EVALUATION OF AGE DIFFERENCES IN LOCOMOTOR ACTIVITY FOLLOWING PSYCHOSTIMULANT ADMINISTRATION IN ADOLESCENT VERSUS ADULT MICE

BY

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DISSERTATION

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ABSTRACT

In humans, the brain continues to develop over the course of adolescence. One implication is that drugs of abuse are likely to impact adolescents differently than adults. Rodent literature has added credibility to this idea. Following administration of an equivalent dose of psychostimulant (e.g. cocaine, amphetamine, methamphetamine), adolescent rodents are commonly observed to locomotor stimulate to a lesser magnitude than that of adults. However, recently conflicting reports have been published showing adolescents to stimulate equally or greater than adults. The cause for variability in the literature is unclear, but may be a result of genetic differences between subjects or other dissimilarities in experimental design. Therefore, in Chapter 2 adolescent (postnatal day 30) and adult (postnatal day 65) mice of both sexes from four different inbred strains (C57BL/6J, BALB/cByJ, DBA/2J, FVB/NJ) were screened for differences in locomotor stimulation following cocaine. The greatest difference in stimulation between age groups was observed in C57BL/6J, but BALB/cByJ and female FVB/NJ mice also showed attenuated stimulation in adolescents as compared to adults. Since locomotor stimulation differences were greatest in C57BL/6J mice, they were used in all subsequent chapters. Chapter 3 tests the hypothesis that attenuated stimulation in adolescents may be caused by lower concentrations of drug in the brain as compared to adults. Concentrations of cocaine and methamphetamine were measured in blood and brain samples of each age group at varying time points following administration. Overall, the pattern of drug concentration levels over time was similar between age groups, suggesting alternative explanations for behavioral differences. Chapter 4 investigates the possibility that developmental changes in the brain may contribute to behavioral differences by examining neural activity as measured by Fos induced from cocaine in 16 different brain areas. Results showed that for a given level of locomotor activity, adolescents had greater levels of Fos expression in the dorsal caudate as compared to adults. This posed the question of how greater Fos expression could relate to relatively lower locomotor stimulation in adolescents. Chapter 5 attempts to answer this question by examining the hypothesis that adolescents experience greater activation of a negative feedback circuit within the caudate called the striosomal pathway. Fos expression was localized using a striosomal marker, MOR1 antibody stain, following cocaine administration. No differences were observed between age groups, suggesting the striosomal pathway is not differentially activated between adolescents and

adults. Overall, the mechanism underlying the phenomenon of attenuated stimulation in adolescents as compared to adults following psychostimulant administration remains unknown, but the dorsal caudate remains an area of interest.

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CHAPTER I

Review of the adolescent rodent brain and behavior literature: validity of the model, behavioral response to psychostimulants, and brain development

The adolescent animal

The brain continues to develop over the course of adolescence and is not considered to be fully developed until around 20 years of age (Giedd, 2004). Continuing patterns of neural development are concurrent with other developmental changes, such as puberty, and likely affect behavior (Lange et al., 1997, Galvan et al., 2006). Indeed, several studies have shown adolescents tend to behave in a manner that is qualitatively distinct from adults (for review see Spear, 2000). Some of these behaviors, such as elevated levels of risk taking as compared to adults, can have negative consequences. However, how changes in the nervous system over the course of adolescence relate to behavior is largely unknown. Further research is needed to discover how characteristic behavioral responses in adolescents are associated with developmental changes in the brain.

Animal models can be utilized to study adolescence because the developmental changes and characteristic behaviors that occur in humans during adolescences have analogs in rodents (Spear, 2000). For example, adolescent rodents display many of the same types of behaviors that adolescent humans display. As in humans, adolescent rodents spend more time than adults in social interactions and play behaviors. While both adolescents and adults display social interactions, conditioned place preference for social interactions has been shown to be stronger in adolescents than adults, suggesting an increased emphasis on social behaviors during adolescence (Douglas et al., 2004). Another behavior that is conserved across humans and rodents is an increase in novelty seeking and risk taking behaviors. One way to measure risk taking and novelty seeking is to measure ambulation in an open field chamber. Adolescent rats have been shown to be hyperactive compared to adults in a novel environment (Masur et al., 1980). Additionally, adolescents show greater preference than adults for novel environments (Adriani et al., 1998).

Neurologically, adolescent rodents follow a similar developmental sequence as humans. This is observed at both the macro (structural) level and micro (molecular) level. For example, the prefrontal cortex is known to undergo massive remodeling during both rat and human adolescence (Giedd et al., 1999, Spear, 2000). The volume of the prefrontal cortex decreases during adolescence while dopaminergic input increases (Kalsbeek et al., 1988, Lewis, 1997).

Developmental changes in dopamine signaling have also been observed in the striatum. For example, the number of dopamine receptors in the striatum is greater during adolescence than during adulthood in both humans and rats (Seeman et al., 1987, Teicher et al., 1995, Tarazi et al., 1998, Tarazi et al., 1999). Collectively, the number of homologies between human and rodent development during adolescence suggests that rodents are useful to model aspects of the adolescent time period in humans.

In rodents, adolescence occurs from postnatal day (PN) 28 until adulthood at day 60, with the onset of puberty occurring about 40 days of age (Laviola et al., 2003, Smith, 2003). In order to better understand the changes that occur over this time range, adolescence in rodents is commonly divided into three time periods: early adolescence (PN 21-34), middle adolescence (PN 34-46), and late adolescence (PN 46-59) (Laviola et al., 2003). While this framework is useful, it is important to note that age divisions often vary slightly in the literature (Spear and Brake, 1983, Spear, 2000, Laviola et al., 2003, Smith, 2003).

Psychostimulants and adolescence

Drug use in humans is commonly initiated during adolescence (Chen and Kandel, 1995, Nelson et al., 1995, Mathias, 1996). Given that the brain continues to develop during the course of adolescence (see above), it is possible that drugs of abuse may impact adolescents differently than adults. While most drug research is constrained in adolescence for obvious ethical reasons, limited evidence suggests this may be true for psychostimulants such as cocaine, amphetamine, and methamphetamine. For example, juveniles reported lower feelings of euphoria and had greater decreases in motor activity as compared to adults following dextroamphetamine administration (Rapoport et al., 1980). Furthermore, escalation of cocaine use is more rapid in adolescents than adults and adolescents have been suggested to be at a greater risk for addiction than adults (Estroff et al., 1989, O'Brien and Anthony, 2005). Despite these observations, the extent to which psychostimulants impact adolescents differently from adults and the mechanisms underlying those differences are largely unknown.

Animal models have proven useful in examining behavioral differences to psychostimulants between adolescents and adults. One of the earliest reported differences was

that adolescent rats are less sensitive to the locomotor stimulating properties of amphetamine than adults (Lanier and Isaacson, 1977). Since that time, the phenomenon of attenuated locomotor stimulation in adolescents has been observed for cocaine and methamphetamine as well (Spear and Brake, 1983, Laviola et al., 1995, Maldonado and Kirstein, 2005, Zakharova et al., 2009), although conflicting observations have been reported for cocaine (Catlow and Kirstein, 2005, Caster et al., 2007, Camarini et al., 2008) (see chapter 2 page 16 for a review of adolescent versus adult locomotor stimulation). The reason why this phenomenon occurs is not known.

Mechanisms of locomotor stimulation to psychostimulants

Differential locomotor stimulation between adolescents and adults is a good model for exploring differences to the effects of psychostimulants between age groups because locomotor stimulation is well studied in adults. A major component of locomotor activation from stimulants is increased dopamine signaling in the striatum (Rebec, 2006). The striatum is part of the basal ganglia circuit, which is a network of brain regions involved in the control of movement (Graybiel et al., 1994). Cocaine increases dopamine signaling in the striatum by acting as an antagonist at the dopamine transporter (amphetamine and methamphetamine have similar modes of action) (Sulzer et al., 2005). Knowledge of this system, and other mechanisms of psychostimulant induced locomotor activity, can be leveraged to formulate hypotheses for why age differences exist (See chapters 4 & 5 for a more complete review of the basal ganglia circuit and how this information was used to create specific hypotheses).

Fos

Immediate early genes, such as c-Fos, are genes that can initiate cascades to induce other gene expression (Herrera and Robertson, 1996). They encode for one of two broad categories: transcription factors or direct effector proteins (Clayton, 2000). c-Fos is a transcription factor in the Fos family. It binds with other members of the Fos and Jun family to create the activator protein-1 complex which promotes the expression of downstream genes (Glover and Harrison, 1995, Hughes and Dragunow, 1995). This has led researchers to consider immediate early genes such as c-Fos to play an important role in the processing of information on the cellular level.

c-Fos has been shown to be a good marker of cellular activity. Basal levels of c-Fos expression are relatively low, making increases in expression relatively easy to detect (Morgan and Curran, 1991). Increases in c-Fos expression have been shown following a wide variety of paradigms including behavioral paradigms, primary sensory stimuli, and pharmacological administration (Clayton, 2000). It is thought that increased expression represents increased neural activity. In vitro studies have shown that depolarization of a neuron increases Fos expression (Greenberg et al., 1986, Morgan and Curran, 1986). However, Fos is not a perfect correlate to electrophysiological or metabolic activity. Areas of the brain which are known to be electrophysiologically and/or metabolic active based on knowledge of neural circuits and 2-deoxyglucose maps, do not always show correlated c-Fos activation (Sagar et al., 1988, Kaczmarek and Chaudhuri, 1997). However, disconnects between neural activation and c-Fos are primarily observed in regions of high basal activity and c-Fos is generally considered to reflect the functional activity of neurons in most novel contexts (Kovacs, 1998).

Examining Fos immunoreactivity has been useful in correlating patterns of expression in the brain to experimental treatments. For example, Zombeck et al. (2008) examined patterns of Fos activation in the brain between mice placed in an environment previously paired with food versus mice placed in an environment previously paired with cocaine. Researchers successfully identified the paraventricular hypothalamic nucleus as an area which showed context specific activation for food but not cocaine. One of the advantages of examining Fos in this study was that a large number (17) of brain regions could be surveyed and that the number of Fos positive cells could be correlated with behavioral measures. Similar techniques are employed in chapters 4 & 5 of this dissertation (see respective chapters for details).

Potential relevance for humans

In humans, initial subjective experience is predictive of later use. Positive subjective experiences with drugs have been associated with increased use later in life (Davidson et al., 1993, Fergusson et al., 2003), while negative experiences are associated with decreased later use (Volkow et al., 2002). Discovering the mechanisms involved in variation in initial responding to a drug is of great interest for understanding why some people struggle with drug abuse while others do not.

The relevance for how acute locomotor stimulation to psychostimulants in rodents relates to subjective experience is controversial. Some have argued that locomotor stimulation is positively correlated with dopamine release in the accumbens and therefore can be thought of as a proxy for relative pleasure from the drug. Conversely, others have shown that low levels of locomotor responding are predictive of behavioral demonstrations of high rewarding effects of the drug. Specifically, low levels of initial locomotor response are correlated with increased place preference and increased self administration to cocaine (Allen et al., 2007, Mandt et al., 2008). Furthermore, genetic approaches have suggested that there may be an association between locomotor stimulation in rodents and subjective experience in humans. Variation in a gene associated with high or low acute locomotor stimulation to methamphetamine in mice, *Csnk1e*, was discovered to influence subjective sensitivity (i.e. self report of drug effects) to amphetamine in humans (Veenstra-VanderWeele et al., 2006). More evidence is needed before definitive claims on how locomotor stimulation in rodents relates to subjective experience can be made. However, collectively the current evidence suggests using animal models of acute locomotor stimulation can be useful for discovering mechanisms underlying behavioral variation in response to psychostimulants.

Approach to understanding age differences in locomotor stimulation

The focus of this dissertation was the phenomenon of attenuated locomotor stimulation to psychostimulants in adolescents versus adults. The aim was to extend the understanding of the biological basis for this phenomenon. Put another way, the goal of this dissertation is to develop a foundation for answering the question of "why adolescents locomotor stimulate less than adults following psychostimulant administration?" Therefore, the chapters presented herein should be viewed as introductory studies toward discovering the mechanism with the purpose of providing evidence for or against plausible causes for age differences in stimulation.

My approach was to start as broadly as possible, then refine and narrow my hypotheses based on the results in a top-down manner. It was unclear from the literature how broadly the phenomenon of attenuated locomotor stimulation in adolescents as compared to adults extends across genotype. Therefore, chapter 2 examines the locomotor response to cocaine between

adolescent and adult mice in multiple inbred strains. The purpose was to discover possible genetic variation in the trait and to identify in which strains age differences are greatest. After discovering C57BL/6J as a strain that demonstrates robust age differences in stimulation, the question shifted to potential underlying mechanisms.

One possible explanation for why adolescents stimulate less than adults is that adolescents experience lower concentrations of drug in the brain than adults. Few studies have examined the hypothesis that pharmacokinetics could be different between age groups (McCarthy et al., 2004, Caster et al., 2005, Frantz et al., 2007). Of those that have, none have correlated concentrations of drug in the brain with locomotor behavior. Therefore, in chapter 3 I measured concentrations of cocaine and methamphetamine in the brains of adolescent and adult mice following behavioral analysis of locomotor stimulation. The aim was to determine the extent to which pharmacokinetic differences between adolescents and adult C57BL/6J mice could account for differential acute locomotor stimulation to methamphetamine and cocaine between the age groups.

An alternative possibility for attenuated stimulation in adolescents is that the continued development of the adolescent brain influences the behavioral response to psychostimulant administration. However, the number of changes occurring in the brain over the course of adolescence makes it difficult to assess which differences influence behavioral differences and which do not. Evidence supporting regional differences is desirable so that the list of candidate mechanisms can be narrowed. One technique to screen where in the brain functional differences may exist is to examine regional Fos expression. By using Fos as a marker of neural activity, a number of brain regions can be examined for differential Fos activation between age groups. The assumption is that Fos will correlate with behavior and therefore areas of the brain that are involved in differential locomotor stimulation will also show differential Fos expression. Therefore in chapter 4 I measured patterns of Fos expression in 16 different brain regions between adolescent and adult mice following drug (cocaine or methamphetamine) or saline. The aim was identify key brain regions associated with differential locomotor stimulation between age groups.

The advantage of identifying a candidate brain region or regions is that information about the distribution of cell types, receptor signaling systems, and principle afferent and efferent connections in the regions becomes available from the literature. This knowledge is valuable in postulating hypotheses on which biological substrates are involved in differences in locomotor responding between age groups. In chapter 5, I hypothesize that adolescents display increased inhibitory feedback from the striatum to dopaminergic neurons in the substantia nigra via the striosomal pathway based on results from chapter 4. The aim was to identify if the striosomal pathway from the striatum is differentially activated from cocaine between adolescents and adults.

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CHAPTER II

Acute locomotor responses to cocaine in adolescents versus adults from 4 divergent inbred mouse strains

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Abstract

Growing evidence suggests that adolescent mice display differential sensitivity to the acute locomotor activating effects of cocaine as compared to adults, but the direction of the difference varies across studies and the reasons are not clear. Few studies have directly examined genetic contributions to age differences in locomotor stimulation from cocaine. The goal of this study was to determine the extent to which reduced stimulation in C57BL/6J adolescents as compared to adults generalizes to other strains. Therefore, we examined male and female mice from four genetically divergent inbred stains (BALB/cByJ, C57BL/6J, DBA/2J, FVB/NJ) at two ages, postnatal day 30 and postnatal day 65. Mice received either saline or cocaine (15 or 30 mg/kg), and then immediately were placed back into their home cages. Locomotor activity was recorded continuously in the home cage by video tracking. Adolescents displayed reduced stimulation as compared to adults for C57BL/6J, BALB/cByJ, and female FVB/NJ mice. No age differences were observed for DBA/2J or male FVB/NJ. No main effects of sex were observed. Strain differences in pharmacokinetics, neural development or physiology could contribute to the observed differences between ages across strains. Future comparative studies could discover biological differences between strains that explain age differences in cocaine sensitivity.

Introduction

Drug use in humans is commonly initiated during adolescence (Chen & Kandel 1995; Mathias 1996; Nelson *et al.* 1995). The brain is still undergoing substantial development during this time (e.g., prefrontal cortex (Giedd *et al.* 1999; Spear 2000), dopamine neurotransmitter system, (Seeman *et al.* 1987; Tarazi *et al.* 1998, 1999; Teicher *et al.* 1995)). Differences in brain morphology and physiology likely contribute to differential behavioral responses to drugs in adolescents as compared to adults (Zombeck *et al.* 2009). This is concerning because initial drug responses can predict future patterns of use (Davidson *et al.* 1993; Haertzen *et al.* 1983; Lambert *et al.* 2006).

In rodent models, distinct behavioral responses to drugs in adolescents as compared to adults have been observed using a variety of experimental paradigms (Belluzzi *et al.* 2004; Hollstedt *et al.* 1980; Levin *et al.* 2003; Shram *et al.* 2006; Silveri & Spear 2001; Torres *et al.* 2008; Vastola *et al.* 2002; Zakharova *et al.* 2009b). One clear example is locomotor activity following acute psychostimulant administration. Adolescent rodents typically display reduced sensitivity to the locomotor activating effects of cocaine (Laviola *et al.* 1995; Maldonado & Kirstein 2005a; Spear & Brake 1983; Zombeck *et al.* 2009; Zombeck *et al.* 2010), amphetamine (Adriani & Laviola 2000; Bolanos *et al.* 1998; Lanier & Isaacson 1977; Mathews & McCormick 2007; Mathews *et al.* 2009; Spear & Brake 1983), and methamphetamine (Zakharova *et al.* 2009a; Zombeck *et al.* 2009; Zombeck *et al.* 2010), using a wide range of doses and experimental paradigms. However, conflicting findings, particularly for cocaine, have been reported. For example, some studies have observed no differences between age groups (Adriani *et al.* 1998; Camarini *et al.* 2008; Collins & Izenwasser 2002; Niculescu *et al.* 2005; Parylak *et al.* 2008), while others have observed *greater* stimulation in adolescents as compared to adults in response to psychostimulants (Badanich *et al.* 2008; Caster *et al.* 2007; Catlow & Kirstein 2005). The explanation for this variability is not known, but probably involves both environmental sources, including differences in handling (Maldonado & Kirstein 2005a, b) and testing environment (Masur *et al.* 1980), as well as genetic, including species and strain differences between studies.

Genetic contributions to psychostimulant-induced locomotor activity among adult mice is well established (e.g., Bryant *et al.* 2009; Marley *et al.* 1998; Phillips *et al.* 2008). However, genetic contributions to variability between adolescent and adult sensitivity to psychostimulants are not well understood. Balda et al. (2008) demonstrated that the nNOS gene is involved in behavioral sensitization to cocaine in adult, but not adolescent, mice. This suggests that genes influencing behavioral effects of psychostimulants in adolescents may not be the same as adults. Further research is needed to determine the extent to which environmental and genetic background influence age differences in psychostimulant induced locomotor activity.

Comparing inbred mouse strains is one method for discovering genetic influences on behavior. Of the studies in mice, age differences in locomotor activity following psychostimulant administration have been examined in only a few different strains. Adolescent C57BL/6J mice consistently display reduced stimulation to cocaine and methamphetamine as compared to adults (McCarthy *et al.* 2004; Zombeck *et al.* 2009). Outbred CD1 mice commonly show no age differences in stimulation to cocaine or amphetamine (Adriani *et al.* 1998; McCarthy *et al.* 2004; Niculescu *et al.* 2005), but see Adriani & Laviola (2000) who found reduced stimulation in adolescents as compared to adults following 2 mg/kg amphetamine. Inbred DBA/2J mice also apparently show no age differences (Camarini *et al.* 2008). However, these data do not include information on females for either the C57BL/6J or DBA/2J strains. Information on many of the other inbred strains (e.g., as represented it the Mouse Phenome Database) is also lacking.

The goal of this study was to determine the extent to which the phenomenon of attenuated stimulation in adolescents as compared to adults in C57BL/6J mice extends to other inbred strains. We hypothesized that adolescents would stimulate less than adults across strains, but that the magnitude of this difference would depend significantly on genotype.

Methods

Subjects

A total of 192 animals were used in this study from 4 different inbred stains: C57BL/6J, FVB/NJ, BALB/cByJ, and DBA/2J. All 4 strains are listed as Tier 1 priority in the Mouse

Phenome Database and were chosen because they each represent different branches of the phylogenetic tree for inbred mouse strains, as represented in Rhodes et al. (2007).

Male and female mice (n=4/sex/age/strain/dose) arrived from Jackson Laboratory (Bar Harbor, ME, USA) in two different age groups: postnatal day 21 and 56. Mice were initially housed in groups of 3-4 for 5 days before being transferred to custom-made acrylic home cages conducive for video tracking (Fig. 2.1) where they remained for 4 additional days (see Zombeck *et al.* 2009; Zombeck *et al.* 2010). All mice were housed on a 12:12 reverse light/dark cycle (lights off at 7 AM and on at 7 PM) with the room temperature maintained at 21±1℃. Mice had ad libitum access to food and water at all time. Adolescent mice were tested at postnatal day 30 and adults at day 65. This is a commonly accepted period for adolescents and adults in rodents (Spear 2000; Spear & Brake 1983). All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and adhered to NIH guidelines. The Beckman Institute Animal Facility where the mice were held is AAALAC approved.

Behavioral testing

All behavioral testing was conducted in the animal's home cage using custom-made home cages with clear plastic lids and food and water delivered from the side (Zombeck *et al.* 2009). Two different types of bedding were used depending on whether the mouse had a white or a dark coat color. Corncob bedding (Harlan 7097) was used for dark mice, whereas Sheppard Paperchip® bedding was used for white mice (see Fig. 2.1). Following Zombeck et al. (2009; 2010), horizontal distance traveled in the home cage was recorded continuously using TopScan (Clever Sys, Vienna, VA, USA) video tracking software. Behavioral testing began at the onset of the dark cycle. Red lights were placed in various positions in the room overhead to illuminate the cages during the dark phase for continuous video tracking (mice cannot see red light). All mice were tracked under baseline conditions without being disturbed for 1 hr. Following the 1 hr baseline, all mice received an intraperitoneal injection of saline and then immediately returned to their home cage to monitor the response to injection. After 1 hr, mice were administered another saline injection or cocaine (15 or 30 mg/kg) and locomotor activity was recorded for an additional 1 hr. Doses were chosen based previous studies that have shown reliable behavioral differences between age groups in C57BL/6J males (Zombeck *et al.* 2009; Zombeck *et al.* 2010).

Statistics

Statistical analysis was preformed using SAS version 9.1 (SAS Institute, Cary, NC, USA). ANOVAs were performed separately for each strain with age, sex, and dose as factors, and across strains with strain, age, and dose as factors. Pair-wise differences were evaluated using Tukey post hoc tests. Heritability was estimated by one-way ANOVA for each age and dose with strain as the factor (Belknap *et al.* 1993; Crabbe *et al.* 1990; Rhodes *et al.* 2007). For all tests, a *P* value of <0.05 was considered significant.

Results

Saline

The response to a saline injection was measured 1 hr following onset of the dark cycle, prior to cocaine administration. Total distance traveled was summed over the 1 hr following injection. Adolescent and adult mice displayed similar levels of locomotor activity in all strains except BALB/cByJ. This was reflected in a significant Age x Strain interaction in the 3-way ANOVA with age, strain and sex as factors ($F_{3,174}$=4.1, *P*=0.007). Posthoc analysis showed that BALB/cByJ adolescent mice traveled less far than adults (main effect of age, $F_{1,44}$=15.2, *P*=0.0003). No significant age effects were observed in the other strains. No significant strain or sex differences were observed collapsed across age (*P*>0.05).

Cocaine

The magnitude and direction of the difference in locomotor stimulation between adolescents and adults differed depending on genotype (Fig. 2.2). All main effects and interactions were significant in the overall 3-way ANOVA including the Age x Strain interaction ($F_{3,166}$=8.8, *P*<0.0001) and the Age x Dose x Strain interaction ($F_{6,166}$=2.4, *P*=0.03). C57BL/6J and BALB/cByJ adolescents showed the predicted pattern of reduced locomotor stimulation as compared to adults (C57BL/6J main effect of age, $F_{1,36}$=19.7, *P*<0.0001; BALB/cByJ main effect of age, $F_{1,36}$=15.5, *P*=0.0004). Within BALB/cByJ mice, the Age x Sex interaction was marginally non-significant ($F_{1,36}$=3.5, *P*=0.07). This is because for the high dose, 30 mg/kg, adolescents displayed attenuated stimulation only in females (*P*=0.01), not in males (*P*=0.46) (Fig. 2.3). However, this difference was not large enough to produce a significant Age x Dose x

Sex interaction ($F_{2,36}$=1.5, P=0.23). Age differences were significantly sex dependant within FVB/NJ mice (Age x Sex interaction, $F_{1,36}$=10.4, P=0.003). Female FVB/NJ mice showed the typical attenuated response in adolescents compared to adults (P=0.0002), but no age difference was observed in males (Fig. 2.4). No significant age or sex differences in locomotor stimulation from cocaine were observed in DBA/2J mice (all P>0.05).

Magnitude of locomotor stimulation varied greatly between strains (main effect of strain, $F_{3,166}$=43.0, P<0.0001) (Fig. 2.2). C57BL/6J displayed the greatest increase in locomotor activity in response to cocaine administration (all pair-wise comparisons with C57BL/6J were P<0.01), followed by DBA/2J (DBA/2J > FVB/NJ and BALB/cByJ, both P<0.0001). FVB/NJ and BALB/cByJ stimulated the least and were not statistically different from each other (P>0.05). Heritability estimates were conducted separately for each age group because the Age x Strain interaction was significant (above). Strain accounted for 48-74% of the variation in locomotor stimulation in adults and 30-65% in adolescents (Table 2.1). Smaller and non-significant heritability estimates were observed following a saline injection.

Discussion

The purpose of this study was to determine the extent to which attenuated locomotor stimulation to cocaine in adolescent versus adult male C57BL/6J mice (Zombeck *et al.* 2009; Zombeck *et al.* 2010) could be generalized to other genotypes. The major finding is that the phenomenon extends to BALB/cByJ, but DBA/2J and FVB/NJ showed a qualitatively different result (Fig. 2.2). This is consistent with a previous study that found no age differences in stimulation from cocaine in DBA/2J (Camarini *et al.* 2008). To the best of our knowledge, this is the first comparison of locomotor stimulation between adolescents and adults for FVB/NJ, though this strain is related to the outbred CD-1 strain that has been tested (Adriani *et al.* 1998; Adriani & Laviola 2000; McCarthy *et al.* 2004; Niculescu *et al.* 2005).

The finding that age differences varied among strains is interesting and implies that there are tractable biological differences between strains that underlie the age differences in behavior. By tractable, we mean that it should be possible to identify common biological features consistently altered in the strains showing the age differences but not in the others, though the

features could have disparate biological explanations. For example, in certain strains, adolescents might experience lower concentrations of cocaine in their brains as compared to adults for reasons related to distribution of the cocaine in the whole animal (i.e., the pharmacokinetic hypothesis) (Zombeck *et al.* 2009). Alternatively, the differences could be related to developmental, biochemical or molecular changes in the brain associated with the transition in age (pharmacodynamic) (Zombeck *et al.* 2010). A third possibility is that the ontogeny for reduced sensitivity to cocaine could vary depending on genotype. For example, it is possible that had we compared slightly younger or older adolescents, we might have observed reduced sensitivity in FVB/NJ males and DBA/2J males and females. Previous studies have found age differences to change over the course of adolescence in rats (Badanich *et al.* 2008; Lanier & Isaacson 1977).

With respect to the pharmacokinetic hypothesis, the literature suggests that it may contribute to some of the differences shown in Figure 2.2, but probably is not a major factor. In C57BL/6J, an extensive analysis suggested that large differences in stimulation occur between adults and adolescents at equivalent doses of cocaine in the brain (Zombeck *et al.* 2009). With respect to strain differences among adult mice, slightly lower concentrations of cocaine were observed in BALB/cByJ mice compared to C57BL/6ByJ which is consistent with reduced stimulation in BALB/cByJ versus C57BL/6J (Fig. 2.2) (Wiener & Reith 1990). On the other hand, C57BL/6J displayed much greater stimulation than DBA/2J in our study, particularly at the 15 mg/kg dose (Fig. 2.2) even though cocaine concentrations in the brains of C57BL/6J and DBA/2J are reported to be similar (Ruth *et al.* 1988; Tolliver *et al.* 1994).

The reduced locomotor stimulation in adolescents as compared to adults in C57BL/6J, BALB/cByJ, and FVB/NJ females, most likely reflects reduced sensitivity to the drug rather than increased sensitivity or transition into stereotypy (i.e., repetitive behaviors that would compete with horizontal movement). First, higher doses are needed to produce stereotypy in mice (Atkins *et al.* 2001; Schlussman *et al.* 2003; Tilley & Gu 2008; Tolliver & Carney 1994a, b). Second, 30 mg/kg cocaine produced greater locomotor activity than the 15 mg/kg dose in all strains except FVB/NJ, suggesting that both age groups were on the ascending limb of the inverted U-shape dose response curve. Stereotypy contributes more to the descending limb of the curve (Shuster *et*

al. 1977; Tolliver & Carney 1994a). It is important to note that since FVB/NJ did not show a difference in locomotor response between the two doses of cocaine, it is not clear if mice are on the ascending or descending limb of the dose response curve. Further research is needed to make definitive conclusions about sensitivity to cocaine in adolescent and adult FVB/NJ mice.

Sex differences

It was surprising to observe age differences in female but not male FVB/NJ mice (Fig. 2.4). None of the other strains showed sex-dependant effects across both doses. BALB/cByJ demonstrated a trend for sex differences at 30 mg/kg, but not 15 mg/kg cocaine, suggesting subtle differences in sensitivity between the two sexes (Fig. 2.3). Previous studies report mixed results as to whether age differences in sensitivity to psychostimulants is sex dependant. Parylak et al. (2008) and Mathews et al. (2007) have both reported attenuated locomotor stimulation in females adolescents as compared to adult at doses that do not produce similar age differences in males for cocaine and amphetamine respectively. However, these findings have not been replicated in other studies of age differences to cocaine and amphetamine in male and female rodents (Adriani & Laviola 2000; Laviola *et al.* 1995; Mathews *et al.* 2009). The cause for inconsistencies among the findings is unclear, however differences in handling procedures may contribute. Maldonado et al. (2005a; 2005b) discovered that the direction of age differences (i.e., whether adolescents show more, less, or no differences compared to adults) in locomotor stimulation to cocaine varies depending on both sex and whether or not the rats were habituated to handling prior to the experiment.

The lack of an overall main effect for sex is consistent with other mouse studies that have observed no difference in locomotor activity between males and females following cocaine administration (Kikusui *et al.* 2005; Wahlsten *et al.* 2003). But see Morse et al. (1993) who found male C57BL/6J and DBA/2J mice to travel greater distances than females following cocaine. It is interesting that in rat studies, females are often found to stimulate more than males to cocaine (Craft & Stratmann 1996; Heyser *et al.* 1994; Laviola *et al.* 1995; Parylak *et al.* 2008; van Haaren & Meyer 1991). Variability in sex differences among rats and mice have been reported before (Jonasson 2005) and highlight the importance of accounting for species when considering sex differences.

Heritability

Many previous studies have established significant genetic influences on locomotor responses to cocaine in adult mice. These include selective breeding experiments (Marley *et al.* 1998) and comparisons across inbred strains (Reith & Selmeci 1992; Ruth *et al.* 1988; Wiener & Reith 1990). Consistent with previous reports, BALB/cByJ adults displayed lower levels of locomotor stimulation in comparison to adult C57BL/6J and DBA/2J related strains (Reith & Selmeci 1992; Ruth *et al.* 1988; Wiener & Reith 1990). However, unlike previous studies (Cook *et al.* 1998; Rocha *et al.* 1998; Tolliver & Carney 1994a, 1995), C57BL/6J displayed greater stimulation than DBA/2J (Fig. 2.2), but see Kalkafi et al. (2003) who also found greater stimulation in C57BL/6J than DBA/2J. Two major differences between our study and many previous studies could explain discrepancies. The first is that we tested our mice at night during their normal active period whereas a majority of previous studies tested animals during the light cycle. Another major difference is that we measured locomotor activity in the animal's home cage using continuous video tracking. To the best of our knowledge, all previous studies placed animals into a new cage or arena during measurement of locomotor activity. Placing animals into a new environment or one that is different from home induces a state of arousal and increases locomotor activity on its own. Moreover, the response is strongly strain dependent (Lad *et al.*; Orsini *et al.* 2004). Therefore, testing in the home cage may reduce noise in the data from reaction to novelty, and explain discrepancies in heritability estimates reported for adults in Table 2.1, as compared to previous studies.

The heritability estimates in Table 2.1 must be interpreted with caution. They are equal to R-square values from a 1-way ANOVA with strain as the factor. The common assumption when using a panel of inbred strains to estimate heritability is that all animals experienced the same environment (Crabbe *et al.* 1990; Hegmann & Possidente 1981). However, this assumption is blatantly false. Most studies, including this one, did not transfer embryos or cross foster pups. In most cases including the current study, all C57BL/6J mice were raised by C57BL/6J dams and reared around other C57BL/6J mice whereas all BALB/cByJ mice were raised by BALB/cByJ dams and reared around their own kind. If social environment matters for cocaine responses, then the heritability estimates in Table 2.1 and many others of the same kind

(Rhodes *et al.* 2007; Turner *et al.* 2005) will be inflated. Another note of caution is that heritability as measured from R-square using a panel of inbred strains does not represent a narrow-sense or a broad-sense estimate. The estimate is not narrow-sense because it includes genetic variance from epistasis, but it is not broad-sense either because it excludes genetic variance from dominance. Finally, the higher estimates of heritability in adults as compared to adolescents (Table 2.1), is a direct result of greater stimulation in adults. The proportion of variation attributed to strain is magnified when the range in phenotypes is increased and individual variance within strains is relatively uniform (see Fig. 2.2).

Summary

The main finding was that differential locomotor stimulation from cocaine between adolescents and adults is strongly dependent on genetic background. Certain strains including C57BL/6J and BALB/cByJ, showed the typical pattern of reduced stimulation in adolescents as compared to adults. However, others such as DBA/2J and male FVB/NJ, showed no differences between ages. Sex differences were only apparent as interactions with age for BALB/cByJ and FVB/NJ strains, suggesting a minor contribution of sex in explaining the age or strain differences. Disparate biological explanations could contribute to the strain differences including developmental differences in brain physiology, morphology, or pharmacokinetics. Future studies testing these specific hypotheses as well as examining more strains at multiple time points during adolescence are needed to develop a richer understanding of the phenomenon of age differences in cocaine-induced stimulation.

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Tables

Table 2.1. Heritability estimates of locomotor activity following saline or cocaine administration

	Saline	15 mg/kg	30 mg/kg
Adults	0.05	**0.48**	**0.74**
Adolescents	**0.30**	**0.30**	**0.65**

Note: Bold font represents R^2 values were significant at $P<0.05$ level.

Figures

Figure 2.1. Photograph of the custom-made home cages where animals were tested for cocaine-induced locomotor stimulation by continuous overhead video tracking. Note that FVB/NJ and BALB/cByJ were tested in cages with dark type bedding (Shepherd Paperchip®) whereas C57BL/6J and DBA/2J were tested with light colored bedding (Harlan Corncob) to facilitate video tracking (dark object on light background or light object on dark background). Red light was used to illuminate the cages during the dark cycle when locomotor activity testing occurred.

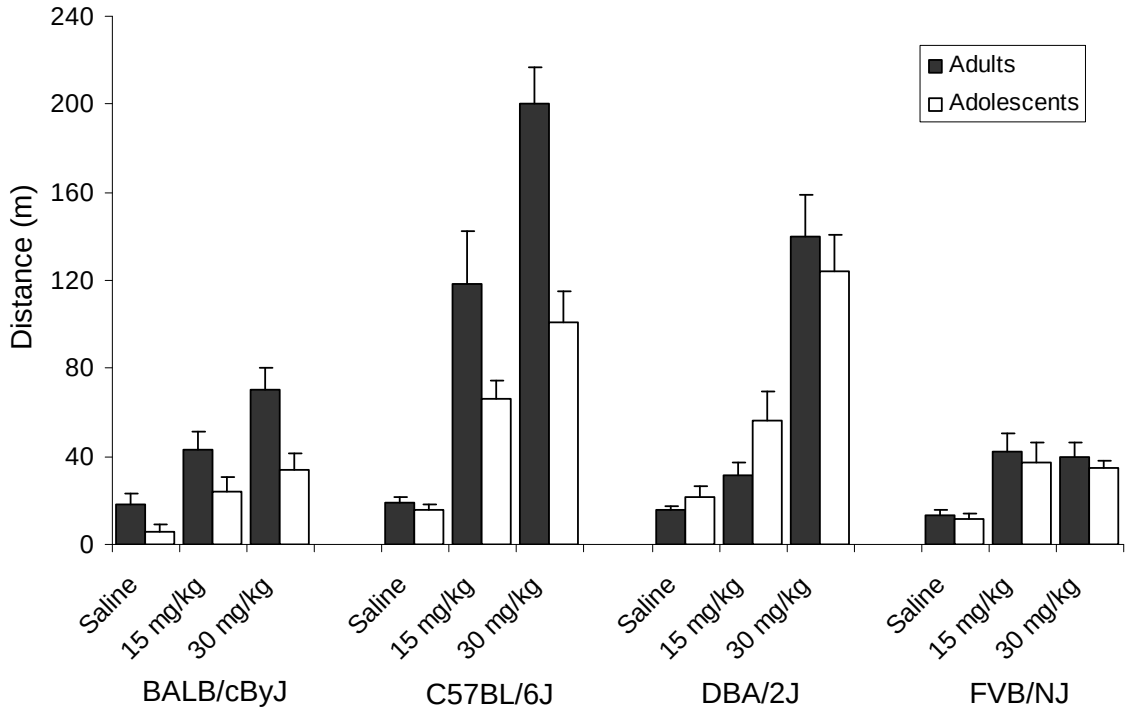

Figure 2.2. Average (± SE) distance traveled summed over 60 min following acute i.p. injection of saline or cocaine. Each bar represents the average of 8 individuals (collapsed across sex; 4 males, 4 females). Adults are shown as solid bars and adolescents open bars.

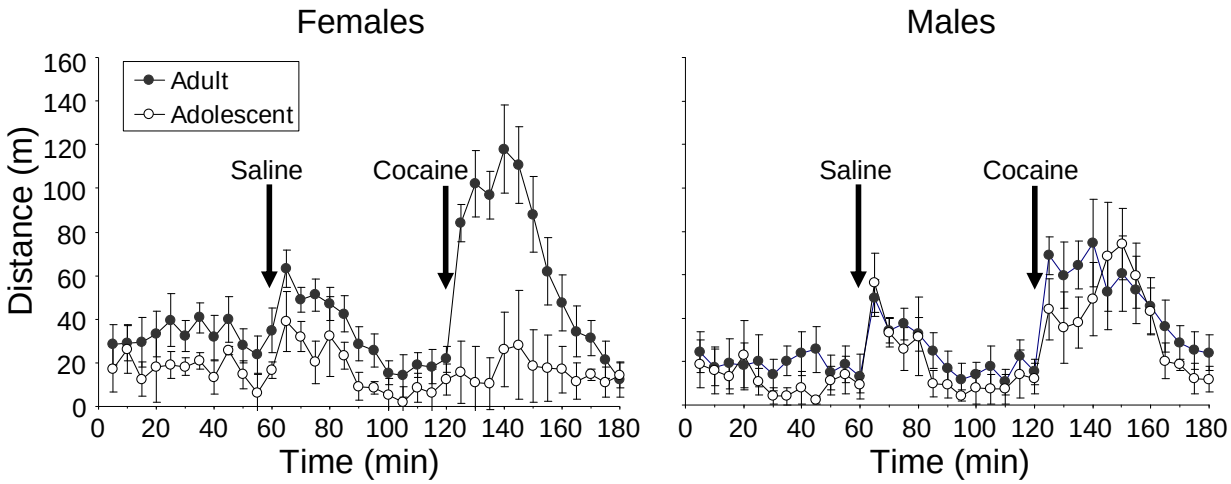

Figure 2.3. Comparison of female and male BALB/cByJ mice following 30 mg/kg cocaine. Average distance traveled in 5 min bins (± SE) is plotted against time for adults (filled symbols) and adolescents (open symbols). Animals were given a saline injection at 60 min, and 30 mg/kg cocaine injection at 120 min. Each data point represents the average of 4 individuals. Both graphs share the same y-axis.

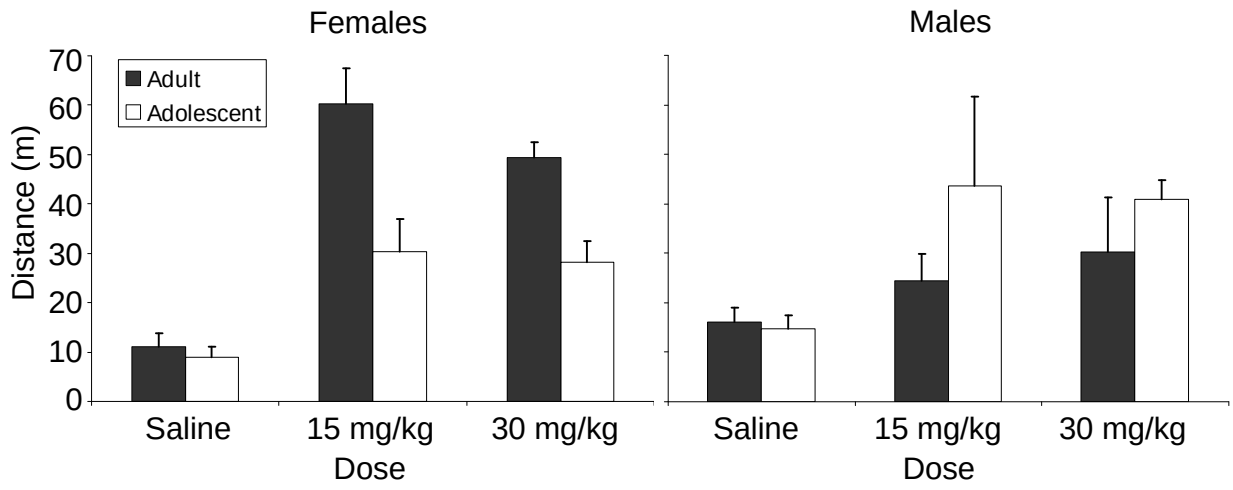

Figure 2.4. Comparison of female and male FBV/NJ mice following cocaine injection. Average (± SE) distance traveled summed over 60 min following acute i.p. injection of saline or cocaine plotted separately for adults (solid bars) and adolescents (open bars). Each bar represents the average of 4 individuals. Both graphs share the same y-axis.

CHAPTER III

Examination of a pharmacokinetic hypothesis for why adolescents stimulate less than adults to cocaine and methamphetamine

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Abstract

Adolescent mice display reduced locomotor stimulation to cocaine and amphetamine as compared to adults, but the mechanisms are not known. The primary aim of the current study is to test a possible pharmacokinetic explanation for the attenuated locomotor stimulation seen in adolescents. A secondary aim is to extend the current literature for acute methamphetamine in adolescents. Male, adolescent (PN 30-35) and adult (PN 69-74) C57BL/6J mice were administered an intraperitoneal injection of cocaine (5, 15, 30 mg/kg) or methamphetamine (1, 2, 4 mg/kg) and euthanized 5, 10, 15, 30, 60, 120, or 240 minutes later. Home cage locomotor activity was recorded by video tracking and drug concentration levels in brain and blood from the infraorbital sinus were measured using liquid chromatography combined with mass spectroscopy. Both methamphetamine and cocaine increased locomotor activity in a dose response fashion, but the magnitude of the increase was less in adolescents than adults. Concentration of methamphetamine in the brain was similar between ages across time points. Concentration of cocaine in the brain was significantly higher in adolescents than adults at 5 minutes, but similar at all other time points. Results suggest pharmacokinetics may make a small contribution to differential stimulation between adolescents and adult mice, but are unlikely the only factor. Developmental differences within the brain that effect pharmacodynamic properties of psychostimulants (e.g., number of receptor or transporters) represent alternatives.

Introduction

Human adolescents are prone to engage in risky behaviors such as taking drugs. This is disturbing since adolescents may be at a greater risk for addiction to drugs of abuse than adults (Estroff et al. 1989). Differential behavioral responses to psychostimulant drugs in adolescents versus adults have also been observed in rodents. For example, adolescent rodents display greatly reduced locomotor stimulation than adults after amphetamine or cocaine administration (Bolanos et al. 1998; Collins and Izenwasser 2002; Frantz et al. 2006; Lanier and Isaacson 1977; Laviola et al. 1999; Laviola et al. 1995; Spear and Brake 1983)but see (Parylak et al. 2008). Although neural adaptations associated with progressive increases in locomotor stimulation (i.e., sensitization) are widely regarded relevant for addiction, the contribution of individual differences to the first acute locomotor response is not known and has been debated (Kalivas et al. 1998; Robinson and Berridge 2001). In any case, the differential behavioral response between adult and adolescent rodents is robust and for that reason it provides a useful model to explore potential mechanisms for differential behavioral responses to drugs between age groups.

One possible explanation for differential locomotor stimulation in adolescent versus adult rodents is that the two ages experience different levels of drug in their brain. Even though animals are given the same dose per kg body weight, cocaine and methamphetamine pharmacokinetics might change with age. In fact, concentrations of amphetamine in adolescent rats have been found to be lower than adults up to 60 minutes after administration (Spear and Brake 1983). However, researchers concluded that attenuated stimulation in adolescents were not related to brain concentration levels based on the finding that younger rats had even lower concentrations in the brain, but no behavioral differences than adults.

Similar data are not available for methamphetamine. To the best of our knowledge, locomotor stimulation to methamphetamine between adolescents and adults has not been studied. Moreover, only one study has examined methamphetamine levels in the brains of adolescent versus adults and that was after four 10 mg/kg subcutaneous (s.c.) injections in Sprague–Dawley rats (Kokoshka et al. 2000). Using these data to generalize about possible pharmacokinetic contributions to differential locomotor stimulation is problematic, given that only 1 high dose was investigated, and that the route of administration was different than the intraperitoneal (i.p.)

route used in most other studies. Additionally, in Kokoshka et al. (2000), locomotor behavior of the rats was not examined making brain drug concentration and behavior correlations impossible.

Although more information is available about pharmacokinetics of cocaine in adolescents versus adult rodents, the data are still incomplete (see Table 3.1). First, no study has examined early time points (less than 10 min) when rapid changes are taking place. Second, the effect of different i.p. doses has not been examined within a single study. Third, only one study has looked at the time course of cocaine concentrations in the brain between age groups. In this study, dialysate levels of cocaine from the striatum after 20 mg/kg i.p. cocaine were compared between adolescents and adults and no differences found (Frantz et al. 2006). However, this conflicts with other studies showing lower cocaine levels in adolescents when whole brains are examined, but only for singular time points (Caster et al. 2005; McCarthy et al. 2004). Forth, only one study has been preformed in mice (McCarthy et al. 2004). Given that strain and species differences in metabolism can occur (Azar et al. 1998; McCarthy et al. 2004), caution must be taken before generalizing the above findings for mice. Finally, to the best of our knowledge, no study has measured adolescent behavior and brain drug concentrations within the same animal. Therefore within subject correlations have not been possible, only between subjects.

The aim of this study was to determine the extent to which pharmacokinetic differences between adolescents and adult C57BL/6J mice could account for differential acute locomotor stimulation to methamphetamine and cocaine between the age groups. Based on generalizations of past studies in mice and rats (see Table 3.1), we predicted adolescents would have lower drug concentrations relative to adults and that these lower concentrations would coincide with lower locomotor stimulation to cocaine and methamphetamine in adolescents versus adults.

Methods

Subjects

Male, C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were used. Adolescent mice were 21 days old at arrival and tested at 30-35 days of age. Adult mice were 60 days old at arrival and tested at 69-74 days of age. These are accepted time periods for adolescent and adult mice and rats (Spear and Brake 1983). Mice were housed in groups of 4 for 6 days after arrival and then housed singly for 5 days prior to the experiment. Single housing was necessary for

video tracking (see below). Mice were not handled prior to the test day except for routine cage changes. Food and water were available at all times. All mice were housed on a 12 hr reverse light dark cycle (lights off at 10AM and on at 10PM). All testing was done at the onset of the dark cycle when animals are typically active. Room temperature was maintained at 21 ± 1 ℃. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and adhered to NIH guidelines. The University of Illinois is AALAC approved.

Drugs

Both cocaine hydrochloride and methamphetamine hydrochloride were obtained from Sigma Aldrich (St. Louis, MO). Injection solutions were prepared according to the salt, not the base form. Drugs were dissolved in 0.9% saline and administered in a volume of 5 ml/kg. Doses and time points were chosen based on the literature (Azar et al. 1998; Benuck et al. 1987; Brien et al. 1978).

Equipment

Mice were housed in custom made home cages. Cages (18.5 cm x 33.5 cm x 16 cm) were constructed out of clear plastic with food and water access mounted on the side. Horizontal distance traveled in the home cage was recorded using TopScan (Clever Sys Inc, Vienna, VA) video tracking software. TopScan software was run on a Dell Precision 380 workstation (Dell Computer Corp., Round Rock, TX) which was connected to a Nuvico digital color quad interface (Nuvico, Englewood, NJ) and an Osprey-2000 (Viewcast Corp., Dallas, TX) or WinTV (Hauppauge Computer Works, Hauppauge, NY) capture card. Four Panasonic WV-CP244 cameras (Panasonic Corp., Secaucus, NJ) mounted 152 cm above the cages were used to capture the video used for analysis.

<u>Experiment 1 – time course</u>

Previous studies have not established the time course for cocaine or methamphetamine concentrations in brain or blood in adolescents as compared to adult mice. Therefore, one of the goals was to obtain these data for C57BL/6J. The purpose of these data was to identify time points when adolescent and adults display peak concentrations of drug in the brain, as well as time points when brain drug levels are similar between age groups.

Methamphetamine

Mice were given a 2 mg/kg i.p. injection of methamphetamine and sampled at 5, 30, 60, 120, and 240 minutes post injection (n = 5, 3, 7, 4, 3 adults and 5, 4, 8, 4, 3 adolescents per time point, respectively). In each case, samples were taken from the infraorbital sinus and brain. Infraorbital sinus blood samples were collected using 44.7 µl heparinized capillary tubes and placed in microcentrifuge tubes containing 50 µl $ZnSO_4$ (5% in H_2O) and 50 µl $Ba(OH)_2$ (0.3 N). Immediately after taking the blood sample, mice were decapitated and each hemisphere of the brain was collected and placed in microcentrifuge tubes containing 150 µl $ZnSO_4$ and 150 µl $Ba(OH)_2$. The $ZnSO_4$ and $Ba(OH)_2$ were added to precipitate out proteins and lyse cells. All tubes were kept on wet ice until processed.

Cocaine

Mice were given a 30 mg/kg i.p. injection of cocaine and sampled at 5, 15, 30, or 60 minutes post injection (n = 6, 5, 6, 5, adults and 6, 6, 6, 6 adolescents per time point, respectively). Brain and blood samples were collected as before except 45 µl sodium fluoride (1% in H_2O) was added to each tube. Sodium fluoride was added to reduce molecular degradation of cocaine (Caster et al. 2005).

Experiment 2 – locomotor activity

Separate groups of animals (n=8 adolescents and 8 adults) were used to measure the time course of locomotor stimulation to methamphetamine and cocaine. For this TopScan was used to continuously record the home cage activity of animals before and after a series of two i.p. injections of saline or drug. The test began when TopScan was turned on at the beginning of the dark cycle. After 30 minutes of recording animals undisturbed, animals were given an i.p. injection of saline (0.9%) and immediately returned to home cages. After 60 minutes, animals were given another injection, either 2 mg/kg methamphetamine or 30 mg/kg cocaine (n=4 adults and 4 adolescents per group).

Experiment 3 – dose response

The 10 minute time point for cocaine and 15 minute time point for methamphetamine were chosen for further analysis of drug concentrations in brain and blood for different doses because the pharmacokinetic data indicated concentration of drug in the brain would be close to

the peak at these times in both age groups. A total of 96 additional animals were given 5, 15, or 30 mg/kg cocaine or 1, 2, or 4 mg/kg methamphetamine (n = 8 / dose / age) and sampled after 10 or 15 minutes, respectively. In addition to collecting the drug concentration data, we also collected locomotor activity in the home cage up until the time of sampling.

Liquid Chromatography/Mass Spectrometry

Preparation of stock solutions and standards

Stock cocaine and methamphetamine solutions were prepared in MilliQ water (Millipore Milli-Q Biocel water purification system, Billerica, MA) to create 250 and 100 µg/mL solutions respectively. Stock solutions were used to prepare standard solutions of concentrations 0.01, 0.05, 0.1, 0.25, 0.5, 1, 2, 3, 5, and 7 µg/mL for cocaine and 0.1, 0.5, 1, 5, 10, and 15 µg/ml for methamphetamine. All solutions were kept at 4 ℃ and prepared fresh before each LC/MS run. Standard solutions were run in tandem with samples.

Sample preparation

Blood samples were centrifuged using an Eppendorf 5417R centrifuge (Hamburg, Germany) at 20,000 x *g* for 10 minutes and the supernatant transferred to a LC/MS vial (Agilent, Santa Clara, CA). Brain samples were first homogenized for approximately 10 seconds with a motorized pestle (Kontes, Vineland, NJ). 300 uL MilliQ water was added to brain cocaine samples before centrifuging in order to increase the volume of fluid for sampling. In order to aid in detecting low concentrations, water was not added to brains of mice that received methamphetamine. Brain samples were centrifuged at 20,000 x *g* for 15 minutes.

Instrumentation and Chromatographic Conditions

General LC/MS procedures followed Concheiro et al. (2006). An Agilent 1100 series LC system (Santa Clara, CA) was used for sample separation and introduction to mass spectrometry. Samples were placed in a cooled sample tray (4 ºC) and injected (5 µL) into the Agilent column ZORBAX Eclipse XDB-C8 (4.6 × 150 mm, 5-micro) (Santa Clara, CA, USA) for cocaine and a Phenomenex Onyx Monolithic C18 column (100 × 4.6 mm) (Torrance, CA, USA) for methamphetamine. The column was equilibrated with 95% solvent A (0.1% formic acid in H_2O) and 5% solvent B (ACN), and eluted at ambient temperature with a 300 µL/minute flow rate. The linear gradient for cocaine is as follows: 1 minute, 5% B; 8 minutes, 50% B; 13 minutes, 5%

B. The linear gradient for methamphetamine was: 1 minute, 5% B; 8 minutes, 100% B; 13 minutes, 5% B. A clean run of 100% ACN for 5 minutes, and then 5% ACN for 5 minutes was preformed after every 10 samples followed by a blank run to ensure optimum column performance.

Positive ion mass spectra were acquired using an Agilent MSD Trap XCT Plus mass spectrometer equipped with an ESI source (Santa Clara, CA, USA). For best sensitivity, positive ESI signals from standard cocaine and methamphetamine solutions were tuned with the use of a Kd Scientific 789100A model syringe pump (Holliston, MA, USA) connected directly to ion source via PEEK tubing. Nitrogen was used as nebulizer gas (30 psi) and drying gas (9 l/minute). The capillary voltage was set to 4.5 kV. The heated capillary of ESI source was kept at 350 ºC during the analysis. Software Chemstation for LC 3D system Rev B.01.03 (Agilent Technologies, Santa Clara, CA) was used for LC/MS system control and data acquisition.

Data Analysis

Statistical analysis was preformed with SAS 9.1 (SAS Institute Inc., Cary, NC). Adolescents and adults were compared for body mass and baseline locomotor activity using un-paired t-tests. The correlation between body mass and brain mass was analyzed using simple linear regression and polynomial regression. Distance traveled within 10 or 15 minute periods following injections and drug concentrations in the brain and the blood were analyzed using 2-way analysis of variance with age and dose or age and time as factors. Pair-wise differences were evaluated using Tukey or Scheffe post hoc tests. The relationship between locomotor stimulation and brain drug concentration was analyzed by analysis of covariance. In this model, locomotor activity was analyzed as the response, brain concentration as the continuous predictor (covariate), and age as the factor. For all tests, a p-value of $<.05$ was considered significant.

Results

Body mass

Adolescent body mass was 74% of adult body mass [16.4 ± 0.20 g SEM versus 22.3 ± 0.16, $t(198)= 23.1$, $P<0.0001$]. Adolescent brain mass was 95% of adult brain mass [404.4 mg ± 2.45 SEM versus 425.0 ± 2.31 mg, $t(205)=6.1$, $P<0.0001$]. Brain mass was significantly correlated with body mass within each age group [adolescents: $R^2=0.20$, $t(103)=1.1$, $P<0.0001$)

adults: R^2=0.06, t(100)=2.4, P=0.017] and among all individuals R^2=0.26, [t(205)=8.44, P<0.0001]. The second order coefficient of the polynomial regression was significant [t(204)=-1.99, P=0.048] suggesting a curve or plateau in brain mass after approximately 20 g of body mass (Fig. 3.1).

Methamphetamine

Experiment 1 – time course

Methamphetamine levels in the brain peaked slightly earlier and were slightly lower at 30 or 60 minutes post injection in adolescent relative to adult animals though these effects were not significant (Fig. 3.2a). The effect of time was highly significant [F(5,54)=4.23, P=0.003], but there was no main effect of age [F(1,54)=1.98, P=0.17] or interaction.

Experiment 2 – locomotor activity

Under baseline conditions in home cages, in the absence of any injections, animals moved negligible distances as compared to after injections of cocaine or methamphetamine (see Fig 3.3). After a saline injection, there was a small increase in activity that returned to baseline within approximately 20 min. Baseline differences before or after saline between adolescents and adults were small but nonetheless statistically significant (adolescents slightly lower than adults, all P<0.0001). Note that because of the difference in the magnitude for locomotor activity after the drug doses, these small baseline differences have negligible quantitative effects on drug-induced locomotor activity (i.e., locomotor activity after drug minus after saline).

The distance traveled within a 90 minute period after 2 mg/kg methamphetamine injection was significantly less in adolescents compared to adults [F(2,18)=116.72, P<0.0001] (Fig. 3.2d). The result was the same after subtracting distance traveled after saline within subjects.

Experiment 3 – dose response

Concentrations of methamphetamine in both brain and blood samples at the 15 minute time point increased as a function of dose [brain, F(2,42)=13.43, P<0.0001; blood, F(2,36)=12.63, P<0.0001], however no age differences were observed at any dose [brain, F(1,43=0.07, P>0.05; blood F(1,36)=0.96, P>0.05] (Fig. 3.2b,c).

Locomotor activity at the 15 minute time point significantly increased with dose for both age groups [$F(3,38)=66.34$, $P<0.0001$] (Fig. 3.2e). The main effect of age was significant [$F(1,38)=9.34$, $P=0.004$] (Fig. 3.2e). Although the interaction between age and dose was not significant, Scheffe post hoc analysis indicated that adults differed from adolescents at 2 mg/kg ($P=0.002$), and there was a trend for 1 mg/kg ($P=0.09$) but not at 4 mg/kg ($P=0.29$), where the greatest amount of stimulation occurred.

Locomotor activity was significantly correlated with concentration of methamphetamine in the brain at the 15 minute time point across all individuals and all doses [$R^2=0.25$, $P= 0.005$]. Results of linear regression show a trend for lower activity for a given concentration of methamphetamine in the brain in adolescents as compared to adults [$F(1,44)=2.64$, $P=0.11$] (Fig. 3.2f). Results were similar when locomotor activity was adjusted by subtracting distance traveled after saline within subjects.

Cocaine

Experiment 1 – time course

Concentration of cocaine in the brain reached a higher peak and the peak occurred earlier in adolescents as compared to adults after 30 mg/kg i.p. injection (Fig. 3.4a). Because of the potential importance for the result at 5 minutes, that time-point was repeated on three separate occasions. On each occasion, mean concentration was higher in adolescents than adults [overall t-statistic for 5 minute time point was $t(22)=3.08$, $P = 0.005$]. In the analysis with all other time points, where adolescents and adults did not differ (Fig. 3.4a), the interaction between age and time was non-significant [$F(4,66)=2.02$, $P=0.10$] but the Tukey or Scheffe post hoc difference between adolescent and adult at the 5 minute time point was significant [$P<0.05$]. The main effect of time was significant [$F(4,66)=14.20$, $P<0.0001$] and age non-significant [$F(1,66)=1.48$, $P=0.07$].

Experiment 2 – locomotor activity

The distance traveled within a 90 minute period after 30 mg/kg cocaine injection was significantly less in adolescents compared to adults [$F(1,18)=12.67$, $P=0.002$] (Fig. 3.4b). The result was the same after subtracting distance traveled after saline within subjects.

Experiment 3 – dose response

Concentrations of cocaine in both brain and blood samples at the 10 minute time point increased as a function of dose [brain, $F(2,42)=39.32$, $P<0.0001$; blood, $F(2,42)=37.89$, $P<0.0001$], however no age differences were observed at any dose [brain, $F(1,42)=0.04$, $P>0.05$; blood $F(1,42)=0.83$, $P>0.05$] (Fig. 3.4b,c).

Locomotor activity significantly increased with dose for both age groups [$F(3,42)=34.39$, $P<0.0001$] (Fig. 3.4e). Adults showed significantly greater locomotor stimulation from increasing doses than adolescents as evidenced by a significant main effect of age [$F(1,42)=10.55$, $P=0.002$] and interaction between age and dose [$F(3,42)=3.61$, $P=0.021$]. Scheffe post hoc analysis indicated that adults differed from adolescents at 15 mg/kg ($P=0.006$) and 30 mg/kg ($P=0.001$).

Locomotor activity in response to cocaine was significantly correlated with concentration of cocaine in the brain across individuals [$F(1,46)=36.98$, $P<0.0001$]. Results of analysis of covariance show that for a given concentration of cocaine in the brain, the level of activity was significantly lower in adolescents as compared to adults [$F(1,45)=4.57$, $P=0.038$] (Fig. 3.4f). As before, baseline corrections did not change results.

Blood-Brain Correlations

Concentration of cocaine in the brain was strongly correlated with infraorbital blood among individuals ($R^2 = 0.85$, $P<0.0001$; Fig. 3.5b). The correlation between blood and brain methamphetamine concentrations was significant but not as strong in cocaine samples ($R^2 = 0.22$, $P=0.004$) (Fig. 3.5a).

Discussion

These data extend previous reports of reduced locomotor stimulation to cocaine (Collins and Izenwasser 2002; Frantz et al. 2006; Laviola et al. 1995) or amphetamines (Bolanos et al. 1998; Lanier and Isaacson 1977) in adolescents as compared to adults. To the best of our knowledge, this is the first demonstration that the phenomenon is also true for methamphetamine in adolescent versus adult male C57BL/6J mice. Given that cocaine and methamphetamine share

similar mechanisms of action, the result is not surprising. Regarding the primary question of whether pharmacokinetic differences could contribute to the behavioral differences, for methamphetamine, results suggest the pharmacokinetic contribution is likely small. The behavioral differences between the age groups were large and statistically significant (Fig. 3.2d,e) whereas the differences in brain concentrations were small and not significant (Fig. 3.2a,b). Moreover, levels of activity were lower in adolescents than adults for a fixed concentration of methamphetamine in the brain (Fig. 3.2f). Taken together, these results argue against the pharmacokinetic hypothesis for differential locomotor stimulation to methamphetamine.

Results for cocaine were more complex. Results establish for the first time, that adolescents have a higher concentration of cocaine in their brain as compared to adults, 5 minutes after an i.p. injection. The observation was replicated three times to confirm the new finding (Fig. 3.4a). The reason cocaine accumulated in the brain of adolescents more rapidly than adults may be because brain weight is a larger percentage of body weight in adolescents than adults (Fig. 3.1). Since cocaine is lipophilic, the initial peak in adolescents may represent a rapid redistribution of a liophilic molecule in a highly perfused organ. Furthermore, this initial peak could indirectly contribute to differential locomotor stimulation. For example, it is possible that the higher peak and earlier rise in cocaine concentrations in the brains of adolescents as compared to adults resulted in greater acute functional tolerance to cocaine. Acute tolerance refers to rapid neuroadaptations that occur leading to greater behavioral response when drug concentrations are rising than when falling. It is a well documented phenomenon in alcohol exposure and has also been observed for the subjective effects of cocaine in humans (Foltin and Fischman 1991) as well as cardiovascular responses to cocaine in rats (Tella et al. 1999) and humans (Foltin and Fischman 1991). The higher peak in adolescents may act comparably to what would be a higher dose in adults. Since acute tolerance to alcohol has been shown to increase with dose in mice (Ponomarev and Crabbe 2004), the effect of the higher peak in adolescents may increase acute tolerance therefore decreasing locomotor stimulation.

An alternative explanation is that the higher peak concentration of cocaine produced stereotypic behavior in adolescents, resulting in reduced locomotor activity (Caster et al. 2005). Although stereotypy was not measured in the present study, indirect evidence suggests

adolescents were not engaged in stereotyped behaviors. First, differences in locomotor activity between age groups were seen shortly after drug administration and stereotyped behaviors typically occur after an initial locomotor activation phase (Rebec and Bashore 1984). Second, the dose response analysis showed that both adolescents and adults increased responding with dose (Fig. 3.2e, 3.4e). Given that the dose response curve for the locomotor stimulating effects of cocaine is known to follow an inverted U-shape with stereotypy contributing to the descending limb (Shuster et al. 1977; Tolliver and Carney 1994), if adolescents were engaged in stereotypy, an increase of dose would be expected to further decrease locomotion. Rather, the evidence suggests that adolescents are less sensitive than adults to the locomotor stimulating effects of cocaine and methamphetamine. This is consistent with the conclusions of previous studies (Bolanos et al. 1998; Collins and Izenwasser 2002; Frantz et al. 2006; Lanier and Isaacson 1977; Laviola et al. 1999; Laviola et al. 1995; Spear and Brake 1983). It also explains why adolescents and adults showed similar levels of locomotor activity after the high methamphetamine dose (4 mg/kg; Fig. 3.2e). We have other unpublished data on the time course for locomotor stimulation to 4 mg/kg in adolescents versus adults and believe that this dose of methamphetamine is probably near the intersection of the descending limb of the U-shape curve in adults with the ascending limb in adolescents. Probably had we used a higher dose of cocaine, we would have eventually reached the point of intersection where stimulation in adolescents is comparable to adults.

The finding that cocaine concentration in the brain is similar between adolescents and adults after 10 minutes is consistent with Frantz et al. (2006) and Caster et al. (2005) in rats. However our results do not replicate the finding of McCarthy et al. (2004) which suggested adolescent male C57BL/6J mice have lower cocaine concentrations in the brain than adults 15 minutes after cocaine administration (See Table 3.1). The explanation for this discrepancy is not clear.

The current study used custom made home cages conducive for video tracking to monitor locomotor activity in the animal's home environment. The finding that locomotor stimulation to cocaine was attenuated in adolescents as compared to adults under these conditions is consistent with previous studies where animals were transferred to a new cage for activity measurements (Bolanos et al. 1998; Collins and Izenwasser 2002; Frantz et al. 2006; Lanier and Isaacson 1977;

Laviola et al. 1999; Laviola et al. 1995; Spear and Brake 1983). Although this suggests the phenomenon of differential locomotor stimulation is robust across these two environments, it is important to note the methodological differences that could impact generalization of results. First, mice were singly housed for video tracking. Single housing has been shown activate the hypothalamic-pituitary-adrenal axis and affect behavior in a number of tasks (Schrijver et al. 2002; Schrijver and Wurbel 2001). On the other hand, the single housing in these studies lasted over 50 days whereas in this study animals were singly housed for only 5 days. Another methodological consideration is that mice were not handled, other than to change cages, prior to the testing day. Handling has been shown to increase locomotor stimulation to cocaine in adolescent, but not adult, rats (Maldonado and Kirstein 2005a; b). Therefore, it is possible that had we handled the mice, the adolescents might have displayed higher levels of locomotor stimulation, more comparable to adults. Finally, testing in the home cage as opposed to a novel environment might have affected the magnitude of the behavioral difference between the age groups. While locomotor stimulation to psychostimulants has been observed in both types of environments (Ganea et al. 2007), adolescent rats have been shown to ambulate more than adults when placed in a novel environment in the absence of drug administration (Spear and Brake 1983).

Blood and brain sample correlations

The finding that cocaine concentration in the brain is higher than in the blood is consistent with previous studies examining trunk blood and brain drug concentrations in adult mice (Benuck et al. 1987; Patrick et al. 1993; Reith et al. 1987) and rats (Nayak et al. 1976) but see McCarthy et al. (2004) who found higher concentrations of cocaine in blood than brain in C57BL/6J mice. Results suggest sampling blood from the infraorbital sinus after cocaine administration accurately reflects concentrations of cocaine in the brain. The weaker correlation between blood and brain samples for methamphetamine as compared to cocaine can be explained by a few key differences. First, the dose range was lower for methamphetamine than cocaine (1, 2, 4 mg/kg methamphetamine versus 5, 15, 30 mg/kg cocaine). Second, concentrations of methamphetamine in blood were lower than in brain and some of the blood (but not brain) samples reached the limit of detection for LC/MS/MS.

Limitations

It is important to note that the techniques used in this study do not differentiate pharmacologically active drug (e.g., drug bound to dopamine transporters) and the amount of drug that is inactive (e.g., drug that is not affecting cellular processes). It has been established that depot binding (e.g., drug binding to plasma protein, muscle, and fat), can affect the magnitude and duration of drug action (Fasano et al. 2005; Nayak et al. 1976). Given that white matter is increasing throughout adolescence (Giedd 2004), it is conceivable that there is a difference in depot binding within white matter between adolescents and adults. If so, then it is possible adults and adolescents could have the same absolute concentration of drug in the brain while having differential amounts of pharmacologically active drug.

Another limitation for whole brain sampling is that it does not differentiate drug levels in areas of the brain that are more directly involved in locomotor stimulation from other regions. For example, dopaminergic projections from the ventral tegmental area to the striatum have been shown to be important in the motor activation effects of cocaine and methamphetamine (Rebec 2006). If there are any differences in distribution of cocaine or methamphetamine within the brain between adolescents and adults, then that could also explain behavioral differences.

Caution should be taken before generalizing results to other strains or other species given that previous studies have noted differences in pharamacokinetics between strains (Azar et al. 1998; McCarthy et al. 2004) and between rats and humans (Cho et al. 2001). While dose, strain, and type of administration all affect pharmacokinetics, our estimates of cocaine and methamphetamine concentrations in the brain and blood are within the range observed in other studies for methamphetamine (Brien et al. 1978; Fornai et al. 1999; Hendrickson et al. 2004; Won et al. 2001) and cocaine (Azar et al. 1998; Benuck et al. 1987; Miller et al. 1996; Pan and Hedaya 1998; Reith et al. 1987). Additionally, the primary routes of metabolism in mice (Boyer and Petersen 1992; Shuster et al. 1983) are similar to those in rats (Estevez et al. 1977) and humans (Bencharit et al. 2003; Brzezinski et al. 1997).

Summary

These results add to the growing rodent literature on adolescents documenting differential behavioral responses to drugs of abuse as compared to adults (Badanich et al. 2006; Bolanos et al. 1998; Collins and Izenwasser 2002; Laviola et al. 1995). We show, for the first time, that

adolescents display reduced locomotor stimulation to methamphetamine, similar to cocaine and amphetamine that share similar mechanisms of action. Results do NOT support the pharmacokinetic hypothesis for differential locomotor stimulation to methamphetamine because locomotor stimulation was significantly reduced in adolescents versus adults even though concentrations of drug in the brain were similar at all time points. However, for cocaine, the story was more complex. Adolescents experienced higher concentrations of cocaine in their brain as compared to adults 5 minutes after an i.p injection. This is an important discovery because a higher concentration in adolescents as compared to adults has never previously been reported (see Table 3.1), but no other study, to our knowledge sampled that early. The possible role this might have on affecting behavior at later time points is not clear, but acute functional tolerance represents a possible mechanism for future exploration. Nonetheless, given that the pharmacokinetic difference was only for the early time point and that locomotor activity differed at many later time points, the possible pharmacokinetic contributions are likely small.

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Table 3.1. Summary of the literature comparing psychostimulant pharmacokinetics in adolescent vs. adult rodents

Author(s)	Drug and dose mg/kg	Strain	Age	Time point	Route admin.	Sampling technique	Results
Caster et al. (2005)	Cocaine 15 mg/kg given every hour for 3 h	Sprague–Dawley rats	PN 28, 42, 65	30 min after each injection	i.p.	Whole brain	No differences in brain cocaine levels, but blood plasma cocaine concentrations were higher in PN 65 than PN 28 and 42
McCarthy et al. (2004)	Cocaine 20 mg/kg	C57BL/6 mice	PN 35, 63	15 min	i.p.	Whole brain	PN 35 had lower brain and blood cocaine concentrations
	Cocaine 20 mg/kg	CD-1 mice	PN 35, 63	15 min.	i.p.	Whole brain	PN 35 had lower blood, but not brain cocaine concentrations
Frantz et al. (2006)	Cocaine 20 mg/kg	Wistar rats	PN 37–52 and PN 75–90	Every 10 min for 180 min	i.p.	Microdialysis from nucleus accumbens	No differences in brain dialysate levels of cocaine between age groups
	Cocaine 0.37, 0.74 and 2.92 mg/kg	Wistar rats	PN 37–52 and PN 75–90	Every 10 min for 60 min	i.v.	Microdialysis, from nucleus accumbens	Adults showed higher dialysate levels of cocaine at 10 and 20 min
Kokoshka et al. (2000)	Methamphetamine 4 administrations of 10 mg/kg in 2 hr intervals	Sprague-Dawley rats	PN 40, 90	1 hr. after last injection	s.c.	Striatal tissue	PN 90 had higher methamphetamine concentrations in striatal tissue and blood plasma
Spear and Brake (1983)	Amphetamine 5 mg/kg	Sprague–Dawley rats	PN 25, 35, 45, adult	15, 30, and 60 min.	i.p.	Whole brain	PN 25 and 35 had lower brain amphetamine levels than PN 45 at all time points

Figures

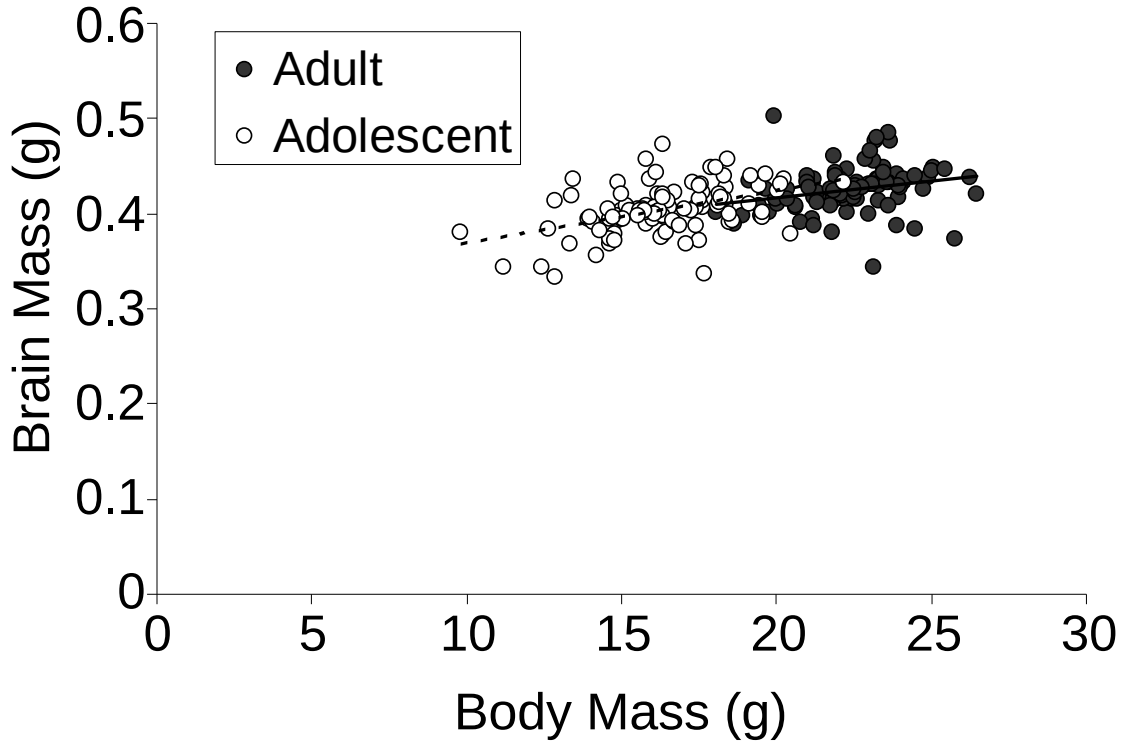

Figure 3.1. Brain - body mass allometry. Brain mass plotted against body mass for adolescent (open circles) and adult (filled circles) C57BL/6J male mice. Simple linear regression lines are shown separately for adults (solid line) and adolescents (dashed line).

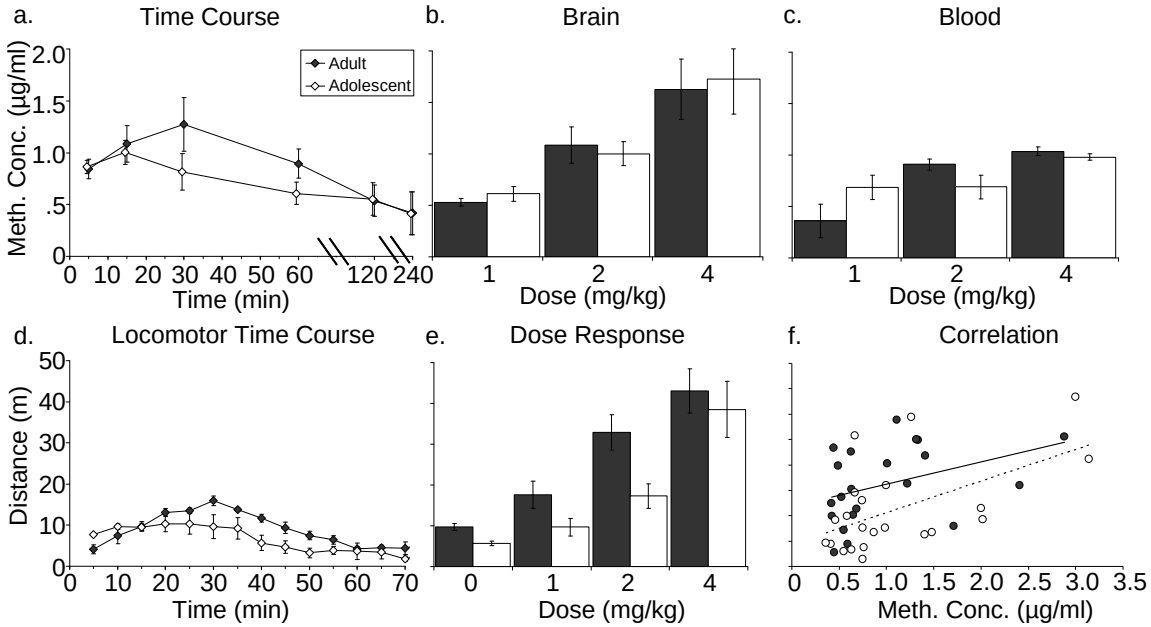

Figure 3.2. Methamphetamine data. (a) Time course for methamphetamine concentrations in the brain after i.p. injection of 2 mg/kg (adults closed symbols, adolescents open symbols; data from experiments 1 and 3 combined; n = 5, 8, 3, 7, 4, 3 adults and 5, 8, 4, 8, 4, 3 adolescents per time point, respectively). Methamphetamine concentrations in the brain (b) and blood (c) at the 15 min time point after i.p. injection of 1, 2, or 4 mg/kg (adults filled bars, adolescents open bars; n=8 per bar). (d) Time course for locomotor activity after i.p. injection of 2 mg/kg (n=4 per age group). Only the first 70 minutes are shown to facilitate comparison with the pharmacokinetic data above. (e) Distance traveled in 15 minutes after 0, 1, 2, or 4 mg/kg (n=8 per bar). (f) Distance traveled plotted against brain-methamphetamine concentration, separately for adults (open symbols) and adolescents (filled symbols). The simple linear regression lines are shown separately for adults (filled line) and adolescents (dashed line). All graphs in a row share the same y-axis labels. Standard error bars shown.

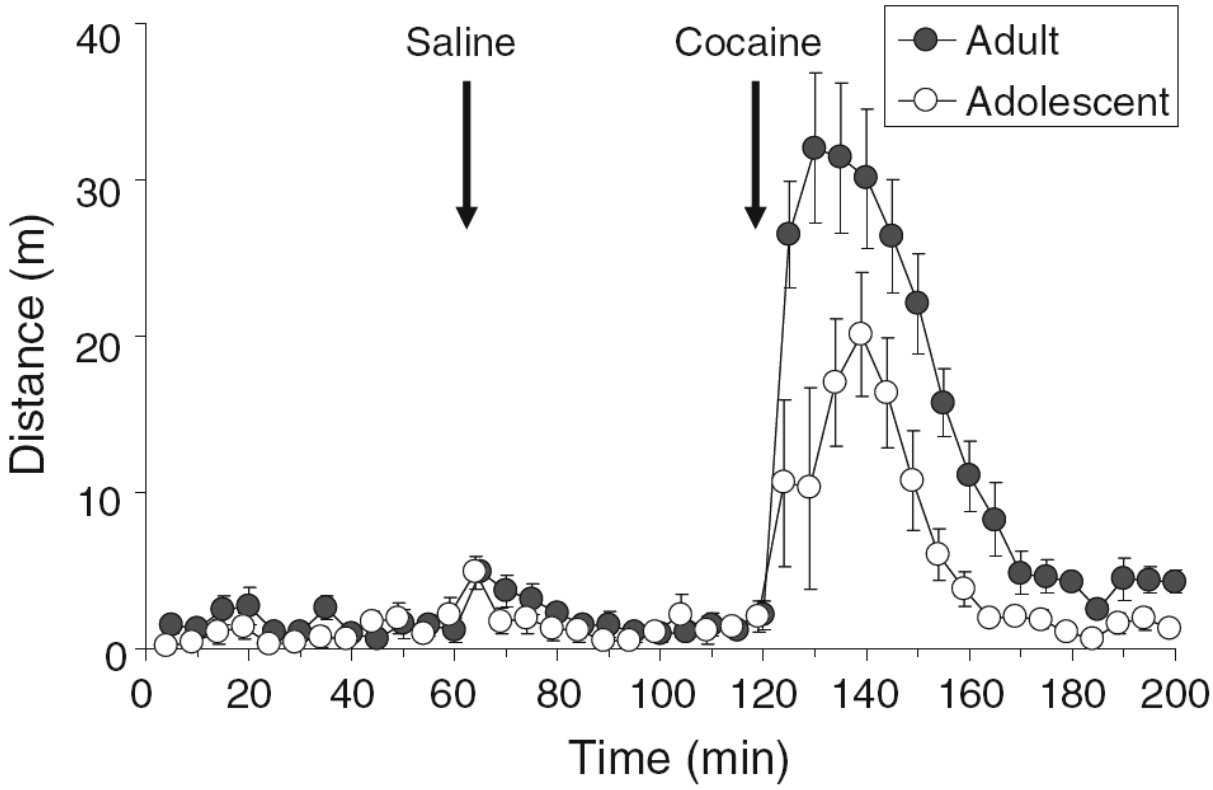

Figure 3.3. Locomotor activity before and after cocaine. Average distance traveled (meters, in 5 min bins; ± standard error; n=4 per age group) in the home cage of adolescent (open circles) or adult (filled circles) mice starting at the onset of the dark cycle (when lights shut off) and ending 200 minutes later. The first 60 minutes shows baseline activity when animals were left undisturbed. An i.p. injection of saline was administered at 60 min (1st arrow). At 120 min (2nd arrow) an i.p. injection of 30 mg/kg cocaine was administered. Other than removing animals for the injections they were left undisturbed and distance was measured continuously using video tracking software (see methods).

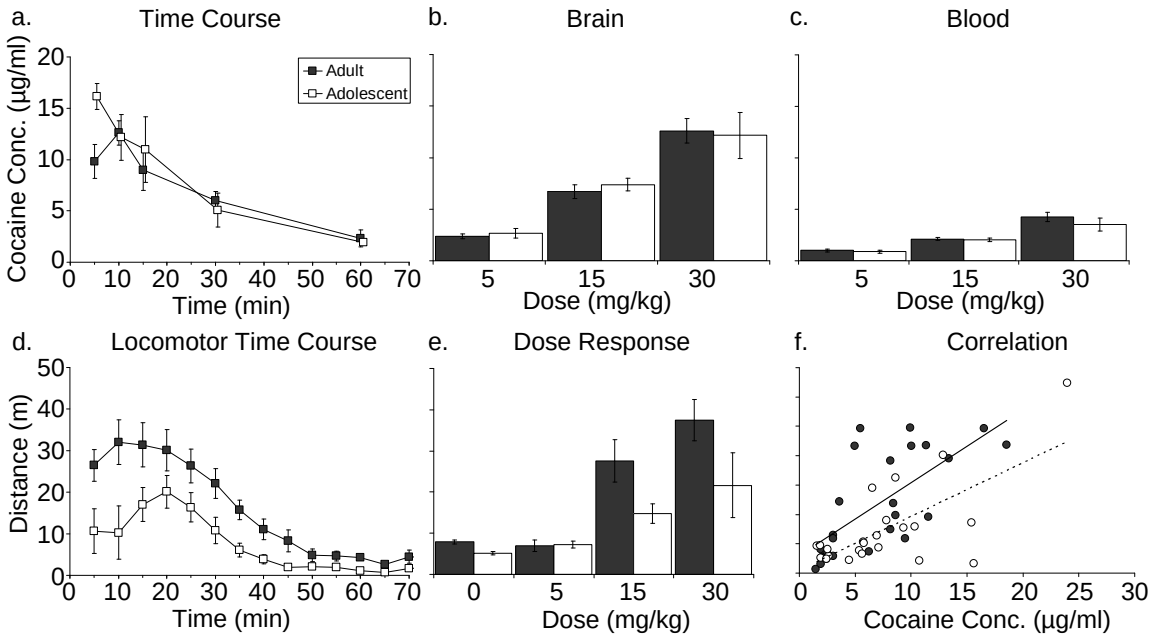

Figure 3.4. Cocaine data. (a) Time course for cocaine concentrations in the brain after i.p. injection of 30 mg/kg (adults closed symbols, adolescents open symbols; data from experiments 1 and 3 combined; n = 6, 8, 5, 6, 5, adults and 6, 8, 6, 6, 6 adolescents per time point, respectively). Cocaine concentrations in the brain (b) and blood (c) at the 10 min time point after i.p. injection of 5, 15, or 30 mg/kg (adults filled bars, adolescents open bars; n=8 per bar). (d) Time course for locomotor activity after i.p. injection of 30 mg/kg (n=4 per age group). Note this is the same data as shown in Figure 3.3, minutes 125-190. Only the first 70 minutes are shown here to facilitate comparison with the pharmacokinetic data above. (e) Distance traveled in 10 minutes after 0, 5, 15, or 30 mg/kg (n=8 per bar). (f) Distance traveled plotted against brain-cocaine concentration, separately for adults (open symbols) and adolescents (filled symbols). The simple linear regression lines are shown separately for adults (filled line) and adolescents (dashed line). All graphs in a row share the same y-axis labels. Standard error bars shown.

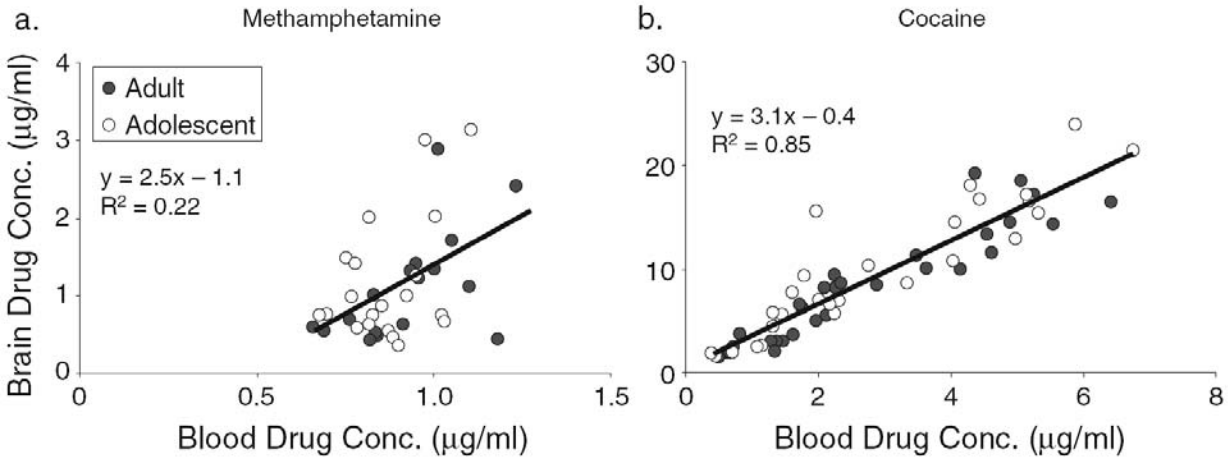

Figure 3.5. Correlation between brain and blood. Concentration of methamphetamine (a) and cocaine (b) in the brain plotted against infraorbital blood for adolescents (open circles) and adults (filled circles). Simple linear regression lines are shown for the combined adolescent and adult data.

CHAPTER IV

Patterns of neural activity associated with differential acute locomotor stimulation to cocaine and methamphetamine in adolescent versus adult male C57BL/6J mice

(Published in *Neuroscience*, 2010, 165:1087-1099)

Abstract

Adolescence is a time period when major changes occur in the brain with long-term consequences for behavior. One ramification is altered responses to drugs of abuse, but the specific brain mechanisms and implications for mental health are poorly understood. Here, we used a mouse model in which adolescents display dramatically reduced sensitivity to the acute locomotor stimulating effects of cocaine and methamphetamine. The goal was to identify key brain regions or circuits involved in the differential behavior. Male adolescent (PN 30-35) and young adult (PN 69-74) C57BL/6J mice were administered an intraperitoneal injection of cocaine (0, 15, 30 mg/kg) or methamphetamine (0, 2, 4 mg/kg) and euthanized 90 minutes later. Locomotor activity was monitored continuously in the home cage by video tracking. Immunohistochemical detection of Fos protein was used to quantify neuronal activation in 16 different brain regions. As expected, adolescents were less sensitive to the locomotor stimulating effects of cocaine and methamphetamine as indicated by a rightward shift in the dose response relationship. After a saline injection, adolescents showed similar levels of Fos as adults in all regions except the dorsal and lateral caudate where levels were lower in adolescents. Cocaine and methamphetamine dose dependently increased Fos in all brain regions sampled in both adolescents and adults, but Fos levels were similar in both age groups for a majority of regions and doses. Locomotor activity was correlated with Fos in several brain areas within adolescent and adult groups, and adolescents had a significantly greater induction of Fos for a given amount of locomotor activity in key brain regions including the caudate where they showed reduced Fos under baseline conditions. Future research will identify the molecular and cellular events that are responsible for the differential psychostimulant-induced patterns of brain activation and behavior observed in adolescent versus adult mice.

Introduction

The literature on acute locomotor stimulation from cocaine and amphetamines in animal models dates back more than 80 years (Tatum and Seevers, 1929). Key brain regions (e.g., caudate, nucleus accumbens), neural circuits (e.g., natural reward, basal ganglia, motor), and specific cellular and molecular events (e.g., dopamine neurotransmission, DARPP-32 signaling) have been identified that contribute to increased physical activity, arousal, reward and other behaviors induced by these drugs (Gold et al., 1989, Uhl et al., 2002, Rebec, 2006, Zachariou et al., 2006, Zombeck et al., 2008). However, it has proven much more difficult to identify the features that contribute to individual differences in sensitivity to locomotor responses or other behavioral effects (Volkow et al., 2002, Klein and Gulley, 2009). This is important because it has been argued that individual differences in sensitivity to initial drug experience are related to vulnerability for future drug abuse (Lambert et al., 2006).

Recently, an important gap in the literature is being filled that could make a significant contribution to the field. Several studies using rodent animal models have discovered that sensitivity to the acute locomotor response to psychostimulant drugs such as cocaine and amphetamines is strongly dependent on age. We previously reported that adolescent C57BL/6J mice (age range 30-35) are significantly less sensitive to the locomotor stimulating effects of cocaine and methamphetamine as compared to young adults (age range 60-67) (Zombeck et al., 2008). This general observation of reduced acute locomotor response has been observed in previous studies mostly using rats (Lanier and Isaacson, 1977, Laviola et al., 1995, Bolanos et al., 1998, Maldonado and Kirstein, 2005a, Frantz et al., 2007, Zakharova et al., 2009) but it is not always observed (Camarini et al., 2008, Parylak et al., 2008) and some studies show increased stimulation in adolescent rats as compared to adults (Catlow and Kirstein, 2005, Caster et al., 2007, Caster and Kuhn, 2009).

Many potential mechanisms could explain differential locomotor stimulation between ages. Developmental changes during adolescents include increased dopamine receptors in the caudate in adolescent rodents as compared to adults (Teicher et al., 1995, Tarazi et al., 1998, Tarazi et al., 1999), immature prefrontal cortex (Rosenberg and Lewis, 1995, Giedd et al., 1999), decreased white matter (Giedd, 2004), among others (for review see Spear, 2000). One method

to refine the search for a mechanistic explanation is to examine the patterns of Fos activation that occur throughout the brain after an injection of drug in each age group. The idea is that neuronal activation, as indicated by Fos induction, will reflect behavior and therefore identify the key brain regions and circuits involved in differential behavioral responses (Rhodes et al., 2003, Rhodes et al., 2005, Zombeck et al., 2008). By virtue of knowing the key brain regions, information about the distribution of cell types, receptor signaling systems, and principle afferent and efferent connections in the regions becomes available from the literature. This can help refine hypotheses about specific cellular or molecular mechanisms underlying the behavioral difference between the age groups.

Only a few studies have compared Fos or other related molecular responses to psychostimulants in adolescent as compared to adult rodents. Caster and Kuhn (2009) found higher levels of *c-fos* gene expression in the caudate of adolescent (age 28 days) as compared to adult (65 days) Sprague Dawley rats in response to 10 mg/kg cocaine, but the reverse for 40 mg/kg. In this study, adolescents displayed greater locomotor stimulation than adults for the 10 mg/kg dose and the reverse for the 40 mg/kg dose. Another study found elevated ΔFosB expression in the nucleus accumbens and caudate of adolescent versus adult male CD-1 mice following chronic administration of cocaine (20 mg/kg/day for 7 days) or amphetamine (5 mg/kg/day for 7 days) (Ehrlich et al., 2002). ΔFosB accumulates after repeated administration of psychostimulants and is thought to mediate longer lasting transcriptional regulation that is directly induced from the immediate early gene responses that occur during the initial drug administration (Nestler et al., 2001). This would suggest that cells in the striatum may display a greater initial genomic response than adults for a given dose of drug associated with decreased sensitivity to the locomotor activating effects. However, Ehrlich et al. (2002) did not measure locomotor activity or immediate early gene responses, so this hypothesis requires further investigation. Consistent with the idea, Anderson et al. (2001) found a greater percentage of Fos positive cells in the striatum of adolescent (age 35 days) versus young adult (age 60) Sprague Dawley rats following acute amphetamine (1 or 5 mg/kg). However, lower *c-fos* mRNA levels were observed in the ventral caudate of adolescent compared to adult Sprague Dawley rats following two intravenous doses of cocaine (Cao et al., 2007).

Taken together the evidence reviewed above points to the striatum as a location where cellular or molecular differences occur in adolescents as compared to adults that could mediate reduced sensitivity to locomotor activating effects of psychostimulants between the age groups. That is not surprising given that cocaine and amphetamines increase dopamine in extracellular spaces and the striatum is a major site of dopamine innervation (Wise, 2002). But there are a number of remaining questions. First the direction of the difference in immediate early gene induction, i.e., whether adolescents show greater or reduced Fos response to the drugs as compared to adults, is not consistent between the studies. Second, to the best of our knowledge, none of the previous studies examined Fos induction from methamphetamine between adolescents and adults. Given current methamphetamine use (Winslow et al., 2007), and the potential differences during adolescence, we thought it would be important to investigate. Third, to our knowledge, none of the previous studies analyzed the Fos responses using locomotor activity as a covariate in the statistical analysis. We have found, as others have in previous studies, that level of locomotor activity is strongly correlated with Fos levels throughout the brain (Rhodes et al., 2005, Caster and Kuhn, 2009). Therefore, one of the aims of this study was to determine whether the differential Fos induction from cocaine and methamphetamine in adolescents as compared to adults could be explained merely based on the level of physical activity displayed by the animals. Fourth, other areas besides the striatum receive dopamine innervation, and cocaine and methamphetamine affect signaling of other neurotransmitters including serotonin (Cunningham and Callahan, 1994, Muller et al., 2003) and norepinephrine throughout the brain (Uhl et al., 2002). Moreover, many other brain regions are involved in the locomotor activating effects of cocaine and amphetamines besides the striatum (e.g., ventral pallidum, motor cortex). The adolescent brain significantly differs from adults in these brain areas as well and that could contribute to differential locomotor activity. Therefore, we examined 16 different regions throughout the brain that we hypothesized might be involved in the differential locomotor activating effects of cocaine and methamphetamine in adolescents as compared to adults.

As compared to adults, we predicted adolescents would have reduced levels of Fos in response to cocaine and methamphetamine in most brain areas because we expected Fos would reflect the reduced locomotor activity (Zombeck et al., 2009). After locomotor activity was

removed as a covariate, we expected the differences in Fos between the age groups would no longer be apparent except in key brain regions involved in the differential behavior. We reasoned that reduced or enhanced signaling in response to the same stimulus in adolescents versus adults could modulate the motor circuit and contribute to reduced sensitivity to locomotor stimulation in adolescents.

Methods

Subjects

A total of 96 male C57BL/6J mice were used. The experiment was conducted in 4 separate batches consisting of 2 replicates for cocaine and 2 for methamphetamine. Each batch or replicate, consisted of 12 adults and 12 adolescents evenly distributed among the doses. After arrival from The Jackson Laboratory (Bar Harbor, ME), mice were housed in group of 4 for 6 days to habituate and then housed singly in custom-made acrylic home cages (18.5 cm x 33.5 cm x 16 cm) with clear plastic lids conducive for video tracking from above. Adolescent mice were 21 days old at arrival and tested at 30-35 days of age. Adult mice were 60 days old at arrival and tested at 69-74 days of age. All mice were housed on a 12:12 reverse light/dark cycle (lights off at 10 AM and on at 10 PM) with the room temperature maintained at 21 ± 1 ºC. Free access to food and water was available at all times. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and adhered to NIH guidelines.

Drug solutions

Cocaine hydrochloride or methamphetamine hydrochloride (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 0.9% saline and was administered at a dose of 0, 15, 30 mg/kg or 0, 2, 4, mg/kg respectively via intraperitoneal injections in a volume of 10 ml/kg. Dose was chosen based on the literature (Azar et al., 1998, Vorhees et al., 2005, Zombeck et al., 2009) and was prepared according to the salt not the base form.

Locomotor activity

Locomotor activity was continuously recorded using Topscan software (Clever Sys Inc, Reston, VA, USA). Mice were video-tracked in custom-made home cages where they were acclimated for 3-8 days prior to any injections (see Subjects section). Recording began at the

onset of the dark phase (i.e. active period). First, baseline locomotor activity was monitored for 1 hr. All mice then received a saline injection in order to measure the behavioral response to an injection. Activity was again measured for 1 hr after which an injection of cocaine (0, 15, 30 mg/kg) or methamphetamine (0, 2, 4 mg/kg) was administered. For each dose of each drug, 8 adolescent and 8 adult mice were sampled. Locomotor activity recorded for 1.5 hrs before animals were sacrificed by decapitation. Brains were quickly dissected and placed in 5% acrolein in phosphate buffered saline (PBS) solution overnight (Zombeck et al., 2008).

Immunohistochemistry

Following Zombeck et al. (2008), brains were transferred into 30% sucrose solution for 24 hours at 4 ºC and then transferred into a fresh 30% sucrose solution for storage until sectioning. Brains were then sectioned (40 μm thick) using a cryostat. Sections were placed into a 24 well plate containing tissue cryoprotectant, then stored at -20 ºC. Alternate sections were transferred into PBS, 24 hrs before beginning immunohistochemistry. Free-floating sections were pretreated with sodium borohydride (100 mg per 20 ml PBS) for 30 min, washed with PBS-X (PBS containing 0.2% v/v Triton X-100), and blocked with 6% v/v Normal Goat Serum (NGS) for 1 hr at room temperature. Sections were then incubated in rabbit antibody against c-Fos at a dilution of 1:20,000 (Calbiochem, San Diego, CA, USA) in PBS-X containing 2% NGS for 48 hrs at 5 ºC. After primary incubation, sections were washed in PBS-X followed by incubation in secondary biotinylated antibody against rabbit immunoglobulin made in goat (Vector Labs, Burlingame, CA, USA) at a dilution of 1:500 in PBS-X with 2% NGS for 90 min at room temperature. The peroxidase method (ABC system, Vector Labs, Burlingam, CA, USA; 37 ul A, 37 ul B in 15 ml PBS-X) and diaminobenzidine (DAB) as chromogen enhanced with nickel chloride (Sigma, St. Louis, MO, USA) was used to visualize the antibody complex. The reaction was stopped by washing the sections in PBS. Sections were mounted onto subbed slides, allowed to air dry, and then were dehydrated and coverslipped using Permount (Sigma, St. Louis, MO, USA).

Image analysis

Following Zombeck et al. (2008), microscopic images of the sections were captured via a Zeiss Axiocam digital camera (Zeiss, Germany) interfaced to a personal computer. ImageJ

software (NIH, Bethesda, MD) was used to automatically count Fos-positive cells at 100X total magnification within a frame (1.0 X 0.63 mm) placed at the locations shown in Figure 4.1 redrawn from Paxinos and Franklin (2001). For brain regions that were smaller than the frame, as was the case for the piriform cortex and the dentate gyrus, the region was outlined by hand and particles were counted only within the outlined structures. The counting was done unilaterally, in three alternate sections for each brain region, to obtain an average cell count per brain region for analysis.

Statistical analysis

Statistical analysis was preformed using SAS version 9.1 (SAS Institute, Cary, NC, USA). Locomotor activity was analyzed two ways. First, total distance traveled was summed over each epoch; the 60 minutes before any injections were given, the 60 minutes after the saline injection, and the 90 minutes after the saline or drug injections. Baseline total distance traveled (before injections and after saline) was compared between adolescents and adults using an un-paired t-test. Distance following the drug injections was analyzed separately for each drug by two-way ANOVA with dose and age entered as the two factors. Second, locomotor activity was divided into 15 minute bins consisting of summed distance traveled over that period. Adolescents and adults were compared for baseline distance traveled over the 4 time increments (i.e., 1 hour period) following a saline injection using repeated measures analysis of variance with age and time (4 levels) as factors. Distance traveled after cocaine or methamphetamine administration was analyzed separately for each dose of drug over the 6 time increments (i.e., 90 min period) following drug injection using repeated measures analysis of variance with age and time (6 levels) as factors.

Number of Fos positive cells for each brain region was analyzed using analysis of variance with batch, age and dose as factors. Batch was included as a factor to eliminate differences in staining due to variance between immunohistochemisty runs (Zombeck et al., 2008). The Fos numbers were power transformed as needed to decrease skewness and kurtosis in the residual distribution. Least square means (adjusted for batch) and confidence intervals were back-transformed so that the means could be presented in the same units, number of Fos-positive cells, for all regions.

The relationship between Fos staining and locomotor activity was analyzed by analysis of covariance. This was done to determine whether Fos levels differ between adolescents and adults after accounting for the expected positive relationship between acute levels of physical activity and Fos observed in previous studies throughout the brain (Rhodes et al., 2005, Caster and Kuhn, 2009). In this model, Fos staining was analyzed as the response, locomotor activity summed over 90 minutes was the continuous predictor (covariate), and age was entered as a categorical factor. Separate tests were run for each dose. Otherwise, dose would strongly bias the correlation between physical activity and Fos because dose strongly influences both variables. Again, the Fos numbers were power transformed as needed to decrease skewness and kurtosis in the residual distribution. The difference between the least square means, adolescent minus adult, adjusted for locomotor activity, were back-transformed so that the difference could be presented in the same units, number of Fos-positive cells, for all regions.

Two different methods were used to correct for the multiple testing in this study. First we adjusted the cut off p-value so that the global false discovery rate for the entire study was less than or equal to 5% using Qvalue software (Storey, 2002, Rhodes et al., 2005). Second, we extracted the principle components (the linear combinations of Fos levels across all regions that explain 70% of the variation in the data), and analyzed those variables using the same strategy described above for analyzing Fos in individual regions.

Results

Locomotor activity

See Figure 4.2. Baseline locomotor activity summed over 60 minutes preceding injections and over the 60 minutes following the saline injection was not significantly different between adolescents and adults. A saline injection induced a small, brief increase in locomotor activity [time, $F(3, 282)=163.2$, $P<0.0001$; first 15 minutes following injection greater than following three time points, all $P<0.0001$], but no significant differences were observed between ages (i.e., the effect of age and age by time interaction were not significant).

Both cocaine and methamphetamine increased the total distance traveled over the 90 minute period following injections as indicated by a main effect of dose [cocaine, $F(2,42)=24.9$, $P<0.0001$; methamphetamine, $F(2,42)=133.6$, $P<0.0001$]. Adolescents displayed reduced locomotor activity as compared to adults as shown by a main effect of age [cocaine, $F(1,42)=10.0$, $P=0.003$; methamphetamine, $F(1,42)=23.9$, $P<0.0001$]. The interaction between age and dose was significant for methamphetamine [$F(2,42)=4.3$, $P=0.02$] but not cocaine. To examine this difference in more detail, distance traveled in 15 minute bins following the drug injections was analyzed separately for each dose of each drug. The magnitude of the difference in locomotor activity between adolescents versus adults was similar at all time points for both doses of cocaine and for the 2 mg/kg dose of methamphetamine (i.e., the adolescent and adult curves were parallel following injection of cocaine or methamphetamine in Fig. 4.2b, c and e, and the interaction between age and time was not significant). However, the time-course of locomotor stimulation after 4 mg/kg, revealed an interesting difference between the age groups. Initially, during the first 45 minutes, both adolescents and adults displayed similar levels of locomotor activity, but the effect wore off between 45 to 90 minutes in adolescents whereas in adults, the high level of locomotor activity was maintained up to 90 minutes following the ip injection of 4 mg/kg (see Fig. 4.2f) [the interaction between age and time was significant, $F(5,70)=8.4$, $P<0.0001$].

Fos positive cells

Analysis of all the p-values collected from the tests reported in Tables 4.1-4.4 indicated that the standard cut off p-value, 0.05, would result in a global false discovery rate of 6 %. Qvalue calculated that if the cut off was set at 0.04 then the false discovery rate was 5%. Therefore, we considered a p-value less than or equal to 0.04 as evidence for statistical significance for the individual tests. For the results of the principle component analysis, we used the standard 0.05 cut off because the data were reduced across the 16 brain regions to 3 principle components.

Baseline differences

After a saline injection, Fos levels were similar in adolescents and adults in all regions except the dorsal and lateral caudate, where adolescents displayed significantly reduced Fos as

compared to adults [dorsal caudate, $F(1,26)=4.6$, $P=0.04$; lateral caudate, $F(1,26)=5.9$, $P=0.02$]. Locomotor activity was significantly correlated with Fos in the globus pallidus, and similar trends were observed for the dentate gyrus ($P=0.08$), and nucleus accumbens core region ($P=0.06$).

Cocaine

Cocaine increased Fos as indicated by a significant main effect of dose (see Fig. 4.3, Table 4.1). Inspection of the least square means in Table 4.1 shows a strong dose response for most regions. Levels of Fos were similar in adolescents and adults in all regions except the lateral caudate and the somatosensory cortex where adolescents displayed slightly lower Fos across all treatments including the baseline saline injection.

When Fos was analyzed with locomotor activity (distance traveled 90 min after drug injection up to the point of euthanasia) as a covariate, 3 of the 16 brain regions sampled showed a significant correlation between numbers of Fos positive cells and level of locomotor activity after the 30 mg/kg dose: the cingulate cortex, dorsal caudate, and dentate gyrus (Table 4.2). After the 15 mg/kg dose only 1 region showed a significant correlation, the motor cortex. After correcting for differences in locomotor activity among subjects using analysis of covariance, the dorsal caudate and the bed nucleus of the stria terminalis showed significant differences between the age groups, with adolescents showing greater Fos in this region as compared to adults for a given level of locomotor activity after the 30 mg/kg dose (Fig. 4.4a, Table 4.2).

The first principle component accounted for 50% of the variation in the data and was strongly correlated (Pearson's $r > 0.50$) with number of Fos positive cells in 6 of the 16 brain regions (ventral caudate, dentate gyrus, globus pallidus, nucleus accumbens core and shell, and piriform cortex). Congruent with the results in Tables 4.1 and 4.2 for most of the high loading brain regions, the first principle component showed significant effects for dose [$F(2,28)=3.8$, $P=0.04$] and locomotor activity [$F(1,30)=4.2$, $P=0.05$] but not age. The second and third principle components loaded on different regions but none of them showed significant effects of age. Together the first 3 components accounted for 70% of the variation in the data.

Methamphetamine

As with cocaine, methamphetamine significantly increased Fos in all regions (Table 4.3). Inspection of the least square means in Table 4.3 shows that the induction of Fos is strongly dose dependent for a majority of regions (Table 4.3). Levels of Fos were similar in adolescents and adults in all regions except the visual cortex and ventral pallidum where adolescents displayed slightly higher Fos across all treatments including the baseline saline injection.

Locomotor activity was positively correlated with Fos in the dorsal caudate and the prefrontal cortex after the 2 mg/kg dose, and negatively correlated with Fos in the nucleus accumbens core region after the 4 mg/kg dose, but no other statistically significant locomotor correlations were detected. When locomotor activity was included as a covariate, the dorsal caudate showed a trend for increased Fos in adolescents as compared to adults for a given level of locomotor behavior in response to the 2 mg/kg dose (Table 4.4). This difference was greater and significant for the 4 mg/kg dose (Table 4.4). In a post hoc analysis for the 2 mg/kg dose, after removing one outlier (an adolescent animal that moved 123 meters yet displayed only 4 Fos cells, see Fig. 4.4b), the covariate, locomotor activity, remained significant [$F(1,10)=18.9$, $P=0.002$] , and age was statistically significant [$F(1,10)=10.7$, $P=0.009$] with adolescents showing greater Fos than adults for a given level of locomotor activity (Fig. 4.4b).

In addition to the dorsal caudate, 3 other brain regions showed elevated levels of Fos in adolescents as compared to adults for a given level of locomotor activity after the 4 mg/kg dose of methamphetamine: the lateral caudate, shell of the nucleus accumbens and cingulate cortex (Table 4.4). Of these, there was a significant interaction between age and locomotor activity in the lateral caudate, with adolescents showing a negative correlation between locomotor activity and number of Fos positive cells whereas adults showed a positive correlation.

The first principle component accounted for 58% of the variation in the data and was strongly correlated (Pearson's $r > 0.50$) with number of Fos positive cells in 8 of the 16 brain regions (cingulate cortex, motor cortex, nucleus accumbens core and shell, prefrontal cortex, somatosensory cortex, visual cortex, and ventral pallidum). Congruent with results in Tables 4.3 and 4, for most of the high loading brain regions, the first principle component showed

significant effect of dose [$F(2,24)=6.54$, $P=0.0054$] and locomotor activity [$F(1,26)=10.71$, $P=0.003$] but not age. The second and third principle components loaded on different regions but none of them showed significant effects of age. Together the first 3 components accounted for 78% of the data.

Discussion

Despite significantly reduced locomotor response to cocaine and methamphetamine in adolescents as compared to adults (see Fig. 4.2), the Fos response was largely similar between the two age groups in the brain regions sampled (Fig. 4.3, Tables 4.1 and 4.3). This was a surprise because in previous studies, we and others have found strong correlations between locomotor activity and Fos throughout the brain (Rhodes et al., 2005, Caster and Kuhn, 2009). Therefore, we predicted that the Fos response generally would be reduced in adolescents as compared to adults because their level of locomotor activity was reduced. In fact, the opposite was true. In a majority of regions adolescents tended to display greater Fos than adults for a given amount of locomotor activity (Tables 4.2 and 4.4). After correcting for locomotor activity using analysis of covariance, the striatum stood out among all other brain areas as consistently showing significantly greater numbers of Fos cells for a given level of locomotor activity in adolescents as compared to adults (Fig. 4.4). This was not a result of baseline differences in Fos between the two age groups, because after a saline injection, adolescents had significantly fewer rather than greater numbers of Fos cells in the dorsal and lateral caudate as compared to adults. The number of Fos positive cells in the dorsal caudate in response to 30 mg/kg cocaine, and in the dorsal caudate, lateral caudate, and nucleus accumbens shell in response to 4 mg/kg methamphetamine were all significantly greater in adolescents than adults for a given level of locomotor activity (Tables 4.2 and 4.4). Overall, these findings confirm previous studies that have identified the striatum as a location where molecular or developmental changes likely occur that mediate the reduced locomotor response to psychostimulants in adolescents.

Caster and Kuhn (2009) recently examined locomotor activity, *c-fos* and *zif268* gene expression in various subregions of the striatum and cortex of Sprague Dawley rats after acute administration of 10 or 40 mg/kg cocaine. Our results are consistent with Caster and Kuhn (2009) in identifying the striatum as a key brain region involved in the differential locomotor

response between age groups. However, the direction of the differences in locomotor behavior and induction of immediate early genes (i.e., whether adolescents displayed a greater or lesser response than adults) and the relationship between immediate early gene induction and locomotor activity were not consistent between the two studies. First, in Caster and Kuhn (2009), adolescent rats displayed greater locomotor activity than adults after 10 mg/kg and reduced locomotor activity after 40 mg/kg, whereas adolescent male C57BL/6J mice displayed reduced locomotor activity after 15, or 30 mg/kg cocaine and no significant locomotor stimulation in either age group from 5 mg/kg (Zombeck et al., 2009). Second, Caster and Kuhn (2009) found that striatal *c-fos* levels changed in parallel with locomotor activity between the age groups whereas we did not. In Caster and Kuhn (2009), *c-fos* levels were greater in adolescents than adults for the 10 mg/kg dose when adolescents were more physically active than adults, and greater in adults than adolescents for the 40 mg/kg dose when adults were more physically active than adolescents. Therefore, in Caster and Kuhn (2009), the differences in *c-fos* and *zif268* between adolescents and adults could have been a reflection of the relationship between *c-fos* and locomotor activity, whereas in our study, analysis of covariance suggested that Fos is significantly greater in adolescent mice as compared to adults in the striatum for a given level of locomotor behavior (Fig. 4.4).

Only a few other studies besides Caster and Kuhn (2009) examined immediate early gene induction from cocaine or amphetamines in adolescents versus adults, but to the best of our knowledge none of these other studies measured locomotor activity. Our results are generally consistent with Ehrlich et al. (2002) who found greater ΔFosB expression in the caudate and nucleus accumbens after repeated administration of cocaine or amphetamine in adolescent versus adult CD-1 mice. Greater ΔFosB expression in the striatum is expected if Fos responses are greater during initial exposure to the drugs, because Fos contributes to the build up of the more stable transcription factor, ΔFosB. Results are also consistent with Anderson et al. (2001) who found slightly greater numbers of Fos-positive cells in the striatum of adolescent as compared to adult Sprague Dawley rats in response to 1 or 5 mg/kg amphetamine. On the other hand Kosofsky et al. (1995) and Cao et al. (2007) found reduced *c-fos* mRNA in the striatum of adolescent as compared to adult Sprague Dawley rats after i.p. 40 mg/kg cocaine (Kosofsky et al., 1995), or after two 100 μl intravenous injections of 750 μg/kg cocaine spaced 1 min apart

(Cao et al., 2007). The explanation for the difference is not clear, but relatively large doses and varying routes of administration may have played a role. Additionally, knowing the level of locomotor behavior displayed by the animals before they were sampled in the different studies might help clarify some of the differences.

In our study, the results of the principle component analysis confirmed that the central (correlated) patterns of neural activation across the different brain regions significantly reflected locomotor activity but none of the first three principle components showed age differences. This result suggests that a large background pattern of neural activity during the test is unrelated to the age difference in locomotor behavior. Fos levels in the striatum, which differed significantly between the age groups in the individual tests, were also partially correlated with the principle components. That suggests only a subset of the signal represented by Fos in the striatum contributes to age differences in locomotor stimulation. One of the limitations of using the immunohistochemical detection of Fos to reflect neuronal activation, is that Fos labels many different types of neurons and even some glial cells that happen to be transcriptionally activated over a relatively large time window (e.g., 90 min) following drug administration (Nestler et al., 2001, Edling et al., 2007). In this analysis, cells were not labeled with other markers (i.e., double or triple labeled) because the goal was to first identify key brain regions or circuits implicated in the differential behavior.

Future studies will be needed to identify the phenotype of the cells in the striatum that display greater activation for a given level of locomotor stimulation in adolescents than adults. The majority of neurons in the striatum are GABAergic neurons that project to either the globus pallidus (internal or external segment) or substantia nigra. These projections are referred to as the direct, indirect, and striosomal pathways, each of which has implications for movement and can be individually identified with neuronal markers (e.g., dynorphin, enkephalin, substance P, etc.) (Graybiel, 1990).

Previous studies using adult rats tested in their home cage (i.e., as opposed to a novel environment), suggest that a majority of the Fos-positive cells in the striatum induced from acute cocaine or amphetamines contain dynorphin and express D1 as opposed to D2 receptors (Badiani

et al., 1999, Uslaner et al., 2001, Ferguson et al., 2003, Gross and Marshall, 2009). A subset of these D1-expressing GABA/dynorphin neurons project back to the substantia nigra pars compacta and inhibit the dopamine output neurons (negative feedback). These neurons occur in discrete regions of the striatum called the striosomes that can be histologically differentiated from the surrounding regions, known as the matrix (Bolam et al., 1988, Graybiel et al., 1990). Increased Fos in striosomes in adolescents as compared to adults could explain reduced locomotor activity because that would suggest the dopamine output neurons were receiving stronger negative feedback. Alternatively, increased Fos in D1-expressing GABA/dynorphin neurons in the matrix that project to the internal portion of the globus pallidus and substantia nigra pars reticulata would not be expected to result in decreased locomotor activity because activation of this pathway increases motor activity via the ventral thalamus and cortical motor output neurons. Therefore, our current working hypothesis is that adolescents are less sensitive to the locomotor stimulating effects of cocaine and methampethamine in part because of greater negative feedback from the striatum back to dopamine output neurons.

Another interesting discovery was the difference in time course for locomotor stimulation in adolescents and adults in response to the 4 mg/kg dose of methamphetamine (Fig. 4.2f). The results show that for a high dose of methamphetamine, the effect on locomotor activity is initially the same for adolescents as adults, but wanes much more quickly in adolescents. This pattern was not apparent for any other dose of cocaine or methamphetamine that we tested. All the other doses showed a similar time course of locomotor stimulation and return to baseline between ages even if the amplitudes of stimulation were different. To the best of our knowledge we are the first to report this interesting difference for 4 mg/kg methamphetamine. In previous studies, we conducted a careful analysis of the concentrations of methamphetamine in the brain after a 4 mg/kg dose and found no statistically significant differences between the ages (Zombeck et al., 2009). One possibility for the rapid return to baseline in adolescents as compared to adults is that there is a ceiling effect preventing a greater peak response in adults. An alternative explanation is that the molecular, developmental or neurological changes in the brain that differentiate adults from adolescents in locomotor responses to psychostimulants are highly dynamic, capable of changing within the time-course of acute administration of the drug.

This study extends the current literature on psychostimulant induced locomotor activity in adolescents. Consistent with previous reports, adolescents stimulated less than adults to acute methamphetamine treatment (Zakharova et al., 2009, Zombeck et al., 2009). The literature for cocaine is mixed. Some studies are consistent with ours and have found attenuated stimulation to acute cocaine in adolescents as compared to adults (Laviola et al., 1995, Maldonado and Kirstein, 2005a, Frantz et al., 2007, Zombeck et al., 2009), while others have found no difference (Camarini et al., 2008, Parylak et al., 2008) or increased stimulation (Catlow and Kirstein, 2005, Caster et al., 2007, Caster and Kuhn, 2009) in adolescents. The cause for the discrepancies in the findings is unclear, however variations in age ranges within adolescents (Snyder et al., 1998), dose (Caster and Kuhn, 2009), route of administration, strain (McCarthy et al., 2004), and handling (Maldonado and Kirstein, 2005a, Maldonado and Kirstein, 2005b), may contribute.

Although it is possible that reduced locomotor stimulation in adolescents is a result of increased stereotypy (i.e., suggesting adolescents are more sensitive to the drugs), that is not likely for a number of reasons. First, we observed the mice during the tests, and although we did not record our observations in any formal way, we did not see evidence for increased stereotypy in any of the drug groups relative to saline controls. Moreover, that would not be expected because typically a higher dose of cocaine and methamphetamine is used to induce stereotypy in mice (Tolliver and Carney, 1994a, Tolliver and Carney, 1994b, Atkins et al., 2001, Schlussman et al., 2003, Tilley and Gu, 2008). Another reason is that both adolescents and adults increased activity at the higher doses of the drug relative to the medium doses. This suggests that both age groups are on the ascending limb of the dose response curve. Stereotypy is thought to contribute more to the descending limb of the curve (Shuster et al., 1977, Tolliver and Carney, 1994a).

In summary, results show that adolescent male C57BL/6J mice display greater Fos response to cocaine and methamphetamine in the striatum as compared to adults for a given level of locomotor activity. It is possible that the greater Fos reflects a greater negative feedback or inhibitory signal in adolescents. Future studies examining the phenotype of c-fos activated cells in the striatum and other brain regions of adolescents as compared to adults are needed to test these ideas.

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Tables

Table 4.1. Mean number of Fos positive cells and associated statistics after saline, 15 or 30 mg/kg cocaine

Brain region	Age	Least squared means			Statistics	
		0	15	30	Dose	Age
Cortex						
CG	Adults	75.0 (29.6, 120.4)	122.4 (77.0, 467.9)	157.0 (111.5, 202.4)	***P*<0.01**	*P*=0.97
	Adolescents	66.8 (21.4, 112.3)	122.5 (73.9, 171.2)	162.6 (117.2, 208.0)		
M1	Adults	6.19 (2.9, 12.4)	10.1 (5.0, 19.6)	26.9 (14.2, 49.2)	***P*<0.01**	*P*=0.53
	Adolescents	5.2 (2.5, 10.6)	14.9 (7.2, 29.4)	38.3 (20.7, 68.6)		
PFC	Adults	107.0 (56.0, 158.0)	161.8 (110.8, 212.8)	209.4 (154.8, 264.1)	***P*<0.01**	*P*=0.22
	Adolescents	69.6 (18.5, 120.6)	136.9 (82.3, 191.5)	190.3 (135.7, 244.9)		
Pir	Adults	38.1 (17.4, 69.2)	104.8 (63.9, 158.5)	156.1 (99.1, 229.4)	***P*<0.01**	*P*=0.89
	Adolescents	53.4 (28.8, 87.7)	87.8 (51.4, 136.5)	137.7 (88.7, 200.1)		
SX	Adults	14.9 (5.9, 34.7)	80.0 (36.9, 164.4)	75.0 (32.5, 162.3)	***P*<0.01**	***P*=0.04**
	Adolescents	6.19 (2.3, 15.5)	35.8 (14.5, 81.9)	41.1 (17.9, 88.5)		
V1	Adults	24.2 (5.9, 65.2)	105.0 (45.3, 205.5)	134.4 (62.0, 251.8)	***P*<0.01**	*P*=0.77
	Adolescents	16.8 (3.3, 49.9)	127.3 (57.9, 240.7)	106.8 (46.2, 208.3)		
Basal ganglia						
CPuD	Adults	19.2 (−54.5, 92.9)	177.0 (103.3, 250.7)	304.3 (225.4, 383.3)	***P*<0.01**	*P*=0.49
	Adolescents	8.8 (−64.9, 82.5)	152.3 (73.4, 231.2)	275.3 (201.6, 349.0)		
CPuL	Adults	4.8 (1.6, 12.0)	19.4 (9.1, 37.5)	60.3 (33.2, 102.6)	***P*<0.01**	***P*=0.04**
	Adolescents	0.9 (0.2, 2.9)	17.9 (7.7, 36.6)	30.4 (15.3, 55.7)		
CPuV	Adults	20.8 (−55.9, 97.5)	176.7 (100.0, 253.4)	296.8 (220.2, 373.5)	***P*<0.01**	*P*=0.84
	Adolescents	17.3 (−59.4, 93.9)	166.4 (84.3, 248.5)	290.6 (213.9, 367.3)		
GP	Adults	1.1 (1.0, 1.2)	1.2 (1.1, 1.3)	1.4 (1.3, 1.5)	***P*<0.01**	*P*=0.60
	Adolescents	1.0 (0.8, 1.1)	1.3 (1.2, 1.4)	1.3 (1.2, 1.4)		
NACC	Adults	21.1 (10.6, 38.5)	81.3 (48.7, 129.5)	116.5 (72.4, 178.0)	***P*<0.01**	*P*=0.81
	Adolescents	20.5 (10.3, 37.6)	86.5 (49.8, 142.1)	125.8 (78.8, 193.1)		
NACS	Adults	55.1 (30.1, 94.7)	146.2 (89.2, 229.1)	194.7 (122.3, 297.9)	***P*<0.01**	*P*=0.74
	Adolescents	60.3 (33.2, 102.6)	157.3 (96.7, 245.0)	135.7 (66.8, 252.4)		
VP	Adults	32.7 (11.4, 54.1)	42.0 (22.1, 61.9)	52.0 (32.1, 71.9)	***P*=0.04**	*P*=0.95
	Adolescents	28.8 (8.8, 48.7)	34.1 (14.1, 54.0)	62.2 (42.3, 82.2)		
Septum						
BNST	Adults	72.2 (43.5, 112.1)	86.4 (53.5, 131.3)	93.4 (56.3, 144.8)	*P*=0.05	*P*=0.23
	Adolescents	61.5 (36.0, 97.4)	119.5 (77.7, 175.1)	147.1 (98.3, 210.9)		
LS	Adults	62.7 (31.8, 93.5)	75.0 (44.2, 105.9)	114.0 (83.1, 144.8)	***P*<0.01**	*P*=0.96
	Adolescents	29.2 (−1.7, 60.0)	98.4 (65.5, 131.4)	125.9 (92.9, 158.9)		
Hippocampus						
DG	Adults	4.7 (2.5, 7.8)	10.9 (7.1, 15.7)	9.69 (6.2, 14.2)	***P*<0.01**	*P*=0.57
	Adolescents	3.8 (1.9, 6.6)	8.6 (5.3, 12.8)	10.6 (6.8, 15.3)		

Confident intervals are shown in parentheses after the means. The interaction between age and dose was not significant for any brain region and therefore these *P* values are not shown. *n*=8 per group.

Table 4.2. The difference in cocaine-induced Fos between adolescents and adults after correcting for locomotor activity

Brain region	Dose	Locomotor activity	Adolescents-adults	Statistics	
				Locomotor activity	Age
Cortex					
CG	15	−	−38.8	P=0.70	P=0.69
	30	+	69.6	**P=0.01**	P=0.07
M1	15	+	−13.0	**P=0.04**	P=0.20
	30	+	21.0	P=0.45	P=0.67
PFC	15	+	−39.2	P=0.92	P=0.35
	30	+	48.0	P=0.05	P=0.56
Pir	15	+	−20.7	P=0.82	P=0.46
	30	+	−69.2	P=0.91	P=0.43
SX	15	+	−4.1	P=0.49	P=0.35
	30	+	−80.7	P=0.18	P=0.07
V1	15	+	30.8	P=0.64	P=0.87
	30	+	48.1	P=0.23	P=0.73
Basal ganglia					
CPuD	15	+	−12.8	P=0.76	P=0.82
	30	+	197.7	**P<0.01**	**P<0.01**
CPuL	15	+	−8.7	P=0.87	P=0.62
	30	+	−11.8	P=0.13	P=0.73
CPuV	15	+	0.8	P=0.97	P=0.77
	30	+	170.1	P=0.14	P=0.29
GP	15	+	11.3	P=0.53	P=0.32
	30	+	4.2	P=0.16	P=0.68
NACC	15	+	18.9	P=0.77	P=0.94
	30	+	32.0	P=0.68	P=0.60
NACS	15	+	35.5	P=0.69	P=0.59
	30	+	40.8	P=0.31	P=0.83
VP	15	+	9.9	P=0.36	P=0.96
	30	+	32.2	P=0.31	P=0.19
Septum					
BNST	15	+	31.0	P=0.48	P=0.54
	30	+	111.9	P=0.91	**P=0.04**
LS	15	−	2.6	P=0.23	P=0.86
	30	+	23.7	P=0.73	P=0.48
Hippocampus					
DG	15	+	1.1	P=0.31	P=0.85
	30	+	6.5	**P=0.02**	P=0.10

In the locomotor activity column, the + or − sign indicates whether the correlation between locomotor activity and Fos was positive or negative. The column adolescents-adults shows the difference in mean number of Fos cells at the average level of locomotor activity among both age groups. The interaction between age and locomotor activity was not significant for any brain region or dose and therefore these P values are not shown.

Table 4.3. Mean number of Fos positive cells and associated statistics after saline, 2, or 4 mg/kg methamphetamine

Brain region	Age	Least squared means			Statistics	
		0	2	4	Dose	Age
Cortex						
CG	Adults	15.9 (4.8, 42.1)	76.4 (32.6, 158.0)	118.8 (52.5, 239.8)	***P*<0.01**	*P*=0.87
	Adolescents	14.2 (4.1, 38.2)	63.0 (24.6, 138.8)	178.3 (87.8, 331.6)		
M1	Adults	6.1 (−14.8, 27.0)	28.8 (7.9, 49.8)	71.8 (50.9, 92.7)	***P*<0.01**	*P*=0.19
	Adolescents	19.5 (−12.9, 31.9)	48.2 (25.8, 70.6)	89.9 (69.0, 110.9)		
PFC	Adults	11.5 (−38.3, 61.4)	130.8 (87.9, 173.6)	175.7 (129.9, 221.6)	***P*<0.01**	*P*=0.91
	Adolescents	9.6 (−29.9, 68.1)	70.9 (25.1, 116.8)	220.7 (177.8, 263.5)		
Pir	Adults	40.3 (5.7, 74.9)	85.0 (48.0, 122.1)	97.0 (62.4, 131.6)	***P*<0.01**	*P*=0.17
	Adolescents	25.8 (−11.3, 62.9)	97.3 (62.7, 131.9)	161.2 (126.6, 195.8)		
SX	Adults	2.4 (0.1, 13.1)	19.0 (3.9, 55.8)	95.0 (41.5, 184.3)	***P*<0.01**	*P*=0.45
	Adolescents	17.3 (3.3, 52.0)	24.5 (6.4, 63.4)	66.3 (26.0, 137.7)		
V1	Adults	7.1 (−96.6, 110.9)	65.4 (−54.4, 185.2)	268.5 (164.8, 372.3)	***P*<0.01**	***P*<0.01**
	Adolescents	15.0 (−88.7, 118.8)	234.0 (123.0, 345.1)	478.3 (374.6, 582.1)		
Basal ganglia						
CPuD	Adults	8.3 (−28.1, 44.7)	85.1 (42.7, 121.5)	138.2 (99.2, 177.2)	***P*<0.01**	*P*=0.54
	Adolescents	5.2 (−31.2, 41.6)	61.4 (25.0, 97.8)	193.2 (156.8, 229.6)		
CPuL	Adults	3.5 (0.8, 9.9)	9.6 (3.4, 21.2)	18.3 (7.4, 37.2)	***P*<0.01**	*P*=0.99
	Adolescents	1.3 (0.2, 5.0)	9.3 (2.9, 21.7)	29.8 (14.7, 53.5)		
CPuV	Adults	26.8 (14.8, 45.5)	103.7 (66.4, 156.0)	175.2 (117.5, 254.6)	***P*<0.01**	*P*=0.30
	Adolescents	24.1 (13.2, 41.4)	125.8 (82.0, 186.6)	259.6 (179.7, 365.7)		
GP	Adults	4.5 (1.9, 9.4)	13.0 (6.5, 23.8)	45.9 (27.1, 74.1)	***P*<0.01**	*P*=0.83
	Adolescents	4.5 (1.7, 10.0)	11.2 (5.5, 20.8)	59.0 (35.7, 93.0)		
NACC	Adults	4.66 (1.5, 10.0)	20.1 (10.4, 33.7)	53.7 (37.3, 73.8)	***P*<0.01**	*P*=0.28
	Adolescents	2.4 (0.5, 6.4)	27.4 (16.3, 42.1)	86.0 (63.9, 112.2)		
NACS	Adults	22.1 (−10.8, 54.9)	75.3 (40.1, 110.4)	134.8 (102.0, 167.7)	***P*<0.01**	*P*=0.07
	Adolescents	34.2 (−1.0, 69.3)	81.0 (45.8, 116.1)	195.9 (163.0, 228.7)		
VP	Adults	5.4 (3.0, 9.0)	10.1 (5.7, 16.4)	38.1 (27.6, 51.1)	***P*<0.01**	***P*<0.01**
	Adolescents	12.8 (7.9, 19.7)	14.3 (8.6, 22.2)	50.8 (37.9, 66.6)		
Septum						
BNST	Adults	25.7 (1.1, 50.2)	93.0 (64.4, 121.6)	74.5 (50.0, 99.1)	***P*<0.01**	*P*=0.41
	Adolescents	38.6 (7.5, 69.7)	86.1 (59.8, 112.3)	96.5 (70.3, 122.8)		
LS	Adults	33.0 (−4.5, 70.5)	90.7 (53.2, 128.1)	127.0 (86.2, 167.8)	***P*<0.01**	*P*=0.55
	Adolescents	58.5 (13.1, 103.8)	69.9 (29.1, 110.7)	151.7 (116.7, 186.7)		
Hippocampus						
DG	Adults	12.2 (6.1, 22.6)	40.8 (23.8, 66.6)	31.2 (16.6, 54.6)	***P*<0.01**	*P*=0.52
	Adolescents	11.9 (5.9, 22.0)	20.0 (10.6, 35.0)	39.9 (23.1, 65.2)		

Confident intervals are shown in parentheses after the means. The interaction between age and dose was not significant for any brain region and therefore these *P* values are not shown. *n*=8 per group.

Table 4.4. The difference in methamphetamine-induced Fos between adolescents and adults after correcting for locomotor activity

Brain region	Dose	Locomotor activity	Adolescents-adults	Statistics	
				Locomotor activity	Age
Cortex					
CG	2	+	−22.2	P=0.86	P=0.75
	4	+	140.9	P=0.07	**P<0.01**
M1	2	+	12.2	P=0.32	P=0.37
	4	−	20.6	P=0.81	P=0.38
PFC	2	+	8.3	**P=0.01**	P=0.99
	4	+	96.8	P=0.61	P=0.71
Pir	2	+	41.4	P=0.70	P=0.28
	4	−	37.8	P=0.09	P=0.11
SX	2	+	−7.5	P=0.29	P=0.67
	4	+	−9.2	P=0.61	P=0.71
V1	2	+	173.2	P=0.08	P=0.11
	4	−	24.0	P=0.09	P=0.50
Basal ganglia					
CPuD	2	+	46.6	**P<0.01**	P=0.11
	4	+	100.6	P=0.60	**P=0.03**
CPuL	2	+	−3.2	P=0.94	P=0.90
	4	#	10.9	P=0.61	**P<0.01**
CPuV	2	+	14.5	P=0.95	P=0.64
	4	−	54.4	P=0.05	P=0.19
GP	2	+	−7.0	P=0.96	P=0.42
	4	−	−17.3	P=0.16	P=0.67
NACC	2	+	−0.4	P=0.36	P=0.96
	4	−	9.2	**P=0.03**	P=0.45
NACS	2	+	41.7	P=0.98	P=0.24
	4	+	77.7	P=0.16	**P<0.01**
VP	2	+	4.0	P=0.52	P=0.36
	4	+	12.0	P=0.66	P=0.17
Septum					
BNST	2	+	−7.6	P=0.79	P=0.75
	4	−	22.8	P=0.95	P=0.30
LS	2	+	12.0	P=0.13	P=0.59
	4	+	73.4	P=0.40	P=0.12
Hippocampus					
DG	2	+	−19.9	P=0.27	P=0.37
	4	−	12.4	P=0.64	P=0.39

In the locomotor activity column, the + or − sign indicates whether the correlation between locomotor activity and Fos was positive or negative. # a significant interaction between age and locomotor activity was observed for CPuL (P<0.01), adolescents showed a negative correlation between Fos and locomotor activity whereas adults showed a positive correlation. No other significant interactions between age and locomotor activity were observed. The column, adolescents-adults shows the difference in mean number of Fos cells at the average level of locomotor activity among both age groups.

Figures

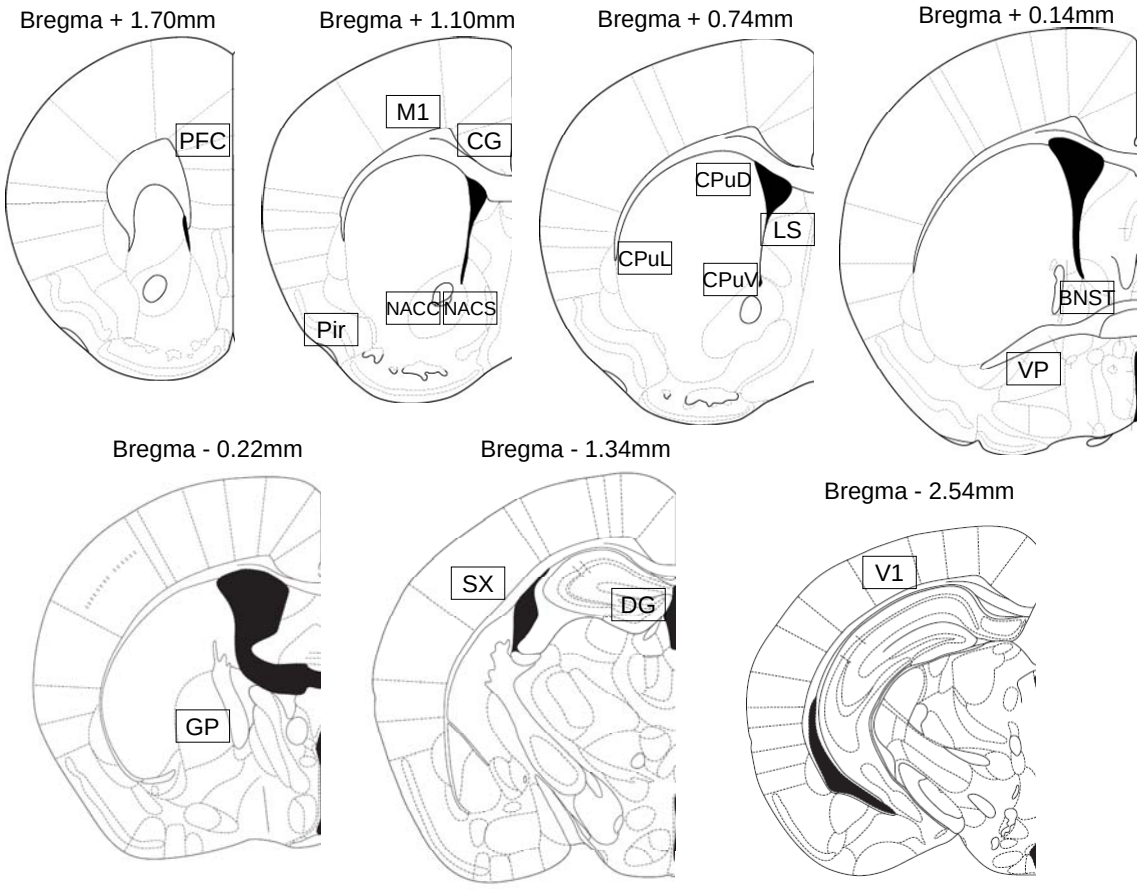

Figure 4.1. Locations where Fos positive cells were counted (boxes, shown roughly to scale, were 1 X 0.63 mm). Reprinted from The Mouse Brain in Stereotaxic coordinates, 2nd edition, Paxinos G and Franklin K, Figures 17, 22, 25, 30, 33, 42, 52, Copyright 2001, with permission from Elsevier. As noted, for the piriform cortex and the dentate gyrus, the nucleus was outlined by hand and particles were counted only within the outlined structures. Legend: PFC=prefrontal cortex, M1=motor cortex, Cg=cingulate cortex, NACC=nucleus accumbens core, NACS=nucleus accumbens shell, Pir=piriform cortex, CPuD=dorsal caudate, CPuL=lateral caudate, CPuV=ventral caudate, LS=lateral septum, BNST=bed nucleus of the stria terminalis, VP=ventral pallidum, GP=globus palidus, SX=somatosensory cortex, DG=dentate gyrus, V1=visual cortex.

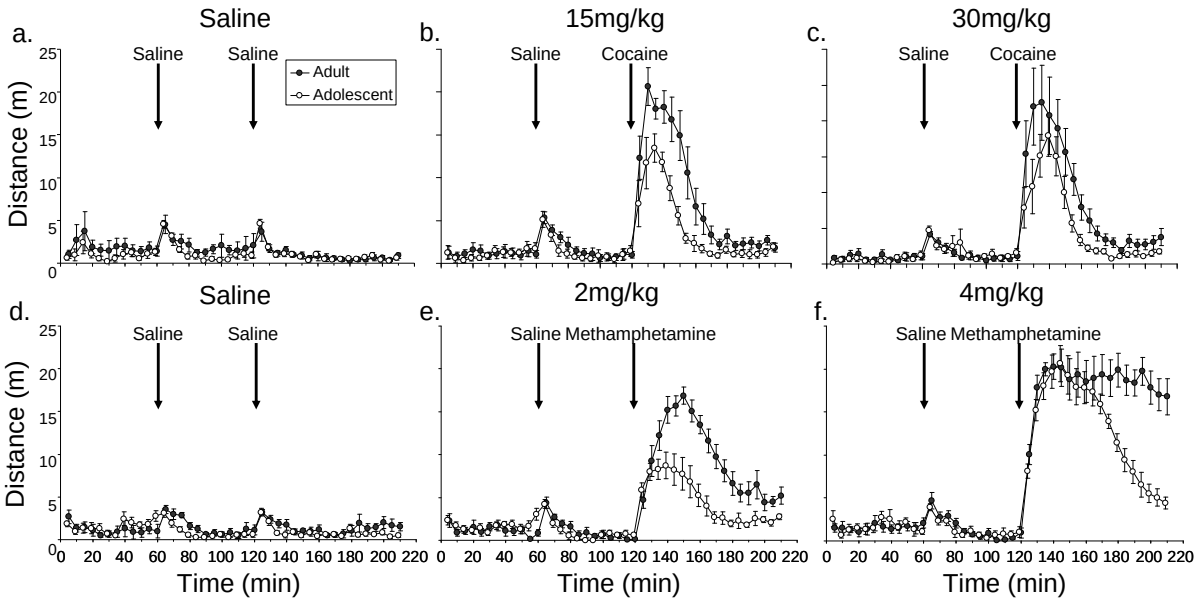

Figure 4.2. Reduced locomotor response to cocaine and methamphetamine in adolescent male C57BL/6J mice as compared to adults. Average distance traveled in 5 min bins (± SE) is plotted against time separately for adults (filled symbols) and adolescents (open symbols). Animals were given a saline injection at 60 min, and either saline or drug injection at 120 min. Data for the cocaine trials are shown on top and methamphetamine on the bottom. Each data point represents the average of 8 individuals. All graphs share the same x- and y-axis labels.

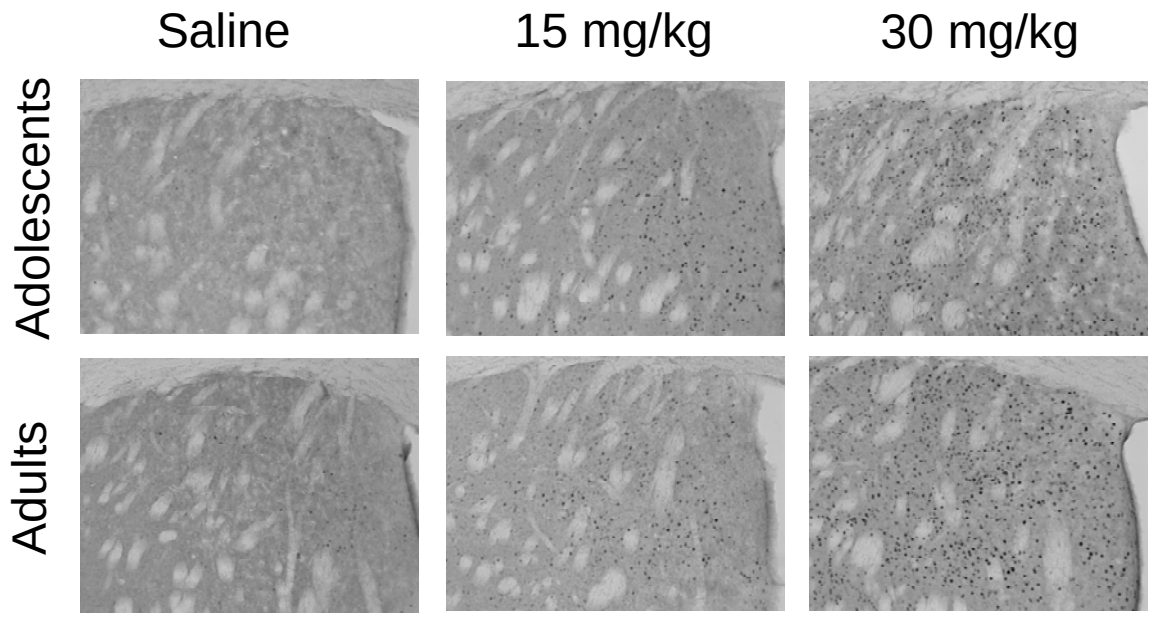

Figure 4.3. Acute cocaine increases Fos in a dose-dependent fashion in adolescents and adults. Representative sections stained for Fos showing the dorsal caudate of adolescents and adults 90 min after an intraperitoneal injection of saline, 15, or 30 mg/kg cocaine. The dots represent Fos-positive nuclei, total magnification was 100X.

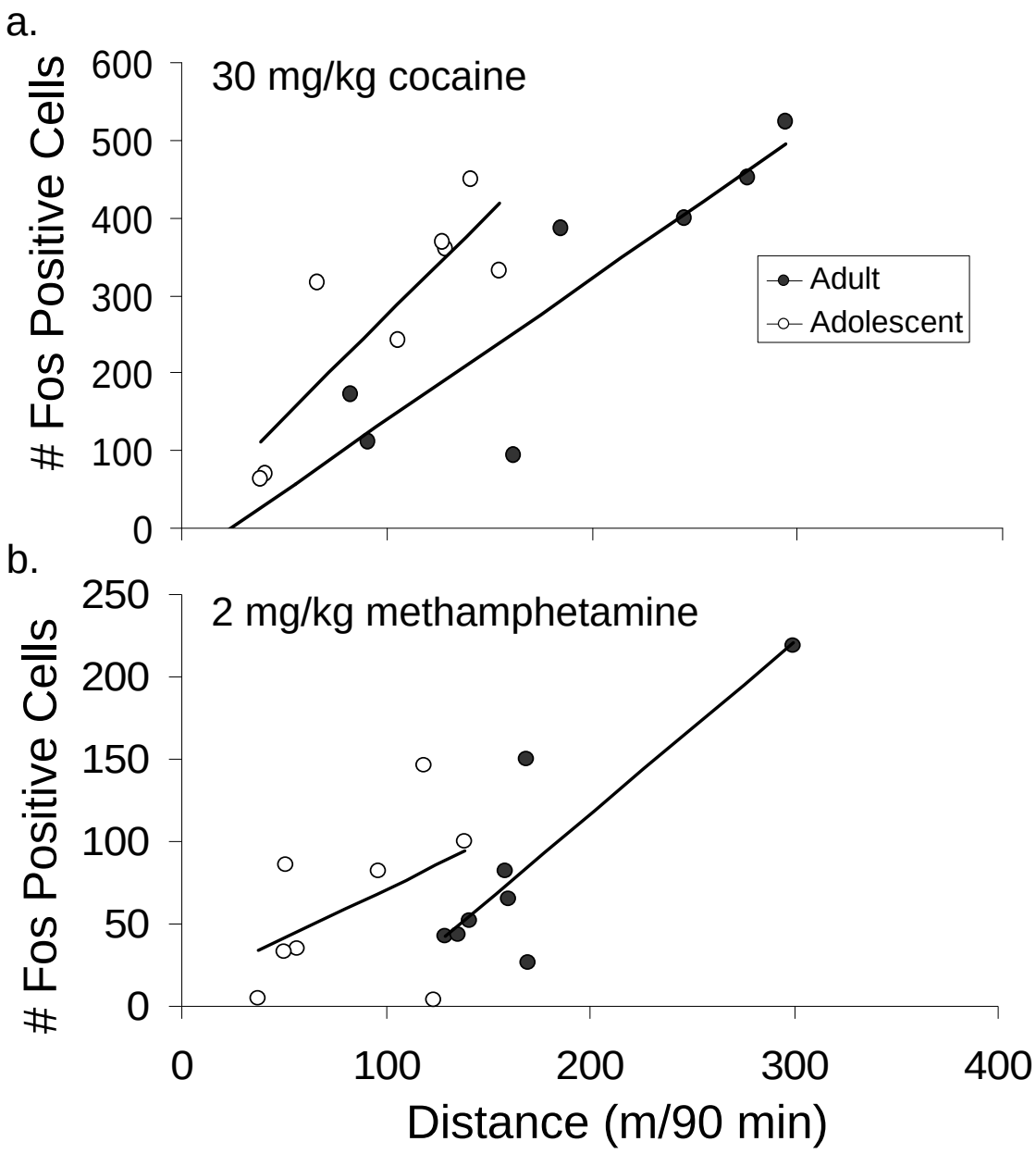

Figure 4.4. Increased Fos response from cocaine and methamphetamine in the dorsal caudate for a given level of locomotor activity in adolescents as compared to adults. Number of Fos positive cells in the dorsal caudate is plotted against distance traveled in the 90 minute period following an injection of either 30 mg/kg cocaine (top) or 2 mg/kg methamphetamine (bottom). Adolescents (open symbols) are shown separately from adults (filled symbols). The simple linear regression lines are shown separately for each age group. Both graphs share the same x-axis label.

CHAPTER V

Neuroanatomical distribution of psychostimulant-induced Fos in striosome versus matrix: a possible explanation for reduced locomotor stimulation in adolescents versus adults

Abstract

In the previous chapter, adolescent mice displayed elevated Fos immunoreactivity for a given level of locomotor activity in the dorsal caudate following cocaine administration as compared to adults. This presented the question of how relatively higher Fos in adolescents could be associated with lower locomotor activity. The current chapter tests the hypothesis that adolescent mice experience greater inhibitory feedback from the caudate to the substantia nigra by examining Fos immunoreactivity in the striosomal regions of the caudate. Home cage locomotor activity in adolescent and adult C57BL/6J mice was recorded for 90 min following 30 mg/kg cocaine or saline administration. Adjacent brain sections were immunostained for Fos and the striosomal marker, MOR1. Despite significant differences between age groups in locomotor stimulation, no differences were observed in the number of Fos positive cells within striosomal regions. Results suggest that striosomal signaling does not differ between age groups. Alternative signaling pathways in the striatum represent additional explanations for Fos differences between age groups. For example, increased cholinergic signaling from striatal interneurons in adolescents may act to oppose dopaminergic signaling and depress locomotor activity. Future studies are needed to test alternative hypothesis for mechanisms underlying attenuated stimulation in adolescents as compared to adults.

Introduction

In chapter 4, adolescents displayed increased Fos relative to adults after correcting for differences in locomotor activity in the dorsal caudate following the highest doses of cocaine and methamphetamine (Zombeck et al., 2010). Surprisingly, few other brain regions showed significant differences in Fos, despite a large difference in locomotor stimulation between age groups. We interpreted these findings as evidence that portions of the striatum display increased neuronal activation in adolescents compared to adults in response to psychostimulants. This claim is substantiated by other papers that have also found greater immediate early gene expression in the striatum of adolescents as compared to adults (Andersen et al., 2001, Ehrlich et al., 2002, Cao et al., 2007, Caster and Kuhn, 2009).

The striatum is a logical area to investigate given its known role in psychostimulant induced locomotor stimulation (Rebec, 2006). Cocaine and methamphetamine are known to increase dopamine in extracellular spaces in the striatum (Wise, 2002). Dopamine release in the striatum is thought to be a major contributor to psychostimulant induced increases in locomotor activity (Wickens, 1990). This idea stems from myriad studies examining the role of dopamine in motor activation and specifically how disruptions in dopamine signaling can prevent psychostimulant induced locomotor activation. For example, mice lacking the dopamine D1 receptor gene do not show locomotor stimulation to cocaine (Drago et al., 1998). Furthermore, in vivo electrophysiology studies show that administration of cocaine results in widespread activation of striatal neurons and antagonism of dopamine receptors by haloperidol blocks this effect (White et al., 1998). However, dopamine is not purely excitatory within the striatum and there are a number of mediating factors dictating the role of striatal dopamine in locomotor activation. For example, dopamine D1 receptors are commonly excitatory, while dopamine D2 receptors are commonly inhibitory. The role of dopamine in locomotor stimulation is further complicated by interactions with other neurotransmitter systems. The striatum receives glutamatergic inputs from the cortex and ablation of the cortex attenuated amphetamine induced excitation of motor-related neurons (Tschanz et al., 1994). Therefore it is thought that dopamine and glutamate are synergistic in the induction of locomotor response (Haracz et al., 1998). Together, the evidence suggests psychostimulant effects on dopamine transmission in the striatum are involved in the locomotor stimulatory effects of the drug.

The striatum is part of the basal ganglia which are a group of subcortical structures involved in motor control. The basal ganglia is complex and involves many pathways, however the classical model describes two main efferent pathways from the striatum (Graybiel, 1990, Graybiel, 2004). The first is called the direct pathway and activation is thought to increase locomotor activity. The second is called the indirect pathway and activation is thought to decrease locomotor activity. This basic theory of basal ganglia function is widely published, however until recently, in vivo evidence supporting the theory was lacking and electrophysiological studies incongruent with the theory cast doubt on the validity of the model (Gulley et al., 2004, Surmeier et al., 2005). Two groups have demonstrated support for the classic model of the basal ganglia by selectively activating the direct and indirect pathways using genetic engineering (Bateup et al., 2010, Kravitz et al., 2010). Both studies used the knowledge that dopamine D1 receptors are expressed primarily in striatal neurons comprising the direct pathway, while dopamine D2 receptors are expressed primarily in striatal neurons of the indirect pathway. Kravitz et al. (2010) used an optogenetics approach by virally introducing channelrhodopsin-2 and controlling its' expression via the regulatory elements of the D1 or D2 receptor gene. The result was the ability to selectively activate D1 or D2 containing neurons using light. Kravitz et al. (2010) discovered that when D1 containing neurons were activated (direct pathway) in vivo, mice exhibited reductions in freezing behavior and elevated locomotor activity. In contrast, when D2 containing neurons were activated (indirect pathway), mice exhibited increases in freezing behavior and reductions in locomotor activity. Bateup et al. (2010) employed a similar genetic engineering approach, but instead of optogenetics to control D1 and D2 neurons, they selectively disrupted DARPP-32. The result was impairment in striatonigral (direct pathway) or striatopallidal (indirect pathway) neurons. Mice lacking DARPP-32 in striatonigral neurons exhibited lower basal and cocaine induced locomotor activity than controls. Conversely, mice lacking DARPP-32 in striatopallidal neurons exhibited greater basal and cocaine induced locomotor activity than controls. Collectively, these data support the direct and indirect pathway model for striatal involvement in locomotor activity in mice.

Which cell types and which pathways Fos is localized in is important for understanding how relatively greater Fos in adolescents could relate to lower locomotor stimulation. One

hypothesis is that decreased activation of the direct pathway is responsible for attenuated adolescent stimulation. However, this hypothesis is not consistent with the observation of relatively greater Fos in adolescents. Therefore, alternative pathways and cell types need to be explored to determine how higher Fos could result in lower locomotor stimulation. An alternative hypothesis is that adolescents experience greater activation of the indirect pathway than adults. That is, the increased Fos observed in adolescents compared to adults occurs in D2 containing neurons of the striatum. However, Fos induction from cocaine has been shown to primarily occur in D1 containing neurons (Graybiel et al., 1990, Moratalla et al., 1993, Bertran-Gonzalez et al., 2008). Fos induction in D2 containing neurons has only been shown is certain contexts. Specifically, D2 containing neurons show immunoreactivity to Fos when the animal was placed in a novel environment following drug injection (Bertran-Gonzalez et al., 2008). In Chapter 4 (see methods), mice were placed in their home environment following drug administration. Therefore, D2 containing neurons would not be expected to show Fos induction in the results presented in Chapter 4. Additionally, cocaine and methamphetamine increase dopamine in extracellular spaces (Sulzer et al., 2005) and dopamine is thought to inhibit the indirect pathway (Fisone et al., 2007). Therefore, if increased activation of the indirect pathway does occur in adolescents, it would unlikely be from the dopamine signaling enhancing properties of these drugs.

Striatal mechanisms that modulate dopamine release in the striatum may also alter psychostimulant induced locomotor stimulation. The striatum contains a negative feedback loop to the substantia nigra pars compacta, called the striosomal pathway (Graybiel, 1990). Striosomes are areas of the striatum that are histologically distinct from the surrounding area. They were first identified by Graybiel et al. (1978) as areas of sparse acetylcholine esterase staining. Since then, many other neural markers have been identified that differentiate the striosomes from the surrounding matrix (e.g. MOR1, calbindin) (Graybiel, 1990, Graybiel et al., 1990, Bernard et al., 1993). In this pathway, excitatory inputs from cortex and substantia nigra synapse on GABAergic neurons which primarily project to the substantia nigra pars compacta and immediate surrounds (Gerfen 1984). The result is inhibition of dopaminergic signaling to striosome and matrix portions of the striatum. Therefore, it is possible that the increased Fos in adolescents as compared to adults in the previous chapter was neuroanatomically located

primarily in the striosomal region. If true, that could explain why adolescents displayed attenuated locomotor stimulation from cocaine as compared to adults.

While further studies are needed to fully elucidate striosomal impact on locomotor activity, the ratio of striosome to matrix immediate early genes expression has been found to predict motor stereotypies (Canales, 2005). Activation of the striosomal pathway can be identified by examining GABAergic neurons in striosomes. One plausible hypothesis is that adolescents have greater activation of the striosomal pathway than adults in response to cocaine. This is consistent with the Fos data, in that greater activation could lead to decreased locomotor stimulation. Furthermore, striosomal neurons have been shown to have predominately D1 binding sites as opposed to D2 (Graybiel, 1990). Fos in primarily activated in D1 containing neurons (Graybiel et al., 1990, Moratalla et al., 1993, Bertran-Gonzalez et al., 2008). Therefore, greater Fos signaling in the striosomes in adolescents versus adults could contribute to reduced locomotor stimulation in adolescents.

Microdialysis studies examining dopamine release in adolescent and adult rodents lends some support for the idea that dopamine signaling is altered during adolescence. Basal levels of extracellular dopamine in the striatum have been shown to be lower in adolescents than adults, suggesting inhibitory tone on dopamine signaling may be greater in adolescents (Andersen and Gazzara, 1993). However, Frantz et al. (2007) observed no differences in levels of extracellular dopamine in the nearby region of the nucleus accumbens following cocaine administration. Furthermore, D1 and D2 dopamine receptors have been shown to be expressed greater in adolescents than adults suggesting even similar levels of extracellular dopamine may have different effects between the two age groups (Tarazi et al., 1998, Tarazi et al., 1999). While much research is still needed to understand differences in dopamine signaling between age groups and the potential functional significance thereof, these data show it is conceivable that dopaminergic pathways may be differentially activated between adolescents and adults.

The goal of this chapter is to identify if the striosomal pathway from the striatum is differentially activated from cocaine between adolescents and adults. The hypothesis is that

adolescents will display increased Fos immunoreactivity in neurons in striosomes as compared to adults.

Methods

Subjects

Male C57BL/6J (n=32) mice arrived from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in groups of 4 for 5 days to habituate and then housed singly in custom made acrylic home cages (18.5 cm x 33.5 cm x 16 cm) with clear plastic lids conducive for video tracking from above. Adolescent mice were 21 days old at arrival and tested at 30 days of age. Adult mice were 56 days old at arrival and tested at 65 days of age. All mice were housed on a 12:12 reverse light/dark cycle (lights off at 10 AM and on at 10 PM) with the room temperature maintained at 21 ± 1 ºC. Free access to food (Harlan Teklad 7012, Madison, WI, USA) and water was available at all times. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and adhered to NIH guidelines.

Drug solutions

Cocaine hydrochloride (Sigma Aldrich, St. Louis, MO, USA) and sodium pentobarbital (Sigma Aldrich, St. Louis, MO, USA) were prepared by dissolving in 0.9% saline and were administered at a dose of 30 mg/kg and 100 mg/kg respectively via intraperitoneal (i.p.) injections in a volume of 10 ml/kg. The cocaine solution was prepared according to the salt, not the base, form. Dose was chosen based on a previous study showing age differences in Fos immunoreactivity in the dorsal caudate following 30 mg/kg cocaine (Zombeck et al., 2010).

Locomotor activity

Locomotor activity of the mice in their home cage was recorded using Topscan software (Clever Sys Inc, Reston, VA, USA) following Zombeck et al. (2009, 2010). All mice received a saline injection 1 hr following the onset of the dark phase (i.e. active period) in order to measure the behavioral response to an injection. Activity was measured for 1 hr after which an injection of cocaine (30 mg/kg) was administered. Locomotor activity recorded for 1.5 hrs before animals were sacrificed.

Immunohistochemistry

Brain fixation

Following Clark et al. (2008) mice were anesthetized with 100 mg/kg sodium pentobarbital (i.p.) and then perfused transcardially with 4% paraformaldehyde in phosphate buffer saline (PBS; 0.287% sodium phosphate monobasic anhydrous, 1.102% sodium phosphate dibasic anhydrous, 0.9% sodium chloride in water). Brains were postfixed overnight, and then transferred to 30% sucrose in PBS. Brains were coronally sectioned (40 µm thick) using a cryostat. Sections were placed into a 24 well plate containing tissue cryoprotectant (30% ethylene glycol, 25% glycerin, 45% PBS), then stored at -20^{o}C.

Fos

One in 6 series sections were transferred into PBS, 24 hrs before beginning immunohistochemistry. Free-floating sections were pretreated with sodium borohydride (100 mg per 20 ml PBS) for 30 min, washed with PBS-X (PBS containing 0.2% v/v Triton X-100), and blocked with 6% v/v Normal Goat Serum (NGS) for 1 hr at room temperature. Sections were then incubated in rabbit antibody against c-Fos at a dilution of 1:20,000 (Calbiochem, San Diego, CA, USA) in PBS-X containing 2% NGS for 48 hrs at 5 ºC. After primary incubation, sections were washed in PBS-X followed by incubation in secondary biotinylated antibodies against rabbit immunoglobulin made in goat (Vector Labs, Burlingame, CA, USA) at a dilution of 1:500 in PBS-X with 2% NGS for 90 min at room temperature. The peroxidase method (ABC system, Vector Labs, Burlingam, CA, USA; 37 ul A, 37 ul B in 15 ml PBS-X) and diaminobenzidine (DAB) as chromogen enhanced with nickel chloride (Sigma, St. Louis, MO, USA) was used to visualize the antibody complex. The reaction was stopped by washing the sections in PBS. Sections were mounted onto subbed slides, allowed to air dry, and then were dehydrated and coverslipped using Permount (Sigma, St. Louis, MO, USA).

MOR1

Adjacent sections rostral to those stained for Fos were stained for MOR1. Free-floating sections were washed with PBS-X and pretreated with hydrogen peroxide (3% in PBS-X) for 10 min. Sections were again washed with PBS-X and then blocked with 10% NGS (PBS-X plus). Sections were incubated in rabbit antibody against MOR1 at a dilution of 1:8000 (Immunostar, Stillwater, MN) in PBS-X plus for 48 hrs. The remainder of the procedure followed as was used with Fos.

Image analysis

Microscopic images of adjacent sections (one stained for Fos, the other MOR1) were captured via a Zeiss Axiocam digital camera (Zeiss, Germany) interfaced to a personal computer. Images were taken at 100X total magnification in the dorsal caudate region following Zombeck et al. (2008). Images were aligned and analyzed using ImageJ software (NIH, Bethesda, MD). First, the striosome region was outlined by hand and particles were counted only within the corresponding outlined structures on the adjacent Fos stained section. Next, the entire caudate was outlined and total Fos counts in the caudate and total area of the sampled caudate were obtained. The counting was done unilaterally, in three sections for each brain region, to obtain an average cell count per brain region for analysis.

Statistical analysis

Distance traveled summed over the 60 minutes post saline injection was analyzed using unpaired t-tests comparing adults and adolescents. Locomotor activity 90 minutes following cocaine administration was analyzed using analysis of variance (ANOVA) with age (adolescent versus adult), dose (saline or 30 mg/kg), and age by dose interaction as factors.

Volume of the striosome and matrix, number of Fos positive cells within striosome and matrix, and density of Fos positive cells per volume for the striosome and matrix subregions were analyzed using ANOVA with age, dose, and age by dose interaction as factors. Fos counts were also analyzed using analysis of covariance. This was done to determine whether Fos levels differ between adolescents and adults after accounting for the expected positive relationship between acute levels of physical activity and Fos observed in previous studies throughout the brain (Rhodes et al., 2005, Caster and Kuhn, 2009). In this model, Fos staining was analyzed as the response, summed locomotor activity over 90 minutes as the continuous predictor (covariate), and age as the factor. To examine density differences between striosome and matrix regions in adolescents and adults following cocaine administration, repeated measures ANOVA was performed for Fos density with age and location (striosome or matrix) and the interaction as factors.

Results

Locomotor activity

One hour after the onset of the dark cycle, mice received a saline injection to assess the behavioral response to an injection. No differences in locomotor activity following a saline injection were observed between adolescents and adults ($t_{(23)}$=0.78, P>0.05). The pattern of locomotor activity was consistent with previous chapters (see Fig. 2.3, 3.3, 4.2)

Cocaine administration caused approximately 8-fold increase in locomotor activity relative to saline (main effect of dose, $F_{(1,28)}$=117.2, P<0.0001) (Fig 5.1). However the magnitude of locomotor stimulation to cocaine was attenuated in adolescents as compared to adults (main effect of age, $F_{(1,28)}$=10.3, P<0.003; age*dose interaction, $F_{(1,28)}$=6.4, P<0.02).

Fos localization

The caudate displayed patches of MOR1 stain consistent with previous studies identifying the areas as striosomes (Capper-Loup et al., 2002) (See Fig. 5.2). In general, cocaine administration significantly increased Fos in both striosomes and matrix subregions. However adolescents did not differ statistically across the dependant variables. Age differences were not observed in volume of the striosomes or matrix, number of Fos positive cells within striosomes or matrix, or the density of Fos positive cells in the striosomes or matrix. See Table 1 for a summary of means and statistics.

We were interested in examining if the pattern of Fos density shifted between predominately striosome or matrix portions between age groups following cocaine. Therefore, repeated measures ANOVA was performed with Fos density as the repeated variable, and location (striosome or matrix), age (adolescent or adult), and the interaction as factors. There was a nonsignificant trend for greater density in striosomes relative to matrix (main effect of location, $F_{(1,14)}$=3.9, P=0.07); adolescents showed 17% greater Fos density in striosomes compared to matrix, while adults showed a 9% greater difference. However the difference between age groups was not statistically significant (main effect of age, F(1,14)=0.7, P>0.05), nor was a main effect of location or the age by location interaction (age*location interaction, $F_{(1,14)}$=0.15, P>0.05).

Previous studies have shown strong correlations between number of Fos positive cells in the caudate and locomotor stimulation from cocaine (Rhodes et al., 2005, Caster and Kuhn, 2009). Therefore, locomotor activity was included as a covariate in an analysis of Fos density in striosomes and in an analysis of total Fos (striosomes + matrix) between ages following cocaine. Locomotor activity was positively correlated with total Fos (main effect of locomotor activity ($F_{(1,12)}$=8.5, P=0.01). Similar trends were observed for Fos density in the striosomes ($F_{(1,12)}$=4.4, P=0.06), and matrix ($F_{(1,12)}$=7.84, P=0.02). Fos activation was slightly greater in adolescents compared to adults for a given level of locomotor activity, but this was not statistically significant for either total Fos, Fos density in striosomes, or Fos density in matrix subregions (P>0.05).

Discussion

The major finding of this chapter is that Fos expression in striosomal regions of the dorsal caudate did not significantly differ between adolescent and adult mice, despite behavioral differences between ages in cocaine stimulation (Fig. 5.1). The failure to show age differences for Fos expression in striosomal regions provides evidence against the hypothesis that elevated striosomal signaling following cocaine administration causes reduced locomotor stimulation seen in adolescents as compared to adults.

A trend for greater Fos density in striosome regions as compared to the matrix was present in both adults and adolescents. This observation is consistent with previous studies showing similar trends for predominately striosomal Fos expression following pharmacological treatment. For example, relatively greater Fos expression in striosomes versus matrix has been demonstrated following coadministration of dopamine D1 and D2 agonists (Capper-Loup et al., 2002). Consistent with the idea that dopamine signaling activates striosomal regions, Graybiel et al. (1990) found greater striosomal Fos following amphetamine administration. However the same study showed no regional differences in Fos following cocaine administration. Overall, the data suggest dopamine signaling increases Fos expression biased toward striosome regions over matrix portions of the striatum.

Fos expression is correlated with locomotor activity in a number of brain regions, including the caudate (Rhodes et al., 2005, Caster and Kuhn, 2009). Previously, we demonstrated that adolescents displayed increased Fos relative to adults after correcting for differences in locomotor activity (Zombeck et al., 2010). Therefore, distance traveled following cocaine administration was included as a covariate in an analysis of total Fos counts (matrix and striosomes combined). Similar main effects, although not statistically significant, were observed with adolescents displaying elevated Fos counts for a given distance traveled than adults. Therefore, the question of how elevated Fos immunoreactivity in the caudate of adolescents as compared to adults is associated with *lower* levels of locomotor stimulation remains unanswered.

Dampening of dopamine induced locomotor stimulation by interneurons within the caudate represents one possibility for how relatively greater Fos immunoreactivity in adolescents could result in lower levels of locomotor stimulation. In addition to medium spiny projection neurons, the caudate contains large aspiny cholinergic interneurons. These neurons oppose dopaminergic function and generally act to depress motor activity (Graybiel, 1990, Wickens, 1990). Therefore, one hypothesis for attenuated stimulation in adolescents is that they display elevated acetylcholine signaling relative to adults. Similar ideas have been expressed by Bolanos et al. (1998). Multiple explanations are possible for how cholinergic interneurons could be differentially activated from cocaine between age groups. One theory for how dopamine and cholinergic systems interact in the caudate is that dopamine inhibits cholinergic interneurons via D2 dopamine receptors located on interneurons (Wickens, 1990). However, cholinergic interneurons may also contain D1 dopamine receptors which increase acetylcholine release (Damsma et al., 1990, Zhou et al., 2002). Therefore, adolescents may display increased D1 dopamine receptors on cholinergic interneurons which results in increased cholinergic inhibition of GABAergic projection neurons and ultimately attenuated locomotor stimulation. An alternative explanation is that differential interneuron stimulation could be driven by cortical projections. To test the hypothesis that elevated interneuron signaling in adolescents contributes to stimulation differences, the caudate could be double labeling for choline acetyltransferase and Fos as one method for estimating cholinergic interneuron activity (Robertson and Staines, 1994). Greater number of choline acetyltransferase and Fos colabeled cells in adolescents would suggest

a role for elevated cholinergic inhibition of motor activity in attenuated stimulation displayed in adolescents.

So far, the hypotheses postulated have all been predicated on the assumption that Fos is reflective of excitatory signaling within a neuron. An alternative idea is that adolescents display increased Fos relative to adults because those cells have been inhibited to a greater degree. Greater inhibition of GABAergic neurons of the direct pathway is consistent with the behavioral observation of relatively lower locomotor activity in adolescents. However, Fos expression is primarily thought to represent excitatory signaling within the cell. Pharmacological agents that increase signaling (e.g. cocaine) elevate Fos (Zombeck et al., 2010), while agents that decrease signaling (e.g. diazepam) lower Fos (Beck and Fibiger, 1995). Therefore, it is unlikely that elevated Fos in adolescents is reflective of greater neural inhibition.

Non-neuronal induction of Fos represents an alternative explanation for differential Fos immunoreactivity between ages. For example, astrocytes play an important role in glutamate metabolism (Kondziella et al., 2007). Glutamate signaling in is increased following cocaine administration and is thought to augment dopamine induced locomotor activity (Pierce et al., 1996, Reid et al., 1997). For example, intra accumbens infusion of NMDA antagonist reduces cocaine induced locomotor stimulation (Pulvirenti et al., 1991). While Fos is primarily expressed in neurons, cell culture studies have demonstrated c-fos expression in astrocytes following cocaine (Malaplate-Armand et al., 2005). It is conceivable that adolescents may have increased astrocyte activation compared to adults. If metabolism of glutamate by astrocytes is greater in adolescents, then excitatory glutamatergic signaling in the caudate would be expected to terminate faster, possibly resulting in decreased locomotor stimulation observed in adolescents. This hypothesis could be tested by examining Fos activation in astrocytes or using microdialysis to measure glutamate release in the caudate following cocaine administration.

In summary, the current study failed to show evidence for the hypothesis that elevated striosomal signaling contributes to attenuated locomotor stimulation to cocaine in adolescents as compared to adults. However, the dorsal caudate remains an area of interest for examination of age differences to cocaine. Alternative explanations, such as differences in interneuron signaling

or astrocyte functions, represent possible future avenues of exploration for age differences in stimulation.

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Tables

Table 5.1 Histology summary

		Mean				Statistics	
		Adults		Adolescents			
		0	30	0	30	Dose	Age
Fos	Striosomes	0.8±0.2	28.4±5.1	1.3±0.4	24.0±2.1	**F(1,28)=81.8, P<0.0001**	F(1,28)=0.5, P>0.05
	Matrix	26.2±5.2	366.9±32.8	24.9±7.7	313.6±26.9	**F(1,28)=210.4, P<0.0001**	F(1,28)=1.6, P>0.05
Area	Striosomes	62.4±8.3	74.9±8.1	69.1±5.9	75.5±9.5	F(1,28)=1.4, P>0.05	F(1,28)=0.2, P>0.05
	Matrix	1092.4±11.4	1090.0±8.9	1080.7±7.0	1091.0±9.1	F(1,28)=0.2, P>0.05	F(1,28)=0.3, P>0.05
Fos Density	Striosomes	0.01±0.003	0.37±0.050	0.02±0.009	0.34±0.038	**F(1,28)=111.3, P<0.0001**	F(1,28)=0.1, P>0.05
	Matrix	0.02±0.005	0.34±0.029	0.02±0.007	0.29±0.024	**F(1,28)=218.8, P<0.0001**	F(1,28)=1.6, P>0.05

Figures

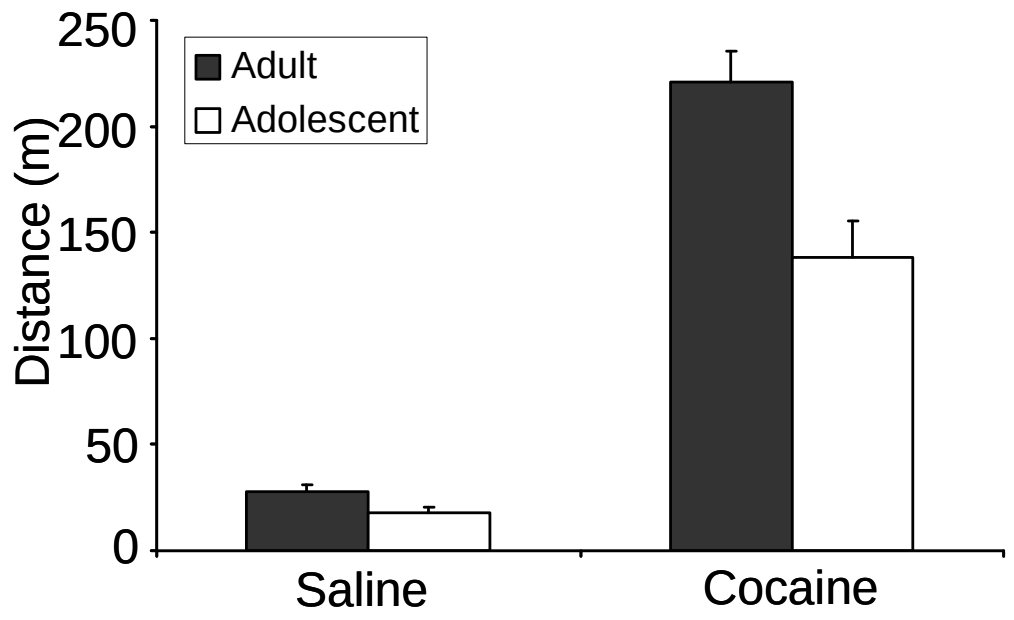

Figure 5.1. Sum distance traveled in 90 minutes following 30 mg/kg cocaine or saline in adolescent and adult mice. Error bars represent SEM.

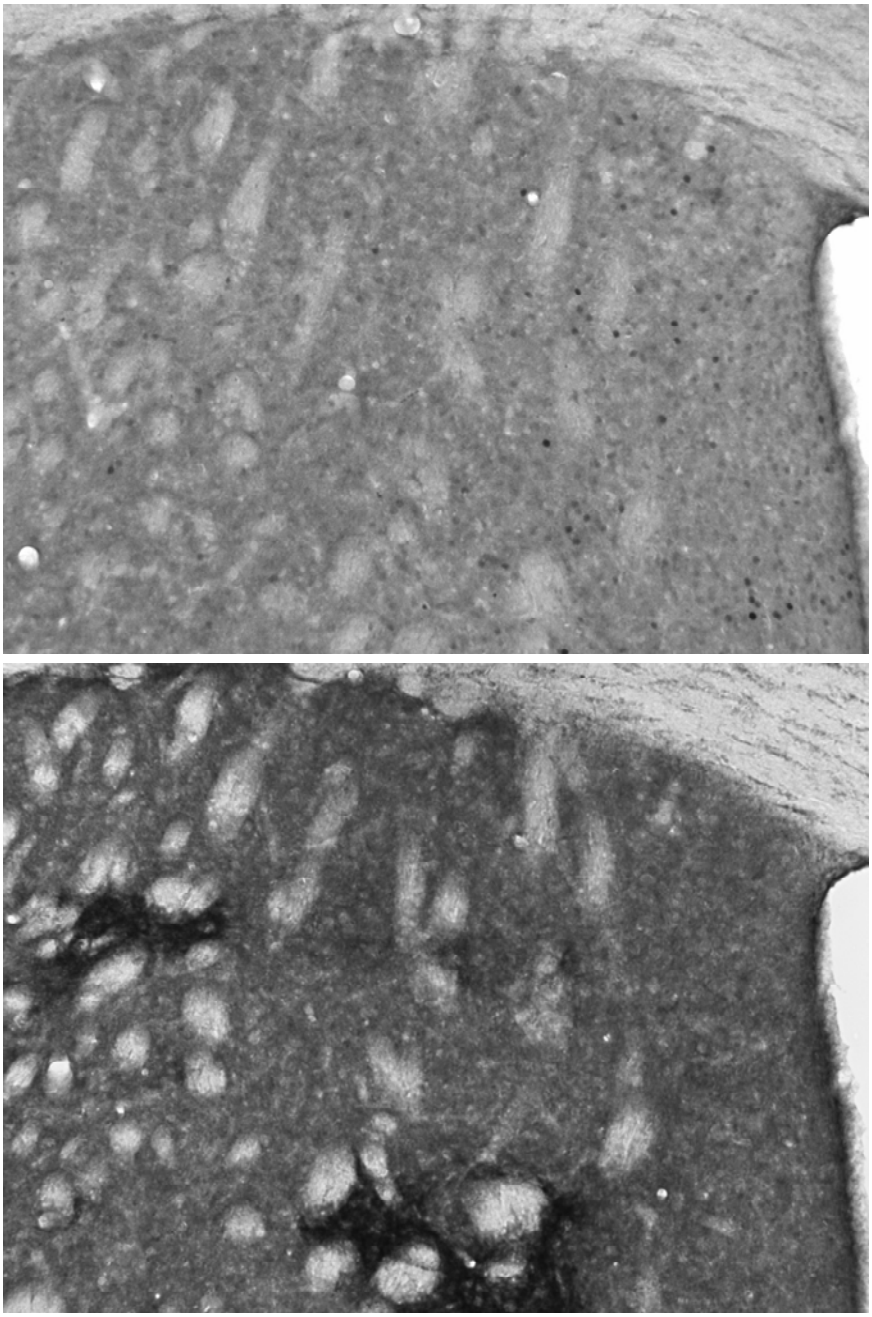

Figure 5.2. Shown here are adjacent sections, the top photograph shows Fos positive nuclei and the bottom photograph shows striosomes by MOR1 immunostain.