidence to the conclusion that cocaine is a reinforcer in this model. Finally, low levels of lever-pressing among these saline controls extended into extinction and reinstatement tests 1 and 60 days later, whereas cocaine self-administration groups exhibited more extinction responding and/or more cue-induced reinstatement than saline controls. On the other hand, relatively low levels of extinction responding among cocaine self-administration groups was followed by significant reinstatement upon re-presentation of the previously drug-paired cues after 1 day of abstinence, but only in the adults. Although this contrasts our previous report in which cue-induced reinstatement did not differ across age groups after such a short (1 day) abstinence period, the direction of the age difference is the same, with adults responding more than adolescents. After 60 days of abstinence from cocaine, both extinction and reinstatement were more robust in adults than adolescent-onset groups, as expected (Li and Frantz, 2009). These behavioral results replicate the phenomenon in which we are interested, and provide appropriate basis from which to test for age differences in basal, cocaine-, and/or reinstatement-stimulated expression of two important neuroplasticity-related genes, *arc* and *bdnf*.

The present patterns of basal and cocaine-related *arc* expression in the mPFC of adult male rats are consistent with existing reports from several research groups. Both non-contingent and self-administration of cocaine up-regulate *arc* expression in the mPFC, and the up-regulation endures into abstinence (Fumagalli et al., 2006, Fumagalli et al., 2009). Specific cue-induced reinstatement testing also increases *arc* in the mPFC of adult rats (Zavala et al., 2008) as well as mice (Ziolkowska et al., 2011). In the absence of extinction and reinstatement testing, however, *arc* expression in the mPFC appears to decrease 1-day after cocaine self-administration (Freeman

et al., 2008, 2010). On the other hand, our novel findings with regard to the effects of cocaine and cocaine-associated cues on *arc* expression in the mPFC of adolescent-onset groups contrast two related reports. Whereas we observed similar levels of both basal and cocaine- or cue-stimulated *arc* in the mPFC of both age groups, basal and nicotine-stimulated *arc* in the cortex of adolescent rats is higher than adults (Schochet et al., 2005), while amphetamine-stimulated *arc* in the cortex and hippocampus is conversely lower in juvenile vs. adults rats (Banerjee et al., 2009).

With regard to *arc* in the NAc, our results with adult rats contrast prior reports on elevations in NAc *arc* induced by experimenter-administered cocaine (Fumagalli et al., 2006) or striatal *arc* induced by experimenter-administered amphetamine (McCoy et al., 2011). It is possible that in the NAc, behaviorally-contingent drug exposure (e.g. self-administration) fails to trigger *arc* expression. The overall higher levels of *arc* mRNA among adolescent-onset groups compared with adults, regardless of drug treatment or time point in our study, does support the concept we proposed that ongoing plasticity in younger but not older subjects may attenuate some effects of cocaine. Yet the differences in expression levels, while significant, are not robust. Future experiments will be necessary to test whether the age difference is meaningful for plasticity across these age ranges. Moreover, delineation of particular subregions of the NAc (e.g. core vs. shell) or specific neural ensembles even within core or shell (Bossert et al., 2011) that may account for the age-dependent *arc* expression in this region overall should be investigated in the future.

Cocaine and cocaine-associated cues increased *bdnf* expression in the mPFC of the present adult rats, which is consistent with other studies. After cocaine self-administration, both BDNF mRNA and protein are up-regulated in mPFC (Sadri-Vakili et al., 2010). A drug-associated context can also induce reinstatement of cocaine seeking and up-regulate *arc* and *bdnf*

expression in the mPFC after short or prolonged abstinence (Hearing et al., 2008). However, neither a single session of cocaine self-administration nor yoked cocaine alters *bdnf* expression in the mPFC (Fumagalli et al., 2009), perhaps suggesting that context and cues, but not the drug itself stimulate *bdnf* expression. In adolescents, higher levels of *bdnf* mRNA than in adults were recorded regardless of drug treatment group or time point. As with NAc *arc*, this finding could represent higher levels of ongoing plasticity early in life. Indeed, BDNF declines in several brain regions across the lifespan (Silhol et al., 2005). Yet we had predicted higher levels in the mPFC of younger rats than in adults, in particular, immediately after the last self-administration session, given that exogenous BDNF injected into this region at this time decreases later cue-induced reinstatement of cocaine-seeking in adult male rats (Berglind et al., 2007). The absence of this predicted age difference selectively at this time point could suggest that BDNF in the mPFC does not contribute to the age differences in cue-induced reinstatement or that only specific subregions or cellular ensembles in the mPFC express *bdnf* mRNA differentially across ages.

In the NAc, *bdnf* mRNA did not change at any time point. This could be explained by the fact that *bdnf* mRNA is transcribed and translated in the mPFC, then the protein is transported in an anterograde direction to the dorsal and ventral striatum (McGinty et al., 2010). Therefore, *bdnf* mRNA should not necessarily be detected in the NAc. BDNF protein in the NAc increases in a time-dependent manner along with abstinence after cocaine self-administration (Grimm et al., 2003), and future protein assays would be required to test for age differences in these effects.

Notably at the 60-days abstinence period, some element(s) of extinction and reinstatement testing increased *arc* and *bdnf* expression more than at 1-day abstinence testing for all age and drug-treatment groups. While some studies show that drug-associated cues or context increase *arc* and *bdnf* expression in cocaine-experienced animals only (Hearing et al., 2008,

Zavala et al., 2008), the present results may relate to remote memories and/or novelty. In a contextual fear conditioning model, activity-dependent genes in the anterior cingulate cortex are up-regulated by remote memory, but not recent memory (Frankland et al., 2004). Thus the up-regulation of *arc* and *bdnf* in both cocaine groups and saline controls may be due to the activation of the mPFC by remote memory of visual and auditory cues, even though those cues were not paired with behavior or drug exposure**.** Alternatively, after a long period of housing in home cages during abstinence, the self-administration chambers and environmental cues may instead be experienced as novel for rats in both cocaine and saline groups, thus inducing gene expression in the mPFC. Novelty-exposure does up-regulate *arc* expression in frontal cortex and hippocampus (Klebaur et al., 2002, Santini et al., 2011). Finally, *arc* expression in frontal cortex is also induced by stress (Ons et al., 2004), and the present 7 h procedure for extinction and cue-induced reinstatement could be stressful. This is a less likely explanation, though, because the same procedure was used after 1-day abstinence.

In summary, age differences in expression of *arc* and *bdnf* were brain region specific and depended to some degree on drug treatment group and time point of analysis. Overall expression levels of *arc* in the NAc and *bdnf* in the mPFC were higher in adolescent-onset groups than adults. Several studies show that these two genes promote synaptic plasticity and synaptic consolidation. For example, Arc can regulate actin cytoskeletal dynamics, which in turn mediate plasticity at glutamatergic synapses (Bramham et al., 2008), specific cocaine-induced changes in spine morphology (Toda et al., 2010), and drug-primed reinstatement of cocaine seeking (Toda et al., 2006). Arc also contributes to long-term depression mediated by endocytosis of AMPA receptors, which is known to be impaired by cocaine (Huang et al., 2007). Finally, Arc participates in homeostatic forms of plasticity (Bramham et al., 2008). Among other functions,

BDNF drives transcription and translation of *arc* (Bramham et al., 2010). In addition, BDNF in the mPFC can normalize cocaine-induced neuroadaptations that alter glutamate neurotransmission in the NAc (Whitfield et al., 2011). Considering these important roles of *arc* and *bdnf* in neuroplasticity, higher levels of these and possibly other neuroplasticity-related genes in younger animals than in adults may speed the turnover of cocaine-induced synaptic changes and dampen some of the long-term effects of cocaine self-administration.

5.6 Figures

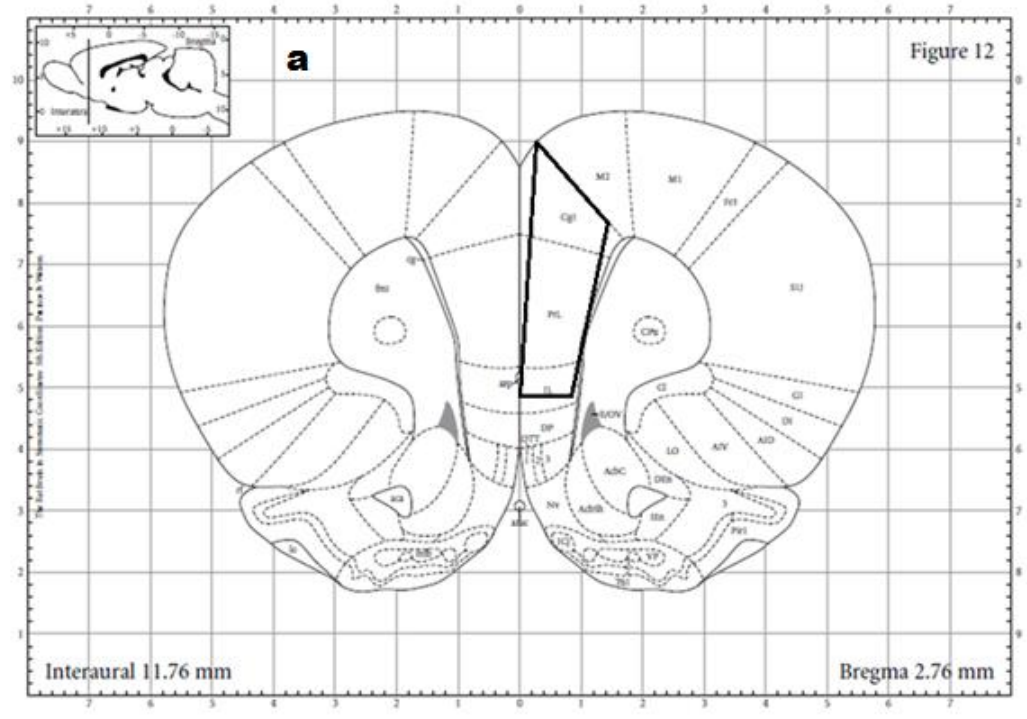
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Figure 12
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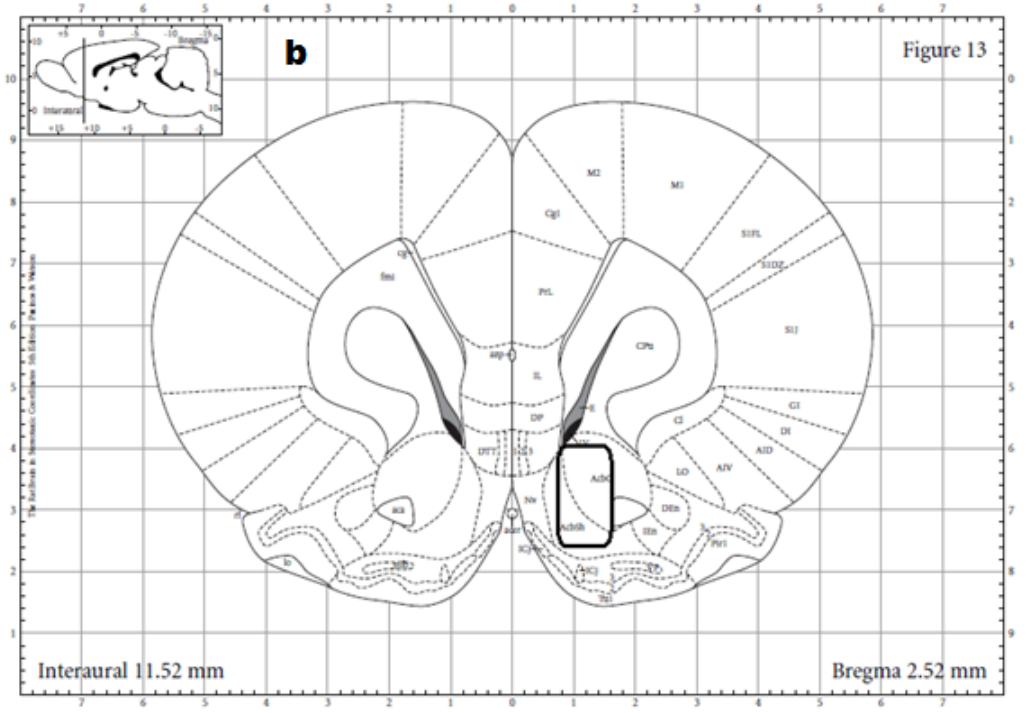
Bregma 2.76 mm


b
Figure 13
Interaural 11.52 mm
Bregma 2.52 mm

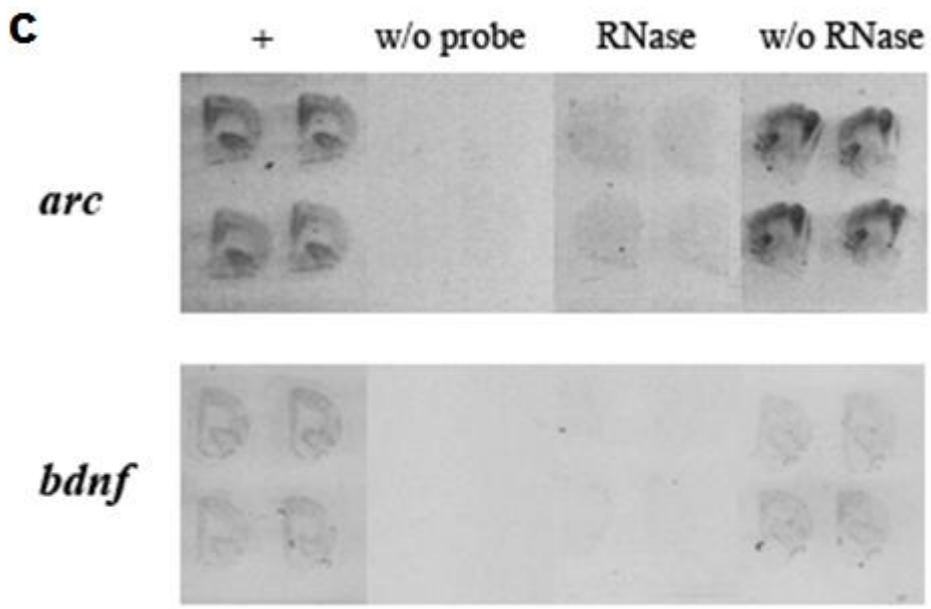


Figure 5.1. Regions for measurement in mPFC (a) and NAc (b). Controls for *in situ* hybridization (c). mPFC sections known to have targeted mRNAs for *arc* and *bdnf* showed normal labeling (+). Without *arc* or *bdnf* probes added (w/o probe), digesting mRNA with RNase before hybridization showed no labeling (RNase). Slides that went through additional steps for digestion but without the RNase added showed normal labeling (w/o RNase).

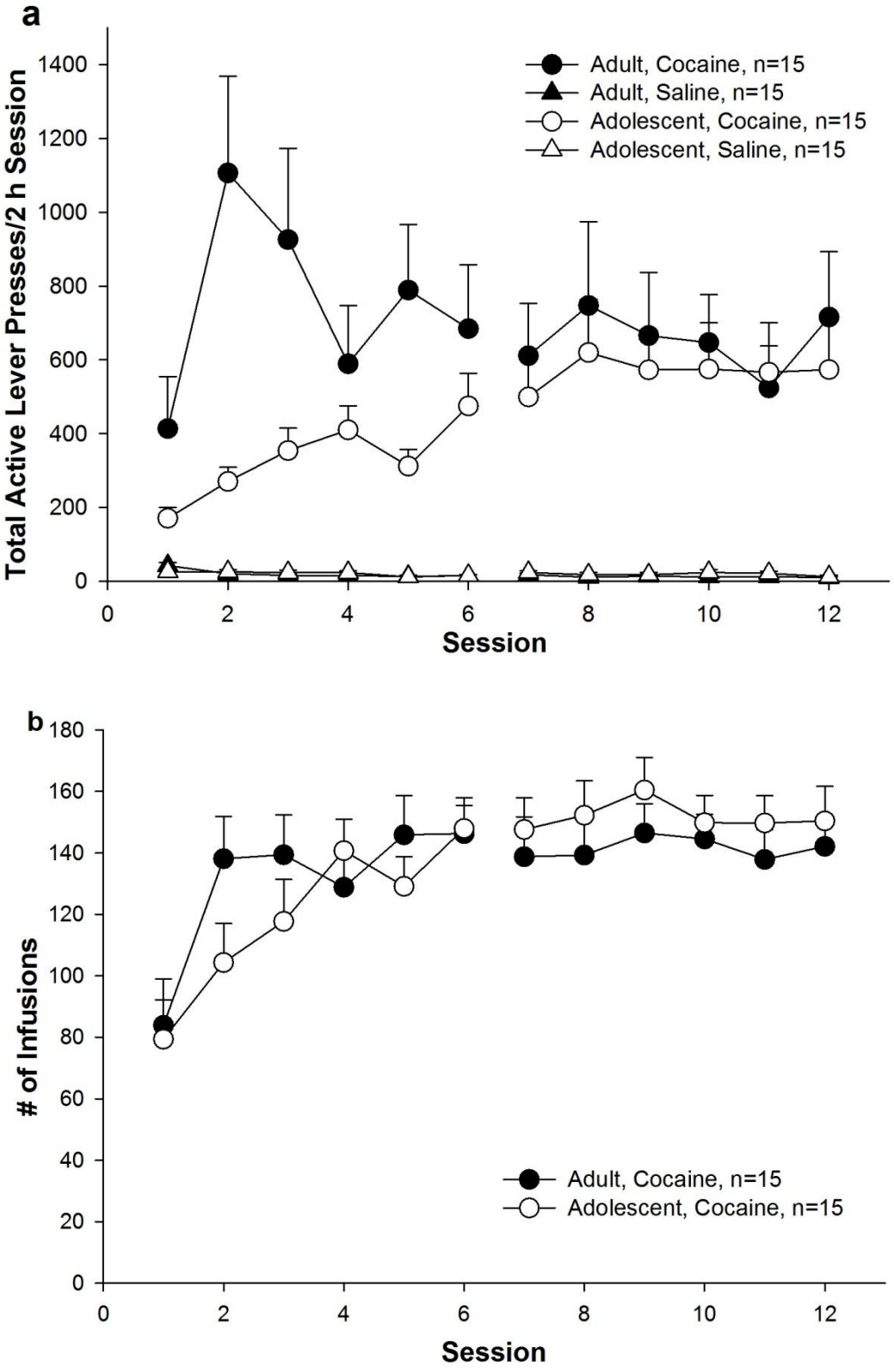

a
Total Active Lever Presses/2 h Session
1400
1200
1000
800
600
400
200
0
0
2
4
6
8
10
12
Session
Adult, Cocaine, n=15
Adult, Saline, n=15
Adolescent, Cocaine, n=15
Adolescent, Saline, n=15
b
# of Infusions
180
160
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
Session
Adult, Cocaine, n=15
Adolescent, Cocaine, n=15

Figure 5.2 Behavioral data from Self-administration. (a) Number of total active lever presses during 12 2-h self-administration sessions in cocaine and yoked saline groups. Cocaine groups pressed at a much higher level than saline controls. (b) Number of cocaine infusions taken during 12 2-h self-administration session in cocaine adolescent vs. adult rats. Points represent mean ± SEM. No statistically significant age differences were observed.

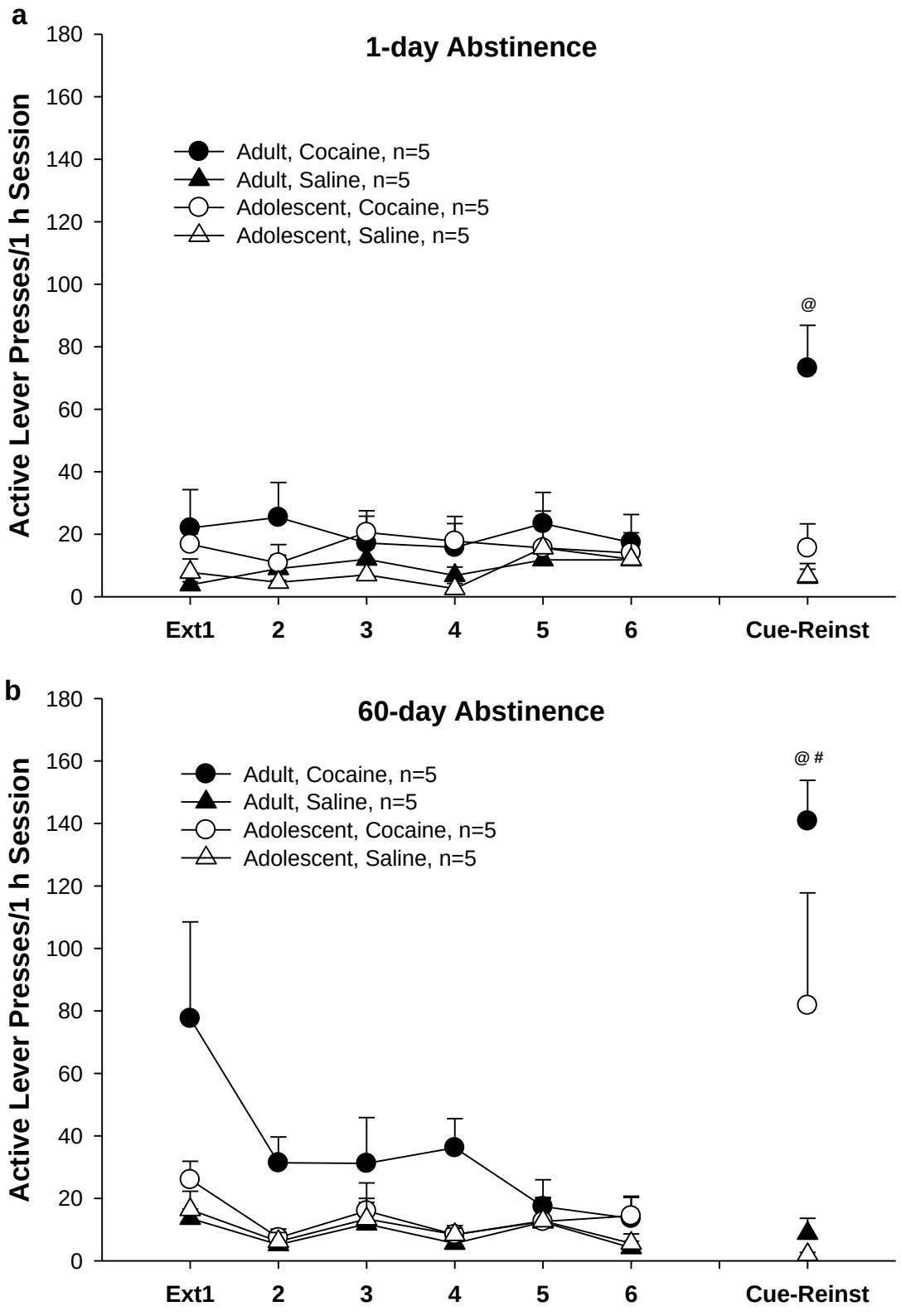

a
1-day Abstinence
Active Lever Presses/1 h Session
Adult, Cocaine, n=5
Adult, Saline, n=5
Adolescent, Cocaine, n=5
Adolescent, Saline, n=5
@
Ext1
2
3
4
5
6
Cue-Reinst
b
60-day Abstinence
Active Lever Presses/1 h Session
Adult, Cocaine, n=5
Adult, Saline, n=5
Adolescent, Cocaine, n=5
Adolescent, Saline, n=5
@ #
Ext1
2
3
4
5
6
Cue-Reinst

Figure 5.3. Active lever pressing in extinction and cue-induced reinstatement after 1-day (a) or 60-days abstinence (b). Only cocaine groups showed cue-induced reinstatement. Adolescent-onset groups responded at a lower level than adult-onset groups (@$p<0.05$), and cue-induced reinstatement increased over abstinence period in both age groups (#$p<0.05$). Points represent mean $\pm$ SEM lever presses.

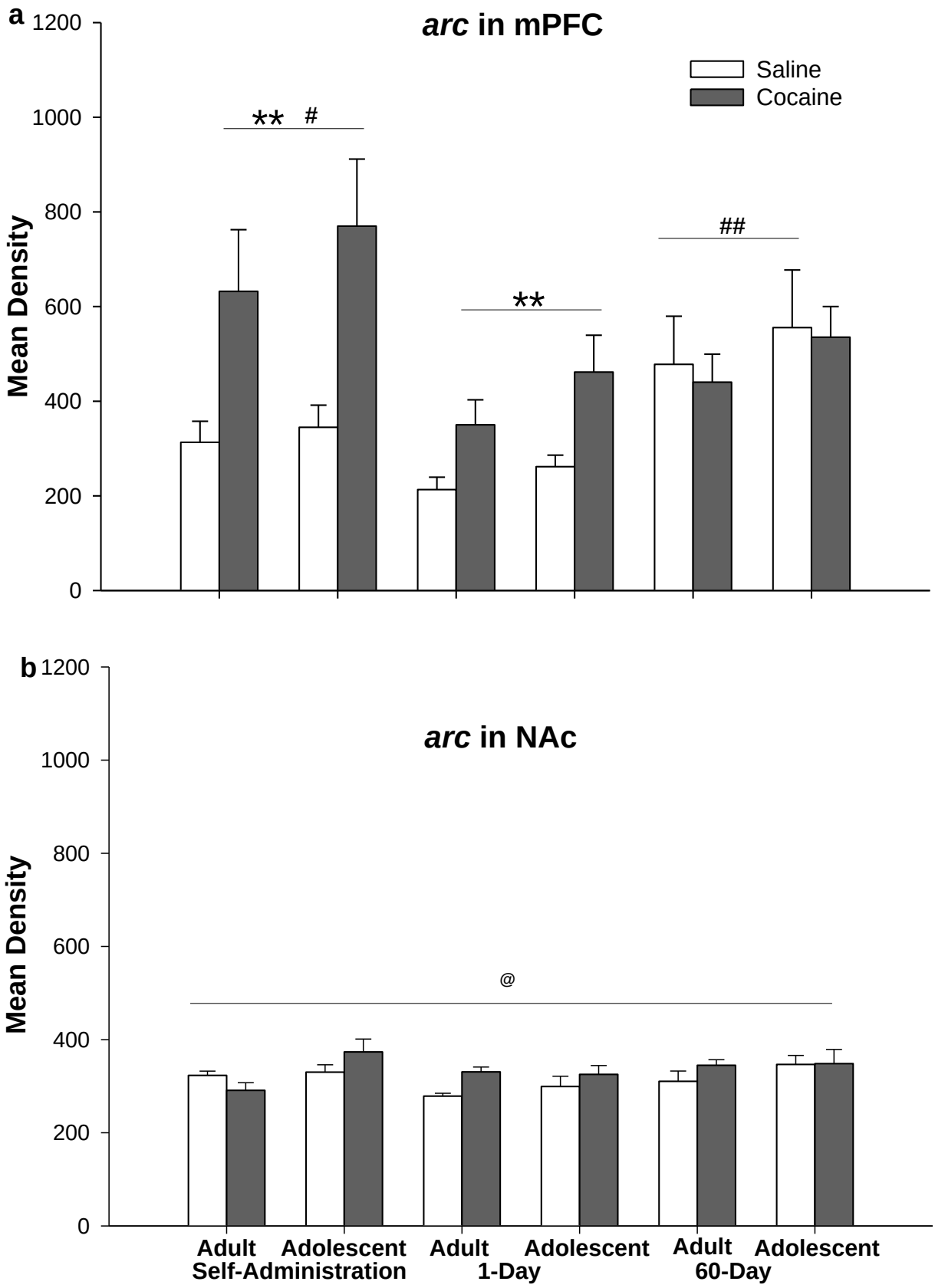

a
arc in mPFC
Saline
Cocaine
Mean Density
1200
1000
800
600
400
200
0
** #
**
##
b
arc in NAc
Mean Density
1200
1000
800
600
400
200
0
@
Adult
Adolescent
Adult
Adolescent
Adult
Adolescent
Self-Administration
1-Day
60-Day

Figure 5.4 Age differences in *arc* expression. (a) In the mPFC, no age differences were found for *arc* expression. *Arc* expression in cocaine groups was higher than in saline groups after the last self-administration session and after 1-day abstinence (**p<0.01). Also in cocaine groups, *arc* expression was higher after the last self-administration session than after cue-induced reinstatement at 1-day and 60-days abstinence (#p<0.05). In saline controls, *arc* expression was higher after cue-induced reinstatement at 60-days abstinence than after self-administration and 1-day abstinence (##p<0.01). (b) In the NAc, overall expression of *arc* was higher in adolescent-onset groups than in adults (@p<0.05). Bars represent the mean density ± SEM.

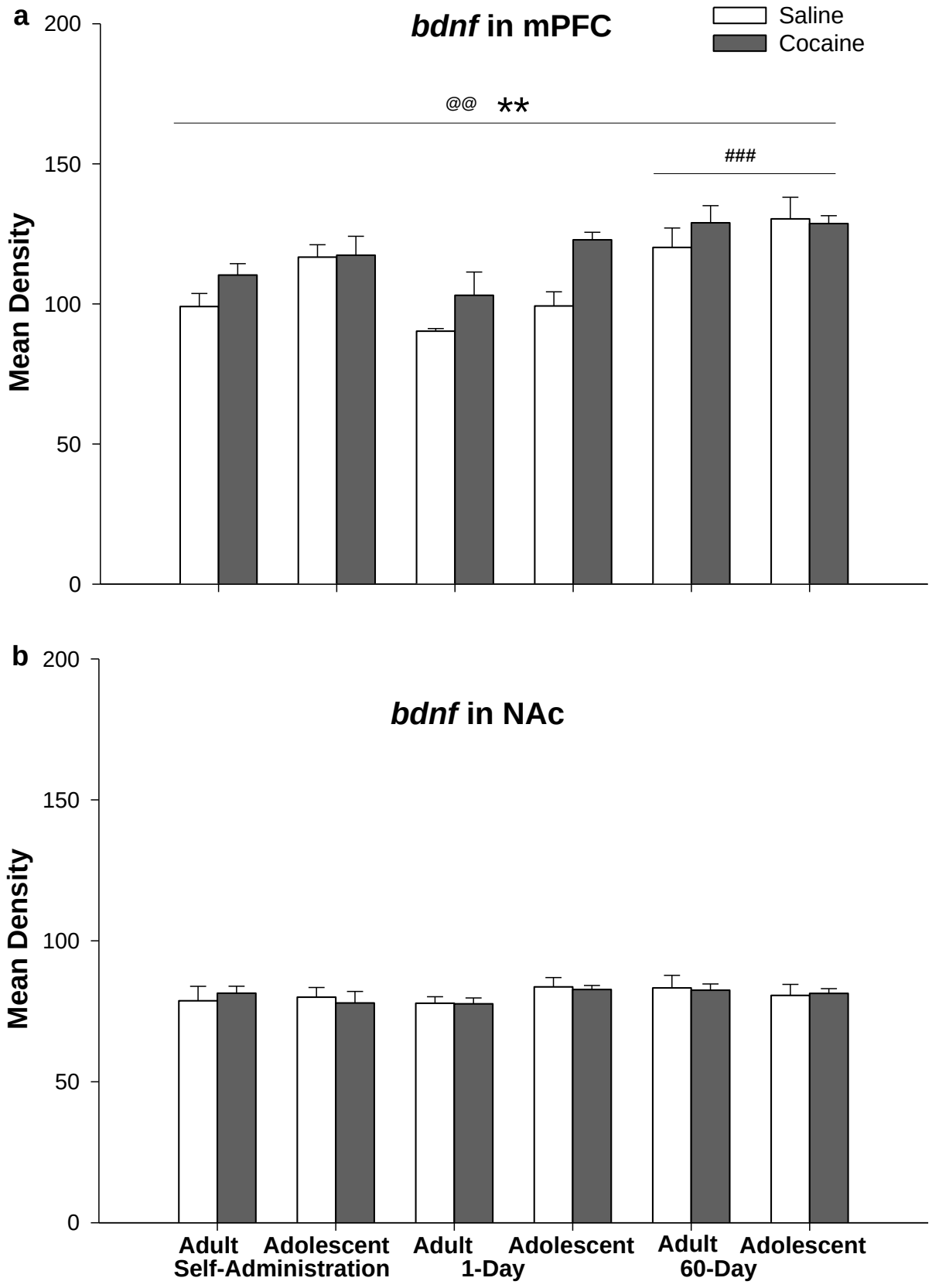

a
bdnf in mPFC
Saline
Cocaine
@@ **
###
Mean Density
200
150
100
50
0
b
bdnf in NAc
Mean Density
200
150
100
50
0
Adult
Adolescent
Adult
Adolescent
Adult
Adolescent
Self-Administration
1-Day
60-Day

Figure 5.5 Age differences in *bdnf* expression. (a) In the mPFC, overall expression of *bdnf* was higher in adolescent-onset groups than in adults (@@p<0.01). Overall expression of *bdnf* was also higher in cocaine animals than in saline groups (**p<0.01). *Arc* expression was higher after cue-induced reinstatement at 60-days abstinence than after self-administration and 1-day abstinence regardless of drug treatment (###p<0.01). (b) In the NAc, no effects of age or treatment group on *bdnf* expression were observed. Bars represent the mean density ± SEM.

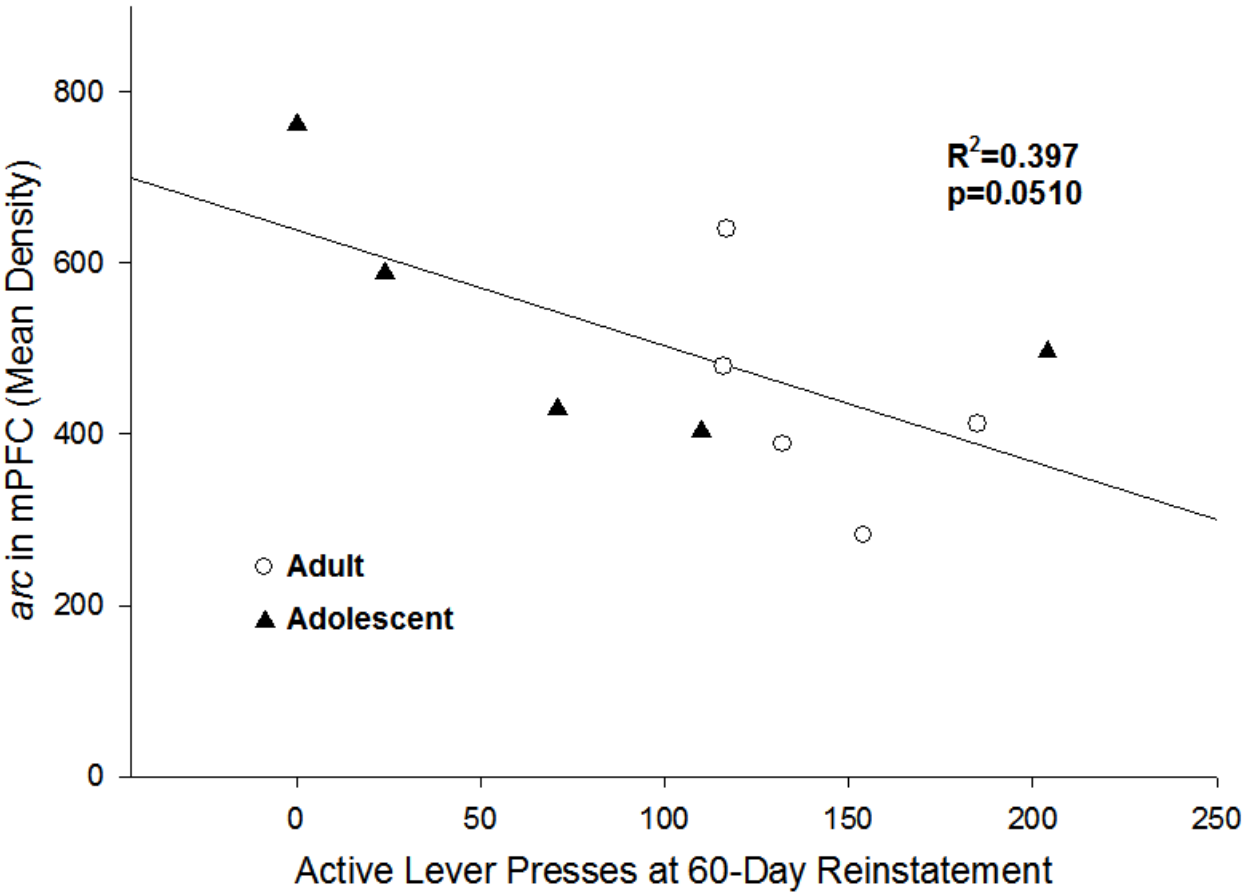


Figure 5.6 Total active lever presses during reinstatement after 60 days of abstinence and *arc* expression in the mPFC tended to be negatively correlate (p=0.051). Points represent individual subjects in each age group.

6. GENERAL DISCUSSION

6.1 Summary

This dissertation focused on the influence of adolescent-onset of cocaine self-administration on the long-term vulnerability to relapse to cocaine seeking behavior. Adolescence male rats were tested with behavioral assessment of i.v. cocaine self-administration, extinction and reinstatement, and we reported that, although taking similar amount of cocaine, male rats trained to self-administer cocaine during adolescence showed attenuated cue-induced reinstatement of cocaine seeking compared with adults. This attenuated cue-induced reinstatement did not generalize to a natural reward, sucrose pellets. Then we asked whether the attenuated reinstatement may be due to rapid developmental re-organization of reinforcement circuits (high levels of plasticity) during abstinence from cocaine in adolescent-onset groups but not in adult group. To stimulate or inhibit neuroplasticity, subjects experienced environmental enrichment or impoverishment during abstinence. Whereas our experiment confirmed previous reports that environmental enrichment during abstinence decreased cue-induced reinstatement in adult rats, environmental manipulations failed to affect cue-induced reinstatement in adolescent-onset groups. Thus, we turned to internal factors for neuroplasticity that may contribute to age-differences in reinstatement of cocaine seeking. Using *in situ* hybridization to quantify the mRNA for two neuroplasticity-related genes, activity-regulated cytoskeletal-associated gene (*arc*) and brain-derived neurotrophic factor (*bdnf*), we identified age differences in *arc* and *bdnf* expression after cocaine self-administration or cue-induced reinstatement of cocaine seeking were brain region specific. Overall, *arc* expression in the nucleus accumbens (NAc) and *bdnf* expression in the medial prefrontal cortex (mPFC) was higher in adolescent-onset than in adult groups. Together our data suggest that adolescence in rodents may be a period of relative

biological resistance to some long-term drug effects. In this general discussion, the significance and limitations of this dissertation research will be discussed, as well as future directions to further understand some potential mechanisms underlying age differences in reinstatement of cocaine seeking.

6.2 Adolescent resistance to long-term drug effects

Our studies support other emerging evidence showing that adolescent subjects, at least in rodents, may actually be less affected by some enduring effects of drug use than adults (Schramm-Sapyta et al., 2009). Similar to our results, a different laboratory shows that cue-induced reinstatement of cocaine seeking is lower in adolescents than in adults, although cocaine- and stress-induced reinstatement is higher among the adolescents in that study (Anker and Carroll, 2010). Adolescent-onset groups with a history of nicotine self-administration also show less resistance to extinction of nicotine than adults (i.e. they stop seeking nicotine faster than adults) (Shram et al., 2007). Using other drugs, research from our lab also shows the same age-dependent effects as this dissertation; after morphine self-administration by adolescent or adult rats, cue-induced reinstatement is attenuated in the younger cohort (Doherty et al., 2009), and context- and cue-induced reinstatement is also attenuated in the younger rats that self-administer heroin (Doherty et al., in press).

Both acute and enduring aversive effects of abused drugs also appear less intense in adolescent than in adult rats (Schramm-Sapyta et al., 2009). For example, conditioned taste aversion induced by a single injection of a high dose of cocaine is less profound in adolescent rats than in adults (Schramm-Sapyta et al., 2006). In terms of somatic and affective signs of aversive withdrawal, cocaine withdrawal has not been compared between adolescent and adult rodents. Opioid, nicotine and ethanol produce less intense withdrawal in adolescent rats than

adults. For nicotine, less withdrawal-associated conditioned place aversion and anxiety-like behavior are also observed in adolescent rats than in adults (O'Dell et al., 2004, 2007). In particular, our lab members show that after heroin exposure, withdrawal signs are less robust in adolescents, and their food intake and body mass are less affected by drug abstinence than are these physiological signs in adults (Doherty et al., in preparation). For ethanol, similar less severe withdrawal symptoms are reported in adolescents than in adults, such as withdrawal-induced reduction in social interaction (Doremus et al., 2003, Varlinskaya and Spear, 2004).

Finally, some of the cognitive impairments induced by prolonged cocaine exposure are less severe in adolescent than adult rats. For example, performance on an amygdala-dependent task remains intact after cocaine self-administration during adolescence, but is impaired after cocaine self-administration during adulthood (Kerstetter and Kantak, 2007). On the other hand, a greater impairment in an orbitofrontal cortex-related cognitive task occurs after cocaine intake during adolescence than in adulthood (Harvey et al., 2009). Moreover, the cognitive impairments produced by the depressant drugs ethanol and THC are more severe in adolescent than adults (Sircar and Sircar, 2005, Quinn et al., 2008). These finding suggests that drug-induced cognitive impairments are brain-region and drug-specific.

Despite the limitations of animal models to mimic complex human behavior, our results suggest that the adolescent phase of development may be associated with biological resistance to some of the long-term effects of drugs. This tentative conclusion contradicts common interpretations of human survey data, as high rates of initiation of drug use during the teenage years are often cited to support the idea that adolescent subjects are more sensitive to illicit drugs than adults (Smith, 2003, SAMHSA, 2009). A more conservative conclusion from our studies and related literature might be that age differences in acute and enduring drug effects depend on

drug type, brain regions most affected, specific age at exposure or self-administration, and potential interactions with environmental parameters.

6.3 Biology x environment interactions in drug addiction

It is now generally accepted that both biological (e.g. genetic) and environmental influences account for mental disorders, including drug addiction (Wermter et al., 2010). The relative influence of biological vs. environmental factors may vary by developmental stage. For example, human twin studies suggest that for nicotine, alcohol, and cannabis, young adolescents are more heavily influenced by familial environmental factors than adults, and remain resistant to genetic factors. As individuals age into adulthood, however, genetic factors become more influential than environmental factors (Kendler et al., 2008).

Animal models also support the biology x environment interaction. Using genetic mutations, many central nervous system disorders, such as neurodegenerative diseases, developmental abnormalities and psychiatric disorders, have been modeled in rodents. In some cases, housing the mutant animals in enriched environments can prevent, alleviate or delay the symptoms of the modeled disorder (Nithianantharajah and Hannan, 2006), providing solid evidence for an the interaction between biology and environment. Regarding drug addiction, rat lines selectively bred for high levels of cocaine self-administration take five times more amount of cocaine than lines bred for low levels of intake, suggesting that genetic factors can determine cocaine intake (He et al., 2008). Yet environmental enrichment has both "preventive" and "curative" effects on drug-taking and drug-seeking behavior (Stairs and Bardo, 2009, Solinas et al., 2010).

In our environmental enrichment study, we expected younger groups to be more affected by environmental changes during abstinence from cocaine self-administration than adults.

Instead, we found that the cue-induced reinstatement in the adolescent-onset group, which was already low even in impoverished conditions, could not be further attenuated by enriched conditions. In fact, when both adolescent and adult age groups were housed in enriched conditions, they exhibited similar levels of cue-induced reinstatement, i.e. the adults came down to the level of the younger group. This raised the question whether some internal neuroplasticity associated with biological development could prevent or reverse the neuroadaptations thought to be induced by cocaine in adolescent-onset groups, and thus limit the influence of environmental manipulations. Indeed, cocaine induces neuroplastic changes in dopamine and glutamate systems in brain motivational circuitry, including prefrontal cortex, nucleus accumbens, amygdala, etc, that mediate the long lasting behavior of addiction at least in adults (Kalivas and McFarland, 2003, Kalivas and O'Brien, 2008, Thomas et al., 2008). This circuitry is also undergoing robust developmental organization during adolescence (Smith, 2003, Crews et al., 2007) (Figure 6.1). For example, cortical regions show hypermetabolism during adolescence, suggesting higher levels of neuronal activity (Kim et al., 2009). Many excitatory glutamatergic connections are lost during development into adulthood (van Eden et al., 1990). Dopamine input to the prefrontal cortex is at its highest level during adolescence, and dopamine receptor numbers peak during adolescence, then prune to adult levels in the prefrontal cortex (Teicher et al., 1991, Andersen et al., 2002b, Brenhouse et al., 2008). Therefore, any cocaine-induced neuroplasticity may be prevented, overshadowed or perhaps normalized by this developmental organization in adolescent brains, and environmental enrichment may not be able to further minimize the effects of cocaine.

Supporting this hypothesis that ongoing neuroplasticity is higher in younger rats than adults, our data showed that expression of *arc* and *bdnf*, two effector genes, was higher in

younger animals than in adults. The protein products of *arc* and *bdnf* are important in helping to form, strengthen, and stabilize synapses during learning and memory, and they promote survival and differentiation of neurons (Tzingounis and Nicoll, 2006). Arc and BDNF are suggested to induce counteradaptations against some cocaine-induced behavioral and biological changes. For example, suppression of *arc* in caudate putamen blunts extinction learning in the absence of cocaine after self-administration (Hearing et al., 2010), and this effect may through Arc-dependent actin cytoskeletal dynamics (Toda et al., 2006, Messaoudi et al., 2007). Arc also contributes to long-term depression mediated by endocytosis of AMPA receptors, which is known to be impaired by cocaine (Huang et al., 2007). Among other functions, BDNF actually drives transcription and translation of *arc* (Bramham et al., 2010). In addition, BDNF in the mPFC can normalize cocaine-induced neuroadaptations that alter glutamate neurotransmission in the NAc (Whitfield et al., 2011). Considering these important roles of *arc* and *bdnf* in neuroplasticity, higher levels of these genes in younger animals than adults may speed the turnover of cocaine-induced synaptic changes and dampen some of the long-term effects of cocaine self-administration. Future experimentation is necessary in order to test this idea (See Future Direction in section 6.6).

6.4 Additional neuroplasticity genes and brain regions that may contribute to age differences in reinstatement of drug seeking

As discussed above, we tested for age differences in expression of two genes associated with neuroplasticity, *arc* and *bdnf*, in two brain regions, the mPFC and NAc. However, numerous other genes and brain regions are involved in drug-taking and seeking behavior. For example, *c-fos, zif268, c-jun,* and homer1 could contribute to age differences in drug addiction. As an immediate early gene, *c-fos* and its protein product are extensively used as indicators of neural

activity (Chaudhuri, 1997). *C-fos* expression induced by cocaine exposure promotes and maintains long-term neuroplasticity associated with drug use (Nestler, 2001). Re-exposure to cocaine-associated environment induces *c-fos* expression in mesolimbic and mesocortical brain regions, even after long period of abstinence (Freeman et al., 2008, Hearing et al., 2008). Another immediate early gene, z*if268*, is induced in several brain regions, such as prefrontal cortex and the NAc, by re-exposure to cocaine-associated contexts (Freeman et al., 2008, Hearing et al., 2008). Moreover, *zif268* in the amygdala is required for reconsolidation of drug memory to associate environmental cues with cocaine reward (Lee et al., 2005). Finally, cocaine induced *c-fos* and *zif268* expression are age-dependent. For example, baseline expression of *c-fos* is higher, but nicotine-induces *c-fos* is lower in adolescent cortex than adults (Schochet et al., 2005). Less *zif268* expression is induced by acute cocaine exposure in striatal cortical regions of adolescent rats than adults (Caster and Kuhn, 2009).

Although we currently report expression of two effector genes in two of the most important brain regions for cue-induced reinstatement of cocaine seeking, the mPFC and NAc, finer-grain analysis of our data will enhance our contributions to this field significantly. For example, particular subregions of the mPFC and NAc (e.g. core vs. shell), or even specific neural ensembles within these regions (Bossert et al., 2011) can be investigated in the future. Whereas we measured gene expression in the entire mPFC and the entire NAc for this report, subregions of these brain areas may play different roles in cocaine seeking. For example, the prelimbic and infralimbic subregions of the mPFC are suggested to promote or inhibit cocaine-seeking, respectively (Peters et al., 2009). Data from our lab showed that adolescent-onset rats had similar numbers of neurons in the prelimbic region, but more neurons in the infralimbic region than adults, although neither of them show c-Fos activation during heroin seeking in adolescent-onset

groups. With regard to the NAc, it contains two anatomically and functionally distinct subregions: core and shell (Doherty et al., in press). The core receives inputs from the prelimbic cortex and may promote drug-seeking, while the shell receives inputs from the infralimbic cortex and may inhibit drug-seeking (Kalivas, 2008). Furthermore, emerging data suggest that drug and drug-associated cues also active specific neural ensembles, but not all neurons, within the mPFC and the NAc (Carelli, 2004, Bossert et al., 2011). Measuring the signal density of entire brain regions may thus overshadow the changes in these subregions or neural ensembles. Therefore, constraining the measurement of mPFC and NAc may give us more precise information regarding age differences in neuroplasticity-related gene expression. In the future, laser capture microdissection could be used to isolate then analyze gene expression in only those neurons activated during reinstatement testing.

For additional brain regions of interest, a prime example is the amygdala, part of the limbic system that performs a primary role in the formation and retrieval of emotional-related memory. The amygdala receives input from and projects to several brain regions known for mediating drug addiction, such as the ventral tegmental area, the NAc and the prefrontal cortex (Buffalari and See, 2010). Inactivation of the basolateral amygdala immediately prior to a conditioning session or before reinstatement testing abolishes the ability of cocaine-associated cues to reinstate drug-seeking, but has no effects on self-administration, thus suggesting a specific role of amygdala in establishing and retrieving the memory of conditioned stimuli (Kruzich and See, 2001). Furthermore, the central amygdala is critical to the time-dependent increases in cue-induced reinstatement known as incubation (Lu et al., 2005, Lu et al., 2007). Thus, to explore the mechanisms for attenuated incubation in our adolescent-onset groups, the amygdala is the most obvious next target. Based on our hypothesis that higher developmental

plasticity in younger animals than adults contributes to attenuated cocaine seeking, we could test whether there are higher rates of plasticity in the amygdala in younger animals than adults, in terms of higher levels of expression of several neuroplasticity-related genes.

6.5 Limitations of using rodent models for complex human behavior

Our results, countering predictions based on common interpretations of human survey data, suggested that adolescence might be a developmental stage associated with some resistance to the long-term motivation to seek cocaine or resistance to the salience of drug-associated cues. Though numerous studies validate the reinstatement model for investigating relapse behavior (Katz and Higgins, 2003, Shaham et al., 2003), many others factors known to influence drug-taking and seeking in human are not included in the current rodent model. Besides the dramatic biological differences, social factors such as peer-pressure and parental involvement significantly influence drug-related behavior in teenagers. Education factors, such as exposure to substance use prevention messages and program, can dramatically decrease drug use in human adolescents (SAMHSA, 2009). Environmental factors are also important for drug use in human. Though we have somewhat introduced environmental factors in our study, the environment faced by human subjects are much more complex than laboratory conditions. Therefore, based on the evidence above, we may at best conclude that adolescence in rodents is a period of relative biological resistance to some long-term drug effects.

Another factor of consideration is how to define drug addiction in rodents. The Diagnostic and Statistical Manual for Mental Disorders version IV (DSM-IV, 1994) provides criteria for drug addiction including loss of the ability to adjust drug intake as a function of environmental contingencies. For example, addicts cannot stop taking drugs despite physical ailments and social or interpersonal problems caused by drug use, neither can they stop seeking

drugs under the situation when drug is not available or excessively high effort is required to get it. The people who become addicted only represent a small subset of recreational drug users. Human studies report a correlation between early onset of drug use and more chance to develop addition in later life (SAMHSA, 2009), which is not supported by studies in adolescent rodents showing resistance to long-term effects of drugs (Schramm-Sapyta et al., 2009). One explanation is that the current used self-administration, extinction and reinstatement model may be not equivalent to drug addiction. Similar to drug use in human, in rodent model, only a restricted number of subjects develop addiction-like behavior after prolonged drug exposure, and there is a persistent impairment in synaptic plasticity in addict rats only (Kasanetz et al., 2010). To better represent addiction-like behavior, more complex testing should be used (Schramm-Sapyta et al., 2009), such as progressive ratio schedule to assess motivation to seek drug (Roberts et al., 1989), punished responding to model compulsivity (Vanderschuren and Everitt, 2004), and extended access to model binge use (Mantsch et al., 2004). Though these models began to be used in studies comparing adolescents and adults, for example, using extended access model, our lab showed that escalation of morphine intake was similar across ages (Doherty et al., 2009), thus the results are mixed. Therefore, more research needs to be done in order to fully answer the question whether early onset of drug use will lead to vulnerability to addiction.

6.6 Future directions

For immediate next-steps, some technical consideration could be addressed to confirm the results in the current dissertation. For example, as mentioned above, more complex schedules of reinforcement in the self-administration model, e.g. progressive ratio, punished responding and extended access schedules, could be used to characterize addiction-like behavior more completely in adolescent rats compared with adults. Protein products of *arc* and *bdnf* could be

measure by Western blot or immunohistochemistry in tissues obtained from the mPFC and the NAc to confirm and explore the age differences in expression of the neuroplasticity-related genes. As discussed in section 6.4, age differences in *arc* and *bdnf* mRNA and protein expression in the subregions and specific neuronal ensembles in mPFC and the NAc, as well as other brain regions, e.g. amygdala, could be tested.

Beyond these immediate suggestions for future research, mechanisms underlying the attenuated cue-induced reinstatement in adolescent-onset groups compared with adults should be investigated. We hypothesize that the developmental changes in the mesolimbic and mesocortical pathways reduce cue-induced reinstatement of cocaine seeking in younger animals by preventing or reversing the pathological neuroplasticity induced by cocaine. First, we could test whether some specific cocaine-induced pathological changes in dopamine and glutamate systems in adults either fail to occur in adolescent-onset groups or occur but resolve quickly back to basal activity during drug abstinence in younger subjects. Then, we could ask whether higher levels of developmental neuroplasticity in younger animals than adults attenuate cue-induced reinstatement. For example, we could test whether higher levels of *arc* in the NAc and *bdnf* in the mPFC in younger animals than adults are responsible for age differences in cue-induced reinstatement of cocaine seeking. Finally, we could explore age differences in DNA methylation and histone modifications in regulation of neuroplasticity-related gene expression.

Cocaine induces neuroplastic changes in dopamine and glutamate systems in brain motivational circuitry, including prefrontal cortex, nucleus accumbens, amygdala, etc, that mediate the long lasting behavior of addiction (Kalivas and McFarland, 2003, Kalivas and O'Brien, 2008, Thomas et al., 2008). This circuitry is also undergoing robust developmental organization (Smith, 2003, Crews et al., 2007) (see section 6.3 and Figure 6.1). With regard to

the dopamine system, its role in reinstatement of cocaine seeking is clear in adult animals. Infusions of dopamine D1 receptor agonists in the NAc dose-dependently reinstate cocaine seeking, while D1 receptor antagonists prevent cocaine seeking and decrease cue-induced activity in mPFC and basolateral amygdala (Ciccocioppo et al., 2001). Meanwhile, the dopamine system undergoes extensive postnatal development. Dopamine input to the prefrontal cortex is at the highest level during adolescence, and specifically dopamine D1 receptor numbers peak during adolescence, then prune to adult levels in the prefrontal cortex (Brenhouse et al., 2008). In the caudate putamen and NAc, dopamine receptors also peak during adolescence (Tarazi et al., 1999). Therefore, the age differences in cocaine seeking may be due to the developmental differences in the dopamine system. One way to test this hypothesis would be to infuse a dopamine D1 receptor agonist, such as SKF 81297, into the mPFC or the NAc during, after cocaine self-administration, or before cue-induced reinstatement. If developmental overexpression of D1 receptors in the mPFC or the NAc somehow attenuates the effects of cocaine and cocaine-associated cues in younger vs. adult rats, then elevating D1 receptors activity with the exogenous agonist around the time of cocaine self-administration or before reinstatement should increase subsequent reinstatement of cocaine seeking to adult levels.

The importance of the glutamate system in reinstatement of cocaine seeking has also received remarkable attention recently. A decrease in basal levels of glutamate in the NAc after repeated drug exposure and an enhanced release of glutamate during reinstatement are common among cue-, stress-, and drug-primed reinstatement (Knackstedt and Kalivas, 2009). Decreased extracellular glutamate is caused by down-regulation of the cystine-glutamate antiporter, which normally exchanges extracellular cystine for intracellular glutamate (Baker et al., 2002). Changes in postsynaptic glutamate receptors also play role in reinstatement. Increases in Ca^{2+} permeable

GluR2-lacking AMPA receptors in the NAc contribute to incubation of cocaine-seeking (Conrad et al., 2008a). Moreover, NAc neurons show an LTP-like state, as well as impaired LTD during abstinence from cocaine (Martin et al., 2006), suggesting prolonged changes in glutamate transmission in the NAc after cocaine exposure. Linking these results with adolescent drug sensitivity, the glutamate system undergoes dramatic developmental organization during adolescent brain maturation, like the dopamine system (Crews et al., 2007). For example, NMDA and AMPA receptors decline in cortical and limbic system during peri- and post-weaning development (Insel et al., 1990), and LTP is more frequently recorded in the NAc in adolescents compared with adults, suggesting higher levels of plasticity in younger animals (Schramm et al., 2002). Moreover, chronic ethanol exposure and withdrawal can induce age-dependent expression of NMDA receptor subunits (Pian et al., 2010). Therefore, either or both baseline and drug-related age differences in glutamate transmission could contribute to the age differences in cocaine seeking we observed. For example, whereas cocaine self-administration leads to greater expression of Glu-R2-lacking AMPA receptors in the NAc of adult rats, these AMPA receptor characteristics might not change in younger rats, or perhaps those that change are also those that are pruned during development, leaving only normal glutamate function behind. If this is the missing element in adolescent subjects, then we should be able to drive up levels of cue-induced reinstatement expression of Glu-R2-lacking AMPA receptors by deleting the GluR2 subunit using small-interfering RNA (siRNA) in the NAc during abstinence from cocaine.

Then, we would ask whether higher levels of developmental neuroplasticity in younger animals than adults prevent or reverse some of the cocaine-induced neuroadaptations in the brain, and thus attenuates cue-induced reinstatement in younger animals. In this dissertation, we

showed *arc* expression was higher in the NAc of younger animals than adults. *Arc* is one of the effector genes that contribute to formatting, strengthening, and stabilizing synapses (Bramham et al., 2008, 2010, Shepherd and Bear, 2011). Arc is suggested to evolve counteradaptations against cocaine-induced biological and behavioral changes. For example, Arc can regulate actin cytoskeletal dynamics, which in turn mediate plasticity at glutamatergic synapses (Bramham et al., 2008), specific cocaine-induced changes in spine morphology (Toda et al., 2010), and drug-primed reinstatement of cocaine seeking (Toda et al., 2006). Arc also contributes to long-term depression mediated by endocytosis of AMPA receptors, which is known to be impaired by cocaine (Huang et al., 2007). Finally, suppression of *arc* in the caudate putamen blunts extinction learning in the absence of cocaine after self-administration in adults (Hearing et al., 2010). Therefore, we hypothesize that higher *arc* expression in the NAc of younger animals than in adults during abstinence could prevent or reverse some of the cocaine-induced pathological changes in the glutamate system, and decrease reinstatement of cocaine seeking in younger animals. Arc protein expression in the NAc in adolescent-onset groups could be inhibited by infusions of antisense oligodeoxynucleotides (Ploski et al., 2008). We predict that inhibition of Arc would increase reinstatement of cocaine seeking in adolescent-onset groups to adult levels, and cocaine would induce similar pathological changes in glutamate system in adolescent-onset groups as adults, e.g. elevated expression of GluR2-lacking AMPA receptors.

We also observed higher *bdnf* expression in the mPFC of younger animals than adults. Similar to Arc, BDNF is another important factor for modulating activity-dependent synaptic plasticity. Among other functions, BDNF actually drives transcription and translation of *arc* (Bramham et al., 2010). BDNF administered to the mPFC can normalize cocaine-induced neuroadaptations that alter glutamate neurotransmission in the NAc (Whitfield et al., 2011), and

it attenuates reinstatement in adults (Berglind et al., 2007). Therefore, we hypothesize that higher *bdnf* expression in the mPFC in younger animals than in adults during abstinence could decrease reinstatement of cocaine seeking in younger animals and might actually induce higher *arc* expression in the NAc. To test this hypothesis, we would decrease BDNF expression in the mPFC in adolescent-onset groups by infusions of antisense oligodeoxynucleotides during abstinence (Reibel et al., 2000), and we predict that this manipulation would increase reinstatement of cocaine seeking in adolescent-onset groups to adult levels, and may also decrease *arc* expression in the NAc.

Finally, we could ask why higher levels of neuroplasticity-related gene expression occur in younger animals than adults. A common method of gene regulation is modification of chromatin structure via DNA methylation and/or histone modifications, such as acetylation. DNA de-methylation and histone acetylation "relax" chromatin and facilitate gene expression (Martinowich et al., 2003). Repeated cocaine dynamically regulates chromatin structure (Anier et al., 2010, Maze et al., 2011), which contributes to some neural changes induced by cocaine (Im et al., 2010, Wong et al., 2011). Therefore, we hypothesize that higher levels of neuroplasticity-related gene expression in younger animals than adults could be related to higher baseline levels of DNA de-methylation and histone acetylation in younger animals. We predict that decreasing DNA de-methylation (i.e. increasing DNA methylation) by infusions of DNA methyl transferase (Roth and Sweatt, 2009), or decreasing histone acetylation by infusions of histone deacetylase (Romieu et al., 2008), during abstinence in younger animals would decrease expression of neuroplasticity-related genes and increase reinstatement of cocaine seeking to adult levels.

6.7 Overall summary and conclusion

In summary, using a rodent model of adolescence and behavioral assessments of intravenous (i.v.) cocaine self-administration, extinction, and reinstatement of cocaine-seeking we found that younger animals might have biological prevention against long-term effects of cocaine. Although taking similar amounts of cocaine during self-administration, adolescent-onset groups surprisingly exhibited attenuated time-dependent increases in cue-induced reinstatement of cocaine-seeking compared with adults, and this attenuated incubation did not extend to a natural reward, sucrose pellets. Then, we showed that environmental manipulations during abstinence had no effect in adolescent-onset groups, whereas the enriched environment attenuated cue-induced reinstatement in adult-onset groups compared with their impoverished counterparts. Finally, we showed that age differences in *arc* and *bdnf* expression were brain region specific, and overall expression of *arc* in the NAc and *bdnf* in the mPFC were higher in adolescent-onset than adults. Together our data suggest that adolescence in rodent may be a period of relative biological resistance to some long-term drug effects, and thus that intervention programs targeting adolescent drug abusers may have high success rates. Also, our study suggests that adolescent rats may provide a natural rodent model for protection against long-term effects of cocaine. Exploring the neural and molecular substrates that mediate the age differences could provide insights into finding the therapeutic targets for treatment of addiction.

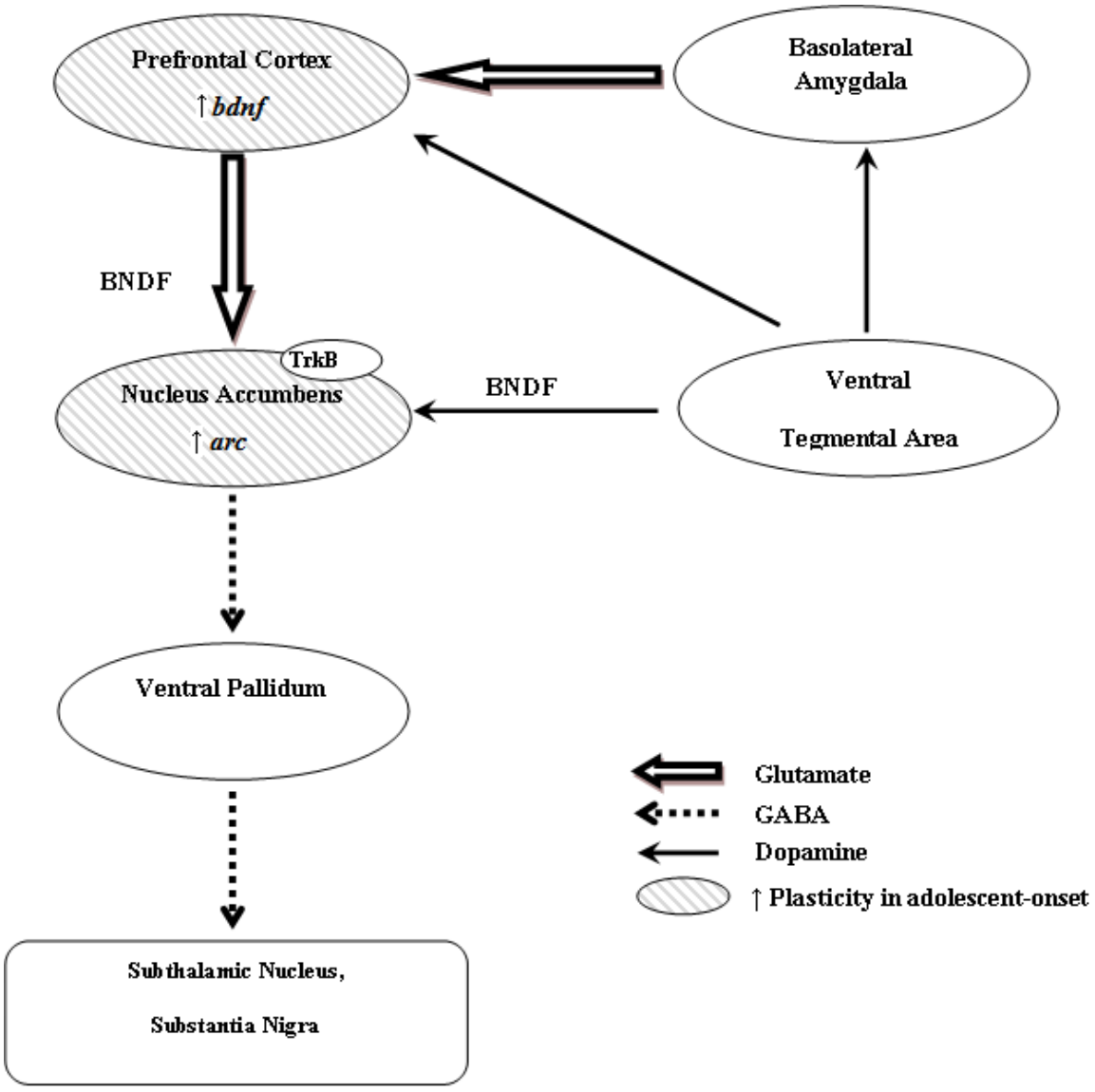


Figure 6.1 Age differences in neuroplasticity-related gene expression in the mesolimbic and mesocortical pathways. Cocaine induces neuroplastic changes in brain motivational circuitry that mediate the long lasting behavior of addiction, including prefrontal cortex, nucleus accumbens, amygdala, etc. This circuitry is also undergoing robust developmental organization. We hypothesize that the developmental neuroplasticity may prevent or reverse cocaine-induced neuroadaptations. In the dissertation, we showed that overall *arc* expression in the NAc and *bdnf* expression in the mPFC were higher in adolescent-onset groups than in adults.

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APPENDIX: PUBLICATIONS

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