Adolescent Vulnerabilities to Cocaine: Assessing Locomotor and Transcriptional Responses to Acute Cocaine and Cocaine-Induced Behavioral Plasticity During Adolescence.

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctorate of Philosophy in the Department of Pharmacology in the Graduate School of Duke University

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ABSTRACT

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Abstract

Adolescence is a critical period for drug addiction in humans. Most lifelong drug addiction is initiated during adolescence and the progression from initial drug use to the expression of addictive behaviors occurs more rapidly during adolescence than in adulthood. The purpose of this work was to examine if the adolescent brain uniquely responds to the addictive stimulant cocaine. This was accomplished by comparing the following measures in adolescent and adult male rats: locomotor responses to cocaine across a range of doses in two acute cocaine binge models, plasma cocaine and brain concentrations, locomotor responses to apomorphine, the relative magnitude of locomotor sensitization induced by a single high dose of cocaine (40 mg/kg), and cocaine-induced c-fos and zif268 expression. We determined that young adolescent (PN 28) rats had greater stereotypy responses to all doses of a repeated dose cocaine binge (15 mg/kg), the highest dose of an escalating dose binge (25 mg/kg), and low dose apomorphine. In addition to showing exaggerated acute locomotor responses to cocaine, young adolescents demonstrated a form of intrabinge sensitization that was absent in adults. Exaggerated adolescent locomotor responses could not be attributed to cocaine metabolism as we did not observe greater cocaine plasma or brain concentrations in adolescents compared to adults. A single high dose of cocaine (40 mg/kg) induced more ambulatory and stereotypy sensitization in young adolescents.
than adults. Further, the magnitude of the acute locomotor response to cocaine predicted the magnitude of locomotor sensitization in individual adolescents. We also showed that cocaine dose-dependently caused age-specific increases in the expression of the plasticity-associated immediate early genes \textit{c-fos} and \textit{zif268}: low dose (10 mg/kg) cocaine caused greater increases in striatal \textit{c-fos} expression in adolescents whereas high dose (40 mg/kg) cocaine caused greater increases in striatal \textit{c-fos} and \textit{zif268} expression in adults. Both doses of cocaine stimulated bigger increases in cortical \textit{zif268} expression in adults compared to adolescents. Finally, we demonstrated that the coordinated expression of striatal \textit{c-fos} and \textit{zif268} develops during adolescence: there was no correlation between striatal \textit{c-fos} and \textit{zif268} expression in individual adolescents but a strong correlation was seen in adults. The results of these experiments demonstrate that adolescents have unique molecular responses to acute cocaine and may help explain how adolescents show unique adaptive changes following continued cocaine use.
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1. CHAPTER 1: Introduction

Adolescence is a critical period for drug addiction in humans. Most lifelong drug addiction is initiated during adolescence (reviewed in Laviola et al. 1999; Spear 2000; Chambers et al. 2003). Longitudinal and retrospective studies consistently demonstrate that early exposure to drugs and alcohol is one of the strongest predictors of adult substance abuse (Barnes and Welte 1986; Deykin et al. 1987; Robins and Przybeck 1985; Robins and McEvoy 1990; Grant and Dawson 1997). Further, the onset of drug addiction during adolescence is correlated with an increased severity of addiction including higher rates of morbidity and mortality (reviewed in Chambers et al. 2003; Spear 2000; Yu and Williford 1992). Adolescent drug use is frequently associated with problematic patterns of administration: adolescents are more typically polydrug users (Brown 1993; Stewart and Brown 1995) and a few studies suggest that adolescents may more frequently be classified as heavy or binge users of marijuana (Ellickson et al. 2004), cocaine (Estroff et al. 1989), and alcohol (Wechsler et al. 1995; Johnston et al. 2004). Finally, the progression from initial drug use to the expression of addictive behaviors occurs more rapidly during adolescence than in adulthood (Estroff et al. 1989; Chen and Kandel 1995; Chen et al. 1997; Clark et al. 1998). Although numerous studies such as these demonstrate the importance of adolescence in human drug use, the biological basis for these vulnerabilities is still largely unclear.
While a number of social and environmental factors have been shown to increase an individual’s risk of drug addiction (Baer et al. 1987; Deykin et al. 1987; DeWit et al. 1999; Kirisci et al. 2006; Chapman et al. 2007), there is good evidence that the unique maturational state of the adolescent brain may contribute to this vulnerability. Adolescents are inherently risk-taking (Moffitt 1993; Trimpop et al. 1999). Elevated risk-taking in adolescence is not restricted to humans, but widely conserved across vertebrate animals ranging from mice to primates (Spear et al. 1980; Kraemer et al. 1982; Adriani et al. 1998; Crockett and Pope 1993). It has been proposed that risk-taking behaviors during adolescence are beneficial as they encourage maturing animals to learn how to maximize utility of their environment and emigrate to new social groups in order to prevent inbreeding (Arnett 1992, Maggs et al. 1995; Kelley et al. 2004). Even though risk-taking during adolescence may be evolutionarily advantageous, these behaviors are also strong predictors of drug and alcohol abuse (Baumrind 1987; Andrucci et al. 1989; Moffitt 1993). Forebrain dopamine systems contribute to risk-taking behaviors and the rewarding effects of psychostimulants (Le Moal and Simon 1991; Kelley 1999). These systems undergo dramatic functional and structural reorganization during adolescence (Seemen et al. 1987; Gelbard et al. 1989; Lidow et al. 1991; Teicher et al. 1995; Andersen and Teicher 2000; Andersen 2002). It is possible that potentially beneficial neural adaptations during this developmental window may also render adolescents vulnerable to drug addiction. Elucidating how these systems
initially respond to addictive substances like cocaine during adolescence will broaden our understanding of biological vulnerabilities to drug abuse in adolescent humans.

The development of drug addiction generally follows a characterized sequence of events. As such, it is important to consider the specific parts of the process to which populations like adolescents are most vulnerable to in order to understand the biological basis of their susceptibility. The process starts with the initial drug experience. If people have a positive experience, then they may be inclined to take the drugs again (Haertzen et al. 1983). Addictive drugs like cocaine and amphetamine are highly rewarding because they activate forebrain dopamine systems which are critical for reward sensation (Le Moal and Simon 1991). A number of individuals may then continue to regularly take drugs from time to time in a non-compulsive manner with little or no negative consequences. However, some individuals will begin to increase their frequency and/or duration of drug use. They then begin to develop neuroplastic changes that lead to compulsive drug craving and seeking behaviors that ultimately lead to addiction (see Gawin 1991). These changes can be long lasting as drug related cues can cause relapse in human users many years after cessation of drug use. A number of animal models have been used to investigate specific aspects of the development of drug addiction. A review of the adolescent literature suggests that understanding how the adolescent brain first responds to stimulants may be especially important.
Since forebrain dopamine systems are involved in reward and they undergo such a dramatic reorganization during adolescence, it has been tempting to speculate that adolescents may simply find drugs more rewarding than adults and therefore take them in greater quantities or with greater frequency. This simplistic view has not consistently been supported by data from human users or animal experiments. At least one study demonstrated that adolescent humans found D-amphetamine less rewarding than adults (Rapoport et al. 1980). Rodent models of drug reward suggest that adolescents may find nicotine more rewarding (Belluzzi et al. 2004; Brielmaier et al. 2007; Torrella et al. 2004; Vastola et al. 2002), but no consensus has been achieved for other addictive compounds including cocaine and amphetamine (Aberg et al. 2007; Adriani and Laviola 2003; Balda et al. 2006; Badanich et al. 2006; Campbell et al. 2000; Schramm-Sapyta et al. 2004). We interpret these studies to suggest that changes in drug reward during adolescence appear to be subtle at best and likely are not sufficient on their own to explain adolescent vulnerability to drug use.

Similar to the reward literature, animal models measuring voluntary drug intake levels have not shown that adolescents as a whole take more drugs than adults. Rodent self-administration models can be used to measure how quickly animals acquire voluntary drug intake, how much they will take, and how hard they will work for them. Several studies have examined drug acquisition and intake in adolescents and adults and produced mostly equivocal results (Frantz et al. 2007; Kantak et al. 2007; Kerstetter
and Kantak 2007; Lancaster et al. 1996, Levin et al. 2003, 2007; Perry et al. 2007; Shram et al. 2007). At least one study found that adolescents may take more nicotine than adults (Levin et al. 2007). However, no consistent age-effects have been seen with any other drugs. One interpretation of these results is that it is possible that not all adolescents are more vulnerable to developing drug addiction than adults. Perhaps individual traits may make individual adolescents particularly vulnerable to drug addiction.

A significant caveat of conditioned place preference (CPP) (a model commonly used to measure drug reward) and self-administration experiments is that they require training and multiple drug exposures. While they may be able to provide information about drug-induced behavioral adaptations or drug incentive, they can not provide information about how the brain first responds to stimulants. It is possible that the way the adolescent brain responds to initial drug exposure(s) underlies adolescent-specific vulnerabilities to drug addiction. Acute drug treatments can be utilized to more directly assess how baseline dopamine function affects initial neural and behavioral responses to stimulants.

Stimulant-induced locomotor activity has frequently been used as a first approximation of the relative reactivity of forebrain dopamine systems to addictive stimulants like cocaine. A few studies have directly compared adolescent and adult psychostimulant-induced locomotor responses and have produced mixed outcomes (Lanier and Isaacson 1977; Spear and Brick 1979; Laviola et al. 1995, 1999; Bolanos et al. 1999).
The results of these acute locomotor experiments, though potentially contradictory, consistently demonstrate that adolescents and adults have distinct acute locomotor responses to psychostimulants. They suggest that adolescent dopamine systems respond differently to pharmacological stimulation than mature adult systems. It is possible that adolescents are not outright hyper- or hyporesponsive to all stimulants, at all doses, or in terms of all behaviors. Carefully examining how factors such as dose and drug administration patterns affect behavioral and neural responses to cocaine during adolescence will enhance our understanding of how stimulants can uniquely activate the adolescent brain.

Whereas drug intake levels may not differ between adolescents and adults, it has consistently been reported that the rate of progression from first drug use to addiction is faster during adolescence in humans (Estroff et al. 1989; Chen and Kandel 1995; Chen et al. 1997; Clark et al. 1998). Locomotor sensitization is one animal model that has been used to measure the progression of drug-induced plasticity. Repeated drug exposures lead to a progressive enhancement of locomotor responses to subsequent drug challenges (reviewed in Robinson and Becker 1986; Post et al. 1988; Wolf 1998; Robinson and Berridge 1993). Even a single high dose of cocaine or amphetamine can induce locomotor sensitization (Robinson et al. 1982; Post et al. 1987; Vanderschuren et al 1999; Grignaschi et al. 2004). Several studies have examined adolescent and adult locomotor sensitization and have inconsistently reported age differences in the magnitude of
sensitization induced (Laviola et al. 1995; Adriani et al. 1998; Laviola et al. 2001; Collins and Izzenwasser 2002, 2004; Belluzzi et al. 2004; Schramm-Sapyta et al. 2004). These studies cumulatively demonstrate that locomotor sensitization can be induced in both adolescents and adults. However, they have all utilized repeated intermittent drug exposures to induce locomotor sensitization. While they may be able to address the relative magnitude of adolescent locomotor sensitization, they can not address the rate at which adolescent sensitization occurs. Utilizing shorter drug exposure regimens to induce locomotor sensitization could better investigate the rate at which cocaine can induce plastic changes in adolescent and adult rodents.

In addition to carefully examining acute locomotor responses and sensitization to stimulants, it will be necessary to investigate postsynaptic responses to pharmacological stimulation in adolescence and adulthood to understand which systems/circuits mediate age-specific locomotor responses to cocaine and how they may affect future responses to cocaine. Few studies have directly examined adolescent neural responses to stimulants but the existing studies have identified developmental changes during adolescence (Andersen et al. 2001a, 2002; Brandon and Steiner 2003; Cao et al. 2007; Ehrlich et al. 2002; Shram et al. 2007). As with above cited acute locomotor studies, these reports have not carefully examined the effects of dose or patterns of administration on their results.

Addictive psychostimulants like cocaine induce locomotor activity and drug reward by modulating postsynaptic membrane potentials in medium spiny neurons of
the striatum (reviewed in Rebec 2006). Postsynaptic receptor stimulation also induces intracellular signaling cascades that result in the rapid and transient expression of various transcription factors including c-fos and zif268 (also known as ZENK, egr1, NGF1-A, and krox-20) (Hope et al. 1992; Mitchell et al. 1989; Moratalla et al. 1992). The induction of these (and other related) immediate early genes (IEGs) have frequently been used as markers of neuronal activity. IEG induction is not required for acute stimulant-induced locomotor activation. However, genomic responses such as these are the basis of neuronal plasticity and they are required for drug-induced “learning” processes such as CPP and locomotor sensitization (Brami-Cherrier et al. 2005; Nestler 2004; Valjent et al. 2006). Adolescents appear to be distinctly sensitive to these learning processes compared to adults (Collins and Izenwasser 2002; Frantz et al. 2007; Laviola et al. 1995; Schramm-Sapyta et al. 2004). Unique molecular responses to drugs of abuse during adolescence could underlie a biological vulnerability to addiction during adolescence.

Finally, it will be important to examine how drug responses like acute locomotor activity, locomotor sensitization, and the expression of plasticity-associated transcription factors including c-fos and zif268 correlate in individual animals. Understanding how these variables relate in individual animals may provide us with a better functional understanding of our results. It will also be interesting to examine if there are any behavioral characteristics that correlate with drug responses in individual animals. The
lack of consistent age-effects on many measures of drug reward and intake (discussed above) suggests that perhaps it is individual adolescents as opposed to the whole population that are especially vulnerable to addiction. Not every adolescent human develops problem behaviors. While adolescence is often characterized by traits such as risk-taking and compulsivity, it is most often individuals that show extreme behavioral traits that actually progress to have drug problems (ex. Hoyle 2000). Examining the relationship between behavioral characteristics and drug responses in adolescents and adults may shed light onto how individual adolescents could be especially vulnerable to some consequences of drug use. Understanding and identifying these correlations could lead to better preventative measures and treatments during adolescence.

The overall goal of this thesis work is to examine locomotor and cellular responses to several acute drug administration paradigms in adolescent and adult male rodents and see how these responses relate to the magnitude of cocaine-induced behavioral plasticity in adolescents and adults. As discussed above, adolescents do not appear to be especially sensitive to the broad range of effects of cocaine. Our experiments will enable us to determine if adolescents may be uniquely sensitive to locomotor and/or transcriptional activation and subsequent locomotor sensitization by specific doses or patterns of cocaine administration that have not been previously examined.
1.1 Adolescence

Adolescence is the transitory period between childhood and adulthood (reviewed in Spear 2000). It is a period that is not defined by discrete actions, but rather a range of “soft events”. Adolescence is the period in which developing animals abandon parental care and learn to join different social groups or survive on their own. In addition to behavioral characteristics such as elevations in risk-taking behaviors and peer affiliation (Steinberg 1989; Schlegel and Barry; 1991), adolescents also experience a number of physiological changes including the dramatic endocrinological events of puberty. Because adolescence is not uniformly characterized by specific events, it is important to carefully consider how to define adolescence in various animal models. In an elegant review, Spear (2000) defined adolescence in the rat as a conservative age range from 28-42 days based largely on the exhibition of “adolescent-typical” neurobehavioral characteristics including growth spurt, prefrontal cortex (PFC) excitatory amino acid changes, and emergence behaviors (Kennedy 1967; Galef 1981; Insel et al. 1990). However, she acknowledged that a number of maturational events indicative of human adolescence occur as late as 55 days of age in rats (Odell 1990; Ojeda and Urbanski 1994). In our studies, we will define PN 28 as early adolescence, PN 42 as middle adolescence, and PN 65 as early adulthood.
1.2 Dopamine

1.2.1 Dopamine Systems and the Basal Ganglia

Dopamine is a monoamine neurotransmitter that can modulate the activity of neurons expressing dopamine receptors. Dopamine neurons of the mesencephalic dopamine system, the largest dopamine system, can be divided into three principle cell groups: A8, A9, and A10 neurons (Dahlstrom and Fuxe 1964). A8 neurons are found in the retrorubral area, A9 neurons are found in the substantia nigra, and A10 neurons are found in the VTA. Mesencephalic dopamine neurons send long projections that link the substantia nigra and VTA to their principle targets: the dorsal striatum, limbic cortex (mPFC, cingulate, and entorhinal areas), and other limbic areas (including the olfactory tubercle, nucleus accumbens septi, amygdala, and piriform cortex). Dopaminergic innervation plays a key role in modulating a number of motor and emotional functions including motivation and risk-taking (Kelley et al. 2004; Le Moal and Simon 1991) and dopamine dysregulation has been implicated in a number of conditions including Parkinson’s disease, schizophrenia and drug addiction (reviewed in Joel and Weiner 2000; Joyce 1993; Le Moal and Simon 1991; Ritz and Kuhar 1993).

The different populations of dopamine neurons project to distinct neuronal populations thereby forming a number of neural circuits that each mediate different cognitive and behavioral processes. Addictive stimulants like cocaine induce a diverse array of cognitive and behavioral effects by increasing dopamine transmission in all of
these circuits. The dopaminergic connections between the A10 dopamine neurons of the VTA and a number of forebrain regions including the amygdala, PFC, and nucleus accumbens are important for mediating motivation and reward (reviewed in Kalivas and Volkow 2005). In the mesolimbic dopamine system, the dopaminergic connections between the VTA and the nucleus accumbens are critical components of the reward pathway. Dopaminergic connections between the VTA and the cortex mediate emotional and cognitive processes important for motivational behaviors. The activation and alteration of these circuits by addictive stimulants is believed to represent the neural basis of addiction (Kalivas and Volkow 2005).

The activation of A9 dopamine neurons is important for mediating psychostimulant action and is also implicated in the addiction process. Dopamine neurons of the substantia nigra primarily innervate the dorsal striatum (caudate-putamen) and these connections are important for movement initiation and mediate the locomotor effects of psychostimulants (reviewed in Joel and Weiner 2000). Dopamine transmission in the caudate-putamen is also important for habit formation and may be important for mediating drug-craving and seeking in addicts (Vanderschuren et al. 2005; Volkow et al. 2006).

Many of the forebrain structures in which dopamine mediates the rewarding and psychoactive effects of addictive stimulants are collectively included as part of a neural network termed the basal ganglia. The basal ganglia is a collection of brain structures
that plays a role in mediating a number of frontal lobe functions (reviewed in Joel and Weiner 2000; Groenewegen 2003). It is comprised of the caudate-putamen, nucleus accumbens, pallidum, subthalamic nucleus, substantia nigra, and the VTA. The striatum represents the principle input of the basal ganglia as it is innervated by excitatory projections from virtually all cortical areas as well as the thalamus, amygdala, and hippocampus (Parent and Hazrati 1995, Wise et al. 1996). Dopaminergic neurons of the substantia nigra and VTA project to most areas of the striatum. The striatum also receives serotonergic input from the mesencephalic raphe nuclei. Dopaminergic and serotonergic transmission in the striatum modulates cortical and thalamic information transfer, thereby affecting motor, cognitive, and emotional functions.

The striatum is a complex structure that has not been consistently defined (in anatomical terms) in the literature. The caudate nucleus, putamen, and nucleus accumbens have frequently been collectively referred to as the striatum because they have similar histological and neurochemical characteristics (Smith and Bolam 1990; Gerfen and Wilson 1996). However, a number of other groups have used a different terminology system that describes the striatum as two distinct compartments: the dorsal (or neostriatum) and ventral striatum. Under this convention, the term dorsal striatum refers to most of the caudate and putamen and the term ventral striatum refers to the ventromedial parts of the caudate-putamen, the nucleus accumbens, and parts of the olfactory tubercle (reviewed in Joel and Weiner 2000). The latter terminology divides
the striatum into two compartments based upon the specific origin of dopaminergic afferents: the dorsal striatum is innervated by the substantia nigra whereas the ventral striatum is innervated by the VTA. We will use the term caudate-putamen (CP) to refer to the dorsal striatum and nucleus accumbens to refer to the ventral striatum. Further, for anatomical studies, the striatum and accumbens will be broken down into more specific subregions defined by their specific cortical afferents.

1.2.2 Dopamine Neurotransmission

Dopamine is synthesized from the amino acid tyrosine and stored in presynaptic vesicles. Terminal invasion by an action potential causes Ca$^{2+}$-stimulated release of dopamine by exocytosis. Upon stimulation, dopamine vesicles fuse with the plasma membrane and release soluble dopamine into the synapse. The extent of dopamine release into the synapse is dependent upon both the rate and pattern of dopamine neuron firing. The dopamine terminal regulates extracellular dopamine levels by monitoring two principle parameters: release and uptake.

Presynaptic dopamine autoreceptor function represents one of the primary time-dependent mechanisms of self-regulation to maintain extracellular dopamine homeostasis. Dopamine-mediated stimulation of D2 receptors on the dopamine cell body (somatodendritic receptors) and the dopamine terminals, termed autoreceptors, can inhibit the subsequent release of dopamine by several mechanisms. The activation of somatodendritic autoreceptors, also known as release regulating autoreceptors, slows
the rate of dopamine neuron firing and impulse flow (Grace and Bunney 1985; Lee and Ellinwood 1989; Silva et al. 1994). Slowed neuron firing results in decreased dopamine release. Terminal autoreceptor activation decreases dopamine synthesis and affects vesicular trafficking, thereby reducing the amount of dopamine readily available for stimulated release (Kehr et al. 1972). There is also evidence that terminal autoreceptor activation can decrease extracellular dopamine levels by increasing DAT-mediated dopamine clearance (Meiergerd et al. 1993; Cass and Gerhardt 1994). By all of these mechanisms, autoreceptor activity provides a means of feedback inhibition to maintain proper dopamine regulation. Several recent studies, including our own, suggest that the inhibition of dopamine release by dopamine D2 autoreceptors is reduced in adolescents compared to adults (Marinelli 2007; Walker and Kuhn 2007).

The dopamine transporter represents the other principle regulator of synaptic dopamine neurotransmission. Catecholamine nerve terminals possess high-affinity transporters that function to bring synaptic amines and indolamines back into the terminal. Their actions are critical in terminating synaptic signaling. Dopamine, norepinephrine, and serotonin, utilize structurally similar Na/K-dependent transmembrane reuptake transporters. Though these transporters are structurally conserved, they demonstrate high selectivity for the transport specific neurotransmitters (Giros et al. 1984). The dopamine transporter is a reversible, concentration-dependent bi-directional transporter that transports dopamine from the extracellular space into the
terminal under normal conditions. Dopamine uptake through DAT can be modulated by increasing or decreasing DAT expression, internalization, plasma membrane surface localization, or post-translational modification including glycosylation. The dopamine transporter is the principle target of several dopaminergic stimulants and a number of clinical pathologies, including drug addiction, are associated with abnormal DAT expression or subcellular localization. Further, there is evidence to suggest that age-specific changes in DAT glycosylation may affect DAT function during post-natal development and possibly during adolescence (Patel et al. 1994).

1.2.3 Dopamine Receptors

Dopamine receptors are G-protein coupled receptors that modulate neuron excitability and intracellular signaling cascades. Dopamine signaling has both immediate and long-term effects. The majority of known dopamine signaling events are coupled to G-protein activation and subsequent downstream effectors including adenylate cyclase and phosphoinositides (Mailman et al. 1986; Undie and Friedman 1990, 1992; Undie et al. 1994). Dopamine receptors can be classified into two principle groups distinguished by how their coupled G-proteins interact with adenylate cyclase: D1 and D2-like receptors (reviewed in Sokoloff and Schwartz 1995, Missale et al. 1998). D1-like dopamine receptors, which include the D1 and D5 dopamine receptors, activate stimulatory Gs/Golf subunits and increase adenylate cyclase activity, thereby increasing intracellular cAMP levels. Conversely, D2-like receptors, which include D2, D3, and D4
dopamine receptors, couple to inhibitory G\textsubscript{i}/G\textsubscript{o} subunits and decrease intracellular cAMP levels. Figure 1 shows a diagrammatic representation of dopamine neurotransmission and the intracellular responses of D1 and D2-like postsynaptic neurons.

Dopamine does not directly stimulate postsynaptic neuron firing or inhibition. Rather, it is a modulatory neurotransmitter that has immediate effects on postsynaptic neurons by increasing or decreasing the excitability of postsynaptic neurons to glutamate (Freund et al. 1984). Determining the specific effects of D1 or D2 stimulation on ionic conductances and synaptic transmission has been difficult and a number of contradictory results exist (reviewed in Nicola et al. 2000). However, a clearer picture is beginning to emerge. By affecting the activity of cAMP-dependent protein kinase (PKA), dopamine receptor activation can phosphorylate/dephosphorylate a number of ion channels including L-type calcium channels, NMDA, and AMPA receptors and thereby modulate ion currents to enhance or decrease neuron excitability (Surmeier et al. 1992; Schiffman et al. 1998; Zhang et al. 1998). Accordingly, D1 and D2 receptor activation has opposite effects on neuronal excitability (Capeda et al. 1993, 1995; Kombian and Malenka 1994; Levine et al. 1996a,b). In the striatum and accumbens, D1 activation enhances glutamaturgic excitatory postsynaptic potentials (EPSP) and D2 activation reduces glutamaturgic EPSPs (see Nicola et al. 2000).
Since D1 and D2 like receptors have opposite effects on postsynaptic activity they are rarely co-expressed on the same neurons. In many cases, the individual subtypes are expressed in close proximity on neurons involved in separate circuits. As a result, the activation of receptors with opposite cellular responses, even within the same brain nuclei, often results in similar behavioral and cognitive responses. Within the striatum, for example, D1 and D2 receptors are primarily localized to two distinct neuronal pathways that oppositely regulate movement (reviewed in Joel and Weiner 2000). Activation of D1 receptors by dopamine activates motor stimulating circuits resulting in increased motor output. Activation of D2 receptors inhibits motor inhibitory circuits which also results in increased motor output.

Other cognitive and behavioral effects of dopamine signaling can be difficult to predict without knowledge of anatomical organization. Administration of both D1 and D2 agonists can enhance cocaine self-administration (Weed et al. 1997; Woolverton and Ranaldi 2002; Rowlett et al. 2007) and activation of both receptor subtypes in the nucleus accumbens is required for reinforcement (Ikemoto et al. 1997). However, D1 and D2 agonists can have opposite effects on drug discrimination, reinstatement, and heroin self-administration (Khroyan et al. 2000; Haile and Kosten 2001; Rowlett et al. 2007). Studies such as these highlight the exquisite complexity of dopamine signaling. Dopamine systems can dynamically regulate numerous cognitive processes by innervating an array of targets and signaling through different classes of receptors.
Dopaminergic psychostimulants such as cocaine activate a myriad of neural circuits and elucidating the anatomical specificity of stimulant action during adolescence will be an important step in understanding how drugs can uniquely activate the adolescent brain.

1.2.4 Dopamine-Mediated Gene Expression

In addition to affecting ion conductances and neuron firing rates, dopamine signaling also induces a number of intracellular signaling events ultimately resulting in the expression of various immediate early genes (IEGs) such as the Fos and Jun families of transcription factors such as c-fos. The induction of transcriptional events comprises the basis of long-lasting neuronal changes by dopamine as they are believed to represent the cellular correlates of learning and memory. Addiction is often viewed as a learning process. Once learned, addicts can experience cue-induced cravings and relapse for decades. Similarly, rodents exposed to stimulants will display drug seeking behaviors and locomotor sensitization months after discontinuation of drug access.

A number of IEGs can be induced by both pharmacological and electrical stimulation (Aronin et al. 1991; Berretta et al. 1992; Graybiel et al. 1990; Fue and Beckstead 1992; Miyachi et al. 2005; Page and Everitt 1993; Robertson et al. 1990, 1992). Their expression is dependent upon dopamine D1 signaling as D1 antagonists can completely ablate stimulant-induced IEG expression (Keefe and Gerfen 1995; Young et al. 1991). As mentioned above, dopamine modulates glutamaturgic excitability and selective glutamate antagonists can also block stimulant-induced IEG expression (Keefe
and Gerfen 1996, 1999; Konradi et al. 1996). The expression of these genes is essential for the development of locomotor sensitization and the acquisition of CPP or self-administration. Their activity is also required for the induction of delayed messages such as δ-fos B. Several studies have demonstrated that the plasticity-associated immediate early genes c-fos and zif268 are induced by psychostimulants in both adolescents and adults (Andersen et al. 2001a; Cao et al. 2007; Brandon and Steiner 2003; Shram et al. 2007). However, none of these studies have examined the expression of more than one gene in adolescents and adults. Several studies have demonstrated that various stimuli including cocaine can induce c-fos and zif268 independent of one another. It is possible that the maturation of combinatorial regulation of different genes by cocaine during adolescence could explain why adolescents are more sensitive to some, but not all, aspects of drug addiction.
Figure 1: Dopamine neurotransmission and intracellular signaling.

D1 and D2 dopamine receptors oppositely regulate intracellular signaling events. Activation of D1 receptors stimulates adenylate cyclase which turns on (via phosphorylation) PKA. Active PKA can then initiate several signaling cascades which ultimately result in the expression of gene products. Intracellular calcium (through CaMKs) normally opposes phosphorylation of DARPP-32, a protein that activates the transcription factor CREB. However, phosphorylated PKA directly activates DARPP-32. PKA also enhances glutamate signaling cascades by phosphorylating NMDA and L-type calcium channels. In contrast, D2 activation results in the inhibition of adenylate cyclase. Following D2 activation, cAMP cascades are attenuated and DARPP-32 remains inactivated by CaMKs.
1.2.5 Dopamine Systems in Adolescence

Dopamine systems undergo a dramatic degree of functional and organizational changes during adolescence. In fact, final maturation is not complete until adulthood. These changes in dopamine systems are thought to be involved in mediating a number of neurobehavioral characteristics of the adolescent period including risk-taking behaviors. The intimate relationship between dopamine signaling and drug reward also presents a number of potential biological mechanisms that could underlie vulnerability to drug addiction in adolescence. Below, we review some of the major maturational changes in forebrain dopamine systems that occur during adolescence.

1.2.5.1 Dopamine Innervation

While some connections between midbrain dopamine neurons and forebrain targets are present early in life, full innervation is not completed until much later. In the rat, TH-positive fibers are densely packed into the characteristic patch-matrix formations in the striatum by E 21 (Huang 1990). DAT and TH expression are also detectable in the striatum by late embryonic development. As innervation proceeds, however, a number of terminal markers continue to increase through early postnatal development, across adolescence, and into adulthood. For example, striatal DAT binding (Coulter et al. 1997; Tarazi et al. 1998) and dopamine content (Porcher and Heller 1971; Giorgi et al. 1987) peak somewhere between PN 50 and PN 70. Similarly, in humans striatal dopamine content also increases during adolescence (Haycock et al. 2003), although TH, DAT, and
VMAT2 all appear to reach peak levels prior to early adolescence (Meng et al. 1999, Haycock et al. 2003). It is possible that ongoing dopaminergic innervation could affect how adolescent animals process and evaluate rewarding/aversive stimuli or how developing dopaminergic systems respond and adapt to drugs like cocaine.

The functional relevance between these ontogenetic changes and stimulant responsiveness in adolescence is not completely clear, although a number of connections have been proposed. Electrically-stimulated dopamine uptake and release rates parallel increases in striatal dopamine content and DAT expression (Stamford et al. 1989). Likewise, extracellular dopamine levels are lower during adolescence (Andersen and Gazzara 1993; Laviola et al. 2001). Long-term decreases in extracellular dopamine levels could increase postsynaptic sensitivity to dopamine agonists during adolescence. This could partially explain why adolescents have greater locomotor responses than adults to the D2 agonist quinpirole (Frantz and Van Hartesveldt 1999). Additionally, age-related increases in dopamine content could be sufficient to explain parallel increases in amphetamine-induced dopamine overflow and locomotor behavior (Laviola et al. 2001; Bolanos et al. 1998). The dramatic changes in substrates of stimulant action during adolescence warrant further investigation to understand how such alterations affect behavioral and cellular responses to stimulants in adolescence.
1.2.5.2 Dopamine Receptor Overproduction

A number of studies have demonstrated that dopamine receptors in forebrain regions, including the CP, nucleus accumbens, and PFC are overexpressed and subsequently “pruned” in a subtype- and regionally-specific manner during adolescence (Huttenlocher 1979; Giorgi et al. 1987; Gelbard et al. 1989; Tarazi et al. 1999; Andersen et al. 2000, 2001b; Tarazi and Baldessarini 2000). This robust phenomenon has been observed in humans, primates, and rodents. Within rodents, D1 and D2 receptor levels peak between PN 30 and PN 40 in the PFC and CP. A similar but less dramatic reduction has been observed in the nucleus accumbens. One might expect that increased numbers of dopamine receptors would produce exaggerated behavioral and cellular responses to dopaminergic stimulation in mid adolescents compared to older or younger animals. However, several existing studies suggest that adolescents may actually be hyporesponsive to the locomotor and biochemical effects of dopamine agonists (Andersen 2002; reviewed in Spear 2000).

Several hypotheses have been proposed to attempt to explain the functional reasoning for the overproduction and subsequent reduction of dopamine receptors during adolescence. One leading theory suggests that this phenomenon represents the biological basis for environmentally directed synaptic plasticity (reviewed in Crews et al. 2007). This idea suggests that neurological circuits can effectively be shaped based on environmental needs and stimuli thus producing mature adult behavior. Proponents of
this theory suggest that pharmacological perturbation of dopamine systems during adolescence could produce lasting effects, for good or for ill, on drug reward and motivation well into adulthood. Accordingly, a number of studies in humans and animal models have identified lasting behavioral and subjective effects of adolescent drug exposure.
1.3 Cocaine

Cocaine is one of the most commonly abused psychoactive stimulants in the United States. According to the Office of National Drug Control Policy, nearly 6 million Americans used cocaine in 2001 and it is estimated that nearly 3 million of them used cocaine chronically (SAMHSA 2001). Cocaine addiction is worrisome as it is associated with significant physical, psychological, and economic costs. Currently there are no effective pharmacotherapies available for the treatment of cocaine (Reviewed in Haile et al. 2007) although a number of promising drugs are being examined. Therefore, prevention and early intervention are especially important for cocaine. As we learn more about the biology of cocaine abuse in vulnerable populations, we may be able to better identify high risk individuals and focus preventative efforts.

Cocaine has been utilized for centuries. Ancient Peruvians first used cocaine by chewing coca plant leaves. Cocaine was isolated and first used pharmacologically as an anesthetic. Local anesthetics, such as cocaine, block the generation and propagation of action potentials by blocking voltage sensitive sodium channels (Catterall and Mackie 1996). In addition to blocking sodium channels, cocaine also blocks the reuptake of dopamine, norepinephrine, and serotonin by binding to their respective transporters. Peripheral blockade of the norepinephrine transporter (NET) causes potent vasoconstriction and improves the efficacy of cocaine’s local anesthetic properties.
However, CNS blockade of NET, DAT, and SERT underlie the psychoactive effects of cocaine and limit its clinical usefulness.

Enhanced dopaminergic signaling in the CNS provides the pharmacological basis for the locomotor and euphoric properties of psychostimulants including cocaine (Reviewed in Volkow et al. 1997, Kelley 1999). While cocaine can interfere with norepinephrine, serotonin, and dopamine uptake, it is specifically dopamine modulation that is directly related to cocaine-stimulated behavior and self-administration (Reith et al. 1987; Ritz et al. 1987). Lesions of dopaminergic midbrain projections to the CP or accumbens or local administration of dopamine antagonists can abolish psychostimulant-induced behaviors and self-administration (Kelly et al. 1975; Kelly and Iverson 1976; Kalivas and Stewart 1991; Koob 1992; Kalivas 1995; Peirce and Kalivas 1995). Cocaine increases extracellular dopamine levels by competitively binding to DAT and preventing dopamine uptake into terminals (Hanson et al. 1987). Some other commonly abused stimulants, including amphetamine and methamphetamine, also increase extracellular dopamine levels. In addition to blocking DAT, these drugs can also cause the release of presynaptic dopamine into the synapse (Sulzer et al 1993; 1995).

The specific pharmacological action of stimulants is important to consider when testing animals with drugs. Responses to stimulants like cocaine represent the sum of presynaptic dopamine perturbation and postsynaptic responsiveness. Figure 2 shows how cocaine and amphetamine act differently at the presynaptic terminal.
Understanding developmental differences in how specific perturbations to dopamine systems can affect the psychopharmacological responses to stimulants could help understand how adolescents may respond differently to specific stimulants.
Figure 2: Presynaptic actions of cocaine and amphetamine.

Cocaine binds to the dopamine transporter (a). Following electrical stimulation (b), dopamine containing vesicles fuse with the plasma membrane and release soluble dopamine into the synapse. The amount of dopamine released depends on the strength of the signal. Extracellular dopamine levels remain high because cocaine prevents the reuptake of dopamine through DAT (c). Amphetamine enters the terminal through DAT and interacts with the vesicular monoamine transporter on the vesicles (d) and causes the release of dopamine from the vesicles independent of impulse flow (e). Dopamine can then exit the terminal through DAT (f).
1.4 Psychostimulant-Induced Locomotor Activity and Sensitization

Psychostimulants such as cocaine dose-dependently induce an array of behaviors ranging from sniffing and grooming to fixed stereotypies and seizures. In general, low doses of cocaine increase the frequency of normal rat behaviors such as grooming and sniffing. Low to moderate doses of cocaine will cause animals to begin locomoting (i.e. moving from place to place). At higher doses, animals will transition into repetitive stereotyped behaviors referred to as stereotypies. These are repetitive behaviors that are rarely observed in normally behaving animals. Specific stimulants induce different stereotypies. Cocaine frequently induces fixed sniffing and repetitive head movements in which the animals swing and rotate their heads from side to side. Direct dopamine agonists, such as quinpirole and apomorphine, induce a number of oral or licking stereotypies. As the dose is increased, repetitive stereotypies can become so frequent and prolonged that they begin to displace locomotor activity (Figure 3). The expression of stereotyped behaviors is believed to represent an intense locomotor response to psychostimulants and the relative sensitivity with which adolescents transition from cocaine-induced locomotion into stereotypies is poorly defined. Several studies have also demonstrated that specific behavioral responses (locomotor vs. stereotypy) are mediated by dopamine transmission in distinct brain regions (Kelly et al. 1975; Kelly and Iverson 1976). Knowledge of the specific behavioral topography to cocaine during
adolescence could provide some insight into the regional sensitivity of adolescent dopamine systems to cocaine.

Repeated drug exposures lead to a progressive enhancement of locomotor responses to subsequent drug challenges (Reviewed in Robinson and Becker 1986; Post et al. 1988; Wolf 1998; Robinson and Berridge 2001). Even a single high dose of cocaine or amphetamine can induce locomotor sensitization (Robinson et al. 1982; Post et al. 1987; Vanderschuren et al 1999; Grignaschi et al. 2004). It has been hypothesized that locomotor sensitization may model the intensification of drug craving and seeking (Robinson and Berridge 1993, 1995) as sensitizing drug regimens increase drug reward/incentive (Schenk and Partridge 1997), subsequent acquisition of drug-self administration (Piazza et al. 1989, 1990; Giles and Schenk 1992; Pierre and Vezina 1997, 1998), and habit formation (Nelson and Killcross 2006). As discussed above, a significant number of studies have examined the effects of repeated cocaine treatments on the expression of sensitization in adolescent and adult rats, but none of them have compared the rate of sensitization during adolescence and adulthood.
Stimulants like cocaine produce a range of psychomotor behaviors depending on the dose. Two of the predominantly measured behaviors are locomotion and stereotypies. Here, we present the frequency of each behavior as a function of dose. Low doses of stimulants induce predominantly locomotor activity (solid line). However, as the dose continues to increase, animals begin to transition into fixed stereotypies (dashed line). If the dose is increased to sufficient levels, animals will begin to engage in almost continuous fixed stereotypies that can displace locomotor activity.
1.5 Specific Aims

This review of the literature indicates that adolescence is a critical time for drug addiction in humans. Forebrain dopamine systems, which mediate the rewarding and locomotor effects of psychostimulants, undergo significant structural and functional alterations during adolescence. Previous studies investigating both acute and sensitized responses to stimulants in adolescents and adults suggest that adolescents respond differently than adults to stimulants such as cocaine. By treating young adolescents, mid-adolescents, and adults with a range of cocaine doses, we can clarify the existing literature and understand how factors such as age, dose, and administration patterns affect acute locomotor responses to cocaine during adolescence. We can then determine how the magnitude of acute locomotor responses to cocaine correlates with the induction of locomotor sensitization and IEG expression in individual adolescents and adults.

The central hypothesis of this work is that adolescents are more sensitive than adults to the locomotor and neural activating effects of cocaine. We predict that enhanced cocaine-induced transcriptional activation makes adolescents more vulnerable than adults to some of the plastic changes induced by a limited number of cocaine exposures. This hypothesis can be divided into three separate ideas: 1) select doses of cocaine will cause greater locomotor responses in adolescents compared to adults, 2) a single high dose of cocaine will cause more locomotor sensitization in adolescents
compared to adults, and 3) adolescents will show greater cocaine-induced increases in c-fos and zif268 expression than adults in regions of the striatum and/or cortex. Specific aims addressing these hypotheses are described as follows.

1. **Characterize locomotor responses to cocaine within acute cocaine binges in adolescent and adult male rats.** To determine how locomotor responses to cocaine change as a function of age and cocaine dose, we treated animals PN 28, PN 42, and PN 65 with one of two acute cocaine binges. We determined if closely spaced cocaine injections within a binge induced any within session sensitization by utilizing a constant dose binge and by comparing escalating dose binge locomotor responses to those observed following acute high dose (25 or 40 mg/kg) cocaine treatment. We also analyzed plasma and brain cocaine concentrations during the repeated dose cocaine binge.

2. **Determine the ontogeny of locomotor sensitization to a single high dose of cocaine.** We examined the qualitative and quantitative effects of pretreatment with a single high dose of cocaine to a challenge dose of cocaine 24 hours later in adolescent and adult rats. Animals PN 28, PN 42, and PN 65 were treated with a single injection of saline or a high dose of cocaine (40 mg/kg) and then challenged with saline or cocaine (10 mg/kg) the following day. Horizontal activity was further resolved into ambulatory and non-ambulatory components. We ran correlational analyses between locomotor activity induced by a novel environment, acute high dose cocaine, and sensitization
within individual animals to determine if any factors could predict the magnitude of locomotor sensitization in individual animals.

3. Evaluate neural responsiveness to dopamine agonists and cocaine during adolescence. *In vitro* [35S]GTPγS assays were used to assess dopamine, D1, or D2-agonist stimulated G-protein activation in PN 28 and PN 65 tissues from the accumbens, striatum, and PFC. Anatomical *c-fos* and *zif268* induction by cocaine was examined by *in situ* hybridization. Following treatment with cocaine (0, 10, or 40 mg/kg), horizontal activity was recorded for 30 min and animals were killed. *C-fos* and *zif268* mRNA levels were assessed in 12 striatal and 15 cortical subregions. We further ran correlational analysis to determine if the magnitude of expression of either gene correlated with the magnitude of locomotor activity in individual animals. We also measured the correlation between the expression of *c-fos* and *zif268* in individual animals to determine if there were any age effects on gene-specific regulation by cocaine.
2. CHAPTER 2: Methods

2.1 General Methods

2.1.1 Subjects

One goal of these studies was to measure locomotor and neural responses to acute cocaine in adolescents and adults. Justified ethical considerations preclude cocaine exposure in naive human subjects necessitating the use of an animal model system to complete these experiments. We have elected to utilize the rat for several important reasons. First, cocaine has been shown to produce physiologically and pharmacologically similar effects in rats and humans. Rats will self-administer cocaine and the metabolism of cocaine is similar in both species. Second, the rat has been extensively used in behavioral studies and both acute and sensitized locomotor responses to cocaine are well characterized in adults. Locomotor responses from some of our treatment groups can therefore serve as comparisons to previous studies and enhance the contextual interpretation of our findings. Third, a number of studies have examined the development of many neural substrates of cocaine action during adolescence in the rat. This information can provide insight into the mechanistic interpretation of our results and a rational basis for the selection of experimental parameters such as age.
All rats in these studies were Sprague-Dawley rats (CD strain) and were obtained from Charles River Laboratories (Raleigh, NC). Rats were shipped and received 7 days prior to experimentation. Young adolescent animals were received on PN 21, mid-adolescents were received on PN 35, and adults were received on PN 58. After receipt, all animals were housed in an adjacent animal housing facility in clear ventilated plastic cages on laboratory corn-cob bedding with *ad libitum* access to food and water under a 12:12 hour light-dark cycle. Young and mid-adolescent animals were housed 4 animals per cage and adults were housed 2 animals per cage. Animals were transported in their cages to our experimental facility 24 hours prior to experimentation. All experimental procedures were approved by the Institutional Animal Care and Use Committee and were in accordance with the National Institute of Health guidelines for the care and use of animals.

### 2.1.2 Drugs

Cocaine HCL was obtained from two sources: the National Institutes of Drug Abuse and Sigma-Aldrich (St. Louis, MO). Cocaine solutions were prepared on the morning of each experiment and stored in the dark at 4°C until use. Cocaine was measured and diluted to the appropriate volume in saline.
2.1.3 Injections

All injections in these studies were given intraperitoneally (i.p.). In addition to acting as a CNS stimulant, cocaine is also a potent vasoconstrictant. This property greatly slows the absorption of cocaine into the bloodstream following subcutaneous (s.c.) injections. I.p. injections result in greater maximal brain cocaine concentrations and faster attainment of peak levels. Both of these factors influence the magnitude of neural and behavioral responses to cocaine. Further, the majority of previous cocaine-induced locomotor studies have also utilized i.p. injection routes. Utilization of this injection route will allow us to more directly interpret our results in the context of previous cocaine-induced locomotor studies.
2.2 Behavioral Measures

As described above, cocaine dose-dependently affects a number of specific behaviors that are mediated to varying degrees by dopamine transmission in different forebrain centers. Knowledge of the specific behavioral topography to cocaine during adolescence could provide some insight into the regional sensitivity of adolescent dopamine systems to cocaine. For these reasons, we monitored a range of behavioral measures that include automated locomotor activity and experimenter observed behaviors.

2.2.1 Automated Locomotor Activity

2.2.1.1 Horizontal Activity

Automated locomotor activity was recorded in open-field Plexiglas chambers using photobeam monitoring software. The acute behavioral responses to cocaine binges were performed using boxes and software from San Diego Instruments (San Diego, CA). These boxes were 50 x 50 cm. Photobeams were located on two crossbars that were situated 3 and 6 cm from the bottom. Fifteen photobeams spaced 3 cm apart were located on each side of the frame. Horizontal activity was recorded as the breaking of photobeams on the bottom arm. The repetitive breaking of a single photobeam was not recorded as horizontal activity. This allowed us to exclude some, but not all,
repetitive fixed stereotypies that may be influenced by the animals size from our automated locomotor data.

2.2.1.2 Ambulations and Fine Movements

Automated behavioral activity from the apomorphine, single dose sensitization, and c-fos/zif268 experiments was obtained using different locomotor boxes and software that was able to further resolve horizontal activity into ambulatory and non-ambulatory (fine movements) components (Kinder Scientific, Poway, CA). These boxes were 40 x 40 cm with 16 photobeams spaced 2.5 cm apart located 3 cm from the base. Total horizontal activity represents the sum of all photobeam breaks. Ambulatory activity refers specifically moving from place to place. It is recorded as the breaking of a forward beam paired with the cessation of breaking an anchor beam. Fine movements are defined as all non-ambulatory beam breaks. These represent behaviors including stereotypies, head movements, grooming and turning. Figure 4 provides a diagrammatic representation of ambulations and fine movements.

The reason for this fine-grained analysis is two-fold. First, ambulatory activity provides a size-independent measure of automated locomotor behavior. One caveat to using horizontal activity to compare adolescent and adult locomotor responses is that total horizontal activity counts can be affected by subject size. At any given interval, adult rats break more beams than adolescents. Further, adults have a significantly larger gait than adolescents and can move greater distances and break more beams with fewer
movements than adolescents. Horizontal activity may provide an efficient measure for monitoring drug effects within a population, but it can be difficult to concisely determine relative activity levels of animals with significant size differences using horizontal activity. Ambulations is largely size independent as it requires the simultaneous breaking of one forward beam paired with the cessation of one anchor beam irrespective of the number of beams in between the two.

These analyses can also provide more insight into the development of specific cocaine-induced behavioral topographies during adolescence. Previous studies have compared different behavioral responses to cocaine in adolescents and adults and produced mixed results. It is possible that adolescents are not outright hyper or hyporesponsive to cocaine relative to adults but show different responses to specific behaviors. The measurement of ambulatory and non-ambulatory horizontal activity, combined with experimenter observed behavioral monitoring, will provide superior detection and resolution of the development of specific behavioral topographies during adolescence.
Automated locomotor activity was recorded in photobeam monitored open field locomotor chambers. We resolved locomotor activity into ambulations and fine movements. The black dots represent photobeams. Dashed circles represent photobeams that are being interrupted by the rat. Ambulations is defined as moving from place to place and is demonstrated by the sequential movement of a rat in (a-c). Ambulations are recorded when an animal concurrently breaks a forward beam and ceases breaking of an anchor beam (compare a-b or b-c). All non-ambulatory beam breaks are counted as fine movements. One example of fine movements is shown in (d-f). Between panels d and e, the rat shifts its head and part of its body to the right and then returns to its original position in f. In this series, the anchor beam is interrupted at all intervals and only fine movements are recorded.
2.2.2 Behavioral Rating

Specific behaviors were scored using a previously defined stereotypy rating scale. Higher scores denote greater cocaine-induced stereotypy responses. All animals were observed for specific stereotypy behaviors from outside the locomotor activity room through a window on the door. Each animal was observed for 3 separate 15 sec intervals per 5 min. Table 1 shows which behaviors were observed and gives their behavioral rating scores. Each 15 sec interval received a score equivalent to the highest value behavior that was observed in the interval. The relative frequency of intervals observed at each stereotypy rating was calculated as the number of intervals observed at a particular score divided by 36 (3 intervals x 12 five min bins) * 100. In all experiments, observers were blinded to the experimental condition of animals they were rating.
Table 1: Rating scale for experimenter observed cocaine-stimulated behaviors.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sleeping, Inactivity</td>
<td>1</td>
</tr>
<tr>
<td>Grooming, Sniffing, Walking (Normal Rat Behavior)</td>
<td>2</td>
</tr>
<tr>
<td>Continuous Sniffing</td>
<td>3</td>
</tr>
<tr>
<td>Stereotyped Behaviors (Head Scanning, Wall Climbing, Oral Stereotypies, etc.) with frequent locomotion</td>
<td>4</td>
</tr>
<tr>
<td>Fixed Stereotypies Confined to One Section of the Chamber</td>
<td>5</td>
</tr>
<tr>
<td>Dyskinesia</td>
<td>6</td>
</tr>
</tbody>
</table>
2.2.3 Velocity Measurements

To measure high velocity horizontal activity (San Diego Instruments) during the acute escalating dose cocaine binge, the intervals used to measure horizontal activity were reduced to from 5 minutes to 6 seconds. The number of intervals with velocities greater than 30 in/6 sec (for the escalating dose binge) was tabulated manually for each rat.
2.3 Behavioral Studies

2.3.1 Acute Cocaine Binges


2.3.1.1 Escalating Dose Cocaine Binge

Animals PN 28, PN 42, and PN 65 were habituated to the locomotor test chambers for 1 hr. Automated horizontal activity was recorded during this hour to measure total activity induced by placement in a novel environment. Animals were then treated with three hourly injections of cocaine (5, 10, and 25 mg/kg) or saline (to measure relative baseline activities). Horizontal activity was recorded following all three hourly injections. Animals were only observed for stereotypies following the third and highest dose of cocaine as previous and subsequent work from our laboratory has demonstrated that animals do not frequently engage in stereotypies following 5 or 10 mg/kg cocaine (Parylak et al. 2008).

2.3.1.2 Escalating Dose Binge vs. Acute High Dose Cocaine

PN 28, PN 42, and PN 65 animals were treated with an escalating dose binge of cocaine (5, 10, and 25 mg/kg) or two injections of saline followed by an injection of 25 or
40 mg/kg cocaine. The first dose (25 mg/kg) is equivalent to the injection received during the third injection of the binge. The second dose (40 mg/kg) was used as it represents the cumulative total of cocaine animals were exposed to in the escalating dose binge. Automated horizontal activity was recorded continuously and stereotypy scores were obtained following the third injection.

2.3.1.3 Repeated Dose Cocaine Binge

Animals PN 28 and PN 65 were habituated to the locomotor chambers for one hour and total horizontal activity was recorded continuously during this time. PN 42 animals were omitted from these experiments to minimize the number of animals used. Following habituation, animals were treated with three hourly injections of cocaine (15, 15, and 15 mg/kg). Automated horizontal activity and experimenter observed stereotypies were continuously recorded/monitored following all three injections.

2.3.2 Apomorphine Behavior

Animals PN 28, PN 42, and PN 65 were habituated to the locomotor test chambers (Kinder Scientific) for 1 hr. Animals were then injected with saline or a single dose of the direct dopamine agonist apomorphine (0, 0.75, 1.0, 1.5, or 2.0 mg/kg). Experimenters were blinded to the identity of injection solutions. Automated locomotor behavior was recorded and animals were observed for specific behaviors. Apomorphine produces a behavioral profile that is distinct from that of cocaine. As such, the recording
of specific behaviors for apomorphine differs somewhat from that used for cocaine. 
Apomorphine induces a high frequency of specific stereotyped behaviors including 
fixed sniffing and oral stereotypies that are manifested by repetitive gnawing or licking.
It is not currently clear if adolescents and adults engage in similar or different specific 
stereotypies following apomorphine. To examine this, we recorded the number of 
intervals observed in specific stereotypies rather than generating an average stereotypy 
score. We reported the total number of intervals in fixed sniffing or oral stereotypies as 
well as the total number of intervals in which either behavior was observed.

2.3.3 Single Dose Sensitization

These published experiments are presented here with kind permission from 
Springer Science+Business Media: Psychopharmacology, A single high dose of cocaine 
causes differential sensitization to specific behaviors across adolescence, 193, 2007, 247-
260.

2.3.3.1 Cocaine Pretreatment (Day 1)

We used the single-dose sensitization paradigm previously developed by Robert 
Post (Post et al. 1987). This paradigm produces marked locomotor sensitization in 
adults. Since it only requires 1 exposure, it will enable us to examine the expression of 
locomotor sensitization in young adolescents. Figure 5 shows a schematic 
representation of the paradigm over time. Animals PN 28, PN 42, and PN 65 were
placed in the locomotor activity chambers (Kinder Scientific) and automated locomotor activity was recorded for 1 hour. Rats were then injected with saline or 40 mg/kg cocaine. Automated locomotor data was collected and animals were also observed for specific behaviors for a 1 hr session immediately following injection. All animals were then returned to their group-housed home cages.

2.3.3.2 Cocaine Challenge (Day 2)

Twenty-four hrs after their first injection, rats were rehabituated to the locomotor test chambers (Kinder Scientific) for 1 hr and then injected with saline or 10 mg/kg cocaine. Automated locomotor data was collected and animals were also observed for specific behaviors for 1 hr immediately following injection.
Figure 5: Schematic timeline of single dose sensitization paradigm.

On day 1, animals were habituated to the locomotor test chambers for 1 hr. All animals were then injected with either saline or a single high dose of cocaine (40 mg/kg). Locomotor activity was recorded for 1 hr and animals were then returned to their home cages. 24 hrs later, all animals were rehabituated to the same locomotor chamber as the day before and challenged with saline or 10 mg/kg cocaine. Automated horizontal activity was continuously recorded and resolved into ambulatory and non-ambulatory (fine movements) activity. Experimenter observed stereotypies were recorded following the injection on both days. Locomotor sensitization was detected as an effect of pretreatment on day 2.
2.4 Plasma and Brain Cocaine Analyses

Animals PN 28, PN 42, and PN 65 were treated with three hourly injections of cocaine (15 mg/kg). Eight animals of each age were killed 30 min after each injection. This time was selected as peak locomotor activity to 15 mg/kg cocaine is observed at 30 min. Animals were killed by decapitation and trunk blood was collected, centrifuged, and stored in 5 M sodium fluoride at -80°C. Brains were snap frozen on dry ice and stored at -80°C. Cocaine levels were analyzed by the National Institute of Drug Abuse (NIDA) laboratory at the Center for Human Toxicology, University of Utah, using liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry. Analysts were blinded to the age and treatment condition of the samples. Animals with plasma cocaine concentrations below 50 ng/ml were assumed not to have received full injections of cocaine and were excluded from analysis as these values were below 2 standard deviations of the mean.
2.5 Homogenate GTPγS Assays

Drug naive PN 28 and PN 65 rats were killed by decapitation and brains were dissected using a brain block. The CP, nucleus accumbens, and prefrontal cortex were weighed, snap frozen on CO₂, and stored at -80°C. Tissues were homogenized into membrane preparations (see below) and G-protein activation was stimulated with nine concentrations of dopamine, the D1 specific agonist dihydrexidine, or the D2 specific agonist quinpirole.

2.5.1 Membrane Preparations

Tissues were homogenized in 10 ml of cold homogenization buffer (50 mM Tris-HCl, 3 mM MgCl₂, 1 mM EGTA, pH 7.4) with a hand-held homogenizer. The homogenizer was then washed with an additional 10 ml of homogenization buffer and the homogenate was diluted to a total volume of 30 ml. Preparations were then incubated at room temperature for 5 min to allow for dissociation of endogenous dopamine. Samples were then centrifuged for 10 min at 48,000g (4°C). The resulting pellet was then washed by homogenization as before and diluted to 30 ml and centrifuged again for 10 min at 48,000g (4°C). The final pellet was resuspended in 100 volumes of assay buffer (50 mM Tris-HCl, 3 mM MgCl₂, 0.2 mM EGTA, 100 mM NaCl, 0.1 mg/ml BSA, pH 7.4) by pipetting and vortexing and stored on ice until the assay or frozen at -80°C for up to 7 days.
2.5.2 \[^{35}\text{S}]\text{GTP} \gamma \text{S Assays}

Adenosine deaminase (3 mUnits/ml) was added to the membrane preparations and incubated at 30°C for 10 min just prior to the assay. Dopamine, quinpirole, or dihydrexidine solutions (0.01 M) were prepared by dissolving HCL powders (Sigma Aldrich, St Louis, MO) in 1 ml of assay buffer on the day of the assay. Solutions of varying concentrations (10\(^{-3}\) - 10\(^{-9}\)) were then generated by serial 1:10 dilutions. Each reaction was comprised of 500 µl assay buffer, 100 µl DTT (10 mM), 100 µl agonist, 100 µl \[^{35}\text{S}]\text{GTP} \gamma \text{S}, 100 µl GDP (200 µM) and 100 µl of membrane (+ adenosine deaminase). Non-specific binding was determined by adding 100 µl of cold GTP\(\gamma\)S to the reaction. All reactions were performed in triplicate. Reactions were mixed and then incubated at 30°C in a water bath for 1 hr and terminated by rapid filtration through Whatman GF/B filters under vacuum followed by three washes with cold assay buffer. Filters were then placed in scintillation vials with 4 ml of Safety Solve scintillation fluid and radioactivity was measured using a scintillation counter.
2.6 Real Time PCR

Rats PN 28, PN 42, and PN 65 were treated with saline or cocaine (40 mg/kg) in the locomotor test chambers. Rats treated with cocaine were killed 15, 30, or 60 min after injection. Brains were removed and the CP was dissected, snap-frozen on dry ice and stored at -80°C. Total RNA was isolated from these tissues, converted to cDNA, and relative c-fos and zif268 levels were quantitated using RT-PCR. These IEG signals are sensitive to perturbation by external stimuli and to reduce day to day variance, one animal of each age and treatment was used in every session and fold-induction levels were calculated by comparing saline and cocaine animals treated within the same session. Relative mRNA levels were determined by comparing the relative levels of c-fos and zif268 with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. GAPDH is a highly expressed enzyme that catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3,bisphosphoglycerate during glycolysis. It was selected as a reference because its expression is unaffected by cocaine and its expression is relatively stable across post-natal life. Further, relative age-effects with RT-PCR were similar to those observed with in situ hybridization.

2.6.1 RNA Isolation

Total RNA was isolated using the Trizol® method (Invitrogen, Carlesbad, CA). Briefly, tissues were homogenized with a handheld homogenizer in 1 ml of Trizol reagent and 0.20 ml of chloroform. Samples were centrifuged (4°C) at 15000 x g for 10
min. The polar phase (clear) was removed and mixed with 1.0 ml of isopropanol and centrifuged as before. The resulting pellet was rinsed in 70% ethanol, centrifuged (4°C), decanted, and dried at room temperature (RT) for 10 min. The pellet was then redissolved in sterile RNAse/DNAse free water at 55°C for 5 minutes. The concentration of RNA in the samples was then quantitated and immediately converted into cDNA prior to storage. RNA concentration and quality (260/280 ratio) were determined using a NanoDrop® UV spectrophotometer (NanoDrop Technologies, Wilmingston, DE). Samples with 260/280 ratios less than 1.6 were not converted to cDNA.

2.6.2 cDNA Generation

Total RNA was converted to cDNA using the Bio-Rad iScript Select® cDNA synthesis kit (BioRad Inc, Hercules, CA) in a Bio-Rad iCycler thermocycler. Briefly, 1 µl of total RNA (diluted to 1 ug/ul) was mixed with 2 µl of oligo dT primer (1 mM) in 13 µl of sterile RNAse/DNAse free water. The mixture was then heated to 65°C for 5 min and snap-chilled on ice for 1 min. Four ml of iScript Select® reaction mix and 1 µl of iScript reverse transcriptase was added and the reaction was then held at 42°C for 90 min. Reverse transcriptase was inactivated by heating the sample to 85°C for 5 min. cDNA was then stored at -20°C.
2.6.3 Real Time PCR

Real-time PCR was performed using a Roche LightCycler® RT-PCR instrument (F. Hoffman-La Roche Ltd, Switzerland). PCR reactions contained 10 µl of Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Carlesbad, CA), 1 µl 20X Bovine Serum Albumin (1 mg/ml), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 2 µl of cDNA template, and 5 ul RNAse/DNAse free water. Reaction cycles proceeded as follows: 50°C for 2 min hold, 95°C for 2 min hold, followed by 45 cycles of 94°C for 5 sec, 55°C for 10 sec, 72°C for 10 sec. Melting curve analysis (to verify single product reactions) of all samples was performed following each reaction by ramping the temperature to 95°C and reducing the temperature at a rate of 0.5°C per sec to 40°C.

2.6.4 C-fos, zif268, and GAPDH PCR Primer Development and Validation

GAPDH, zif268, and c-fos primers for RT-PCR were generated using PrimerQuest© software available from Integrated DNA Technology (Coralville, IA). C-fos primers were determined using the published rat c-fos mRNA (Genebank accession number X06769). The forward primer sequence (5’-3’) was AATGCCGCACTAAAGCGGATGAAC and the reverse primer sequence was TTTGCCAGACAGAGGACAGCGTAT. These primers generate a 171 BP sequence of the c-fos mRNA spanning bases 531-701. Zif268 primers were determined using the published rat zif268 mRNA (Genbank accession number M18416). The forward primer
sequence (5′-3′) was TCTGAATAACGAGAAGGCCGTGGT and the reverse sequence was (5′-3′) ACAAGGCCACTGACTAGGCTGAAA. GAPDH primers were determined using the published rat GAPDH sequence (Genebank accession number NM 017008). The forward primer sequence was ACAAGATGGTGAAAGGTCGGTGTGA and the reverse primer sequence was AGCTTCCCATTTCAGCCTTGACT. These primers generate a 199 BP sequence of the GAPDH mRNA spanning bases 72-270. All primers were synthesized by Integrated DNA Technologies, received as lyophilized powders, reconstituted to 100 µM in sterile RNAse/DNAse free water, and stored in aliquots at -20°C. The size of products from all primer pairs was validated by running standard PCR reactions followed by agarose gel electrophoresis. Melting curve analysis following each RT-PCR reaction further confirmed that all primer pairs produced detectable levels of only one significant product. We also validated the quantitative accuracy of our primer pairs by performing RT-PCR with 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32 template dilutions.
2.7 In Situ Hybridization

Animals PN 28 and PN 65 were habituated to the locomotor test chambers (Kinder Scientific) for 1 hr. They were then treated with saline, 10 or 40 mg/kg cocaine and locomotor activity was recorded for 30 min. Animals were then immediately killed by decapitation and brains were removed and snap frozen in tissue mounting media using a dry ice/ethanol slurry. Brains were stored at -80°C until processing.

2.7.1 Tissue Preparation

Tissues were cut from whole brains frozen in tissue mounting media using a Leica Cryostat (Leica Microsystems, Wetzlar, Germany). Brains were cut at -20°C and allowed at least 1 hr to equilibrate to temperature prior to cutting. 12 µm coronal sections were thaw mounted to pre-charged glass microslides (Superfrost Plus® slides, VWR International, LLC, West Chester, PA), air-dried at RT for 10 min, and stored at -80°C until fixing. Sections were fixed with a 3% a PFA solution in PBS (pH 7.4) for 5 min and then rinsed 3 x in 1 x PBS. To reduce background signal, slides were acetylated in a 0.25% acetic anhydride and 0.1 M triethanolamine in SSPE (pH 7.4) for 10 min, rinsed 3 x in 2 x SSC, dehydrated by a graded ethanol wash (75, 90, and 100% ethanol for 2 min each), and air-dried at RT for 1 hr.
2.7.2 \(^{35}\text{S}\)Riboprobe Generation

Radio-labeled sense and antisense riboprobes for the \(c\text{-}fos\) and \(zif268\) rat mRNAs were generated by \textit{in vitro} transcription. 70mer double-strand DNA oligo sequences were used as templates for these reactions. Each oligo contained a 20 bp T7 (TAATACGACTCTATAGGG) or SP6 (ATTTAGGTGACACTATAGAA) RNA polymerase promotor and a 50 bp sequence of the targeted RNA. Templates for antisense messages had 5’-3’ T7 sequences followed by bases 207-256 of the rat \(c\text{-}fos\) message (Genbank accession number X06769) or bases 352-391 of the rat \(zif268\) message (Genbank accession number M18416). Antisense probes of these sequences have been utilized previously for \textit{in situ} hybridization of these messages (Brandon and Steiner, 2003). Templates for sense messages had 5’-3’ SP6 sequences followed by compliments to bases 207-256 of the rat \(c\text{-}fos\) message or bases 352-391 of the rat \(zif268\) message.

The transcription reactions were carried out using Riboprobe\textsuperscript{®} combination T7/SP6 in vitro transcription reaction kits (Promega Corp., Madison, WI) using cold ATP, GTP, and CTP with \(^{35}\text{S}\)\(\alpha\)UTP nucleotides. Briefly, nucleotides and Promega reaction components were mixed with template, RNAasin and RNA polymerase enzymes, and incubated at 37°C for 3 hr. Probes were then ethanol precipitated in 70% ethanol and 3M sodium acetate at -80°C for 30 min. Samples were then spun at 15000 x g (4°C) for 30 min, washed in 70% ethanol, and then dissolved in hybridization solution (50% formamide, 60 mM sodium chloride, 10 mM tris-HCL, 24 mM EDTA, 10 mM DTT,
1 x Denhart’s Solution, 5 mg/ml tRNA, 100g/ml dextran sulfate) and stored at -80°C for
up to 1 week. Radioactive levels were calculated using a scintillation counter.

2.7.3 In Situ Hybridization

Slides were labeled with 1.0 x 10⁶ CPM of labeled probe, cover-slipped, and
incubated over night at 65°C in mineral oil. Slides were rinsed 2 x in chloroform, 2 x in a
0.2% β-mercaptoethanol SSPE solution (pH 7.4), and incubated in a 0.2% β-
mercaptoethanol SSPE solution (pH 7.4) at RT for 1 hr. Slides were then washed in a
0.2% β-mercaptoethanol 50% formamide SSPE solution at 65°C for 30 min and a 0.1%
SSPE (pH 7.4) solution for 30 min at 65°C, dehydrated by a graded ethanol wash, and
air-dried for at least 1 hr at RT. Dried slides were then apposed to Biomax® MS
autoradiography film (Kodak Company, Rochester NY) for 10-20 days. Exposure times
were adjusted to prevent reaching film saturation.

2.7.4 Analysis of Autoradiograms

C-fos and zif268 mRNA levels were quantitated using a PC based imaging
analysis system (Scion Image, Scion Corp., Frederick, MD). Mean density values for
each region were recorded from both hemispheres and an average value was obtained.
We normalized average mean density values by subtracting background values
measured over corpus callosum white matter. Normalized average values are presented
as corrected $c$-fos and $zif268$ values. Experimenters were blinded to the age and treatment condition of all brain images during quantiation.

### 2.7.5 Sampling Areas

We selected four rostral to caudal coronal sections from which to sample IEG mRNA levels. Figure 6 shows a schematic representation of regions quantified by *in situ* hybridization. We analyzed 12 striatal regions from 3 rostral to caudal sections. These regions were chosen for specific reasons. First, we analyzed from three rostral to caudal sections because dopaminergic innervation occurs in a caudal to rostral manner. It is possible that during final development there could be delayed maturation in rostral but not caudal regions of the striatum. We analyzed multiple striatal regions because they have different functional roles mediating different aspects of addiction. As reviewed above, the nucleus accumbens is implicated in drug reward and the CP is implicated in habit formation. Further, specific subregions within the CP and nucleus accumbens mediate specific psychomotor behaviors (Delfs and Kelley 1990; Kelly and Iverson 1976). The selected subregions also receive glutamaturgic input from distinct cortical areas (below indicated in parentheses). In the most rostral striatal section (+1.60 mm) we measured $c$-fos and $zif268$ mRNA levels in the dorsal caudate (dorsal agranular, sensorimotor cortex), medial caudate (dorsal and ventral anterior cingulate), lateral caudate (sensorimotor cortex), nucleus accumbens core (prelimbic, agranular insular cortex), medial nucleus accumbens shell (prelimbic, infralimbic cortex), and the ventral
nucleus accumbens shell (agranular insular cortex). In the medial section (+0.40 mm) we measured \textit{c-fos} and \textit{zif268} mRNA levels in the dorsal caudate (dorsal agranular, sensorimotor cortex), lateral caudate (sensorimotor cortex), ventromedial caudate (sensorimotor cortex), and the ventrolateral caudate (sensorimotor cortex). From the most caudal section (-0.80 mm) we measured \textit{c-fos} and \textit{zif268} mRNA levels in the dorsal caudate (dorsal agranular, sensorimotor cortex) and the ventrolateral caudate (sensorimotor cortex). We also measured \textit{c-fos} and \textit{zif268} expression in the above listed cortical regions. From the most rostral section (+3.20 mm), we quantitated mRNA levels in the orbital cortex and the cingulate. We measured \textit{c-fos} and \textit{zif268} mRNA levels in five cortical regions in all of the remaining sections: the dorsal agranular cortex, dorsal anterior cingulate, ventral anterior cingulate, sensorimotor, and agranular insular cortex.
Figure 6: Sampling areas for quantitative in situ hybridization analyses.

In our in situ hybridization experiments, we analyzed regional c-fos and zif268 mRNA levels in seven cortical and twelve striatal subregions from 4 rostral-caudal coronal sections. From the most rostral section (+3.20 mm) we analyzed the anterior cingulate (cing) and the orbital cortex (orb). We analyzed five cortical regions from each of the remaining three sections: the dorsal agranular/premotor cortex (AG), dorsal anterior cingulate (ACD), ventral anterior cingulate (ACV), sensorimotor cortex (SM), and the agranular insular cortex (AI). From the +1.60 section we analyzed the following striatal subregions: the dorsal caudate (DC), lateral caudate (LC), medial caudate (MC), nucleus accumbens core (C), medial nucleus accumbens shell (MS), and the ventral nucleus accumbens shell (VS). From the +0.40 section we analyzed the following striatal subregions: dorsal caudate (DC), lateral caudate (LC), ventromedial caudate (VMC), and the ventrolateral caudate (VLC). From the -0.80 section we analyzed the following striatal regions: dorsal caudate (DC) and the ventrolateral caudate (VLC).
2.8 Statistical Analyses

ANOVA and post-hoc analyses were conducted using NCSS 2000 statistical software (NCSS, Kaysville, UT). Linear regressions and ANCOVAs were performed using JMP statistical software (SAS, Cary, NC). Since we had no reason to assume many of our experimental measures would have linear relationships, Spearman rank-order analyses were also performed for all linear regression analyses using NCSS 2000 statistical software. However, we did not gain or lose any significant linear correlations using the rank order analysis, so Spearman correlations are not presented in the results.

2.8.1 Acute Cocaine Binges

We analyzed locomotor responses to acute cocaine binges using three-factor repeated measures ANOVAs utilizing age as a between subject variable and time and injection (dose) as repeated measures. We analyzed the effects of injection within a repeated dose cocaine binge on fixed stereotypy frequency using a two-factor repeated measures ANOVA with age as a between subject variable and injection as a repeated measure. Experimenter observed stereotypies were not recorded during the first two doses (5, 10 mg/kg) of the escalating dose cocaine binge as stereotypies should be minimal following these doses. The effects of age on the number of high velocity intervals observed during the third and highest dose of a repeated dose cocaine binge were analyzed using a one-way ANOVA.
To determine how an escalating dose cocaine binge affected locomotor and stereotypy responses to a high dose of cocaine, we treated animals with an escalating dose binge or two injections of saline followed by 25 or 40 mg/kg cocaine. We analyzed the effects of age and treatment on fixed stereotypy frequency using two-factor ANOVAs with age and treatment as between subject variables. In these (and all subsequent) analyses, main effects and interactions were considered significant at P<0.05. Newman-Keuls post-hoc tests were used to distinguish significant main effects. Significant interactions were further investigated using subsequent ANOVAs filtered by significant effects.

**2.8.2 Blood and Brain Cocaine Concentrations**

The effects of age and injection on cocaine brain and plasma concentrations during a repeated dose cocaine binge were analyzed using two-factor repeated measures ANOVAs with age as a between subject variable and repeated measures of injection number. Brain and plasma cocaine concentrations were analyzed as separate response variables.

**2.8.3 Apomorphine Behavior**

The effects of age and dose on the temporal pattern of locomotor activity induced by acute apomorphine were measured using three-factor repeated measures ANOVAs with age and dose as between subject variables and time as a repeated measure. The
three measures of horizontal activity (basic movements, ambulations, and fine movements) were analyzed separately as distinct responses variables. The effects of dose and age on frequency of fixed stereotypies were analyzed using a two factor ANOVA with age and dose as between subject variables.

2.8.4 Single Dose Sensitization

In these experiments, all animals were habituated to the locomotor chambers for 1 hour prior to treatment with cocaine or saline. Locomotor activity during habituation (fine movements and ambulations) was analyzed using a two-factor repeated measures ANOVA with age as a between subject variable and repeated measures of time. During habituation on day 1, animals were not separated based on their subsequent treatment groups. After treatment with cocaine or saline, we analyzed locomotor responses using three-factor repeated measures ANOVAs with age and treatment as between subject variables and repeated measures of time. We then analyzed the effects of cocaine treatment on average behavioral rating, sniffing frequency, and stereotypy frequency in adolescents and adults using two factor ANOVAs with age and treatment as between subject variables.

Locomotor sensitization to novelty, a saline injection, or cocaine was measured by comparing locomotor responses in animals pretreated with saline or high dose cocaine. Locomotor responses to placement in the locomotor chambers were analyzed using three-factor repeated measures ANOVAs with age and pretreatment as between
subject variables and repeated measures of time. Locomotor response to saline or cocaine were also analyzed using three-factor repeated measures ANOVAs with age and pretreatment as between subject variables and repeated measures of time. The effects of pretreatment on average behavioral ratings, sniffing frequency, and stereotypy frequency were measured using two factor ANOVAs with age and pretreatment as between subject variables. In all of these analyses, sensitization was detected as an effect of pretreatment.

Median-split analyses (high and low responder) were used to compare initial responses to novelty or acute cocaine to each other and the cocaine challenge. We used high dose cocaine-induced locomotor activity and locomotor responses to the cocaine challenge as response variables in two-factor ANOVAs with age and habituation locomotor classification (high or low responder) or high-dose cocaine locomotor classification as between subject variables.

Linear regression analyses were also employed to compare locomotor responses to novelty or high dose cocaine with each other and the cocaine challenge in individual animals. We used acute locomotor responses to cocaine or locomotor responses to the cocaine challenge as response variables in two-factor ANCOVAs with age and habituation locomotor activity or high-dose cocaine locomotor activity as between subject variables. For these measures, locomotor activity represents the sum of ambulations or fine movements during the 1 hr recording sessions.
2.8.5 G-protein Stimulation by Dopamine Agonists

We analyzed the effects of age on G-protein stimulation by dopamine agonists (dopamine, dihydrexidine, and quinpirole) using repeated measures two-factor ANOVAs with age as a between subject variable and repeated measures of dose. The dose-response relationships for each agonist were analyzed separately.

2.8.6 Time Course of Cocaine-Induced \textit{c-fos} and \textit{zif268} Expression

Fold-increase mRNA is calculated by dividing relative mRNA levels at each time point by the average relative levels of animals placed in the locomotor chambers and injected with saline. The potential effects of age were then investigated using a three-factor repeated measure ANOVA with age and time as between subject variables and gene as a repeated measure.

2.8.7 \textit{c-fos} and \textit{zif268} \textit{In Situ} Hybridization

The effects of age, dose, and region on striatal \textit{c-fos} and \textit{zif268} mRNA levels were initially examined using a four-factor repeated measure ANOVA with between subject variables of age and dose and repeated measures of region and gene. Subsequent ANOVAs filtered by significant effects were used to further investigate three and four way interactions.

\textit{C-fos} and \textit{zif268} expression in the cortex were measured using two different ANOVAs for specific regions. Five cortical regions were repeatedly analyzed at three
rostral-caudal coordinates. We employed a four-factor ANOVA with subject variables of age and dose and repeated measures of region and gene for initial analysis. C-fos and zif268 expression in two other cortical regions from the most rostral (+3.20 mm) section were analyzed separately using the same four-factor ANOVA design. The same ANOVAs were used to examine the relative increases (as a percent of saline) in cocaine-stimulated c-fos and zif268 expression.

We also correlated c-fos and zif268 mRNA levels in individual striatal and cortical subregions using linear regression analyses. The effects of age and region on these correlations were analyzed using a four-factor ANCOVAs with age, dose, gene and locomotor activity as variables. Correlations for striatal and cortical subregions were analyzed using separate ANCOVAs.

The effects of age and dose on the correlation between c-fos and zif268 expression in individual regions were analyzed using three-factor ANCOVAs with age, dose, and region as variables. Correlations in striatal and cortical subregions were analyzed using separate ANCOVAs.
3. CHAPTER 3: Characterize Locomotor Responses Within Acute Cocaine Binges in Adolescents and Adults.

Many human drug addicts will commonly take drugs in a “binge” pattern in which they continue to re-administer as the subjective effects of the previous dose begin to wear off. These binges often involve an escalation of dose (Gawin 1991). Clinical reports suggest that binge patterns of alcohol and marijuana use during adolescence predict worse adult outcomes than other use patterns. Despite this evidence, few studies have explored how binge administration affects acute locomotor responsiveness to stimulants. We utilized acute cocaine binges to model one pattern of human drug intake.

**Experiment 1: Compare Locomotor Responses to an Acute Escalating Dose Cocaine Binge in Adolescents and Adults.**

We used an escalating dose binge to mimic one pattern of human cocaine intake and to examine locomotor responses to cocaine across a range of doses. Cocaine caused dose-related increases in locomotion (Figure 7). ANOVA indicated a main effect of dose [$F(1,70)=18.3, P<0.001$], a main effect of treatment [$F(1,70)=18.28, P<0.001$], and a treatment x dose interaction [$F(2,134)=14.93, P<0.001$]. Cocaine (10 and 25 mg/kg) treated animals generated more horizontal activity than saline treated controls. We did not identify a main effect of age for horizontal activity following any dose of cocaine.
However, we observed a subset of these animals for specific stereotypies following the third and highest dose of cocaine and we examined the distribution of stereotypy scores in each age (Figure 8). The distribution of stereotypy scores differed significantly with age as ANOVA indicated an age x score interaction [F(10,120)=5.99, P<0.001]. Adult animals were predominantly observed in stereotypies frequently interrupted by locomotion (stereotypy score 4), whereas young adolescents were most frequently observed in fixed stereotypies (stereotypy score 5) confined to one corner of the cage.

The two behavioral measures (horizontal activity and stereotypy) suggest potentially contradictory results: Young adolescents were observed in fixed stereotypies confined to one corner of the cage for approximately 50% of the observed intervals. Repetitive beam breaks (indicative of fixed stereotypies) were not counted as horizontal activity. Despite this, young adolescents recorded as much horizontal activity as adults. One possible explanation for these findings is that young adolescents could locomote at greater velocities than adults. We estimated the velocity of locomotion by reducing our recording intervals to 6s. A comparison of the number of intervals spent at maximal velocity (>30 in/6 sec) demonstrated that young adolescents locomoted more quickly than adults (Figure 9). ANOVA indicated a main effect of age [F(2,70)=19.2, P<0.001] and post-hoc analysis indicated PN 28 animals had more high velocity intervals than older animals. These results demonstrate age-specific responses to cocaine within an escalating dose binge.
PN 28, 42, and 65 male rats were treated with saline (a) or an escalating dose binge of cocaine (b). Arrows denote times of injection and cocaine doses (in mg/kg) are indicated above the arrows. Cocaine caused significant dose related increases in horizontal activity compared to saline as determined by repeated measures ANOVA. No age differences were identified in either treatment group. Horizontal activity was continuously recorded and symbols represent mean +/- S.E.M. of each 5 min interval. N=14-16 for each cocaine treated age group and N=8 for all saline treated age groups.
Figure 8: Stereotyped behaviors during the third and highest dose of an escalating dose cocaine binge.

PN 28, PN 42, and PN 65 animals were treated with hourly injections of 5, 10, and then 25 mg/kg cocaine. Stereotypies were recorded following the 25 mg/kg dose and we reported the frequency that animals of each age were observed in each behavioral rating score. Adolescents engaged in more fixed stereotypies than adults. ANOVA indicated a main effect of age for average stereotypy scores and an age x score interaction for stereotypy score distribution. Adults (striped bars) were more frequently observed in stereotypies interrupted by locomotion (score 4) than adolescents whereas adolescents were more frequently observed in fixed stereotypies (score 5) than adults. Symbols and bars represent mean +/- S.E.M. N=7-8 for each age group. *indicates greater than PN 65. **indicates greater than PN 28 and 42.
**Figure 9**: High velocity horizontal activity following the third and highest dose of an escalating dose cocaine binge.

PN 28, PN 42, and PN 65 animals were challenged with 3 hourly injections of 5, 10, and 25 mg/kg cocaine and horizontal activity was recorded continuously. To estimate the number of high velocity intervals following the third and highest dose of cocaine, the recording time for each interval was reduced to 6 sec. Intervals with greater than 30 cm traveled per 6 sec were counted as high velocity intervals. Young adolescents had more high velocity intervals than mid adolescents and adults. ANOVA indicated a main effect of age. *indicates greater in PN28 than older animals. N=14-15 for each age group.
Experiment 2: Compare Locomotor Responses to a Repeated Dose Cocaine Binge in Adolescents and Adults.

To determine if repeated cocaine injections affect locomotor responses to subsequent injections within a binge, we treated animals with three hourly injections of the same dose (15 mg/kg) of cocaine. Both adolescents and adults engaged in stereotypies after 25 mg/kg cocaine. We wanted to use a lower dose to examine if adolescents might transition into stereotypies at lower doses than adults. Horizontal activity and stereotypy observations were made following each injection. For horizontal activity, ANOVA indicated a main effect of injection \[F(2,28)=89.92, \ P<0.001\] but no main effect of age (Figure 10). We also measured the frequency of intervals that animals of each age engaged in fixed stereotypies after each injection (Figure 11). Young adolescents were observed in fixed stereotypies following every injection and the frequency of intervals that they engaged in fixed stereotypies increased following each subsequent injection. ANOVA indicated a main effect of age \[F(1,39)=58.38, \ P<0.001\], injection \[F(2,39)=3.78, \ P<0.04\], and an age x injection interaction \[F(2,39)=3.45, \ P<0.05\]. Adult animals did not engage in fixed stereotypies following any injection with 15 mg/kg cocaine.
Figure 10: Locomotor responses to a repeated dose cocaine binge.

PN 28 and PN 65 male rats were habituated to the locomotor test chambers and treated with 3 hourly injections of 15 mg/kg cocaine. Horizontal activity was continuously recorded. Closed symbols represent adolescents animals and open symbols represent adults. Arrows denote times of injection. Symbols represent mean +/- S.E.M. for each five min interval of the binge. N=8 for each age group.
Figure 11: Stereotypy responses to repeated dose binge cocaine.

PN 28 and PN 65 male rats were treated with 3 hourly injections of 15 mg/kg cocaine. Experimenter observed behaviors were recorded and scored by a behavioral rating scale. We recorded the frequency of fixed stereotypies following each injection in adolescents (filled bars) and adults (open bars). Cocaine caused more fixed stereotypies in PN 28 animals than PN 65 and repeated cocaine injections increased the frequency in adolescents. ANOVA indicated a main effect of injection, age, and an age x injection interaction for both measures. N=8 for each age group. *indicates greater than injection 1 in PN 28.
Experiment 3: Compare Stereotypies Induced by an Escalating Dose Binge and Acute Cocaine.

The high frequency of fixed stereotypies observed in young adolescents following the third and highest dose of an escalating dose binge (25 mg/kg) could represent an exaggerated stereotypy response to the high dose of cocaine or adolescent specific behavioral augmentation within the binge. To determine the relative effect of dose and binge treatment, we treated animals with an escalating dose binge or two hourly injections of saline followed by 25 or 40 mg/kg cocaine (the sum of the three doses in the binge). Horizontal activity from these experiments is shown in figure 12. Following the third injection (the first two injections were not included in the ANOVA as two groups received saline) ANOVA indicated a main effect of treatment for horizontal activity [F(2,79)=3.79, P<0.02]. Post-hoc analysis indicated that 40 mg/kg cocaine produced more horizontal activity than 25 mg/kg acutely or within the binge. ANOVA indicated no main effect or interactions with age for horizontal activity. Our results demonstrate that cocaine-induced horizontal activity is a function of dose and is relatively unaffected by age or repeated treatment within a binge.

Binge treatment did significantly increase adolescent stereotypy responses to the third injection (Figure 13). ANOVA indicated a main effect of age [F(2,77)=10.35, P<0.001], treatment [F(2,77)=7.33, P<0.01], and an age x treatment interaction [F(4,77)=3.75, P<0.01] for fixed stereotypy frequency. Post-hoc analysis demonstrated
that binge treated young adolescents engaged in more fixed stereotypies than any other
age x treatment group. Within young adolescents, binge cocaine treatment more than
doubled the frequency of fixed stereotypies compared to either acute high dose of
cocaine.
Figure 12: Comparison of locomotor responses to acute cocaine and the third dose of an escalating dose cocaine binge.

PN 28, PN42, and PN 65 animals were treated with an escalating dose binge of cocaine or two injections of saline followed by 25 or 40 mg/kg cocaine. Horizontal activity was continuously recorded and activity following the third injection (when all animals received cocaine) is presented here. The left panels (a-c) show age comparisons following each of the three treatments. The right panels (d-f) show treatment comparisons for each of the ages. Symbols represent mean +/- S.E.M. N=8-12 for each age x treatment group.
Figure 13: Effect of escalating dose binge treatment on fixed stereotypy frequency.

PN 28, PN42, and PN 65 animals were treated with an escalating dose binge of cocaine or two injections of saline followed by 25 or 40 mg/kg cocaine. Young adolescents treated with binge cocaine had the highest frequency of fixed stereotypies. ANOVA indicated a main effect of age and an age x treatment interaction. Black bars indicated young adolescents. White bars indicate mid-adolescents. Striped bars indicate adults. All bars represent mean +/- S.E.M. *indicates greater than all other groups. N=8-14 for each age x treatment group.
Experiment 4: Measure Plasma and Brain Cocaine Concentrations after Each Injection of a Repeated Dose Cocaine Binge.

One possible explanation for the augmentation of stereotyped behaviors following successive cocaine treatments within a binge could be age-specific accumulation of brain or plasma cocaine. Cocaine is metabolized with a half-life of approximately 28 min in the rat (Hurd et al. 1989). During a 15 mg/kg repeated dose binge, plasma and brain cocaine levels should increase slightly following subsequent injections. However, it has yet to be established that this increase is comparable in adolescents and adults. We measured cocaine brain and plasma concentrations in PN 28, PN 42, and PN 65 animals 30 min after each injection of a repeated dose binge.

Exaggerated adolescent brain or plasma concentrations were not observed. In fact, adolescents (PN 28 and 42) had lower plasma cocaine concentrations than adults (Figure 14a). For plasma cocaine concentrations, ANOVA indicated a main effect of injection $[F(2,67)=3.45, P<0.05]$ and age $[F(2,67)=4.93, P<0.05]$. Post-hoc analysis indicated that adults had higher plasma cocaine concentrations than younger animals. This suggests that adolescent metabolize cocaine faster than adults. Surprisingly however, brain cocaine concentrations did not significantly accumulate with subsequent injections or differ by age as ANOVA indicated no main effect or interactions of injection or age (Figure 14b).
Figure 14: Plasma and brain cocaine concentrations following each injection of a repeated dose cocaine binge.

PN 28, PN 42, and PN 65 animals were treated with three hourly injections of 15 mg/kg cocaine. A subset of animals was killed 30 min after each injection and blood (a) and brain (b) cocaine concentrations were measured. ANOVA indicated a main effect of age and injection (dose) for blood concentrations. Adults had greater plasma cocaine concentrations than adolescents and repeated cocaine injections increased plasma cocaine concentrations. ANOVA indicated no main effects or interactions for brain cocaine concentrations. Symbols represent mean +/- S.E.M. N=7-8 for each age x injection group.
Experiment 5: Measure Locomotor Responses to Apomorphine in Adolescents and Adults.

We determined that young adolescents had greater stereotypy responses than adults to acute moderate (15 mg/kg) but not high dose (25 or 40 mg/kg) cocaine. It is possible that enhanced locomotor responses to cocaine in adolescents could be mediated by enhanced postsynaptic responses or greater perturbation of presynaptic dopamine regulation. To examine if these age-specific behavioral responses were mediated by enhanced postsynaptic neuron activation, we treated adolescent (PN 28 and PN 42) and adult animals with the direct dopamine agonist apomorphine (0, 0.75, 1.0, 1.5, and 2.0 mg/kg). The dose-responsiveness of apomorphine with respect to age largely reflected that of cocaine: low-moderate doses of apomorphine induced more locomotor activity in young adolescents than older animals. For total horizontal activity (Figure 15), ANOVA indicated a main effect of age [F(2,101)=6.34, P<0.003] and an age x dose interaction [F(8,101)=2.26, P<0.03]. Post-hoc analysis indicated that PN 28 animals engaged in more horizontal activity than older animals. This difference was only statistically significant at the 1.0 mg/kg dose when young adolescents had more horizontal activity than older animals.

Resolution of apomorphine-induced horizontal activity into ambulatory and non-ambulatory components demonstrated that the exaggerated adolescent horizontal activity was largely mediated by greater ambulatory activation of young adolescents by
1.0 mg/kg apomorphine. For ambulations, ANOVA indicated a main effect of age [F(2,101)=7.36, P<0.002] and an age x dose interaction [F(8,101)=2.23, P<0.04] (Figure 16). Post-hoc analysis indicated that PN 28 rats ambulated more than older animals and that PN 28 animals challenged with 1.0 mg/kg apomorphine ambulated more than all other age x treatment groups. Higher doses of cocaine did not induce significant levels of ambulatory activity in animals of any age.

Apomorphine also induced non-ambulatory (fine movements) in animals of all ages (Figure 17). ANOVA indicated a main effect of age [F(2,101)=3.84, P<0.03] and dose [F(4,101)=2.52, P<0.05]. Post-hoc analysis indicated that young adolescents had greater responses than older animals and higher doses of apomorphine induced greater numbers of fine movements. The measure fine movements records a number of behaviors including some fixed stereotypies. However, unlike with cocaine, apomorphine-induced fixed stereotypies did not include head weaving or head bobbing. Consequently, many of these behaviors were likely not detected by our automated system. If anything, these fixed stereotypies likely displaced horizontal locomotion and significantly reduced the total number of photobeam interruptions. Therefore, we also observed animals for fixed sniffing and oral stereotypies to completely evaluate their behavioral profile.

Apomorphine induced fixed stereotypies in animals of all ages and lower doses of apomorphine induced more stereotypies in adolescent compared to adult animals.
(Figure 18). For the frequency of fixed stereotypies, ANOVA indicated a main effect of age \( F(2,89)=5.87, P<0.01 \), a main effect of dose \( F(4,89)=39.51, P<0.001 \), and an age x dose interaction \( F(8,89)=2.41, P<0.02 \). Post-hoc analysis indicated that PN 28 animals did more fixed sniffing than PN 65 animals. The age x dose interaction reflects a leftward shift in the dose response relationship in adolescents compared to adults. The dose of 1.0 mg/kg induced significant increases in fixed stereotypies compared to saline in young and mid-adolescents but not in adults. High doses of apomorphine (1.5 and 2.0 mg/kg) induced fixed stereotypies in all observed animals. Our results with apomorphine are similar to those with cocaine: low to moderate doses can induce greater locomotor responses in adolescents compared to adults, but higher doses induce similar levels of activity.
Figure 15: Apomorphine-induced horizontal activity.

PN 28, PN 42, and PN 65 animals were treated with saline (a), 0.75 (b), 1.0 (c), 1.5 (d), or 2.0 mg/kg apomorphine (e). ANOVA indicated a main effect of age and an age x dose interaction. Apomorphine induced more locomotor activity in young adolescents than older animals following 1.0 mg/kg. Symbols represent mean +/- S.E.M. N=7-10 for each age x treatment group.
Figure 16: Apomorphine-induced ambulatory activity.

PN 28, PN 42, and PN 65 animals were treated with saline (a), 0.75 (b), 1.0 (c), 1.5 (d), or 2.0 mg/kg apomorphine (e). ANOVA indicated a main effect of age and an age x dose interaction. PN 28 rats treated with 1.0 mg/kg apomorphine had greater ambulatory responses than all other age x treatment groups. Symbols represent mean +/- S.E.M. N=7-10 for each age x treatment group.

*ANOVA P<0.05 for age
Figure 17: Apomorphine-induced fine movements.

PN 28, PN 42, and PN 65 animals were treated with saline (a), 0.75 (b), 1.0 (c), 1.5 (d), or 2.0 mg/kg apomorphine (e). Higher doses of apomorphine induced more fine movements than lower doses. ANOVA indicated a main effect of dose but no main effect or interactions with age. Symbols represent mean +/- S.E.M. N=7-10 for each age x treatment group.
Figure 18: Apomorphine-induced fixed stereotypies.

Animals PN 28, PN 42, and PN 65 were treated with 0, 0.75, 1.0, 1.5, and 2.0 mg/kg apomorphine. Animals were observed for specific behaviors. The predominant behaviors observed were fixed sniffing and oral stereotypies. The total number of intervals that animals of each age engaged in either fixed stereotypy is reported here. Lower doses of apomorphine induced maximal numbers of behaviors in young and mid adolescents than adults. ANOVA indicated an age x dose interaction. * indicates greater than PN 65. N=7-10 for each age x treatment group.
4. CHAPTER 4: Determine the Ontogeny of Single Dose Sensitization During Adolescence.

Our binge experiments demonstrate that sensitization to cocaine can be observed in adolescents during a single cocaine binge. We predicted that a single high dose of cocaine (40 mg/kg), which induces locomotor sensitization in adults, would induce relatively greater locomotor sensitization in adolescents. To test this, we treated young adolescents, mid adolescents, and adults with saline or 40 mg/kg cocaine in locomotor test chambers (following a 1 hr non-drug habituation), returned them to their home cages, and challenged them with saline or 10 mg/kg cocaine in the locomotor test chambers 24 hrs later. Automated locomotor activity (ambulations and fine movements) was recorded during the 1 hr habituation and 1 hr test sessions on both days for each animal. After several rounds of experiments, we began to observe all animals for specific behaviors. This included >50% of all adolescent and adult animals.

**Experiment 1: Measure Locomotor Responses to High Dose (40 mg/kg) Cocaine.**

Adolescents had greater locomotor responses following placement into the open-field activity chambers (Figure 19, time<65 min). For ambulations, ANOVA indicated a main effect of age \[F(2,195)=3.4, P<0.05\] and an age x time interaction \[F(22,2145)=3.6, P<0.001\]. Post-hoc analysis indicated that PN 28 rats ambulated more than older
animals and that this difference was most evident in the first five min interval. ANOVA indicated no main effects or interactions for fine movements during habituation.

The high dose of cocaine (40 mg/kg) significantly increased both ambulations and fine movements compared to saline as ANOVA indicated a main effect of treatment for both measures \([F(2,201)=120, P<0.001]\) and \([F(2,201)=308, P<0.001]\), respectively (Figure 19, time>60 min). ANOVA indicated a main effect of age for ambulations \([F(2,201)=3.6, P<0.05]\) and post-hoc analysis indicated that young adolescents had greater ambulatory responses to 40 mg/kg cocaine than adults. Additionally, cocaine induced a distinct temporal pattern of ambulatory activity in young adolescents as ANOVA indicated an age x time interaction \([F(22,2211)=3.0, P<0.001]\). Cocaine caused a biphasic increase in ambulatory activity in young adolescents. Young adolescents also had a distinguishable temporal pattern of activity for fine movements as well. ANOVA indicated an age x time interaction for fine movements \([F(22,2211)=3.6, P<0.001]\). Young adolescents recorded significantly few fine movements than older animals for several intervals of the session.

In our previous experiment, we showed that adolescents and adults had similar horizontal activity levels following acute 40 mg/kg cocaine (see figure 12). Horizontal activity is the sum of fine movements and ambulations. In this study, adolescents and adults had similar levels of horizontal activity following 40 mg/kg cocaine. This fine-grained analysis demonstrates that adolescents moved from place to place more than
adults following 40 mg/kg cocaine. Adults may have been doing more stationary behaviors than adolescents although fine movements is also a size dependent measure (see methods and Figure 2).

A subset of animals was also observed for the occurrence of specific behaviors (Figure 20). To generate both quantitative and qualitative data, we generated an overall behavioral rating and reported the frequency of continuous sniffing and stereotypies (including head weaving, gnawing, patterned locomotion, and wall climbing) which represent the most commonly observed behaviors. Cocaine significantly increased the average behavioral rating and frequency of continuous sniffing and stereotypies in animals of all ages. ANOVA indicated a main effect of treatment for average behavioral rating \([F(2,40)=415, P<0.001]\), continuous sniffing frequency \([F(2,40)=2863, P<0.001]\), and stereotypy frequency \([F(2,40)=410, P<0.001]\). Consistent with our previous data, 40 mg/kg cocaine induced similar effects on specific behaviors in animals of all ages as ANOVA indicated no main effect or interactions with age.
PN 28, PN 42, and PN 65 rats were habituated to the locomotor test chambers for 1 hr and then treated with saline or 40 mg/kg cocaine. Automated locomotor data was recorded continuously during the habituation (time 0-60 min) and cocaine (time 65-120) sessions. Horizontal activity was resolved into ambulations (a) and fine movements (b). High dose cocaine induced more ambulations and a distinct temporal pattern of ambulatory activity in young adolescents. ANOVA indicated a main effect of age and an age x time interaction. Filled symbols represent animals treated with cocaine. Open symbols represent animals treated with saline. During habituation (time 0-60 min), animals were not separated by age. All symbols represent mean +/- S.E.M. for each 5 min interval. Arrows denote injection times. Bars represent mean +/- S.E.M. for the cocaine hour. *indicates greater in PN 28 than PN 42 or 65. ! indicates greater in PN 42 and 65 than PN 28. N=24-31 for each age x treatment group.
Figure 20: Stereotypy responses to high dose cocaine.

PN 28, PN 42, and PN 65 rats were habituated to the locomotor test chambers for 1 hr and then treated with saline or 40 mg/kg cocaine. Animals were observed for specific behaviors and given an average behavioral rating score for each interval. Cocaine primarily induced continuous sniffing and stereotypies frequently interrupted by locomotion. The average behavioral rating of each rat is shown in (a). The frequency of intervals in which continuous sniffing were observed is shown in (b). The frequency of intervals in which a stereotyped behavior was observed (head weaving, wall climbing, patterned locomotion, etc.) is shown in (c). ANOVA indicated a main effect of treatment for all three. ANOVA indicated no main effect or interactions with age. *indicates greater than saline. N=7-10 for each age x treatment group.
Experiment 2: Measure Locomotor Sensitization in Adolescents and Adults 24 hours After a High Dose of Cocaine.

Cocaine pretreatment induced sensitization to novelty-induced locomotor activity (Figure 21, time 0-60). During habituation prior to the cocaine challenge (time < 65 min) ANOVA indicated a main effect of pretreatment for ambulations [$F(2,188)=9.6$, $P<0.001$] and fine movements [$F(2,188)=9.6$, $P<0.01$]. As on day 1, ANOVA indicated a main effect of age [$F(2,188)=9.6$, $P<0.01$] and post-hoc analysis indicated young adolescents ambulated more than older animals during habituation. Cocaine caused similar increases in animals of all ages for both measures.

Cocaine pretreatment induced age-specific patterns of locomotor sensitization in adolescents and adults. Ambulatory sensitization to a cocaine challenge is shown in Figure 21a-c. ANOVA indicated a main effect of age [$F(2,155)=11.5$, $P<0.001$], an age x pretreatment interaction [$F(2,155)=9.7$, $P<0.05$], and an age x pretreatment x time interaction [$F(22,1702)=3.4$, $P<0.001$] for ambulations. Post-hoc analysis indicated that young adolescents ambulated more than older animals. This is largely because cocaine-pretreated young adolescents had greater ambulatory responses than all other age x treatment groups. Cocaine pretreatment had smaller effects on ambulatory responses to cocaine in PN 42 and PN 65 rats. However, animals of all ages did sensitize to cocaine-induced fine movements (Figure 21 d-e). ANOVA indicated a main effect of pretreatment [$F(1,155)=15.2$, $P<0.001$] and a pretreatment x time interaction
[F(11,1703)=24.2, P<0.001]. Consistent with previous studies, we also observed a main
effect of pretreatment when we measured total horizontal activity [F(2,155)=13.9,
P<0.001].

Animals were also observed for specific behaviors. The predominant behavior
induced by 10 mg/kg cocaine was continuous sniffing. Brief stereotypies, interrupted by
locomotion were infrequently observed. We reported an average behavioral rating,
continuous sniffing frequency, and stereotypy frequency (Figure 22). Cocaine
pretreatment increased the average behavioral rating in animals of all ages. ANOVA
indicated a main effect of pretreatment [F(1,85)=21.5, P<0.001]. Similarly, cocaine
increased the frequency of continuous sniffing in animals of all ages. ANOVA indicated
a main effect of pretreatment [F(1,85)=14.7, P<0.001]. Neither of these measures differed
by age. In contrast, cocaine pretreatment caused greater increases in stereotypy
frequency in adolescents compared to adults. ANOVA indicated a main effect of age
[F(1,85)=4.1, P<0.05], pretreatment [(F(1,85)=20.2, P<0.001], and an age x pretreatment
interaction [F(1,85)=3.4, P<0.05]. Cocaine pretreatment increased the stereotypy
frequency in animals of all ages, but this increase was more robust in adolescents
compared to adults. The age-specific increase in stereotypies was not reflected in the
average behavioral rating because they were relatively infrequent occurring on average
in less than 8% of all intervals.
To assess the role of injection stress on locomotor sensitization, we also determined the effect of high dose cocaine pretreatment on locomotor behavior following a subsequent injection of saline (Figure 23). We obtained disparate results in terms of ambulations and fine movements. ANOVA indicated a main effect of age for ambulations [F(2,35)=3.5, P<0.05] and post-hoc analysis indicated that PN 28 animals had greater ambulatory responses than PN 42 animals. However, cocaine pretreatment did not significantly alter the ambulatory responsiveness to a saline injection in an age-specific manner. In contrast, ANOVA indicated an age x pretreatment interaction for fine movements in animals challenged with saline [F(2,35)=4.4, P<0.05]. Cocaine pretreatment significantly increased the number of fine movements made by young adolescents challenged with saline. This effect was not observed in older animals.
Animals PN 28, PN 42, and PN 65 treated with saline (open symbols) or 40 mg/kg cocaine (closed symbols) 24 hr earlier were rehabituated (time < 65 min) to the locomotor test chambers and challenged with 10 mg/kg cocaine (time > 60 min). Automated horizontal activity was resolved into ambulations (a-c) and fine movements (d-f). Cocaine pretreatment induced more ambulatory sensitization in the young adolescents (a). In contrast, cocaine pretreatment induced sensitization to fine movements in animals of all ages (d-f). Closed symbols show animals pretreated with 40 mg/kg cocaine (24 hrs prior) and open symbols show animals pretreated with saline. Arrows denote injection times. *indicates greater than saline pretreatment. N=24-31 for each age x pretreatment group.
Figure 22: Stereotypy sensitization 24 hours after a single high dose of cocaine.

PN 28 (black bars), PN 42 (white bars), and PN 65 (striped bars) rats were treated with 40 mg/kg cocaine and challenged with 10 mg/kg cocaine 24 hr later. Animals were observed for specific behaviors and given an average behavioral rating using our stereotypy rating scale (a). We also report the frequencies of fixed sniffing (b) and stereotyped behaviors which included head weaving, head bobbing, patterned locomotion and wall climbing (c). Cocaine pretreatment increased the average behavioral rating and frequency of fixed sniffing in animals of all ages. ANOVA indicated a main effect of pretreatment for average behavioral rating, sniffing frequency, and stereotypy frequency. Cocaine pretreatment caused greater increases in stereotypy frequency in adolescents than adults as ANOVA indicated an age x pretreatment interaction. All bars represent mean +/- S.E.M. N=8-12 for each age x pretreatment group.
Figure 23: Effects of high dose cocaine on locomotor responses to saline.

PN 28, PN 42, and PN 65 animals treated with saline (open symbols) or 40 mg/kg cocaine (closed symbols) 24 hrs earlier were challenged with saline. Automated horizontal activity was resolved into ambulations (a–c) and fine movements (d–f). Cocaine pretreatment had no effect on ambulatory responses to saline in animals of any age. However, cocaine pretreatment increased the number of fine movements young adolescents made when challenged with saline (d). ANOVA indicated an age x pretreatment effect for fine movements. Symbols represent mean +/- S.E.M. N=7 for each age x pretreatment group.

*ANOVA P<0.05 for pretreatment
Experiment 3: Correlate Locomotor Activity Following High Dose Cocaine with Locomotor Activity Following the Cocaine Challenge.

We hypothesized that exaggerated acute ambulatory responses to cocaine in adolescents were related to the exaggerated ambulatory sensitization. We tested this hypothesis with two additional forms of analyses: median split and linear regressions. Animals of each age were designated as high or low responders to acute high dose cocaine based on a median split. We then compared the average sensitized responses of each group (Figure 24). High responding young adolescents had greater ambulatory responses to the cocaine challenge than all other cohorts. ANOVA indicated a main effect of age \[F(2,79)=9.9, \ P<0.01\] and an age x initial response interaction \[F(1,79)=7.5, \ P<0.01\]. Post-hoc analyses showed that there was a significant effect of initial response in PN 28 and PN 65 animals, but the magnitude of this effect was significantly greater in the young adolescents. While animals of all ages sensitized to fine movements, the initial response to high dose cocaine only correlated with the magnitude of sensitization in the young adolescents (Figure 24b) as ANOVA indicated an age x initial response (fine movements after 40 mg/kg) interaction \[F(2,79)=4.7, \ P<0.01\]. There was no effect of initial responses on sensitization in older animals.

We also compared the magnitude of the acute response to cocaine with the magnitude of sensitization in individual animals of each age by using a linear regression analysis (Figure 25). ANCOVA indicated a main effect of age \[F(2,79)=9.8, \ P<0.01\] and
an age x acute response interaction \[F(2,79)=3.2, P<0.001\]. The acute ambulatory response to high dose cocaine significantly correlated with the magnitude of ambulatory sensitization in the young adolescents only \([P<0.001, r^2=0.35]\). Similarly, the magnitude of the acute fine movements response only correlated with the magnitude of sensitization in young adolescents \([P<0.001, r^2=0.45]\).
Figure 24: Relationship between locomotor responses to cocaine pretreatment and sensitization.

PN 28, PN 42, and PN 65 animals were treated with 40 mg/kg cocaine and then challenged with 10 mg/kg cocaine 24 hrs later. Ambulations (a) and fine movements (b) were continuously recorded during both sessions. Animals of each age were separated into high (filled bars) and low (white bars) responders to acute cocaine by median split analysis. High responding young adolescents and adults had greater sensitized ambulatory responses than low responders. This relationship was more pronounced in young adolescents as ANOVA indicated an age x ambulations (on day 1) and an age x fine movements (on day 1) interaction. Bars represent mean +/- S.E.M. N=21-32 for each age group.
Figure 25: Correlation between acute locomotor responses to cocaine and the magnitude of locomotor sensitization 24 hours later.

PN 28, PN 42, and PN 65 male rats were treated with 40 mg/kg cocaine and then challenged 24 hrs later with 10 mg/kg cocaine. Automated locomotor activity was continuously recorded during both sessions. Horizontal activity was resolved into ambulations (a-c) and fine movements (d-f). Data points indicate the acute (x-axis) and sensitized (y-axis) locomotor responses of individual animals to cocaine. There was a significant correlation between the magnitude of acute and sensitized locomotor activity in young adolescents. ANCOVA indicated a significant age x ambulations (on day 1) and age x fine movements (on day 1) interaction. $R^2$ values = 0.35 and 0.45 for ambulations and fine movements, respectively in young adolescents. N=21-32 for each age group.
Experiment 4: Correlate Novelty Exploration with Locomotor Responses to Cocaine.

Another goal of this study was to determine if specific behavioral traits correlated with the magnitude of acute or sensitized responses to cocaine. Several studies have suggested that novelty-induced locomotion correlates with acute behavioral responsiveness to stimulants (Hooks et al. 1991; Chefer et al. 2003) and self-administration levels (Reviewed in Piazza and Le Moal 1996). We predicted that open-field locomotor responses during the first fifteen min of habituation would correlate with the magnitude of locomotor responses to the acute high dose of cocaine and/or the magnitude of locomotor sensitization in individual animals. We used a median split analysis and linear regression analyses to test this prediction (Figure 26). Surprisingly, there were no significant differences in acute locomotor responses to 40 mg/kg cocaine between high and low novelty responding animals of any age. ANOVA indicated no main effects or interactions. However, the initial ambulatory response to novelty (HR vs. LR) did correlate with the sensitized ambulatory response in the youngest adolescents. ANOVA indicated a main effect of age [F(2,81)=8.4, P<0.001] and an age x initial response interaction [F(2,81)=3.2, P<0.05]. Similarly, the linear regression analyses of individual animals only indicated a significant correlation between novelty-induced ambulatory activity and cocaine-stimulated ambulatory sensitization in the youngest adolescents (P<0.002, r²=0.29) (Figure 27). We did not identify any significant correlation
between novelty-induced fine movements and acute or sensitized cocaine-induced fine movements in animals of any age (Figure 28).
Figure 26: Relationship between novelty-induced locomotor activity and locomotor responses to high dose cocaine or sensitization.

PN 28, PN 42, and PN 65 animals were introduced to the locomotor test chambers for 1 hr and then treated with 40 mg/kg cocaine. Twenty four hrs later, animals were challenged with 10 mg/kg cocaine. Locomotor activity was resolved into ambulations and fine movements and was continuously recorded during both habituation and drug challenge sessions. Novelty activity was determined by summing the total number of ambulations and fine movements made during the first 15 min of the initial habituation session. Animals of each age were then separated into high (filled bars) and low (open bars) responders to novelty by a median split analysis. We compared the average responses to the acute (40 mg/kg) cocaine treatment (a-b) and the cocaine challenge (c-d) in high and low novelty responders. Novelty classification did not correlate with acute locomotor responses. However, high responding PN 28 animals had greater sensitized ambulatory responses than low responders. ANOVA indicated a main effect of age and an age x initial response interaction for ambulations. Bars represent mean +/- S.E.M. N=11-16 for each age x initial response group.
Figure 27: Correlation between ambulatory responses to novelty and high dose cocaine or cocaine sensitization.

PN 28, PN 42, and PN 65 animals were introduced to the locomotor test chambers for 1 hr and then treated with 40 mg/kg cocaine. Twenty four hrs later, animals were challenged with 10 mg/kg cocaine. Novelty activity was determined by summing the total number of ambulations made during the first 15 min of the initial habituation. Novelty-induced ambulations were plotted against ambulatory totals following 40 mg/kg cocaine (a-c) or the cocaine challenge (d-f). Novelty-induced ambulations did not correlate with any ambulatory responses to high dose cocaine. However, novelty-induced ambulatory activity positively correlated with the magnitude of ambulatory sensitization in PN 28 rats (d) and ANCOVA indicated an age x initial response interaction. N=25-32 for each age group.
Figure 28: Relationship between fine movements to novelty and high dose cocaine or sensitization.

PN 28, PN 42, and PN 65 animals were introduced to the locomotor test chambers for 1 hr and then treated with 40 mg/kg cocaine. Twenty four hrs later, animals were rehabituated to the test chambers and challenged with 10 mg/kg cocaine. Novelty activity was determined by summing the total number of fine movements made during the first 15 min of the initial habituation. Novelty-induced fine movements were plotted against fine movement totals following 40 mg/kg cocaine (a-c) or the cocaine challenge (d-f). Each point represents an individual animal. Novelty-induced fine movements did not correlate with the magnitude of any high dose cocaine or sensitized response. N=25-32 for each age group.
The above experiments demonstrate that compared to adults, adolescents have exaggerated locomotor responses to acute cocaine and the direct dopamine agonist apomorphine. These results point to exaggerated postsynaptic responsiveness to dopamine stimulation in adolescents. Further, the magnitude of the acute locomotor response to cocaine is correlated with the magnitude of locomotor sensitization induced by a single high dose of cocaine in adolescents. We hypothesize that equal brain concentrations of cocaine cause greater postsynaptic inductions of the plasticity associated genes *c-fos* and *zif268* in adolescents. In the following experiments, we investigated the ability of dopamine agonists to stimulate G-protein activation and cocaine to induce the expression of *c-fos* and *zif268* in forebrain areas.

**Experiment 1: Measure Dopamine Agonist-Stimulated G-Protein Coupling in Adolescent and Adult Tissue Homogenates.**

Enhanced postsynaptic signaling could be mediated by enhanced receptor/G-protein stimulation by dopamine agonists. We examined dopamine receptor/G-protein stimulation by treating homogenates generated with tissues from the CP, nucleus accumbens, and PFC with varying concentrations of dopamine, the D1 specific agonist dihydrexidine, and the D2 specific agonist quinpirole. G-protein activation was detected
using the non-hydrolizable analogue $[^{35}\text{S}]\text{GTP}\gamma\text{S}$. We observed similar levels of D1 and D2 activation by dopamine, dihydrexidine, and quinpirole at all concentrations in both adolescents and adults (Figure 29).
Figure 29: G-protein activation by dopamine agonists in adolescent and adult synaptosomes.

Tissue homogenates from the nucleus accumbens (a-c), CP (d-f), and PFC (g-i) were generated from gross-dissected drug naive PN 28 (black) and PN 65 (grey) male rats. G-protein activation was stimulated using dopamine (top panels), the D2 agonist quinpirole (middle panels), and the D1 agonist dihydrexidine (bottom panels). Activation is reported as % of unstimulated signal. All three agonists induced similar levels of stimulation in adolescents and adults. Symbols represent mean +/- S.E.M. N=6-9 for each group.


**Experiment 2: Measure the Time Course of Cocaine-Stimulated Striatal c-fos and zif268 Induction in Adolescents and Adults.**

In order to use the induction of the IEGs c-fos and zif268 as quantitative markers of neuronal activity in adolescents and adults, it was necessary to determine that both genes show similar temporal patterns of induction by cocaine in adolescents and adults. Maximal cocaine-stimulated c-fos has been reported at 30 min post injection and maximal zif268 has been reported at 45 min to 1 hr. We measured relative cocaine-stimulated c-fos and zif268 mRNA expression in adolescent and adult CP by treating animals PN 28 and 65 with 40 mg/kg cocaine or saline in locomotor test chambers and killed them 15, 30, or 60 min post injection. Relative mRNA levels were measured using qRT-PCR. ANOVA indicated a main effect of age \([F(1,46)=19.5, P<0.001]\), time \([F(3,46)=32.1, P<0.001]\), gene \([F(1,91)=142.9, P<0.001]\), and interactions of age x time \([F(1,91)=4.15, P<0.05]\), and gene x time \([F(3,91)=20.2, P<0.05]\). We then investigated the effects of age and time in each gene separately (Figure 30).

For c-fos, ANOVA indicated a main effect of age \([F(1,46)=17.6, P<0.001]\) and time \([F(3,46)=27.8, P<0.001]\). Post-hoc analysis showed that cocaine caused bigger fold increases in c-fos expression in adults than adolescents. We also confirmed that c-fos expression was maximal at 30 min. For zif268, ANOVA indicated a main effect of age \([F(1,44)=7.9, P<0.05]\) and time \([F(3,44)=17.2, P<0.001]\). As with c-fos, cocaine stimulated greater increases in zif268 expression in adults than adolescents. However, maximal
zif268 expression was observed 60 min after cocaine treatment. Based on these experiments, we conclude that 30 min post-injection represents an appropriate time from which to examine the induction of both genes by cocaine in adolescents and adults. It caused an underestimate of maximal zif268 expression levels, but we could still detect age-effects for cocaine-induced zif268 expression at 30 min.
Adolescent and adult rats were treated with saline or 40 mg/kg cocaine in the locomotor test chambers and killed 15, 30, or 60 min post cocaine treatment. The CP was removed by gross dissection. Relative *c-fos* (a) and *zif268* (b) mRNA levels were determined using RT-PCR and are expressed as fold increases of saline values. Cocaine induced similar temporal increases in *c-fos* and *zif268* expression in adolescents and adults. ANOVA indicated a main effect of age and time for both genes (P<0.05). Cocaine caused greater increases in striatal *c-fos* and *zif268* expression in adults. *C-fos* expression was maximal at 30 min and *zif268* expression was maximal at 60 min. Adolescents are represented by closed symbols and adults are represented by open symbols. All symbols represent mean +/- S.E.M. N=5-8 for all age x time groups.

Figure 30: Time course of cocaine-stimulated *c-fos* and *zif268* induction in adolescents and adults.
**Experiment 3: Measure the Anatomical Patterns of Activation of c-fos and zif268 After Low (10 mg/kg) and High (40 mg/kg) Dose Cocaine in Adolescents and Adults.**

Our apomorphine locomotor data suggest that adolescents have greater postsynaptic responses to lower doses of dopamine agonists. Our G-protein coupling assays demonstrated that this effect may be mediated somewhere downstream of the receptor. The stimulation of several intracellular signaling cascades results in the expression of a number of IEGs. Transcriptional activation provides a measure of the product of multiple signaling events. Here we treated young adolescents and adults with saline, low (10 mg/kg), and high (40 mg/kg) dose cocaine and measured the anatomical patterns of c-fos and zif268 induction in the striatum and the cortex.

Cocaine dose-dependently increased c-fos and zif268 expression in the striatum (CP and nucleus accumbens) in a regionally specific manner. Table 2 shows the mean +/- S.E.M. for background corrected c-fos (top) and zif268 (bottom) values in each striatal brain region. Figure 31 shows representative c-fos (top) and zif268 (bottom) images from each coronal section after saline and both doses of cocaine. ANOVA indicated a main effect of dose \([F(2,66)=125.1, P<0.001]\) and region \([F(11,706)=64.7, P<0.001]\) and interactions of age x dose \([F(2,66)=8.2, P<0.001]\), age x region \([F(11,706)=2.2, P<0.05]\), dose x region \([F(22,706)=18.9, P<0.001]\), and age x dose x region \([F(22,706)=3.7, P<0.001]\). Further, these effects were gene specific as ANOVA indicated interactions of dose x gene \([F(2,37)=21.6, P<0.001]\), age x dose x gene \([F(2,37)=3.9, P<0.05]\), region x gene
[F(11,375)=25.6, P<0.001], dose x region x gene [F(22,375)=11.0, P<0.001] and age x dose x region x gene [F(22,375)=2.7, P<0.001]. To investigate these interactions, we subsequently measured the effects of age, dose, and region in each gene separately.

ANOVA indicated a main effect of dose [F(2,62)=103.6, P<0.001] and region [F(11,642)=67.2, P<0.001] as well as interactions of age x dose [F(2,62)=10.2, P<0.001], age x region [F(11,642)=2.7, P<0.001], dose x region [F(22,642)=21.6, P<0.001], and age x dose x region [F(22,642)=4.5, P<0.001] for corrected c-fos mRNA levels. We then used the post-hoc analyses to compare c-fos mRNA levels after each dose in each striatal subregion in adolescents and adults separately (Table 2, top). As expected, striatal c-fos expression was minimal in all regions of the striatum following an injection of saline and did not differ significantly by age (even when data were log transformed to normalize variance across regions). Drug effects in the nucleus accumbens were much less pronounced than those observed in the CP in animals of all ages. Cocaine increased c-fos expression in the medial nucleus accumbens shell, but had no effect in the ventral shell in either age group. In the nucleus accumbens core, c-fos mRNA levels were only significantly higher than saline following 40 mg/kg cocaine in adults. In contrast to the nucleus accumbens, drug effects were robust throughout most of the CP. 10 mg/kg significantly increased c-fos expression in all regions of the CP in adolescents, with the exception of the ventrolateral caudate (from both the medial and caudal sections). A similar but less robust trend was observed in adults as 10 mg/kg cocaine did not significantly increase c-
fos expression above saline throughout most of the rostral section. Interestingly, 10 mg/kg cocaine did not significantly increase c-fos expression in the ventrolateral caudate in adults either. However, high dose (40 mg/kg) cocaine increased c-fos expression in all regions of the caudate in animals of both ages. These analyses demonstrate that cocaine can induce c-fos expression in all areas of the CP measured in this study in both adolescents and adults and that adolescents may be more sensitive to striatal c-fos induction by lower doses of cocaine than adults.

Cocaine also induced zif268 expression in all regions of the CP in adolescents and adults as well (Table 2, bottom). ANOVA indicated a main effect of dose [F(2,41)=57.5, P<0.001], region [F(11,439)=28.4, P<0.001] and interactions of dose x region [F(22,439)=6.9, P<0.001] and age x dose x region [F(22,439)=2.2, P<0.01] for corrected zif268 mRNA levels. Post-hoc analyses indicated that basal zif268 levels (saline animals) were higher in adolescents than adults. Similar to c-fos, cocaine caused bigger increases zif268 expression in the CP compared to the nucleus accumbens. Cocaine increased zif268 expression in the medial nucleus accumbens shell, but had no effect on expression in the ventral shell at any dose. Cocaine only significantly increased zif268 expression in the nucleus accumbens core of adults following 40 mg/kg cocaine and had no significant effect at any dose in adolescents. Significant increases in zif268 expression were seen following cocaine in all regions of the CP after both doses in both age groups with one exception: the ventrolateral caudate. As with c-fos, 40 mg/kg cocaine significantly
increased ventrolateral zif268 expression above saline whereas 10 mg/kg cocaine had no significant effect in animals of either age.

Cocaine only induced modest changes in the expression of c-fos and zif268 in the cortical areas we measured. Table 3 shows the mean +/- S.E.M. for background corrected c-fos (top) and zif268 (bottom) values in each cortical brain region. For the five cortical areas we measured in three rostral-caudal slices, ANOVA indicated a main effect of age [F(1,66)=27.6, P<0.001], dose [F(2,66)=30.2, P<0.001], region [F(4,264)=127.4, P<0.001], and interactions of age x region [F(4,264)=3.0, P<0.05], dose x region [F(8,264)=6.4, P<0.001], region x gene [F(4,148)=10.9, P<0.001], age x dose x gene [F(2,37)=6.3, P<0.05], and dose x region x gene [F(8,148)=2.3, P<0.05]. Two-factor ANOVAs were then employed to investigate the significant three-way interactions.

We first examined the effects of dose on regional c-fos and zif268 expression separately using dose as a subject variable and region as a repeated measure. For c-fos, ANOVA indicated a main effect of dose [F(2,65)=11.8, P<0.001], region [F(4,260)=71.5, P<0.001] and a dose x region interaction [F(8,260)=5.4, P<0.001]. Post-hoc analysis showed that c-fos induction was not dose dependent. C-fos mRNA levels were higher than saline after both doses of cocaine but 40 mg/kg did not induce higher expression levels than 10 mg/kg. C-fos mRNA levels were higher after cocaine treatment (either dose) than saline in all regions except the agranular insular cortex. Cocaine had very similar dose effects on regional zif268 expression. ANOVA indicated a main effect of
dose \[F(2,44)=14.3, P<0.001\], region \[F(4,176)=104.3, P<0.001\], and a dose x region interaction \[F(8,176)=3.5, P<0.001\]. Similar to \textit{c-fos}, \textit{zif268} expression was not dose-dependent. However, \textit{zif268} mRNA levels were higher after both doses of cocaine (compared to saline) in all five cortical regions including the agranular insular cortex.

We then investigated the effects of age and dose on cortical \textit{c-fos} and \textit{zif268} expression separately. ANOVA indicate a main effect of age and dose for both genes \((P<0.001\) for all). There was no significant interaction between age and dose for either gene. Post-hoc analyses demonstrated that neither \textit{c-fos} nor \textit{zif268} expression were dose-responsive in animals of either age. \textit{C-fos} and \textit{zif268} mRNA levels were higher after cocaine than saline, but similar following both low and high dose cocaine.

Finally, we analyzed \textit{c-fos} and \textit{zif268} expression levels in the orbital cortex and rostral cingulate from the +3.20 mm section (using a 4 factor repeated measures ANOVA as before). ANOVA indicated a main effect of a dose \[F(2,63)=15.6, P<0.001\] and an age x region interaction \[F(1,63)=6.4, P<0.05\]. Consistent with the other cortical regions, neither \textit{c-fos} nor \textit{zif268} expression was dose-dependent. Adolescents had higher \textit{c-fos} and \textit{zif268} mRNA levels than adults in the orbital cortex. This age effect was drug independent as it was observed after saline and cocaine. These analyses demonstrate that, in general, cocaine increases \textit{c-fos} and \textit{zif268} expression in the cortical regions we measured in both adolescents and adults. Further, these effects are independent of cocaine dose in all of the cortical regions we analyzed. It will be important to decipher
the relationship(s) between patterns of cortical and striatal IEG induction following cocaine treatment. Future studies in this laboratory will use computational approaches to investigate network level activation by cocaine.
Rats were treated with saline, 10, or 40 mg/kg cocaine in the locomotor test chambers and *c-fos* and *zif268* mRNA levels were measured using in situ hybridization. Representative images from adults are presented to show dose-related increases in the expression of *c-fos* (top images) and *zif268* (bottom images) in the striatum and cortex.
Table 2: Effects of cocaine on striatal c-fos and zif268 mRNA levels.

Mean +/- S.E.M. background corrected density values are shown for c-fos (top) and zif268 (bottom) in adolescents (left) and adults (right) for each striatal subregion. * indicates P<0.05 greater than saline. ** indicates P<0.05 greater than saline and 10 mg/kg cocaine. # indicates greater than PN 65. ! indicates greater than PN 28. N=6-15 for each age x treatment group.

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Table 3: Effects of cocaine on cortical *c-fos* and *zif268* mRNA levels.

Mean +/- S.E.M. background corrected density values are shown for *c-fos* (top) and *zif268* (bottom) in adolescents (left) and adults (right) for each cortical subregion. *indicates P<0.05 greater than saline. N=6-15 for each age x treatment group.

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**Experiment 4: Measure Effects of Saline Injection on Basal zif268 Expression.**

We showed above that adolescents treated with saline had higher striatal and cortical zif268 mRNA levels than adults. It is possible that these represent either higher constitutive basal expression levels or greater zif268 responses to placement in a novel environment and/or injection stress in adolescents. To determine what these represent, we treated adolescents and adults with saline in the locomotor chambers or removed them from their home cages and killed them immediately. We determined that adolescents have higher basal expression of zif268 than adults throughout the striatum and the cortex (Table 4). In the striatum, ANOVA indicated a main effect of age [F(1,20)=30.1, P<0.001]. In the cortex, ANOVA indicated a main effect of age [F(1,20)=32.8, P<0.001], region [F(14,260)=9.1, P<0.001], and interactions of age x region [F(14,260)=4.2, P<0.001], and age x treatment x region [F(14,260)=2.0, P<0.05]. Adolescents had higher average zif268 levels than adults in both tissues. Treating adolescents with saline in the locomotor chambers had no effect on their basal zif268 expression levels. Further, significant differences between saline-treated and home cage animals were only observed in a limited number of cortical areas in adults.
Table 4: Effects of saline injection on basal zif268 expression.

Adolescent (left columns) and adult (right columns) animals were treated with saline in the locomotor chambers or removed from their home cages and immediately killed. Mean +/- background corrected density values are shown for striatal (top) and cortical (bottom) subregions. *indicates P<0.05 less than saline. N=5-6 for each group.

<table>
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<tr>
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<th>PN 65</th>
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<td>saline</td>
<td>home cage</td>
<td>saline</td>
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<td>Rostral (+1.6 mm)</td>
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Cortex

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<td>Rostral (+1.6 mm)</td>
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<td>36.3 +/- 11.2</td>
<td>18.7 +/- 2.4</td>
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Experiment 5: Evaluate Percent Increases in c-fos and zif268 Expression Following Low and High Dose Cocaine.

We next calculated our c-fos and zif268 mRNA levels as a percentage of baseline (saline) to determine how much the different doses of cocaine increased the relative expression of each gene. The effects of age and dose on % increases in striatal IEG expression were gene specific. ANOVA indicated a main effect of dose \([F(1,46)=56.3, P<0.001]\), region \([F(11,486)=49.6, P<0.001]\), gene \([F(1,30)=725.4, P<0.001]\), and interactions of age x dose \([F(1,46)=12.8, P<0.001]\), age x region \([F(11,486)=6.0, P<0.001]\), dose x region \([F(11,486)=11.2, P<0.001]\), age x dose x region \([F(11,486)=10.3, P<0.001]\), dose x gene \([F(1,30)=187.7, P<0.001]\) age x dose x gene \([F(1,30)=41.1, P<0.001]\), region x gene \([F(11,308)=97.8, P<0.001]\), age x region x gene \([F(11,308)=11.2, P<0.001]\), dose x region x gene \([F(11,308)=21.2, P<0.001]\), and age x dose x region x gene \([F(11,308)=19.7, P<0.001]\). Therefore, we analyzed the effects of age and dose on regional cocaine-induced c-fos and zif268 expression separately.

While cocaine induced similar anatomical patterns of striatal c-fos and zif268 in adolescents and adults, the relative magnitude of cocaine-stimulated increases in c-fos and zif268 mRNAs differed by dose. When expressed as a percent of saline, high and low dose cocaine induced opposite age-effects on c-fos induction in the striatum (Figure 32). ANOVA indicated a main effect of dose \([F(1,45)=93.6, P<0.001]\), region \([F(11,463)=82.7, P<0.001]\), and interactions of age x dose \([F(1,45)=20.1, P<0.001]\), age x
region \[F(11,463)=9.5, P<0.001\], dose x region \[F(11,463)=18.5, P<0.001\], and age x dose x region \[F(11,463)=17.0, P<0.001\]. Post-hoc analyses demonstrated that cocaine dose-dependently increased \textit{c-fos} expression in both adolescents and adults. These analyses also showed that 10 mg/kg cocaine induced more \textit{c-fos} expression in young adolescents than adults whereas 40 mg/kg cocaine induced more \textit{c-fos} expression in adults than young adolescents. Significant effects of age in specific regions are indicated in figure 33a-b.

Cocaine also dose-dependently produced age-specific effects on the relative induction of striatal \textit{zif268} (Figure 33a-b). ANOVA indicated a main effect of age \[F(1,31)=29.2, P<0.001\], dose \[F(2,31)=44.3, P<0.001\], region \[F(11,331)=17.2, P<0.001\], and interactions of age x dose \[F(2,31)=11.6, P<0.001\], age x region \[F(11,331)=5.4, P<0.001\], dose x region \[F(22,331)=4.7, P<0.001\], and age x dose x region \[F(22,331)=4.2, P<0.001\]. Post-hoc analysis showed that 10 mg/kg cocaine stimulated a comparable induction of \textit{zif268} in young adolescents and adults but high dose cocaine induced more \textit{zif268} expression in adults than young adolescents. Significant effects of age at each region are indicated in figure 33b.

Cocaine had different effects on the induction of \textit{c-fos} and \textit{zif268} in the cortex than in the striatum (Figure 34). In the orbital and rostral cingulate cortex (+3.20 mm) ANOVA indicated a dose x gene interaction \[F(1,28)=19.9, P<0.001\] and post-hoc analysis showed that cocaine caused bigger increases in \textit{zif268} than \textit{c-fos} expression in the orbital cortex.
ANOVA indicated a main effect of region \([F(4,184)=8.1, \ P<0.001]\), gene \([F(1,30)=4.9, \ P<0.05]\), and interactions of age x gene \([F(1,30)=12.0, \ P<0.001]\), and region x gene \([F(4,30)=11.8, \ P<0.001]\) for the five regions measured repeatedly in three sections. Cocaine caused similar increases in \(c-fos\) in adolescents and adults. At the same time, cocaine caused bigger increases in the expression of \(zif268\) in adults compared to adolescents. These differences reflect lower basal \(zif268\) expression levels in adolescents but also suggest that cocaine may induce greater neural responses in the adult cortex.
Figure 32: High and low dose cocaine induce opposite effects of age on striatal c-fos induction.

Striatal c-fos results from table 2 were expressed as a percentage of saline to show relative increases in c-fos expression by cocaine. Increases in c-fos following 10 mg/kg are shown in (a) and increases in c-fos expression following 40 mg/kg are shown in (b). As a percent of saline, 10 mg/kg caused greater increases in c-fos expression in adolescents than adults whereas 40 mg/kg cocaine caused greater increases in adults than adolescents. ANOVA indicated a main effect of dose and interactions of age x dose and age x dose x region. X-axis shows the following regions: rostral dorsal caudate (rdc), rostral lateral caudate (rlc), rostral medial caudate (rmc), nucleus accumbens core (core), medial nucleus accumbens shell (mshell), ventral nucleus accumbens shell (vshell), medial dorsal caudate (mdc), medial lateral caudate (mlc), ventromedial caudate (mvc), medial ventrolateral caudate (mvlc), caudal dorsal caudate (cdc), and the caudal ventrolateral caudate (cvlc). Filled bars represent adolescents and open bars represent adults. All bars represent mean +/- S.E.M. A schematic (c) and representative images from the rostral section are shown in (d). * indicates P<0.05 greater than adults. ** indicates P<0.05 greater than adolescents. N=8-15 for each age x treatment group.
Figure 33: High dose cocaine causes greater increases in zif268 expression in adults than adolescents.

Striatal zif268 results from table 2 were expressed as a percentage of saline to show relative increases in zif268 expression by cocaine. Increases in zif268 following 10 mg/kg are shown in (a) and increases in zif268 expression following 40 mg/kg are shown in (b). As a percent of saline, 10 mg/kg caused similar increases in zif268 expression in adolescents and adults whereas 40 mg/kg cocaine caused greater increases in adults than adolescents. ANOVA indicated a main effect of dose and interactions of age x dose and age x dose x region. X-axes show the following regions: rostral dorsal caudate (rdc), rostral lateral caudate (rlc), rostral medial caudate (rmc), nucleus accumbens core (core), medial nucleus accumbens shell (mshell), ventral nucleus accumbens shell (vshell), medial dorsal caudate (mdc), medial lateral caudate (mlc), ventromedial caudate (vmlc), medial ventrolateral caudate (mvl), caudal dorsal caudate (cdc), and the caudal ventrolateral caudate (cvlc). Filled bars represent adolescents and open bars represent adults. All bars represent mean +/- S.E.M. Schematic (c) and representative images of the rostral section are shown in (d). ** indicates P<0.05 greater than adolescents. N=8-15 for each age x treatment group.
Figure 34: Cocaine-induced increases in cortical c-fos and zif268 expression.

Cortical c-fos and zif268 mRNA levels from table 3 were expressed as a percent of saline to show relative increases in c-fos (a-b) and zif268 (c-d) expression by cocaine. Increases following 10 mg/kg are shown on the top panels and those from 40 mg/kg are shown on the bottom. Cocaine caused bigger increases in zif268 expression in adults than adolescents. ANOVA indicated an age x gene interaction. X-axes show the following regions: anterior cingulate (cing), orbital cortex (orb), dorsal agranular cortex (ag), dorsal anterior cingulate (acd), ventral anterior cingulate (acv), sensorimotor cortex (sm), and the agranular insular cortex (ai). Filled bars represent adolescents and open bars represent adults. All bars represent mean +/- S.E.M. N=8-16 for each age x treatment group.
**Experiment 6: Correlate Locomotor Activity with Regional c-fos and zif268 Expression in Individual Animals.**

Locomotor activity was recorded following cocaine or saline injections and we correlated locomotor responses with the expression of *c-fos* and *zif268* in individual animals. Cocaine dose-dependently increased locomotor activity in animals of both ages but the relative magnitude of locomotor activity between adolescents and adults differed by dose. ANOVA indicated a main effect of dose \([F(2,82)=62.7, P<0.001]\) and interactions of time x dose \([F(10,410)=7.4, P<0.001]\) and age x dose \([F(2,82)=7.6, P<0.001]\). Post-hoc analysis demonstrated that while locomotor activity was similar in adolescents and adults following saline, adolescents had greater locomotor responses than adults to 10 mg/kg cocaine and adults had greater locomotor responses than adolescents to 40 mg/kg cocaine (Figure 35). Before, we observed that adults trended to have greater horizontal activity levels following high dose cocaine (Figure 12) although that trend did not reach significance.

We used linear regression analysis to determine if striatal *c-fos* and/or *zif268* expression correlated with locomotor activity in adolescents and adults. For all linear regression analyses, locomotor activity was expressed as horizontal activity, ambulations, and fine movements. However, none of the results of any of these analyses differed by behavioral readout. Therefore, locomotor activity is only presented as horizontal activity for simplification. Locomotor activity during the 30 min session was
summed for each animal. Our initial ANCOVA indicated a main effect of age, [F(1,31)=6.3, P<0.05], dose [F(1,31)=68.0, P<0.001], region [F(11,31)=18.1, P<0.001], and an age x gene x locomotion interaction [F(1,410)=16.3, P<0.001]. Subsequent ANCOVAs were used to compare locomotor activity and the expression of \( c-fos \) and \( zif268 \) separately.

We first examined the correlation between striatal \( c-fos \) and locomotor activity. There was a significant correlation between \( c-fos \) expression and locomotor activity in most regions of the striatum in both adolescents and adults. ANCOVA indicated a main effect of locomotion [F(1,45)=71.9, P<0.001], age [F(1,45)=19.9, P<0.001] dose [F(1,45)=48.5, P<0.001] and region [F(11,45)=28.6, P<0.001] on \( c-fos \) expression. Figure 36a-b shows the correlation between \( c-fos \) expression in the dorsal caudate of the rostral section as a representative image. Table (5) shows the P-value and, when statistically significant, the correlation coefficient for the correlations between locomotion and \( c-fos \) (top) of all striatal regions.

In contrast to \( c-fos \), locomotor activity only correlated with \( zif268 \) expression in individual adults. ANCOVA indicated a significant effect of age [F(1,31)=6.3, P<0.05], dose [F(1,31)=68.0, P<0.001], region [F(11,31)=18.1, P<0.001], and an age x locomotion interaction [F(1,410)=16.3, P<0.001] on \( zif268 \) mRNA levels. Figure 37c-d shows the correlation between \( zif268 \) expression in the dorsal caudate of the rostral section as a
representative image. Table 5 (bottom) shows the P-value and correlation coefficients between locomotion and zif268 in all measured striatal areas.

We also tried to correlate locomotor activity with the expression of c-fos and zif268 in cortical subregions. Figure 37 shows the relationship between locomotor activity and c-fos and zif268 expression in the dorsal agranular cortex in adolescents and adults. We did not identify any significant correlations between locomotor activity and c-fos or zif268 expression in any cortical subregion in individual animals. This is perhaps not surprising as locomotor activity was dose responsive whereas cortical c-fos and zif268 expression were not.
Figure 35: Locomotor responses to 10 and 40 mg/kg cocaine.

Animals used in the c-fos and zif268 expression studies were recorded also recorded for locomotor activity. Animals were treated with saline (a), 10 mg/kg cocaine (b), or 40 mg/kg cocaine (c). Low dose cocaine caused more locomotor activity in adolescents but high dose cocaine induced more locomotor activity in adults. ANOVA indicated a main effect of dose and an age x dose interaction. Closed symbols represent adolescents and open symbols represent adults. All symbols show mean +/- S.E.M. *indicates greater locomotor responses in PN 28 than 65. **indicates greater locomotor responses in PN 65 than 28. N=6-15 for each age x treatment group.
We correlated the expression of \( c-fos \) (a-b) and \( zif268 \) (c-d) in individual adolescents (left panels) and adults (right panels). Striatal \( c-fos \) correlated with locomotor activity in both adolescents and adults but striatal \( zif268 \) only correlated with locomotor activity in adults. ANCOVA indicated a significant age x locomotion x gene interaction. Open symbols show animals treated with 10 mg/kg cocaine and closed symbols represent animals treated with 40 mg/kg cocaine. Each point represents an individual animal. \( N=7-15 \) for each age x treatment group.
We measured the correlation between *c-fos* (top) and *zif268* (bottom) in individual adolescents (left) and adults (right) and locomotor activity. Locomotor activity correlated with *c-fos* and *zif268* expression in most striatal regions in adults. In adolescents, however, locomotor activity only correlated with *c-fos* expression. P-values for all correlations are shown and $R^2$ coefficients are shown for all significant correlations. *indicates $P<0.05$ for significant correlation.

| Striatal Region | c-fos PN 28 | | R$^2$ | | R$^2$ | | zif268 PN 65 | | P- Value | | R$^2$ | | R$^2$ |
|----------------|-------------|---|---|---|-------------|---|-------------|---|---|-------------|---|
| Rostral (+1.6 mm) | | | | | | | | | | | |
| Dorsal Caudate | P<0.01* | 0.31 | | | | | | | | | |
| Medial Caudate | P<0.001* | 0.42 | | | | | | | | | |
| Lateral Caudate | P<0.01* | 0.32 | | | | | | | | | |
| Nuc Acc Core | P>0.05 | | | | | | | | | | |
| Medial Nuc Acc shell | P<0.01* | 0.28 | | | | | | | | | |
| Ventral Nuc Acc shell | P>0.05 | | | | | | | | | | |
| Medial (+0.40 mm) | | | | | | | | | | | |
| Dorsal Caudate | P<0.03* | 0.21 | | | | | | | | | |
| Lateral Caudate | P<0.02* | 0.24 | | | | | | | | | |
| MV Caudate | P>0.05 | | | | | | | | | | |
| LV Caudate | P>0.05 | | | | | | | | | | |
| Caudal (-0.80) | | | | | | | | | | | |
| Dorsal Caudate | P<0.02* | 0.25 | | | | | | | | | |
| LV Caudate | P>0.05 | | | | | | | | | | |

| Striatal Region | c-fos PN 65 | | R$^2$ | | R$^2$ | | zif268 PN 65 | | P- Value | | R$^2$ | | R$^2$ |
|----------------|-------------|---|---|---|-------------|---|-------------|---|---|-------------|---|
| Rostral (+1.6 mm) | | | | | | | | | | | |
| Dorsal Caudate | P>0.05 | | | | | | | | | | |
| Medial Caudate | P>0.05 | | | | | | | | | | |
| Lateral Caudate | P>0.05 | | | | | | | | | | |
| Nuc Acc Core | P>0.05 | | | | | | | | | | |
| Medial Nuc Acc shell | P>0.05 | | | | | | | | | | |
| Ventral Nuc Acc shell | P>0.05 | | | | | | | | | | |
| Medial (+0.40 mm) | | | | | | | | | | | |
| Dorsal Caudate | P>0.05 | | | | | | | | | | |
| Lateral Caudate | P>0.05 | | | | | | | | | | |
| MV Caudate | P>0.05 | | | | | | | | | | |
| LV Caudate | P>0.05 | | | | | | | | | | |
| Caudal (-0.80) | | | | | | | | | | | |
| Dorsal Caudate | P>0.05 | | | | | | | | | | |
| LV Caudate | P>0.05 | | | | | | | | | | |
Figure 37: Correlation between locomotor activity and cortical *c-fos* and *zif268* expression.

We correlated the expression of *c-fos* (a-b) and *zif268* (c-d) in individual adolescents (left panels) and adults (right panels). Neither *c-fos* nor *zif268* expression correlated with locomotor activity in adolescents or adults. Open symbols show animals treated with 10 mg/kg cocaine and closed symbols represent animals treated with 40 mg/kg cocaine. Each point represents an individual animal. N=7-15 for each age x treatment group.
**Experiment 7: Correlate Regional c-fos and zif268 Expression in Individual Animals.**

Our correlational studies between locomotor activity and *c-fos* and *zif268* suggest that the quantitative relationship between *c-fos* and *zif268* should correlate in adults (both measures were highly correlative with locomotion in the same animals) but might not correlate in adolescents. To address this, we plotted corrected striatal *c-fos* vs. striatal *zif268* mRNA levels in individual animals. As predicted, *c-fos* only correlated with *zif268* in adults. ANCOVA indicated a main effect of region \([F(11,31)=3.4, P<0.001]\), *c-fos* \([F(1,31)=73.4, P<0.001]\), and an age x *c-fos* interaction \([F(1,386)=10.1, P<0.01]\) on the expression of *zif268*. Figure 38 shows the correlation between *c-fos* and *zif268* in the dorsal caudate in individual adolescents and adults and table 6 shows the P-value and correlation coefficients between *c-fos* and *zif268* expression in adolescents and adults in all striatal regions.

We also tried to correlate cortical *c-fos* and *zif268* expression in individual adolescents and adults. However, none of these correlations reached statistical significance. Cocaine-stimulated cortical *c-fos* and *zif268* do not correlate in individual animals.
Figure 38: Correlation between striatal c-fos and zif268 in individual animals.

We correlated the expression of striatal c-fos and zif268 in individual adolescents (a) and adults (b). There was only a significant correlation between the two genes in adults. ANCOVA indicated an age x c-fos interaction (P<0.05). Open symbols represent animals treated with 10 mg/kg cocaine and closed symbols represent animals treated with 40 mg/kg cocaine. All symbols represent a single animal.
Table 6: Correlation between striatal c-fos and zif268 in individual animals.

We correlated the expression of c-fos and zif268 in individual adolescents (left) and adults (right). Significant correlations between the two genes were seen in most regions in adults and only a few restricted regions in adolescents. ANCOVA indicated a significant age x c-fos interaction (P<0.05). P-values for all correlations are shown and R² coefficients are shown for all significant correlations. *indicates P<0.05 for significant correlation.

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<th>Striatal Region</th>
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<th>R²</th>
<th>PN 65 P-Value</th>
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6. CHAPTER 6: Discussion

6.1 Cocaine-Stimulated Locomotor Activity

6.1.1 Acute Locomotor Responses to Cocaine

Young adolescents are more responsive to low-moderate doses of acute cocaine than adults. We demonstrated that young adolescents have exaggerated locomotor responses to acute cocaine (10 and 15 mg/kg), every dose of a repeated (15 mg/kg) dose binge, and the highest dose of an escalating dose binge. Further, we showed that young adolescents transition into repetitive stereotypies at lower doses of cocaine than adults. The existing literature suggests that male adolescent rats, relative to adults, are hyporesponsive to the psychostimulants cocaine and amphetamine (Bolanos et al. 1998; Lanier and Isaacson 1977; Laviola et al. 1995, 1999; Spear and Brick 1979; Snyder et al. 1998). A careful examination of these previous studies, in the context of our current results, reveals some inconsistencies that help to clarify the ontogeny of psychostimulant-induced locomotor activity during adolescence.

We must consider the results of studies using cocaine and amphetamine separately. While they both act pharmacologically by increasing extracellular dopamine levels, they do so by distinct mechanisms that could affect the ontogeny of locomotor responses during adolescence in a drug-specific manner. Cocaine increases extracellular dopamine levels by blocking dopamine uptake through DAT. In addition to blocking
dopamine uptake, amphetamine causes the release of terminal dopamine independent of impulse flow. As a consequence, amphetamine causes greater increases in extracellular dopamine levels than cocaine (Hurd and Ungerstedt 1989; Segal and Kuczenski 1997, 1999). Amphetamine-stimulated extracellular dopamine levels are dependent on terminal dopamine content, which is reduced in the adolescent striatum relative to adulthood (Cao et al. 2007; Slotkin et al. 2002; Trauth et al. 2001). Accordingly, Laviola et al. (2001) demonstrated that acute amphetamine causes approximately 2-4× greater increases in extracellular dopamine levels in adults compared to adolescents. The few studies that have directly compared adolescent and adult locomotor responses to amphetamine have consistently reported adolescents as hyporesponsive compared to adults (Lanier and Isaacson 1977; Laviola et al. 1999; Bolanos et al. 1998). Reduced locomotor responses to amphetamine in adolescents likely reflect reduced striatal dopamine content.

While the adolescent amphetamine literature may be concordant, the cocaine literature is less consistent. One early study suggested that both cocaine-stimulated locomotor activity (measured by matrix crossings) and experimenter observed behaviors decreased during the third week of life to a minimum at around PN 30 (Spear and Brick 1979). Another study showed significant cocaine responses in terms of matrix crossings and stereotypy in young adolescent (PN 27) rats, but again reported a reduction in both behavioral measures in animals PN 35-42 (Snyder et al. 1998). Neither of these studies
directly compared the magnitude of cocaine-stimulated behaviors in adolescents and adults. A report by Laviola et al. (1995) demonstrated relatively similar behavioral profiles in adolescent (PN35-39) and adult male rats following 10 and 20 mg/kg cocaine. In males, they detected no effect of age in terms of matrix crossings or a number of other specific behaviors with the exception of stereotyped sniffing (which was greater in adults). Robust age-differences were observed in females but these can likely be attributed to circulating estrogen in adults (Chin et al. 2002; Quinones-Jenab et al. 1999; Sell et al. 2000). Another study showed that mid adolescents (PN 37-52) trended to have lower locomotor responses to acute 10 and 20 mg/kg cocaine compared to adults (Frantz et al. 2007). Two other studies also directly compared adolescents and adults and observed no significant age effects on acute cocaine-stimulated locomotion or stereotypy after doses ranging from 2.5-30 mg/kg (Collins and Izenwasser 2004; Schramm-Sapyta et al. 2004).

The results of the aforementioned studies with cocaine and amphetamine suggest that locomotor responses to both stimulants may reach a nadir at some point midway through adolescence (PN35-45). Ours is the first study to directly compare locomotor responses to cocaine in young adolescents (PN 28), mid-adolescents (PN42) and adults. Consistent with previous studies (Collins and Izenwasser 2004; Laviola et al. 1995; Schramm-Sapyta et al. 2004), we observed similar locomotor responses to cocaine in mid-adolescent (PN42) and adult rats. Our results suggest that young adolescents
(PN 28) are hyperresponsive to some doses of cocaine relative to both mid-adolescents (PN 42) and adults. Snyder et al. (1998) also clearly demonstrated that 15 mg/kg cocaine induced more locomotor activity in PN 27 than PN 34-41 rats. Taken together, the results of all of these studies suggest that adolescent-specific locomotor responses to cocaine may be restricted to early adolescence.

Our study has further demonstrated that this robust age effect is only apparent following low to moderate doses of cocaine (<15 mg/kg). We consistently observed significant effects of age on acute locomotor and stereotypy responses following 10 and 15 mg/kg cocaine, but not 25 or 40 mg/kg cocaine. We showed exaggerated locomotor responses to 10 mg/kg cocaine in young adolescents in three separate studies (Caster et al. 2007, Parylak et al. 2008, and our IEG study). We also treated young adolescents and adults with 40 mg/kg cocaine in three separate studies. Our results with 40 mg/kg were also largely consistent between experiments. Adolescents and adults always had similar stereotypy responses to 40 mg/kg cocaine. In our acute binge experiments, we saw no age-differences in horizontal activity following 40 mg/kg cocaine. In our single-dose sensitization experiments we also observed no significant effect of age on horizontal activity (basic movements) to acute 40 mg/kg cocaine in adolescents and adults although adolescents did have greater ambulatory responses during the second half hour of the cocaine session. In our IEG studies, adults had greater levels of horizontal activity than adolescents following 40 mg/kg cocaine and we saw no age effect on ambulatory
activity. However, in this study animals were killed 30 min post-injection so the second stimulatory phase of ambulatory activity in adolescents was not observed. It should be noted that the single dose sensitization study carries the most statistical power as cocaine-treated cohorts ranged from 28-32 animals per age group. We interpret these results to suggest there may be subtle age-differences in locomotor responses to 40 mg/kg cocaine, but they are not as robust or reproducible as those we observe following lower doses of cocaine. Similar to our studies, Collins and Izenwasser (2004) did not observe any significant effect of age between young adolescent and adult animals treated with 30 mg/kg cocaine.

While our apomorphine experiments demonstrate that postsynaptic activity likely contributes to enhanced adolescent locomotor responses to lower doses of cocaine (see below), it is possible that age-differences in presynaptic dopamine content could partially explain this dose specificity. As discussed above, adolescents have significantly lower striatal dopamine content than adults. Despite this difference, adolescents and adults show similar stimulated extracellular dopamine levels in the caudate and accumbens following 10 mg/kg cocaine (Walker and Kuhn 2008). Striatal dopamine content does not appear limit extracellular dopamine levels following 10 mg/kg cocaine. It is possible that striatal dopamine content could become a limiting factor following saturation of the transporter by high dose cocaine. High dose cocaine may induce greater extracellular dopamine levels in adults compared to adolescents
which could compensate for reduced postsynaptic activation by dopamine in adults and produce similar locomotor responses to high dose cocaine in adolescents and adults.

Our analysis of blood and brain cocaine concentrations demonstrates that exaggerated locomotor responses to 15 mg/kg cocaine in adolescents can not be attributed to higher blood or brain cocaine levels in adolescents. Another group demonstrated that in some strains of mice, adolescents metabolized cocaine faster than adults (McCarthy et al. 2004). Similarly, nicotine and amphetamine are also cleared more rapidly in adolescent than adult rats (Slotkin et al. 2002; Spear and Brake 1983). If anything, these studies demonstrate that adolescents should be hyporesponsive to stimulants because they achieve lower maximal doses and clear the drugs faster. Studies such as these highlight the importance of determining the effects of age on drug metabolism when comparing drug effects in adolescents and adults.

Our careful analyses of locomotor responses to cocaine have demonstrated that young adolescents have greater locomotor responses to low-moderate doses of cocaine and transition into stereotypies at lower doses of cocaine than adults. These analyses demonstrate that young adolescents are more sensitive than older animals to some of the effects of lower doses of acute cocaine. Although the exact relationship has not been established, locomotor activation by addictive drugs has frequently been used as a surrogate for the reinforcing effects of stimulants. One recent study showed that cocaine CPP largely mirrors our locomotor activation by cocaine: young adolescents show
enhanced CPP to low (5 mg/kg) but not high (20 mg/kg) dose cocaine compared to mid adolescents and adults (Badanich et al. 2006). Below, we discuss how age-specific molecular responses to cocaine show a similar dose-specific relationship.

6.1.2 Intrabinge Sensitization

In two separate contexts we showed that repeated injections within a single cocaine binge increase locomotor responses to cocaine in young adolescents. Within the repeated dose binge, each subsequent injection of cocaine (15 mg/kg) increased the frequency of intervals that adolescents engaged in fixed stereotypies. Following the first injection, adolescents engaged in fixed stereotypies (on average) during 15% of observed intervals, compared to 50% and 60% of intervals following the second and third injections. In young adolescents, the second and third injections induced more fixed stereotypies than the first injection. However, cocaine brain concentrations were similar after each dose suggesting that pharmacokinetic mechanisms were not responsible. Young adolescents appear to sensitize to the locomotor effects of cocaine within a single repeated dose binge.

We also compared stereotypy responses to the third and highest dose of the escalating dose cocaine binge (25 mg/kg) with stereotypy responses to 25 and 40 mg/kg (the sum of the 3 doses in the escalating dose binge) administered acutely. The exaggerated fixed stereotypy responses in young adolescent animals were only observed following binge cocaine. The half-life of cocaine in the rat brain is between 25 and 30
min in the rat (Hurd et al. 1988). Therefore, during the third dose of an escalating dose binge the highest drug levels expected should be approximately 28 mg/kg. Despite the lower cocaine brain concentration, escalating dose binge cocaine induced more fixed stereotypies in young adolescents than a single injection of 40 mg/kg. Binge cocaine treatment did not have any additive affect on locomotor or stereotypy responses in adults. These results demonstrate that repetitive drug administration within even a single binge is sufficient to increase locomotor responsiveness to subsequent cocaine injections. Adolescents appear to be more vulnerable to behavioral plasticity induced within a cocaine binge. Our data suggest that binge cocaine abuse may be particularly precarious during adolescence.

6.1.3 Acute Locomotor Responses to Apomorphine

Locomotor responses to apomorphine largely mirrored those with cocaine: young adolescents showed greater ambulatory responses to select doses (1.0 mg/kg) of apomorphine and transitioned into stereotypies at lower doses than adults. Similar to results observed after cocaine injections, age differences in locomotor activity were not seen at higher doses. Our results agree with those of Frantz and Van Hartesveldt (1999) and Shalaby and Spear (1980). Frantz and Van Hartesveldt observed greater locomotor responses to the D2 specific agonist quinpirole in young adolescents (PN 30) compared to adults. Shalaby and Spear treated animals from PN 7 to PN 35 with select doses of apomorphine and found that matrix crossings to apomorphine peaked on PN 21 and
declined with increasing age. It is not surprising then that we observed the most
horizontal activity in PN 28 animals treated apomorphine. The observation that young
adolescents consistently show greater locomotor responses to direct dopamine agonists
suggests that adolescents may have greater postsynaptic responses to dopamine than
adults.

It should be noted that age-differences in locomotor responses to apomorphine
could be attributed to actions other than enhanced postsynaptic dopamine activation.
Recall that dopamine is a modulatory neurotransmitter that affects postsynaptic
responses to glutamate signaling (see introduction). Apomorphine effects could reflect
age-differences in striatal glutamate signaling. It is possible that adolescents have more
cortical glutamaturgic drive in the striatum, release more glutamate coincident with
dopamine, or have greater postsynaptic responses to glutamate. If any of these
possibilities were true, apomorphine could then increase an already enhanced glutamate
signal in adolescents to produce greater locomotor responses in adolescents. However,
one recent study clearly showed that adolescents have significantly fewer cortical
glutamate projections terminating in the nucleus accumbens compared to adults
(Brenhouse et al. 2008). It seems unlikely, therefore, that enhanced locomotor and
transcriptional responses to cocaine reflect greater striatal glutamate signaling in
adolescents.
Apomorphine can also act presynaptically on D2 dopamine autoreceptors to reduce dopamine release (see introduction). It is possible that adults could be more sensitive to autoreceptor inhibition (thus exhibiting lower extracellular dopamine levels) following lower doses of apomorphine than adolescents. Locomotor experiments with the D2 agonist quinpirole suggest that greater autoreceptor sensitivity in adults is not likely sufficient to mediate reduced locomotor responses to apomorphine relative to adolescents. While activation of D2 autoreceptors will decrease extracellular dopamine levels, activation of postsynaptic D2 receptors by agonists directly stimulates locomotor activity (as dopamine normally would). Autoreceptor inhibition appears to have a minimal effect on locomotor responses to the D2 agonist quinpirole. Except at very low doses (<0.05 mg/kg), quinpirole causes locomotor stimulation in both adolescent and adult rats (Eilam and Szechtman 1988; Frantz and Van Hartesveldt 1999; Van Hartesveldt 1997). We saw no evidence of locomotor suppression in any age group following any dose of apomorphine.

Our locomotor studies with cocaine and apomorphine cumulatively demonstrate that young adolescents have greater psychomotor responses to low doses of dopamine agonists than adults. Lower doses of both cocaine and apomorphine induce more locomotor activity in adolescents than adults. Further, adolescents transition into stereotypies following lower doses of cocaine or apomorphine than adults. The homology between locomotor responses with apomorphine and cocaine suggests that
age-specific postsynaptic responsiveness to dopamine may underlie exaggerated adolescent locomotor responses to cocaine. The greater induction of striatal c-fos by 10 mg/kg cocaine in adolescents demonstrates that at least some post-synaptic responses to select doses of cocaine are greater in adolescents than adults. Below, we discuss the results of postsynaptic responses to cocaine in full detail.

### 6.1.4 Single Dose Sensitization

A single high dose of cocaine induced more locomotor sensitization 24 hrs later in young adolescents than adults. Combined with the acute cocaine binge studies, we have demonstrated in multiple paradigms that young adolescents are more vulnerable than adults to locomotor sensitization induced by minimal exposures to cocaine. Our results suggest that adolescents may sensitize faster to cocaine (following fewer exposures) than adults.

Here we showed that while a single high dose (40 mg/kg) of cocaine is sufficient to induce locomotor sensitization 24 hrs later in adolescents and adults, a more dramatic profile emerges in young adolescents. Animals of all ages sensitized to the non-ambulatory (fine movements) affects of cocaine, but ambulatory and stereotypy sensitization were more pronounced in adolescents compared to adults. We also demonstrated that specific behavioral traits correlated with the magnitude of locomotor sensitization in young adolescents. The magnitude of locomotor activity following high dose cocaine correlated well with the magnitude of locomotor sensitization in individual
young adolescents. Ambulatory activity during habituation also correlated with the magnitude of ambulatory sensitization in individual young adolescents. These data suggest that young adolescents are more vulnerable to some of the behavioral alterations induced by a single high dose of cocaine. They further suggest that individual differences in behavior may be more predictive of sensitization in young adolescents.

The ontogeny of locomotor sensitization has been extensively investigated (Reviewed in Tirelli et al. 2003). It is clear that locomotor sensitization is absent very early in postnatal development. Context-dependent sensitization (sensitization to drugs in a novel environment) begins to appear around the first week of life (McDougall et al. 1994, 1999; Tirelli 2001a, 2001b; Wood et al. 1998) but context-independent (pharmacological) sensitization is delayed until between the third and fourth weeks of life (Bowman and Kuhn 1996; Fujiwara et al. 1987; Scalzo and Holson 1992; Tsuchida et al. 1994; Ujike et al. 1995). These developmental studies demonstrate that context-dependent and context-independent sensitization are both inducible prior to the onset of adolescence.

A number of additional studies have directly compared the magnitude and specific patterns of locomotor sensitization induced in adolescents and adults and produced mixed results (Adriani et al. 2006; Belluzzi et al. 2004, Collins and Izenwasser 2002, 2004; Cruz et al. 2005; Faraday et al. 2003; Laviola et al. 1995; 1999; 2001; Schramm-
Sapyta et al. (2004). These studies utilized a number of different stimulants including cocaine, amphetamine, and nicotine to induce locomotor sensitization. The relative magnitude of locomotor sensitization between adolescents and adults is mixed even within studies using the same stimulant. Several groups have suggested that adolescents may sensitize more (Adriani et al. 2006; Belluzzi et al. 2004) or less (Collins and Izenwasser 2004; Cruz et al. 2005; Schochet et al. 2004) than adults to repeated nicotine injections. With cocaine, Schramm-Sapyta (2004) reported enhanced sensitization in adolescents whereas three other studies observed the opposite (Collins and Izenwasser 2002; Frantz et al. 2007; Laviola et al. 1995). This could be rodent specific as Schramm-Sapyta et al. used mice whereas the other groups used rats. Interestingly, all of the studies that reported reduced sensitization in adolescents, regardless of the stimulant used, tested for the expression of sensitization during the PN 35-45 range. Snyder et al. (1998) demonstrated that the expression of locomotor and stereotypy sensitization is reduced on PN 34 and PN 41 relative to PN 27. Similar to acute cocaine-stimulated locomotion, it is possible that the plastic changes induced by repeated stimulant administration that mediate locomotor sensitization are present but masked by reduced activity during the PN 35-45 window. By utilizing the single dose sensitization paradigm, we are the first group to compare the expression of cocaine sensitization in young adolescents and adults.
All of the previous studies comparing sensitization in adolescents and adults have utilized repeated exposure paradigms over 7-10 days to induce locomotor sensitization. While these studies may potentially be useful to measure the magnitude of behavioral sensitization induced by stimulants, they can not address the rate of sensitization. The results of both our single dose sensitization and acute cocaine binges demonstrate that when only a limited number of stimulant exposures are used to induce sensitization, young adolescents show a more robust sensitization than adults.

All animals in our experiments were treated with cocaine in a novel environment. Therefore, the sensitization that we observed reflects sensitization to both the pharmacological cocaine stimulus and novelty. A large literature has highlighted the importance of context in mediating acute and sensitized neurobehavioral responses to stimulants. Treating animals with stimulants in a novel environment induces greater behavioral responses, greater *c-fos* induction, and the activation of more neuronal populations than treatment in the home cage (Badiani et al. 1998, 1999; Carey and Gui 1998; Crombag et al. 2001; Post et al. 1981). We were able to detect cocaine-induced sensitization to environmental cues by measuring sensitization to the non-drugged environment. This was accomplished by measuring locomotor responses during habituation on day 2 and challenging animals with saline. Cocaine pretreatment increased activity levels during habituation in animals of all ages independent of age. Cocaine pretreatment also increased fine movement responses in young adolescents.
challenged with saline. Cocaine pretreatment did not, however, induce ambulatory sensitization in saline challenged young adolescents. While both pharmacological and contextual actions mediating single dose sensitization may be exaggerated in young adolescents, the magnitude of cocaine sensitization was substantially greater than the observed sensitization to injection stress. Although we can not resolve the exact contributions of pharmacological and contextual sensitization, both are relevant to human drug use.

Age-specific interactions between cocaine action and stress responses could also have contribute to age-specific sensitization. Various laboratory stressors clearly affect mesolimbic dopamine function and addictive behaviors (Maldonado and Kirstein 2005a, 2005b; Miczek at Mutschler 1996; Ortiz et al. 1996; Tidey and Miczek 1997). Stressors as benign as handling of the rats can affect locomotor responses to cocaine (Maldonado and Kirstein 2005a, 2005b). Corticosterone, which is induced by stress, affects responses to cocaine and is one potential mediator of age-specific stress and cocaine effects (Reviewed in Goeders 2002a, 2002b; Kreek and Koob 1998; Piazza and LeMoal 1998). One study from our laboratory demonstrates that adolescents are less sensitive to the aversive properties of both cocaine and lithium chloride than adults (Schramm-Sapyta et al. 2006). Future work in this laboratory is aimed at understanding the possible interactions between stress and cocaine-induced behaviors. Regardless of the specific contributions of stress and pharmacologic activity, we have demonstrated in multiple
contexts that fewer exposures to cocaine induce more locomotor sensitization in young adolescents than adults.

To summarize, we have shown in three separate contexts that adolescents show more locomotor sensitization than adults following a limited number of cocaine exposures. The exact mechanism mediating this effect was not investigated in these experiments. However, Laviola (2001) also observed greater locomotor sensitization in adolescents compared to adults following 3 injections of amphetamine. In that study, he also observed significantly greater increases in amphetamine-stimulated extracellular dopamine levels in adolescents. Perhaps limited exposures to cocaine also increase adolescent cocaine-stimulated extracellular dopamine levels. Below, we discuss some other rapid dopamine-mediated molecular responses that could underlie single dose or intrabinge sensitization in adolescents.

6.1.5 Individual Differences in Sensitization

The correlation between acute cocaine responsiveness and sensitization in individual adolescents suggests that individual adolescents, rather than the population as a whole, may be more vulnerable to cocaine-induced behavioral plasticity than adults. The young adolescents that had robust responses to acute high dose cocaine also demonstrated robust cocaine sensitization. At the same time, young adolescents with minimal responses to acute cocaine treatment demonstrated minimal levels of cocaine sensitization. The subpopulation of young adolescents that demonstrate exaggerated
locomotor responses to acute cocaine appears to be particularly vulnerable to cocaine-induced behavioral plasticity. Interestingly, this subpopulation is primarily restricted to the young adolescents: older rats that demonstrated robust acute responses to high dose cocaine were relatively resistant to cocaine sensitization. It will be important to understand how high and low responding adolescents react differently to cocaine on the cellular level to understand why high responding adolescents may be more vulnerable to sensitization.

One of the goals of these experiments was to determine if there are any behavioral characteristics that can predict which individual animals are most vulnerable to the sensitizing effects of cocaine. In this study, novelty-induced ambulations (ie. ambulations during the first 15 min of habituation) correlated with the magnitude of ambulatory sensitization only in young adolescents. Novelty-induced locomotor activity (fine movements or ambulations) did not correlate with acute high dose cocaine-induced activity in animals of any age. Correlations between novelty-induced locomotor activity and stimulant self-administration have previously been described (reviewed in Piazza and Le Moal 1996). Two studies have reported a correlation between novelty-induced behavior and locomotor responses to stimulants (Chefer et al. 2003; Hooks et al. 1991). Our current study compared novelty to a significantly higher dose of cocaine than these previous studies and it is possible that novelty activity in adults correlates with activity stimulated by low and not high doses of cocaine. At the
same time, Gully et al. (2003) demonstrated that high and low responding rats (to
novelty) only differed in their acute locomotor responses to 10 mg/kg cocaine at a single
time point. At least one study (Stansfield and Kirstein 2005) suggests that other novelty
measures, including novel object preference, may correlate better with dopamine
responses to cocaine than novelty-induced open-field activity. Perhaps other measures
of novelty or stress responses could better predict which adolescents are most
vulnerable to acute cocaine and sensitization. Ongoing studies in this laboratory are
aimed at identifying which individual differences correlate with drug-taking in
adolescent rodents.
6.2 Postsynaptic Responses to Cocaine

Postsynaptic activation of striatal IEGs by cocaine also resembled locomotor responses to cocaine: adolescents had greater increases in the expression of *c-fos* than adults following low but not high dose cocaine. In fact, high doses of cocaine caused greater increases in the expression of *c-fos* and *zif268* in the adult striatum. These differences do not appear to be regulated at the level of receptor as G-protein stimulation by dopamine agonists is comparable in adolescents and adults. We further showed that the expression of these two genes only correlated in individual adults. Cumulatively, our results suggest that differences in intracellular responses to cocaine may mediate age-specific locomotor and transcriptional responses to cocaine.

6.2.1 Cocaine-Induced Striatal *c-fos* and *zif268* Expression

Cocaine stimulated nearly identical anatomical patterns of *c-fos* and *zif268* expression in adolescents and adults. All regions of the striatum that were responsive to cocaine-stimulated IEG induction in adulthood were activated during adolescence. As expected, cocaine preferentially induced *c-fos* and *zif268* expression in the CP (Brandon and Steiner 2003; Daunais and McGinty 1994, 1995; Graybiel et al. 1990; Kosofsky et al. 1995; Moratalla et al. 1993; Steiner and Gerfen 1993; Willuhn et al. 2003). We observed robust expression of *c-fos* and *zif268* in most regions of the CP and medial shell of the nucleus accumbens following both low and high doses of cocaine. High dose cocaine induced statistically significant increases in *c-fos* and *zif268* in some areas of the nucleus
accumbens, but these increases were much less pronounced than those observed in the CP. Interestingly, low dose (10 mg/kg) cocaine did not activate the middle and caudal aspects of the ventrolateral caudate in animals of either age. This pattern of activation matches our behavioral findings as dopamine transmission in the ventrolateral caudate mediates the expression of oral stereotypies (Baker et al. 1998; Delfs and Kelley 1990; Dickson et al. 1994) which we rarely observed in any animals after 10 mg/kg cocaine.

The activation of specific IEGs by cocaine in some striatal regions in adolescents may depend upon the route of administration. We and others (Brandon and Steiner 2003; Kosofsky et al. 1995) observed significant increases in c-fos expression in the ventrolateral caudate in adolescents and adults following high doses (>30 mg/kg) of i.p. cocaine. Cao et al. (2007) only observed marginal increases in ventrolateral caudate c-fos expression in adolescents following 1.5 mg/kg i.v. cocaine. However, Cao et al. only utilized a single dose of cocaine. In our experiments, we did not see activation of ventrolateral c-fos by low dose cocaine in animals of either age. It is possible that a higher dose of i.v. cocaine would also show increased ventrolateral c-fos expression in adolescents. Animals used in Cao et al. (2007) were also frequently handled and surgically manipulated. Perhaps prior experience with experimenters affects ventrolateral c-fos expression in adolescents but not adults. It will be important to determine what factors influence region-specific IEG induction by cocaine to understand such differences that arise between studies.
While cocaine induced qualitatively similar patterns of IEG expression in adolescents and adults, we observed robust dose-dependent age differences in the magnitude of cocaine-induced striatal gene expression. Both adolescents and adults had low constitutive levels of striatal \textit{c-fos} expression. Low dose cocaine (10 mg/kg) induced significantly more \textit{c-fos} in the CP of adolescents compared to adults whereas high dose cocaine induced more \textit{c-fos} expression in the CP of adults. Similar to our results, Kosofsky et al. (1995) showed that \textit{c-fos} expression is more diffuse in lateral aspects of the caudate in adolescents compared to adults following high dose (30 mg/kg) cocaine. The effects of cocaine dose on CP \textit{c-fos} mRNA levels in adults have been examined previously (Daunais and McGinty 1994). That study also demonstrated that acute 10 mg/kg cocaine induces small (<20%) increases in \textit{c-fos} mRNA levels compared to high dose cocaine in adults (30 mg/kg). The relatively weak induction of \textit{c-fos} by 10 mg/kg cocaine in adults contrasts with the robust induction of \textit{c-fos} by 10 mg/kg cocaine in adolescents. \textit{C-fos} induction by 10 mg/kg cocaine in adolescents was so pronounced that it was comparable to 40 mg/kg in several striatal regions. The adolescent striatum appears to be more sensitive to \textit{c-fos} activation by cocaine, although the increased doses of cocaine can cause relatively greater increases in \textit{c-fos} expression in adults.

Cocaine also exerted age and dose specific effects on striatal expression of \textit{zif268}. Adolescents had higher levels of striatal \textit{zif268} expression than adults following saline. These likely represent higher constitutive levels in adolescents, as treating adolescents
with an injection of saline in a novel environment did not increase zif268 levels above animals in the home cage. Adolescents also had higher striatal zif268 mRNA levels than adults after 10 mg/kg cocaine. However, this difference largely reflects higher basal zif268 levels in adolescents as 10 mg/kg cocaine caused similar increases in striatal zif268 expression adolescents and adults. As with c-fos, high dose cocaine caused greater increases in striatal zif268 expression in adults compared to adolescents.

6.2.2 Individual Differences in IEG Expression

We showed that striatal c-fos expression correlated with locomotor activity in individual adolescents and adults. One previous study has shown that locomotor activity correlated well with the expression of striatal c-fos in adult rats (Szucs et al. 2005), but ours is the first to show this in adolescents as well. We are also the first group to examine the correlation between locomotor activity and zif268 expression in individual animals. Surprisingly, striatal zif268 expression only correlated with locomotor activity in adults. There was no significant correlation between striatal zif268 and locomotor activity in individual adolescents. Further, we showed that the expression of c-fos and zif268 correlates well in individual adults whereas no such correlation was observed in adolescents. The age-specific correlations between the expression of individual genes suggest that intracellular responses regulating the expression of specific genes are maturing during adolescence.
We had predicted that the expression of c-fos and zif268 would have correlated in individual animals of both ages. The expression of both genes by cocaine occurs predominantly in the same cell types: D1R containing striatonigral neurons (reviewed in Lu et al. 2006). Both genes can be induced by D1R activation and both of them are dose-responsive with respect to cocaine. However, they do contain different combinations of enhancer sequences in their upstream promoters which could enable different intracellular signals to preferentially activate one gene or the other. For example, zif268 has four serum response element (SRE) binding sites in its promotor whereas c-fos only has one (Christy and Nathans 1989). Several studies have been able to selectively modulate dopamine-mediated c-fos and zif268 expression independently in striatal neurons. In the dopamine depleted striatum, low-moderate doses of selective NMDA receptor antagonists blocked D1-stimulated c-fos expression whereas much higher doses were necessary to reduce zif268 expression (Keefe and Gerfen 1996). Mitogen and Stress-Activated Protein Kinase 1 (MSK1) knockout mice show normal cocaine-stimulated increases in zif268 but no cocaine-induced increases in striatal c-fos expression (Brami-Cherrier et al. 2005). Mu opioid receptor blockade attenuates cocaine and methamphetamine-induced striatal zif268 expression without affecting striatal c-fos expression (Horner and Keefe 2006). Investigating how specific intracellular responses to stimulants like cocaine change during adolescence could help understand gene-
specific responses to cocaine in adolescence and potentially why adolescents are more or less sensitive than adults to some of the plastic changes induced by addictive drugs.

The implications for distinct combinatorial regulation of transcription factors by cocaine during adolescence are numerous. This study only examined the induction of two IEGs by cocaine. Dopamine agonists can stimulate an array of IEGs from at least two gene families (Graybiel et al. 1990; Moratalla et al. 1992, 1993; Young et al. 1991). Further, the known genes of these families have different collections of upstream elements in their promotors and their products are capable of acting in different combinatorial patterns (Janssen-Timmen et al. 1989; Treisman 1995; Tsai-Morris et al. 1988; Vanhoutte and Caboche 2002). A number of groups, including Moratalla et al. (1992), have proposed that transcriptional cooperativity underlies the complexity of functional responses produced by dopamine modulation. One study recently showed that the removal of one upstream kinase (MSK1) that differentially affected cocaine-induced \textit{c-fos} and \textit{zif268} regulation resulted in the loss of cocaine-induced locomotor sensitization concomitant with increased sensitivity to cocaine-induced conditioned place preference (Brami-Cherrier et al. 2005). Studies such as this demonstrate how the dysregulation of specific transcription factors by cocaine can have different effects on specific behavioral alterations induced by cocaine.

Differences in the combinatorial induction of plasticity genes during adolescence may partially explain why adolescents are not outright hyper or hyporesponsive to all
cocaine induced behavioral plasticities. Several studies have exposed adolescent and adult male rats to daily repeated injections of cocaine and observed greater levels of locomotor sensitization in adults (Laviola et al. 1995; Collins and Izenwasser 2002; Frantz et al. 2007; see below for discussion of sensitization). At the same time, others have observed similar or even enhanced development of CPP (Aberg et al. 2007; Badanich et al. 2006; Campbell et al. 2000; Schramm-Sapyta et al. 2004) and acquisition of cocaine self-administration (Frantz et al. 2007; Kantak et al. 2007; Perry et al. 2007) in adolescents compared to adults. Identifying the specific drug-induced behavioral alterations to which adolescents consistently demonstrate unique responses will be an important step to understanding the biological basis for vulnerability to addiction in adolescents.

6.2.3 Acute Gene Induction and Single Dose Sensitization

A single high dose of cocaine induced more locomotor sensitization 24 hrs later in adolescents than adults. Based on these data, we had predicted that high dose cocaine would cause greater increases in the expression of plasticity associated genes in adolescents than adults. However, we found that high dose cocaine induced the opposite: greater increases in \textit{c-fos} and \textit{zf268} in adults than adolescents. These significant observations may help us understand how adolescents and adults could be uniquely sensitive to the molecular effects of different drug administration patterns.
Dopamine receptor stimulation can affect neuronal responses to future stimulation in several ways. The activation of adenylate cyclase by D1 stimulation induces a number of intracellular signaling cascades (highlighted in figure 1). Several kinases, including ERK and DARPP-32, rapidly and reversibly alter the activity of a number of proteins mediating locomotor responses to stimulants such as dopamine receptors, ion channels, and synapsins (Ng et al. 1994, Valjent et al. 2000, 2001a, 2003). For example, cocaine treatment causes the rapid phosphorylation of the AMPA receptor GluR1 subunit which enhances striatal AMPA mediated currents in vivo (Price et al. 1999; Snyder et al. 2000; Wolf et al. 2003; Yan et al. 1999). These effects can be attenuated by the activation of opposing phosphatases including PP1a. Post-translational effects such as phosphorylation/dephosphorylation occur independent of transcriptional activation. It is likely that mechanisms such as these may mediate the rapid development of locomotor sensitization following few cocaine exposures, such as within a single cocaine binge. Our acute binges and single dose sensitization experiments suggest that adolescents may be more vulnerable than adults to neural plasticities induced by non-genomic events. Since they are non-genomic, these may represent transient behavioral plasticities.

Cocaine also induces long-lasting changes in neuronal activity by increasing the expression of new proteins. One such protein is δ-FosB. This is a truncated form member of the Fos family of transcription factors that can be induced following multiple
exposures to cocaine, but not acute treatment (see Nestler 2004). This stable form of the protein has a half-life of several months and its expression strongly potentiates cocaine CPP and locomotor stimulation. Repeated cocaine treatments also lead to a number of persistent morphological effects in multiple neuronal populations (Robinson and Kolb 1999, Robinson et al. 2001). Though it has not been directly examined, it is believed that these structural changes require transcriptional activation. Our current results showing greater increases in striatal c-fos and zif268 in adults suggest that adults could be more vulnerable than adolescents to some of the long-lasting plastic effects of repeated high-dose cocaine treatment. This is assuming that age-specific mRNA levels translate into age-specific protein levels, which were unmeasured in this study. At least one study has demonstrated that repeated daily high dose exposures to cocaine induce more locomotor sensitization in adults than young adolescents (Collins and Izenwasser 2002). Several other studies have also reported reduced locomotor sensitization to cocaine in mid-adolescents compared to adults (Frantz et al. 2007; Laviola et al. 1995).

Cumulatively, it appears that adolescents may be more vulnerable than adults to rapid and potentially transient forms of locomotor sensitization induced by a limited number of cocaine exposures. At the same time, adults may be more vulnerable than adolescents to a number of long-lasting changes induced by repeated cocaine exposures. It is not precisely clear which changes are most relevant to drug addiction. In fact, it has recently been shown that some well characterized events, such as nucleus accumbens
LTD, are important for locomotor sensitization but not voluntary drug intake in self-administering rats (Ahmed and Cador 2006). We have demonstrated that adolescents and adults have unique molecular responses to acute cocaine and it will be important to determine how these relate to response variables such as drug reward, acquisition of self-administration, and voluntary drug intake to determine if they are important mediators of adolescent drug addiction or not.

**6.2.4 Dopamine Receptor Activity**

The combined results of our G-protein coupling assays and locomotor studies further suggest that intracellular responses to dopamine receptor stimulation continue to develop during adolescence. We showed that the activation of G-proteins by the dopamine receptor agonists dopamine, quinpirole, and dihydrexidine in tissue homogenates from the CP, nucleus accumbens, and PFC do not differ by age. This result suggests that age-differences in locomotor activation and regional IEG induction by cocaine and apomorphine are not likely mediated at the level of the dopamine receptor or its coupling to G-proteins. However, we can not completely dismiss the possible role of age-differences in dopamine receptor stimulation. It is unclear what magnitude of change in G-protein stimulation by dopamine agonists is necessary to mediate significant changes in locomotor activity. Animals that have been lesioned with 6-hydroxydopamine demonstrate prominent sensitivity to the locomotor effects of dopamine agonists. At the same time, these animals only show relatively small
increases in D2 stimulated G-protein activation in GTPγS assays (Newman-Tencradi et al. 2001). It is possible that changes in receptor activity that are too small to reliably detect by these assays could mediate our age-specific locomotor and transcriptional observations. It is also possible that using tissue homogenates we were unable to detect regional age-differences in G-protein stimulation. In our IEG experiments, we observed different magnitudes of \textit{c-fos} and \textit{zif268} induction between subregions within the CP, nucleus accumbens, and PFC. It is possible that dopamine receptor activity in specific subregions could mature during adolescence and mediate our locomotor and transcriptional observations. While we can not exclude these and other possibilities, our results suggest that developmental changes in intracellular responses downstream of the dopamine receptor could underlie changes in locomotor and transcriptional responses to dopaminergic stimulants like cocaine during adolescence.

The maturation of specific cellular signaling molecules could easily explain age effects on the expression of specific genes by cocaine. However, explaining the effects of age on dose response may be a little more complicated. As previously discussed, it is possible that enhanced adult responses to high dose cocaine could be at least partially mediated by greater striatal dopamine content during adulthood. At the same time, it is also possible that different signaling cascades are preferentially activated by low and high dose cocaine. No studies have directly examined the effects of cocaine dose on the activation of specific intracellular signaling proteins. If high and low dose cocaine
preferentially activate different cascades that are mediated by different proteins then it would be easy to imagine how adolescents and adults could have opposite transcriptional responses relative to one another following different cocaine doses. Clearly further investigation is needed to understand what mediates different molecular responses to cocaine during adolescence and how these relate to drug taking to understand how these changes could render adolescents vulnerable to addiction.

6.2.5 Cortical c-fos and zif268 Expression

We are the first group to carefully examine cortical IEG responses to cocaine stimulation in individual adolescents and adults. We made several important findings. First, cocaine-induced locomotion does not correlate with the expression of either c-fos or zif268 in cortical areas projecting to the striatum in adolescents or adults. Second, basal c-fos and zif268 expression levels in the cortex are significantly higher than those observed in the striatum and, accordingly, we saw much less pronounced increases in c-fos and zif268 expression in the cortex. Further, unlike the striatum, these increases were not dose-responsive. Finally, we showed that in general adolescents have higher cortical c-fos and zif268 expression levels following saline and cocaine treatment.

We had predicted that the expression of c-fos and/or zif268 in some cortical areas, including the premotor (agranular) and sensorimotor cortex, might correlate with locomotor activity in individual animals. This was simply not observed although it is perhaps not surprising. As discussed above, neuronal activity can be increased by
activating a number of excitatory receptors including D1 dopamine, AMPA, and NMDARs. Within the striatum, stimulant-induced increases in IEG induction are largely mediated by increased dopamine D1 receptor stimulation (Berreta et al. 1992; Robertson et al. 1990, 1992). While cocaine does increase dopamine transmission in the frontal cortex, dopamine transporters are much more diffusely expressed in the cortex than the striatum (Leroux-Nicollet and Costentin 1988). Activation of cortical neurons by cocaine in a novel environment involves the integration of a number of cognitive, sensory, and motor processes. Understanding the consequences of cortical activation by cocaine will be an important step in more broadly understanding how addictive drugs can affect the mind.

Compared to the striatum, we observed much higher levels of both c-fos and zif268 expression in the cortex following saline. Basal expression of these genes in the cortex is likely mediated by a number of non-dopaminergic mechanisms. Our experiments clearly show that this expression is not completely the result of locomotor activity, injection stress, or placement in a novel environment. In adolescents, cortical zif268 expression was no different in the home cage than following habituation and a saline injection in the novel locomotor chambers. The injection in the novel environment slightly elevated zif268 expression in adults, although basal levels were still quite high compared to the striatum.
Cocaine caused proportionally smaller increases in both c-fos and zif268 in the cortex compared to the striatum. These increases were also not dose-dependent as 40 mg/kg cocaine did not increase activity above 10 mg/kg cocaine. This is not due to a detection limit, as cortical IEG levels were far below saturation limits of the film following all doses of cocaine. Again, while dopaminergic perturbation may be closely tied to cocaine dose, a number of other neural functions need not be so closely related. Understanding the functional significance of cortical IEG induction by cocaine may be especially important for understanding adolescent cocaine effects as adults showed proportionally greater increases in cortical zif268 expression by cocaine than adolescents.

Finally, we observed that on average adolescents had higher c-fos and zif268 expression levels than adults in the cortex. These differences were relatively independent of cocaine action. Adolescents had higher basal expression levels of both genes and these gross age differences were generally maintained following cocaine treatment. As a percentage of baseline, we saw bigger increases in zif268 expression in adults than adolescents. This finding suggests that cortical neurons are readily responsive to activation by cocaine in both adolescents and adults. Clearly, the present lack of mechanistic information about cortical IEG induction by stimulants represents a significant gap in understanding how addictive drugs affect the brain. However, our results have demonstrated that the quantitative activation of striatal neurons is more closely related to the psychomotor properties of stimulants.
6.3 Significance of findings

The main goal of this work was to investigate how forebrain dopamine systems initially respond to the addictive stimulant cocaine in adolescence. These systems mediate the reinforcing effects of drugs of abuse. It has long been suspected that changes in the responses of forebrain dopamine systems to addictive drugs may be critical in mediating adolescent vulnerability to addiction. A limited number of previous studies have compared locomotor and \textit{c-fos} activation by cocaine in adolescents and adults and produced mixed results. Our thorough investigation has produced several key findings that help to clarify the existing literature and may help understand how adolescents could be more vulnerable to addiction than adults.

One of our major findings is that young adolescents exhibit exaggerated responses to low but not high doses of cocaine. We showed that adolescents have greater locomotor and \textit{c-fos} responses than adults following lower (< 15 mg/kg) but not higher doses of cocaine. Adolescents also appear to be more sensitive to the activation of transcription factors like \textit{c-fos} by lower doses of cocaine than adults. The greater induction of plasticity genes like \textit{c-fos} by lower doses of cocaine in adolescents could make them more vulnerable to neural alterations induced by cocaine that are critical for the progression to addiction. The use of the terms low and high dose cocaine in these studies may be misleading. This terminology is based on the locomotor activating effects of cocaine. Cocaine induces both pleasurable and aversive subjective effects. In
In general, the aversive effects of cocaine increase as the dose is increased (see Schramm-Sapyta et al. 2006). In fact, high doses of cocaine can induce conditioned aversion for natural reinforcers like sucrose. Research from this and other laboratories has shown that adolescents are significantly less sensitive to the aversive properties of 10 mg/kg cocaine than adults. Therefore, adolescents may be better equipped than adults to take doses of cocaine that induce significant levels of transcriptional activity (and therefore induce long-term effects).

We also demonstrated that adolescents are more vulnerable to locomotor sensitization induced by fewer cocaine exposures compared to adults. In the context of the literature, it appears that the relative magnitude of behavioral alterations induced by cocaine may depend on the administration patterns. We showed in that young adolescents are more vulnerable than older animals to locomotor sensitization induced within an acute cocaine binge or 24 hours after a single high dose of cocaine. However, several other studies suggest that multiple high doses of cocaine over a period of days induce more locomotor sensitization in adults than adolescents. Our transcriptional studies suggest that adults could be more vulnerable to long-lasting molecular changes induced by high dose cocaine than adolescents.

Combining the above observations with human epidemiology may help to better understand the hazards of adolescent drug use. Most life-long drug addiction is initiated during adolescence. Very few people begin experimenting with addictive
substances later in life. Adolescents may begin experimenting with addictive drugs and accelerate their drug intake in part because they are relatively insensitive to unpleasant drug effects. Rapid drug-induced plasticities during adolescence, such as those observed in our studies, may also drive accelerated drug intake in adolescents. However, our results suggest that it is possible that many of the long-lasting effects of drugs like cocaine may not be fully induced until the users enter early adulthood. If so, it is possible that interventional treatment could be significantly more effective during adolescence than in adulthood.

Our results also demonstrate that not all adolescents are equally vulnerable to behavioral plasticity induced by limited cocaine exposures. This suggests that perhaps not all adolescents are more vulnerable to addiction than adults. Through our correlational analyses we demonstrated that not all adolescents sensitize more than adults or have greater c-fos responses to low dose cocaine. Rather, there were subpopulations of highly reactive adolescents that were distinguishable from adults and other adolescents. Some adolescent rats demonstrated extreme levels of locomotor responses to cocaine that were never observed in adults. These were the same animals that showed robust single-dose sensitization to cocaine and robust c-fos inductions. It is unclear if these adolescents would also be most vulnerable to becoming addicted to cocaine. However, they demonstrate that certain adolescents are especially sensitive to some of the effects of cocaine. Perhaps the marked vulnerability to addiction in
adolescents is mediated by a subpopulation of individual adolescents that are especially susceptible to the addictive effects of cocaine. Identifying how specific behavioral traits predict different drug responses and how those responses relate to addiction could help to better understand the biology addiction vulnerability and identify high-risk individuals prior to their initiation of drug use.
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