

CHARACTERIZING DOPAMINE FUNCTION IN ADOLESCENT RATS

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Adolescence is a developmental period that coincides with increased exploration, social interaction, risk-taking, and novelty-seeking, as well as the symptomatic onset of psychiatric illnesses. Remodeling of the mesocorticolimbic and nigrostriatal dopamine systems has been implicated in these vulnerabilities. Yet, little is known about functional consequences of this remodeling. This dissertation sought to investigate the dynamics of dopamine neurotransmission in adolescent rats in two functionally distinct dopamine innervated striatal subregions: the nucleus accumbens (NAc), which is implicated in reward processing, and the dorsal striatum (DS), which is involved in habit formation and flexible learning.

The psychostimulant amphetamine was used as a tool to activate dopamine release in behaving rats. Microdialysis was used to measure extracellular levels of dopamine. An acute systemic administration of amphetamine caused smaller amphetamine-induced dopamine efflux in the DS of adolescent rats (postnatal days 35-38) compared to adults. Dopamine in NAc was increased similarly in both age groups. Amphetamine also caused reduced levels of stereotypy behavior in adolescents than adults. Reduced effectiveness of amphetamine in DS was not due to age-related differences in the dopamine transporter (DAT). Though adolescents have lower levels of DAT in the NAc and DS than adults, the DAT inhibitor nomifensine similarly inhibited basal and amphetamine-induced dopamine efflux in both striatal subregions of both age groups. Furthermore, vesicular monoamine transporter-2 (VMAT2) expression was similar in the DS and

NAc of both adolescent and adult rats. In contrast, expression of tyrosine hydroxylase (TH) was reduced in the DS, but not the NAc, of adolescents compared to adults. The adolescent rats also were behaviorally more sensitive to the effects of a TH inhibitor. Together these data suggest that dopamine neurotransmission in the DS of adolescents is hypofunctional compared to adults, resulting in part from reduced TH activity. Given that actions of dopamine on striatal neurons are primarily inhibitory, functions associated with DS, such as action selection and habit formation, may be less responsive to dopamine mediated inhibition during adolescence.

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PREFACE

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1.0 INTRODUCTION

Adolescence is considered to be a transitional period from childhood to adulthood, accompanied by changes in physical, psychological, and social development (Ernst et al., 2006), that will ultimately enable individuals to acquire necessary skills to become independent. These dramatic changes are thought to render adolescents as more prone to engage in deleterious behavior and have increased incidence of several psychiatric illnesses, making this developmental period a time of considerable vulnerability. Thus, understanding normal brain development throughout adolescence and adolescents' increased risk for mental illnesses is an important public health issue to address.

While the timing of puberty and adolescence overlap, these terms are not synonymous. Puberty, a period of sexual maturity, is defined by temporally discrete biological events (Falkner and Tanner, 1986; Pickles et al., 1998), whereas adolescence is considered a gradual maturation process of a series of "soft events" with no finite onset or offset (Pickles et al., 1998; Spear, 2000). It is for these reasons that, in various mammalian species, there is a dispute amongst researchers on the exact age range of adolescence. Sex, for instance, can influence the adolescence time frame, as female mammals across species mature at a younger age than males (Falkner and Tanner, 1986). Human adolescence is typically considered to occur during ages 12 to 18 years, though this developmental time span can include early- to mid- 20s (Baumrind, 1987; Spear, 2000). In rats, adolescence is generally accepted as postnatal days 28-49 (Spear

and Brake, 1983). Unlike other mammalian species, human adolescence is influenced not only by physiological changes, but also by sociocultural and economic conditions. Though these external factors make it difficult to define adolescence in animal models, researchers have been able to distinguish behaviorally and physiologically the adolescent time period apart from childhood and adulthood. Despite different research groups varying in how they classify their adolescent subjects based on age and level of development, for clarity's sake the literature reviewed here typically defines adolescence as the teenage years for humans, two to four years of age for monkeys, and postnatal days 28 to 42 for rats. Also, the use of the term "juvenile" in the reviewed animal studies here typically refers to pre-adolescent animals.

1.1 ADOLESCENT BEHAVIOR

Part of the "soft events" that define the adolescent transitional phase between childhood and adulthood are characteristic behaviors shared across species. In general, these behaviors include increased social behavior (Csikszentmihalyi et al., 1977), increased novelty and sensation seeking (Adriani et al., 1998; Stansfield et al., 2004; Stansfield and Kirstein, 2006), increased risk taking (Steinberg, 2008), emotional instability (Steinberg, 2005), and impulsivity (Adriani and Laviola, 2003; Chambers et al., 2003; Vaidya et al., 2004). In both rodents and humans, adolescents exhibit increased social interactions with peers (Csikszentmihalyi et al., 1977). The quality of these social interactions has also been shown to change in adolescence, for instance with peak levels in play behavior and reductions in aggressive behaviors (Pellis et al., 1992). Studies have also shown that adolescent rodents and humans make more impulsive choices in delay-discounting tasks, preferring smaller rewards occurring sooner over delayed larger rewards

(Adriani and Laviola, 2003; Steinberg et al., 2009). Sensation seeking, defined as the need for varied, novel sensations and experiences, is also seen in both rodents and humans. Adolescent rodents prefer novelty, exhibit greater novelty-induced locomotion, and spend more time exploring open arms in an elevated plus maze than adults (Laviola and Adriani, 1998; Douglas et al., 2003; Adriani et al., 2004; Stansfield and Kirstein, 2006). Similarly, human adolescents score higher on the Sensation-Seeking Scale than adults, with male adolescents scoring higher levels than females (Zuckerman et al., 1978). However, sensation seeking in humans tends to be greatest during early- to mid-adolescence, and declines in late adolescence to adult levels (Steinberg, 2008). Increased risk taking, and novelty and sensation seeking may serve as adaptive behaviors that allow adolescents to become more independent, to explore adult behavior, and to accomplish other normal developmental cognitive tasks, such as abstract reasoning (Silbereisen and Reitzle, 1992; Muuss and Porton, 1998; Spear, 2000).

Unfortunately, risk taking in adolescents can also prove to be maladaptive. Human adolescents, relative to other age groups, are disproportionately more likely to exhibit reckless behavior, sensation seeking, and risk taking (Spear, 2000). Reckless behavior can be considered “normal behavior” when approximately half of all adolescents engage in drunk driving, sex without contraception, illegal drug use, and minor criminal activities (Spear, 2000). This recklessness often has negative consequences such as incarceration, AIDS infection, and unwanted pregnancy (Lerner and Galambos, 1998; Spear, 2000). Furthermore, adolescent morbidity and mortality rates are twice that of pre-pubescent children (Dahl, 2004). Increased novelty and sensation seeking during adolescence is also associated with increased drug and alcohol use. For instance, the escalation of cocaine use is higher in adolescent users compared to

adults (Estroff et al., 1989), and adolescents who become addicted to alcohol have rates of relapse similar to alcoholic adults, despite the shorter length of use (Brown, 1993).

The likelihood of adolescent risk taking and recklessness calls into question how adolescents process risk at this age compared to individuals of other age groups. There is little evidence to suggest that adolescents perceive themselves as invulnerable or underestimate risk, but rather they tend to overestimate risk (de Bruin et al., 2007). In fact, an individual's perceived risk of risky behavior has been shown to decline with age during adolescence (Irwin Jr, 1993). There are some arguments to suggest that adolescents may engage in risk taking in order to gain the positive arousal associated with sensations of novelty, complexity, change or intensity of experience (Zuckerman, 1992). Therefore, adolescents may require a higher level of risk taking in order to achieve the desired feeling or sensation from certain reinforcers. On the other hand, risk taking and sensation seeking may have little to do with the pursuit of positive outcomes from novel or rewarding stimuli, but rather be a result of an attempt to reduce dysphoria or a mechanism to cope with stress (McCord, 1990; Irwin Jr, 1993). Adolescent risk taking can thus be considered "an optimal life-span pattern for a rational decision maker who must gain knowledge of self and environment through experience" (Gardner, 1999).

In addition to the vulnerability of adolescents when it comes to risk taking and "normal" behavioral development, the adolescent period is a time in which the symptoms of psychiatric illnesses, including mood disorders, eating disorders, addictive disorders, and schizophrenia, typically manifest (Weinberger, 1987; Volkmar, 1996; Pine et al., 2002; Chambers et al., 2003; Andersen and Teicher, 2008; Paus et al., 2008). In fact, previous data have indicated that the peak age of onset for any mental health disorder is 14 years (Kessler et al., 2005). It has been suggested that the emergence of these psychopathologies during this developmental time frame

may be due to aberrations of typical adolescent maturation processes combined with psychosocial factors and biological environmental factors (Paus et al., 2008). Furthermore, cognitive control, which continues to improve throughout adolescence, is often times compromised in these psychopathologies (Luna and Sweeney, 2004; Sweeney et al., 2004). The neuronal mechanisms that contribute to psychiatric illness vulnerability during adolescence, however, are not well understood (Thompson et al., 2004; Carlsson and Carlsson, 2006; Tseng et al., 2007; Davey et al., 2008; Nemoda et al., 2011).

1.2 ADOLESCENT NEURODEVELOPMENT

The typically developing adolescent brain undergoes many neural and gross morphological alterations. While the human brain reaches approximately 80 to 90% of its adult size during childhood (Dekaban, 1978), gray and white matter subcomponents of the brain continue to undergo dynamic changes throughout adolescence. Human neuroimaging studies have shown white matter volume increases linearly throughout childhood and adolescence, with maximum volumes reached in adulthood (Pfefferbaum et al., 1994; Gogtay et al., 2004). Diffusion tensor imaging studies suggest that increases in white matter volume may be an indication of the ongoing maturation of axons along with myelination by oligodendrocytes (Snook et al., 2005; Paus, 2010). Unlike white matter changes, the total volume of gray matter in the brain appears to increase prior to puberty with subsequent post-pubertal loss, resulting in an inverted U-shaped developmental trajectory in each lobe (Jernigan and Tallal, 1990; Jernigan et al., 1991; Giedd et al., 1999; Sowell et al., 2001). This maturation appears to occur in a “back-to-front” pattern, with primary sensorimotor areas maturing first, followed by association areas, and lastly higher-order

cortical areas (Huttenlocher, 1979; Gogtay et al., 2004). Nonhuman primate studies using electron microscopy have observed dramatic decreases in the number of synapses in cortical areas during adolescence (Rakic et al., 1986; Bourgeois and Rakic, 1993). Together these data suggest that synaptic pruning occurs in the human and nonhuman primate adolescent brain, and it is speculated that this pruning is an example of developmental plasticity as a way to accommodate environmental needs through experience (Rakic et al., 1994).

In addition to cortical regions, subcortical areas, in particular the basal ganglia, change during adolescent development (Sowell et al., 1999). Gray matter reductions, for instance, have been observed in the striatum and other subcortical structures, following a similar U-shaped developmental trajectory seen in frontal and parietal cortical areas (Sowell et al., 2002). The developmental morphological changes in human subcortical structures such as the amygdala and hippocampus, however, are less well understood. One study observed amygdala volume increased significantly during adolescence only in males and hippocampal volume increased only in females (Giedd et al., 1996).

Studies of animal models of adolescence show that various neurotransmitter systems undergo significant developmental changes. For instance, rodent brain activity, as measured by glucose metabolism, oxygen utilization, and blood flow, reaches developmental peaks during adolescence, before declining in adulthood (Chugani et al., 1987). The adolescent brain also undergoes hippocampal neurogenesis (He and Crews, 2007), axonal growth (Benes et al., 2000), apoptosis (Markham et al., 2007) and myelination (Benes et al., 1994). Receptor overproduction and pruning has been observed in various neurotransmitter systems, such as dopamine, serotonin, acetylcholine, and endocannabinoid in the adolescent forebrain (Lidow et al., 1991; Lidow and Rakic, 1992; Rodriguez de Fonseca et al., 1993). During adolescence, axons and synapses in

select regions are overproduced, followed by rapid pruning later in adolescence (Crews et al., 2007). These regions include limbic and cortical areas, such as dendritic pruning in the amygdala (Zehr et al., 2006) and prefrontal cortex (Andersen et al., 2000), with evidence suggesting that rodent limbic regions mature before prefrontal cortical areas (Teicher et al., 1995; Andersen et al., 2000).

In contrast to synaptic receptor pruning, transporters for the monoamines dopamine and serotonin in the rodent dorsal striatum (DS) and nucleus accumbens (NAc) show increases in expression either prior to or during adolescence (Coulter et al., 1996; Tarazi et al., 1998b). Dopamine transporter (DAT) levels in both the DS and NAc are considered to follow a similar pattern of postnatal development, with higher DAT levels in adulthood compared to adolescence in these regions (Tarazi et al., 1998b). However, there have been other reports that show DAT levels peak in adolescence and gradually decline with age (Meng et al., 1999; Moll et al., 2000). Also, accumbal serotonin turnover is reported to be drastically lower in adolescent rats compared to both younger and more mature adult rats, while dopamine turnover and synthesis in the NAc and other striatal regions continues to increase throughout adolescence into adulthood (Andersen et al., 1997). Collectively, this remodeling is thought to be a form of developmental plasticity that allows adolescents to adapt to environmental needs in order to mature into adulthood (Crews et al., 2007).

Among neurotransmitter systems, several lines of evidence indicate that the mesocorticolimbic and nigrostriatal dopamine systems undergo major remodeling during adolescence. Adolescent rodents have been shown to maintain lower basal extracellular levels of dopamine in the striatum than adults, while dopamine receptor overproduction and pruning in striatal regions has been observed (Gelbard et al., 1989; Andersen et al., 1997; Tarazi et al.,

1998a; Badanich et al., 2006; Cao et al., 2007). Receptor overproduction and pruning of dopamine receptors (D1 and D2) is shown to occur in both human and rat (Spear, 2000). There is conflicting evidence, however, to support receptor pruning in both the NAc and DS. Several studies have found these regions reach peak levels of dopamine receptor binding in adolescence and then prune to adult levels (Gelbard et al., 1989; Teicher et al., 1995; Tarazi and Baldessarini, 2000), while other studies suggest that dopamine receptor levels in the NAc and DS continue to increase throughout adolescence until reaching adult levels (Hartley and Seeman, 1983; Murrin and Zeng, 1986; Zeng et al., 1988; Leslie et al., 1991).

In addition to structural changes of the dopamine system, the modulatory impact of dopamine receptor binding shifts from being mildly inhibitory to strongly excitatory during adolescence into early adulthood (O'Donnell, 2010). The functional consequences of dopamine receptor activation in the NAc and DS also vary depending on age, with adolescents exhibiting greater basal cAMP levels but lower D1 stimulatory and D2 inhibitory effects on adenylyl cyclase production than adults (Spear, 2000). Firing rates of midbrain dopamine neurons have also been shown to peak during adolescence before decreasing over time into adulthood (McCutcheon and Marinelli, 2009). This developmental remodeling may lead to dynamic changes in the sensitivity of the dopamine system to external stimuli that may mediate some of the behavioral changes observed in adolescents.

Along with the changes in the dopamine system, there are shifts in excitatory and inhibitory neurotransmission occurring in adolescence. The cortical binding of the excitatory neurotransmitter glutamate to its NMDA receptor subtype peaks in early adolescence and declines significantly thereafter into adulthood (Insel et al., 1990; Guilarte and McGlothan, 1998). In the prefrontal cortex, the excitability of fast-spiking interneurons changes during

adolescence as demonstrated by reduced NMDA currents (Wang and Gao, 2009). In the NAc, studies have observed a reduction in accumbal NMDA receptors as well as the loss of excitatory glutamate input to NAc during adolescence (Frantz and Van Hartesveldt, 1999b, a). Levels of the inhibitory neurotransmitter GABA in the rat forebrain increase in a linear pattern throughout adolescence (Hedner et al., 1984). In monkeys, significant changes in both pre- and post-synaptic markers of GABA synapses occur in the prefrontal cortex during adolescence (Lewis et al., 2004). Specifically, basal levels of GABA_A receptor-mediated chloride current are greater in the adolescent cortex compared to adults (Kellogg et al., 1993b). Furthermore, the responsiveness of cortical GABA_A neurotransmission to stressors decreases from adolescence to adulthood (Kellogg et al., 1993a; Kellogg, 1998). The modulatory impact of dopamine-receptor activity shifts during adolescence, such that activation of dopamine D2 receptors increases interneuron activity and dopamine D1 receptor activation leads to NMDA receptor changes (Wang and O'Donnell, 2001; Tseng and O'Donnell, 2007).

There may be several important functional consequences of these developmental alterations and shifts in the balance of excitatory and inhibitory neurotransmission. For instance, adolescence is considered a highly plastic time of corticolimbic brain development, as evidenced by the fact that long-term potentiation, which is a form of plasticity measured by increases in synaptic strength, is more frequently found in the NAc of adolescent mice compared to adults (Schramm et al., 2002).

1.3 DOPAMINE NEUROTRANSMISSION

Dopamine is a neuromodulator involved in the regulation of neuroendocrine secretion and various behaviors including learning (Wise, 2004), cognitive flexibility (Stefani and Moghaddam, 2006; Robbins and Roberts, 2007), reward perception (Schultz et al., 1997; Berridge and Robinson, 1998), and goal-directed movements (Beninger, 1983; LeMoal and Simon, 1991). There are four major dopaminergic pathways that have been identified in the mammalian brain originating from groups of dopamine-containing cells in the midbrain: the nigrostriatal pathway originating from A9 (substantia nigra pars compacta), the mesolimbic and mesocortical pathways originating from A10 (ventral tegmental area), and the tuberoinfundibular pathway originating from A8 (Dahlström and Fuxe, 1964). Each of these subgroups of dopamine neurons is anatomically and functionally distinct and innervates the pituitary gland and forebrain structures such as the prefrontal cortex, NAc, and DS (Carlsson et al., 1962).

The NAc and the DS are core structures of the mammalian basal ganglia, a group of large subcortical nuclei, whose projection medium-sized spiny neurons make up the vast majority (95%) of the striatum's neuronal cell population (Gerfen, 2004). The DS is the main input structure of the basal ganglia, and receives vast projections from cortical regions involved in sensory, motor, and cognitive processes (Voorn et al., 2004). The DS is largely innervated by dopamine neurons from the substantia nigra pars compacta (Gerfen et al., 1987). Dopamine signaling in the DS plays an important role in voluntary motor control (Joel and Weiner, 2000), as well as instrumental learning (Balleine et al., 2009). Both dopamine and endocannabinoid signaling in the DS have also been shown to be essential in transitioning from goal-directed behavior to automatic habitual behavior (Hilario et al., 2007; Ashby et al., 2010). The NAc, on the other hand, receives afferent projections from the amygdala (Kelley et al., 1982) and

prefrontal cortex (Powell and Leman, 1976), as well as dopamine innervations from the ventral tegmental area (Gerfen et al., 1987). The NAc is considered the interface between limbic and motor systems and is thought to be important in converting “motivation” to “action” (Mogenson et al., 1980; Morgane et al., 2005). The NAc is thought to play a key role in adolescent risk taking and sensation seeking (Ernst et al., 2006; Casey et al., 2008; Steinberg, 2008). Despite their distinct roles in various behaviors, the NAc and the DS share some functional overlap in cognitive function (Voorn et al., 2004), particularly in the context of reward-mediated behavior. For example, blocking the mesolimbic or nigrostriatal dopamine pathways can attenuate the reinforcing effects of various rewards (Wise, 2005).

The physiological actions of dopamine are mediated by five distinct G protein-coupled receptors that are divided into two major groups: the D1-class dopamine receptors, consisting of D1 and D5 receptors, and the D2-class dopamine receptors, consisting of D2, D3, and D4 receptors (Andersen et al., 1990; Niznik and Van Tol, 1992; Sibley et al., 1992; Civelli et al., 1993). D1 dopamine receptors are highly expressed throughout the DS, NAc, substantia nigra, olfactory bulb, amygdala, and frontal cortex, and are also found in the hippocampus, cerebellum, thalamic areas, and hypothalamic regions (Fremeau et al., 1991; Weiner et al., 1991). Activation of D1 dopamine receptors in the striatum, which are exclusively expressed on the postsynaptic neurons and coupled to G-protein G_s (stimulatory), excite medium spiny neurons in the “direct” striatal output pathway (Surmeier et al., 2007). Conversely, the role of the D2 dopamine receptors is not as straightforward due to their expression both presynaptically and postsynaptically (Missale et al., 1998; Sibley, 1999). D2 dopamine receptors are highly expressed throughout the striatum and the olfactory tubercle (Bouthenet et al., 1991). Presynaptically localized D2 dopamine receptors, or autoreceptors, serve as an important

negative feedback mechanism that adjusts neuronal firing rate, synthesis, and release of dopamine in response to changes in extracellular dopamine levels (Wolf and Roth, 1990; Missale et al., 1998; Sibley, 1999). Therefore, activation of presynaptic D2 autoreceptors generally causes a decrease in dopamine release that results in decreased locomotor activity, whereas activation of postsynaptic receptors stimulates locomotor activity.

Our understanding of the functional integrity of the NAc and DS is largely based on animal studies observing behavioral responses to dopaminergic drugs. Decreasing or depleting dopamine by blocking dopamine receptors or lesioning dopaminergic cells with 6-hydroxydopamine (6-OHDA) reduces spontaneous locomotion, while drugs that enhance dopamine neurotransmission, such as the psychostimulants amphetamine and cocaine, produce hyperlocomotion and stereotypy (Beninger, 1983). Amphetamine disrupts DAT function, causing efflux of a newly synthesized, “releasable” pool of dopamine, as well as vesicular dopamine (Sulzer et al., 1995; Jones et al., 1998). There is a dorsal-ventral relationship in the striatum related to dopamine function in that stereotypy and fine movements are attributed to DS dopamine activation, whereas hyperlocomotion is attributed to dopamine activation in the NAc (Beninger, 1983; Delfs et al., 1990).

Changes in response to dopamine altering drugs on striatal function are also apparent in more complex, conditioned behaviors. Infusing selective dopamine D1 or D2 receptor antagonists into the NAc has been shown to potently suppress locomotor responses associated with the motivational state of hunger (Baldo et al., 2002), and depleting dopamine using 6-OHDA lesions in the NAc has been shown to suppress instrumental responding reinforced with food (Heffner and Seiden, 1983). Inactivating the DS using lidocaine, on the other hand, has been shown to block the ability of animals to shift behavioral control from goal-directed actions

to habits (Packard and McGaugh, 1996). Furthermore, local injections of amphetamine into the NAc, but not the DS, have been shown to potentiate conditioned reinforcement (Taylor and Robbins, 1984).

1.4 DOPAMINE DYSFUNCTION AND ADOLESCENT VULNERABILITY

Numerous studies have shown that adolescents exhibit different pharmacological sensitivity to drugs interacting with the mesocorticolimbic and nigrostriatal dopamine systems. Previous studies have shown that adolescent rats are less sensitive than both juvenile and adult animals to the locomotor activation of psychostimulant drugs such as cocaine and amphetamine (Spear and Brake, 1983; Bolanos et al., 1998; Laviola et al., 1999). Adolescents also show reduced amphetamine-induced taste aversion compared to juvenile and adult animals (Infurna and Spear, 1979). Evidence suggests that this reduced sensitivity to amphetamine in adolescence is not due to age-related alteration in drug levels in the brain (Spear and Brake, 1983). Furthermore, rats exposed to low doses of the stimulant methylphenidate during adolescence have been shown to increase cocaine self-administration in adulthood, suggesting that early age stimulant exposure may lead to an increase in the incentive value of low reinforcers later in life (Brandon et al., 2001). Repeated low-dose exposure to methylphenidate in adolescent rats has also been shown to differentially change ventral tegmental area dopamine neuronal activity, depending on the length of treatment withdrawal (Brandon et al., 2003). Conversely, adolescent rats have been shown to be more sensitive to the cataleptic behavioral effects of neuroleptics, such as the dopamine receptor antagonist haloperidol (Spear et al., 1980; Spear and Brake, 1983; Teicher et al., 1993).

In addition to motor behaviors, dopamine levels are highly responsive to salient stimuli with both appetitive and aversive valence. Several lines of work indicate that dopamine neurons and terminal release of dopamine are activated by rewarding stimuli (Wise, 2005; Schultz, 2007). Various food reward conditions also increase dopamine release in the NAc (Hernandez and Hoebel, 1988; Wilson et al., 1995; Cousins et al., 1999). In addition to reward sensitivity, the mesocorticolimbic and nigrostriatal dopamine systems are sensitive to stress. Glucocorticoid receptors can be found on dopaminergic neurons in the rodent ventral tegmental area and the substantia nigra, as well as the dopamine terminals of the NAc and prefrontal cortex (Harfstrand et al., 1986; Diorio et al., 1993). As such, elevations of corticosterone secretion due to stress leads to the activation of dopamine transmission, where corticosterone treatment causes increases in extracellular dopamine levels in the NAc and prefrontal cortex, and removing the adrenal glands leads to decreases in extracellular dopamine levels in these regions (Imperato, 1989; Piazza et al., 1996b; Piazza et al., 1996a). Additionally, rodent studies have shown that acute external stress increases dopamine release in regions that are involved in the modulation of reward, such as the prefrontal cortex, amygdala, NAc and DS (Abercrombie et al., 1989; Imperato, 1989; Finlay and Zigmond, 1997; Inglis and Moghaddam, 1999), as well as increases in dopamine turnover in these regions (Deutch and Roth, 1990; Finlay and Zigmond, 1997).

Thus, dopamine dysfunction has been implicated in abnormal processing of both stressful and rewarding stimuli. Although the reward system has not been traditionally implicated in the pathophysiology of schizophrenia, it has been the topic of several recent reports suggesting that abnormal functioning of the reward system may be critical for formation of context-inappropriate associations in schizophrenia patients and may predispose them to psychosis (Jensen et al., 2008). The “dopamine hypothesis” of schizophrenia initially made the assertion that dopamine

dysfunction underlies the behavioral abnormalities of the disorder. This hypothesis is supported by evidence showing that anti-psychotic medications alleviate the psychotic (positive) symptoms of schizophrenia. Most clinically effective anti-psychotic treatments are dopamine receptor antagonists (Baldessarini and Tarazi, 1996), though these treatments are less effective on negative symptoms, which include anhedonia, social withdrawal, and affective flattening (Kirkpatrick et al., 2001). There have been imaging studies providing further evidence to support the dopamine hypothesis, where it has been reported that low doses of D-amphetamine cause increases in dopamine efflux in the brains of patients with schizophrenia compared to control subjects (Breier et al., 1997; Abi-Dargham et al., 1998; Laruelle et al., 1999). Depleting dopamine in the brain through the administration of the dopamine synthesis inhibitor alpha-methyl-tyrosine in patients with schizophrenia revealed significant increases in dopamine D2 receptors. This suggested that schizophrenics have higher levels of dopamine, causing increased occupancy of dopamine D2 receptors at baseline, which in turn obscured the difference in receptor densities that exists (Abi-Dargham et al., 2000). Furthermore, recent PET studies have suggested that patients with schizophrenia have elevated dopamine synthesis in the striatum, and that increased striatal dopamine levels in patients with prodromal symptoms of schizophrenia is correlated with the severity of prodromal psychopathologic and neuropsychological impairments (Howes et al., 2009; Nozaki et al., 2009). Overall, these studies suggest that the psychotic symptoms of schizophrenia are associated with increases in the synaptic release of dopamine, and these increased dopamine levels may cause sensitization to the actions of dopamine (Seeman et al., 2005).

Dysfunction of the mesocorticolimbic and nigrostriatal dopamine systems has also been implicated in attention hyper deficit disorder (ADHD). In recent years, there has been an increase

in the use of dopamine-activating psychostimulants, such as amphetamine and methylphenidate (Ritalin¹), to treat children and adolescents with ADHD (Shaywitz et al., 2001). Methylphenidate is a potent inhibitor of the DAT that potentiates the actions of synaptically released dopamine (Kuczenski and Segal, 1997; Volkow et al., 2001). Studies in rats have shown that therapeutic doses of methylphenidate, known to enhance cognition and reduce locomotor activity, actually increase dopamine in the prefrontal cortex (Berridge et al., 2006). Studies have also shown that increases in dopamine in the ventral striatum of adults diagnosed with ADHD were associated with reduced symptoms of inattention with clinical treatment of methylphenidate (Volkow et al., 2012). Moreover, D1 antagonists block the enhancing effects of methylphenidate, and therapeutic doses of methylphenidate have small effects on dopamine release in the NAc, which thereby cause therapeutic doses (taken orally) to have little abuse potential and suggests that they may be used to protect against drug abuse (Volkow et al., 2002; Svetlov et al., 2007). Imaging studies have suggested that an excess of DAT causes a hypodopaminergic state in patients with ADHD (Spencer et al., 2005), and elevated DAT levels have been reported in the striatum of ADHD patients compared to controls (Krause et al., 2000). There are, however, other studies that refute these claims, providing data that show no differences in DAT (van Dyck et al., 2002) or decreases in DAT density in patients with ADHD (Jucaite et al., 2005). Lastly, in addition to studies of stimulant effects on patients with ADHD, there have been several genetic studies that have identified candidate risk factor genes, which include genes that encode the dopamine D4 and D5 receptors, and the dopamine and serotonin transporters (Bobb et al., 2005; Faraone et al., 2005).

1.5 USING AMPHETAMINE TO STUDY DOPAMINE RELEASE

As has been mentioned in previous sections, amphetamine is often used experimentally to examine the function of dopamine and dopamine-modulated behaviors. However, amphetamine exerts diverse physiological and behavioral effects as a result of its mechanisms of action, and may be a useful for gaining further insight into the functions of various neurotransmitter systems and related.

Amphetamine is a stimulant drug whose actions have been used for the treatment of disorders such as narcolepsy (Prinzmetal and Bloomberg, 1935), obesity (Ersner, 1940), and ADHD (Spencer et al., 1996). In fact, in the United States, amphetamine is the most commonly prescribed treatment for juvenile attention deficit disorder (Sulzer et al., 2005), though the mechanism for its efficacy is unclear but is thought to be due to the enhancement of tonic dopaminergic signaling (Knutson et al., 2004). Amphetamine's use for treating obesity and narcolepsy arises from the fact that amphetamine has been shown to suppress food intake (Cole, 1963) and has been used by the military to promote alertness, especially for long missions (Caldwell et al., 2003; Sulzer et al., 2005), respectively. Amphetamine has also been shown to have profound effects on numerous other behaviors such as aggression (Consolo et al., 1965; Welch and Welch, 1966), sexual behavior (Bignami, 1966), learning and memory (Bohdanecky and Jarvik, 1967; Carr and White, 1984), classical conditioning (Evangelista et al., 1970), and operant behavior (Clark and Steele, 1966; Laties and Weiss, 1966).

Aside from its therapeutic uses, amphetamine is also a drug that leads to habitual use and abuse, due to it causing euphoria and stimulation in humans (Seiden et al., 1993). The administration of large doses of amphetamine in humans has been shown to cause psychosis (Angrist et al., 1971; Ellinwood et al., 1973; Angrist et al., 1974). At low doses, amphetamine

increases hyperlocomotor activity and species-specific stereotypies at higher doses (Randrup et al., 1963). More specifically, in rats, low doses of amphetamine (1.0 mg/kg) induce locomotion, sniffing, and rearing, and at higher doses (5.0 mg/kg) induces focused intense licking and gnawing (Randrup et al., 1963; Scheel-Kruger, 1971; Gulley et al., 2004).

The physiologic and behavioral effects of amphetamine were discovered to be due largely to amphetamine's interaction with various catecholamine systems – such as dopamine and norepinephrine – in the brain and peripheral autonomic nervous system (Axelrod, 1972; Groves and Rebec, 1976). Amphetamine is thought to increase extracellular catecholamine concentrations by causing release from presynaptic nerve terminals in addition to inhibiting reuptake by blocking catecholamine uptake (Glowinski et al., 1966; Heikkila et al., 1975; Kuczenski, 1983; Seiden et al., 1993). The reuptake of catecholamines back into the nerve terminal is thought to be the primary mode of inactivation (Iversen, 1974). Amphetamine has also been shown to increase the release of serotonin and acetylcholine (Groves and Rebec, 1976; Pum et al., 2007). As such, drugs that affect serotonergic and cholinergic neurotransmission also affect amphetamine-induced locomotor responses and stereotypies (Armfred and Randrup, 1968; Weiner et al., 1973; Consolo et al., 1974; Goetz and Klawans, 1974; McGeer et al., 1974). The specific mechanisms of action of amphetamine are complex and somewhat controversial, but thought to involve the release, reuptake, and enzymatic inactivation of catecholamines (Seiden et al., 1993).

Amphetamine is thought to increase dopamine levels through impulse-independent (calcium independent) transporter-mediated release, known as reverse transport (Sulzer et al., 1995). Amphetamine is both able to cross plasma membranes via lipophilic diffusion, as well as serve as a substrate for catecholamine transporters (Liang and Rutledge, 1982; Zaczek et al.,

1991a; Zaczek et al., 1991b; Seiden et al., 1993). Once inside the cells, amphetamine can displace dopamine from vesicles into the cytosol and be released through reverse transport by DAT (Sulzer and Rayport, 1990; Floor and Meng, 1996). Amphetamine's action through vesicular depletion and reverse transport are controversial in the literature, and different models have been proposed to explain amphetamine-induced dopamine release. For instance, the exchange diffusion model suggests that as amphetamine is transported into the cell down its concentration gradient, it accelerates the efflux dopamine out of the cell down its concentration gradient (Fischer and Cho, 1979). Once dopamine has been transported out, the high concentration of amphetamine prevents the reuptake of dopamine (Fischer and Cho, 1979). As a substrate, amphetamine increases the number of inward-facing transporter binding sites, increasing the rate of reverse transport. In contrast, the weak base model suggests that amphetamines redistribute dopamine from synaptic vesicles to the cytosol by collapsing the vesicular pH gradient, decreasing the energy that supports neurotransmitter accumulation, thereby increasing the availability of the catecholamine for reverse transport without requiring a mobile site on the transporter (Sulzer and Rayport, 1990; Sulzer et al., 1993). Vesicular dopamine redistributed to the cytosol, in turn, can be rapidly released by reverse transport.

Amphetamine-induced release of dopamine can be blocked by uptake inhibitors such as nomifensine (Raiteri et al., 1979), while blocking impulse-dependent release via action potentials with the drug tetrodotoxin, has no effect on increases in extracellular dopamine induced by amphetamine (Nomikos et al., 1990). These previous studies support the notion that amphetamine is a dopamine releaser that acts independent of cell firing. Moreover, amphetamine-induced behaviors are inhibited by blocking dopamine synthesis with the tyrosine hydroxylase (TH) inhibitor, alpha-methyl-para-tyrosine, but not by reserpine, which blocks

transport of amines into vesicles, suggesting the effects of amphetamine are mediated by a newly synthesized pool of dopamine in the cytoplasm (Randrup et al., 1963; Scheel-Kruger, 1971; Braestrup, 1977). To this effect, low doses of amphetamine are thought to release cytoplasmic stores of dopamine while higher doses of amphetamine are thought to release both cytoplasmic and vesicular pools of dopamine (Liang and Rutledge, 1982; Zetterstrom et al., 1986).

Though amphetamine has been widely used to understand dopamine function in adults (as noted above), little is known about the impact of amphetamine on dopamine neurotransmission in adolescence. Previous studies have shown that adolescent rodents exhibit smaller behavioral activation (such as locomotion) in response to stimulant drugs compared to adults (Bolanos et al., 1998; Laviola et al., 1999). These findings suggest that dopamine, which mediates locomotor activity and stereotypies, may be differently regulated in adolescent and adults rats, and amphetamine may be a useful tool for investigating these developmental differences.

1.6 PURPOSE OF DISSERTATION

Previous studies show that the mesocorticolimbic and nigrostriatal dopamine systems undergo major developmental changes during adolescence. We believe these changes in adolescents may be differently modulated in striatal subregions compared to adults. Remodeling of the mesocorticolimbic and nigrostriatal dopamine systems in the striatum during the adolescent period has been implicated in the symptomatic onset of many psychiatric illnesses, including schizophrenia and drug addiction. The neuronal mechanisms that contribute to such vulnerability

during adolescence are not well understood and little is known about the dynamics of the dopamine system in behaviorally relevant contexts during adolescence.

The purpose of this dissertation was to use a rodent model to gain a better understanding of the function of dopamine neurotransmission during adolescence by characterizing the mechanisms that regulate dopamine in two functionally distinct striatal subregions that are heavily innervated by midbrain dopaminergic cells, the NAc and the DS. These regions are integral to behaviors that are highly relevant to adolescent vulnerability to psychiatric illnesses. We utilized microdialysis to measure extracellular dopamine levels in the NAc and the DS in awake adolescent and adult rats while simultaneously measuring locomotor and stereotypical motor behaviors. The psychostimulant amphetamine was used to examine the mechanisms of dopamine release since this drug is known to cause increases in extracellular dopamine by disrupting the function of DAT, important for dopamine reuptake, and has also been shown to have differential behavioral effects in adolescence and adulthood in rodents.

Upon discovering region-specific differences in dopamine neurotransmission in response to amphetamine administration in adolescents compared to adults (**Chapter 2**), we sought to further elucidate the mechanisms behind these differences, by examining the protein density and function of dopamine transporters – DAT and the vesicular monoamine transporter-2 (VMAT2) – involved in dopamine reuptake and packaging (**Chapter 3**), as well as protein expression and function of the rate limiting enzyme involved in dopamine synthesis – TH (**Chapter 4**). To examine the density of these specific dopamine-related proteins, we used Western blot analysis to measure protein expression levels. Function of DAT was assessed using the DAT-specific blocker nomifensine, which was infused directly into the NAc and the DS. To assess function of

TH, the TH blocker alpha-methyl-DL-tyrosine was administered systemically while assessing amphetamine-induced behavior.

In **Chapter 5**, these mechanisms of dopamine availability are revisited in the context of the findings from this dissertation, and a hypothesis of reduced dopamine neurotransmission in the adolescent DS as a mechanism for adolescent vulnerability to psychiatric illness is introduced, along with suggestions for future work that might further delineate the mechanisms of adolescent behavioral and psychiatric risks.

2.0 THE EFFECTS OF AMPHETAMINE ON BEHAVIOR AND STRIATAL DOPAMINE NEUROTRANSMISSION IN ADOLESCENT AND ADULT RATS

2.1 INTRODUCTION

Adolescence is a transitional period from childhood to adulthood, which overlaps with puberty (Pickles et al., 1998). During adolescence, the brain undergoes major remodeling (Huttenlocher, 1984; Rakic et al., 1994; Andersen et al., 2000), which may account for the behavioral changes associated with this developmental period. These characteristic behavioral changes include increased exploration, social interaction, risk-taking, and sensation- and novelty-seeking (Baumrind, 1987; Primus and Kellogg, 1989; Adriani et al., 1998). These behaviors, albeit a component of normal maturation processes, can have negative consequences that lead to increased injuries and mortality rates (Lerner and Galambos, 1998; Spear, 2000). Furthermore, it is during adolescence that the symptomatic onset of many psychiatric illnesses, such as schizophrenia, occur (Weinberger, 1987). The neuronal mechanisms that contribute to psychiatric illness vulnerability during adolescence are not well understood, although the dopamine system has been implicated (Thompson et al., 2004; Carlsson and Carlsson, 2006; Tseng et al., 2007; Davey et al., 2008; Nemoda et al., 2011).

Dopamine neurotransmission plays a central role in modulating learning, stress reactivity, cognition, goal-directed behaviors, and reward processing (Robbins, 1997; Goldman-Rakic,

1998; Kelley, 2004a; Wise, 2005). Dysfunction of the mesocorticolimbic and nigrostriatal dopamine systems has been associated with schizophrenia (Carlsson and Carlsson, 2006), mood (Schildkraut, 1965) and addictive disorders (Koob, 1992), symptoms of which are manifested during early or late adolescence (Weinberger, 1987; Volkmar, 1996; Pine et al., 2002; Carlson and Meyer, 2006). The mesocorticolimbic and nigrostriatal dopamine systems experience significant remodeling during adolescence (Kelley et al., 2004; Fareri et al., 2008). These include changes in dopamine turnover, synthesis, and reuptake (Teicher et al., 1993; Andersen et al., 1997).

The purpose of the present study was to gain a better understanding of the dynamics of dopamine neurotransmission in behaving adolescents using a rodent model. We focused on two functionally distinct striatal subregions that are heavily innervated by midbrain dopaminergic cells: the nucleus accumbens (NAc) and the dorsal striatum (DS). These regions are integral to behaviors that are highly relevant to adolescent vulnerability to psychiatric illnesses. The NAc is implicated in motivation and reward processing (Mogenson et al., 1980; Robbins and Everitt, 1996; Kelley, 2004b), and the DS is involved in learning, action selection, and habit formation (Yin et al., 2006; Yin and Knowlton, 2006; Balleine et al., 2009). Recent studies have described age related differences in the reactivity of neurons in these two regions to salient events (Sturman and Moghaddam, 2011a). We used amphetamine as a tool to activate dopamine release in the NAc and the DS in behaving adolescent and adult rats. Amphetamine disrupts the function of the dopamine transporter (DAT), causing efflux of vesicular dopamine and newly synthesized dopamine (Sulzer et al., 1995; Jones et al., 1998). Amphetamine-like stimulants are commonly prescribed to human youths with disorders such as ADHD for therapeutic use (Kutcher et al., 2004), though little is known about their impact on dopamine neurotransmission at this age.

These stimulants produce smaller behavioral activation in adolescent rodents compared to adults (Bolanos et al., 1998; Laviola et al., 1999). Behaviors such as locomotor activity and stereotypy are dependent on the functional integrity of NAc and DS dopamine, and can be used as an indirect measure of the functional consequences of increased dopamine neurotransmission. Thus, examining the impact of amphetamine may be important for revealing developmental differences on some of the basic mechanisms that govern dopamine release in behaviorally active animals. This experiment will also generate relevant correlations between dopamine release alterations in response to amphetamine and dopamine-mediated behavioral changes in adolescent and adult rats.

2.2 METHODS

2.2.1 Subjects

Male Sprague-Dawley rats (Harlan, Frederick, MD) were used for this experiment. Pre-adolescent juvenile rats (postnatal days 21) and adults (postnatal days 60-70) were received at least two weeks prior to experimentation. This two week period allowed animals to habituate to the housing, and during this time juvenile rats reached adolescence. By the time of experimentation, adolescent rats used were considered between postnatal days 34-38 (~130-150 g) and mature adults between postnatal days 70-80 (~ 330-400 g). All subjects were pair housed in a 12 hr light/dark cycle (lights on at 19:00). Experiments were conducted during the animals' dark (active) phase. Animals had ad libitum access to water and rat chow. All animal

experiments were performed in accordance with and approval by the ethical guidelines of the Institutional Animal Care and Use Committee at the University of Pittsburgh.

2.2.2 Surgical Procedure

All surgical procedures were performed between the hours 08:00 and 10:00. For microdialysis probe implantation, rats were anesthetized with isoflurane, placed on a heating pad, and had their heads secured in a stereotaxic apparatus using blunt ear bars. A small incision was made in the skin over the skull and the area irrigated with lidocaine. Animals were implanted bilaterally with dialysis probes, with one probe in the DS (for adolescents: AP +0.7 from bregma, ML +2.0 from bregma, and DV -5.0 from skull; and for adults: AP +1.6, ML +2.2, DV -6.0 from skull) and one in the NAc (for adolescents: AP +1.0 from bregma, ML +1.2 from bregma, and DV -7.0 from skull; and for adults: AP +1.2, ML +1.1, DV -8.4 from skull). Coordinates for the dialysis probes were according to the atlas of Paxinos and Watson (Paxinos and Watson, 1998). Dialysis probes were constructed of Hospal AN69 polyacrylonitrile dialysis tubing (Renal Care, Lakewood, CO), with an outer diameter of 330 μm and an exposed tip of 1.0 mm for adolescent rats and 2.0 mm for adult rats. The smaller exposed tip for adolescents was used to account for smaller brain size at this age. Dialysis probes were secured in a head cap consisting of dental acrylic and were fastened to the skull using two skull screws. Immediately following surgery, the probes were connected to a liquid swivel balance arm assembly, and the rats were singly housed in a clear polycarbonate cage with fresh bedding. The cages were situated in a temperature and humidity controlled room on the same light/dark schedule (lights on at 19:00) used in the vivarium. Probes were connected to the liquid swivel balance arm assembly and the dialysis

probes were perfused at a rate of 1.5 $\mu\text{L}/\text{min}$ with a Ringer's solution (in mM: 145 NaCl, 2.7 KCl, 1.0 MgCl_2 , and 1.2 CaCl_2). Rats were given approximately 24 hr to recover from the surgery prior to the collection of microdialysis samples. This recovery time is accepted as a sufficient period of time for brain tissue to normalize after probe implantation (Krebs-Kraft et al., 2007). A 24 hr recovery period is also a sufficient period of time for the overall health of the animals to return to normal, as our laboratory has shown previously (Moghaddam and Gruen, 1991; Moghaddam and Bunney, 1993; Jedema and Moghaddam, 1994; Moghaddam and Bolinao, 1994). As such, animals used in the study did not show obvious signs of post-surgery distress, but instead groomed normally, ate food and drank water, and had no signs of bleeding or infection around the wound or head cap. Rats had ad libitum access to food and water during recovery, but not during the experiment.

2.2.3 Microdialysis

On the day of the experiment, rats remained in their home-cage for collection of samples (and behavioral testing). Probes were perfused with Ringer's solution only (as above) at a flow rate of 2.0 $\mu\text{L}/\text{min}$ during sample collection. Dialysis experiments started between 09:00 and 11:00. Dialysate samples were collected every 20 min for the duration of each experiment and immediately injected into a high-performance liquid chromatography system with electrochemical detection for the analysis of dopamine, with a detection limit of approximately 5 fmol, as described previously (Adams and Moghaddam, 1998). At least 100 min of baseline samples were collected before pharmacological manipulation. Implanted animals were used only once for experimentation. In cases where there were technical issues with one of the two

bilaterally implanted probes, data from the working probe (and the corresponding behavior data) were included in the analysis.

2.2.4 Behavior

As mentioned above, rats remained in their home-cage for the collection behavioral data (and sample collection). Motor behavior during the microdialysis experiments was quantified as described before (Pehrson and Moghaddam, 2010). Briefly, stainless-steel frames with an array of 32 infrared beams arranged in a 16 x 16 formation around the outside of the animals' cages (Hamilton-Kinder, LLC, Poway, CA). For experiments, beam breaks were monitored over the entire course of sample collection by the Kinder Scientific MotorMonitor program. Ambulations (locomotor movements) were defined as movements in which the animal moved its entire body (i.e., a new beam was broken while a previously broken beam was released) and fine movements (stereotyped movements) were defined as smaller movements where a new beam is broken without releasing a previously broken beam. Motor activity data were pooled into 20 min bins, corresponding to the collection of dialysis samples, and expressed in terms of the number of XY-axis ambulations and fine movements.

2.2.5 Drugs

Once a stable dopamine baseline was reached, animals were given a systemic (i.p.) administration of 0.9% saline (1 ml/kg) or amphetamine (1.0 mg/kg; Sigma-Aldrich). Amphetamine was dissolved in 0.9% saline and frozen for a maximum of 1 week prior to use.

The dose of amphetamine used in this study was chosen based on previous studies that demonstrate that low doses of amphetamine (between 0.5 and 1.5 mg/kg) increase locomotion and some stereotyped movements (i.e. sniffing) in both adolescents and adults (Bolanos et al., 1998; Gulley et al., 2004).

2.2.6 Histology

After completion of microdialysis experiments, rats were anesthetized with chloral hydrate (400 mg/kg) and perfused intracardially with 0.9% saline. Brains were removed and stored in a 10% Formalin solution. Serial coronal 250 μ m sections were taken through the regions of interest and then mounted on glass slides and stained with cresyl violet. Stained sections were then evaluated for accuracy of probe placement. Only data from placements within the brain region of interest were used for further analysis (Figure 2-1).

2.2.7 Data analysis

Microdialysis and behavior data were analyzed as described previously (Adams and Moghaddam, 1998; Pehrson and Moghaddam, 2010). The microdialysis data were expressed as a percentage of baseline dopamine efflux (mean \pm sem), where baseline was defined as mean of the three baseline values obtained immediately before a pharmacological manipulation. Baseline means, reported as fmol/ul, for each group were analyzed using student t-tests. Locomotor activity and stereotypy data were separately expressed as the number of infrared beam breaks within a 20 min bin, to correspond with the 20 min microdialysis sample collection bins (mean \pm sem). Statistical analysis of these dependent measures was conducted using two-factor repeated

measures ANOVAs, both with age or drug treatment as the between-subjects factors and time as the within-subjects factor. Significance was set at $p < 0.05$. Five-eight subjects per group were required based on an estimated effect size (considering previous work in our laboratory). All animals used for dopamine analysis in either the NAc or the DS or were combined for the behavior analysis.

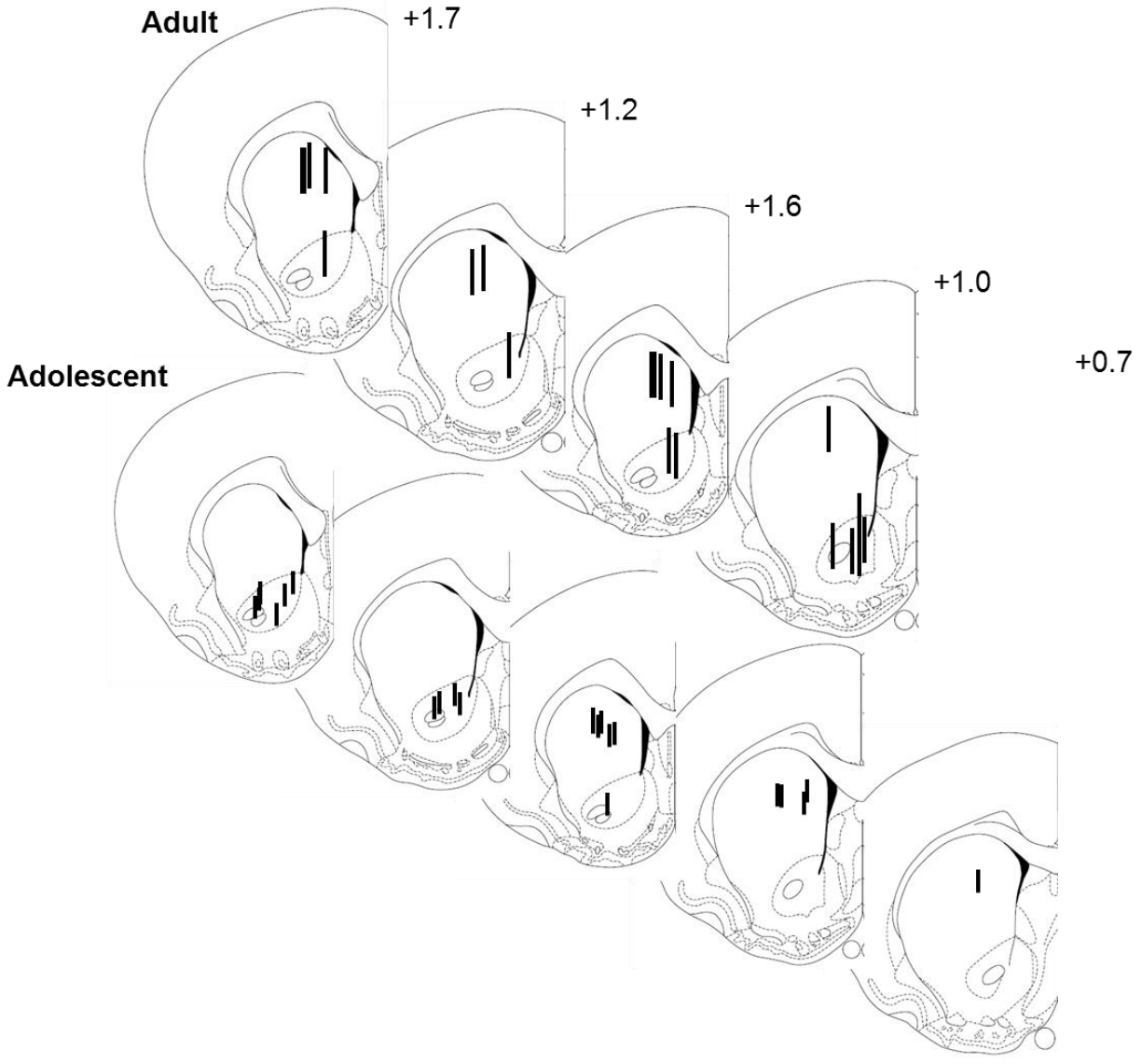


Figure 2-1 Microdialysis probe placements

Microdialysis probe placement for rats included in the study. Probes were placed bilaterally in the NAC and DS of adolescent and adult rats. Black bars represent the approximate location of probe placements, overlaid on standard adult rat atlas images (Paxinos and Watson, 1998). The size of the black bars represents the approximate size of the exposed dialysis tip: 1.0 mm for adolescents, 2.0 mm for adults. Numbers represent distance (in millimeters) anterior to bregma for adult animals.

2.3 RESULTS

2.3.1 Dopamine Efflux

The baseline mean (in fmol/ μ l) for the NAc was 0.48 ± 0.10 in adolescents ($n = 10$) and 0.60 ± 0.14 in adults ($n = 11$), with no significant difference between age groups ($t_{19} = -0.693$, n.s.). The baseline mean (in fmol/ μ l) for the DS was 1.13 ± 0.33 in adolescents ($n = 11$) and 0.95 ± 0.29 in adults ($n = 12$), with no significant difference between age groups ($t_{21} = 0.687$, n.s.). Dopamine efflux was measured in adolescent and adult rats in response to systemic amphetamine (1.0 mg/kg, i.p.). Amphetamine caused significant increases in NAc dopamine levels compared to saline controls in both adolescent (treatment \times age, $F(11, 88) = 8.267$, $p < 0.0001$) and adult (treatment \times age, $F(11, 99) = 22.16$, $p < 0.0001$) rats (Figure 2-2A). Amphetamine also caused significant increases in the DS dopamine levels compared to saline controls in both adolescent (treatment \times age, $F(11, 110) = 5.646$, $p < 0.0001$) and adult (treatment \times age, $F(11, 88) = 36.70$, $p < 0.0001$) rats. Amphetamine had similar effects on increases in dopamine efflux across age groups in the NAc (time \times age interaction, $F(11, 110) = 1.25$, n.s.) (Figure 2-2A). However, amphetamine increased dopamine levels significantly less in the DS of adolescent rats versus adults (time \times age interaction, $F(11, 99) = 27.30$, $p < 0.001$) (Figure 2-2B). In control animals, the saline vehicle injection had no significant effect on dopamine efflux in either the NAc (time \times age interaction, $F(11, 77) = 0.811$, n.s.) (Figure 2-2A) or the DS (time \times age interaction, $F(11, 88) = 0.64$, n.s.) (Figure 2-2D) of either age group.

2.3.2 Behavior

Age-related behavioral differences were also measured simultaneously in animals used for microdialysis analysis in response to amphetamine. Systemic amphetamine administration caused significant increases in locomotor behavior in adolescent (treatment \times age, $F(11, 165) = 1.97$, $p < 0.05$) and adult (treatment \times age, $F(11, 132) = 5.79$, $p < 0.0001$) rats compared to saline controls (Figure 2-2C). Amphetamine also produced increased levels of stereotypies in both adolescents (treatment \times age, $F(11, 165) = 3.59$, $p < 0.0001$) and adults (treatment \times age, $F(11, 132) = 5.19$, $p < 0.0001$) compared to saline controls. Amphetamine produced similar changes in locomotor behavior in adolescents and adults (time \times age interaction, $F(11, 77) = 0.29$, n.s.) (Figure 2-2C). However, amphetamine caused lower increases in fine motor movements (time \times age interaction, $F(11, 66) = 3.40$, $p=0.001$) (Figure 2-2D) in adolescents compared to adults. Administration of the saline vehicle in control animals showed no effect on either locomotion (time \times age interaction, $F(11, 77) = 1.65$, n.s.) (Figure 2-2C) or fine movements between groups (time \times age interaction, $F(11, 88) = 1.68$, n.s.) (Figure 2-2D), though adolescent rats had a transient response in fine movements right after the injection.

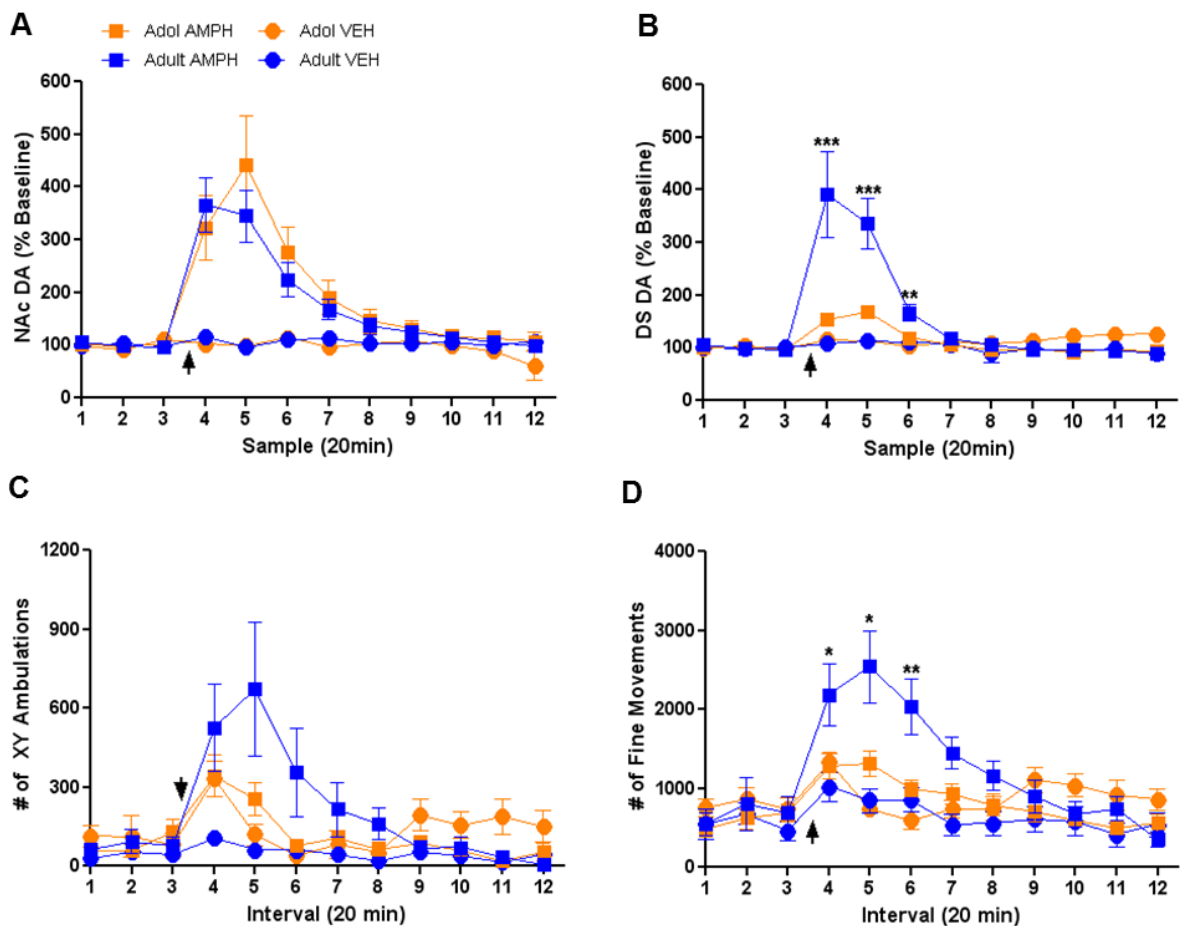


Figure 2-2 Dopamine efflux and behavior in response to amphetamine

Amphetamine caused lower increases in DS dopamine and fine movements in adolescent rats compared to adults. Adolescent (orange) and adult (blue) rats were given an i.p. injection of 1.0 mg/kg amphetamine (squares) or 0.9% saline vehicle (circles). Dopamine efflux was measured in the A) NAc (adolescent amphetamine group, n=6; adult amphetamine group, n=6; adolescent vehicle group, n=5; adult vehicle group, n=5) and B) DS (adolescent amphetamine group, n=6; adult amphetamine group, n=5; adolescent vehicle group, n=6; adult vehicle group, n=5). Motor behavior was also measured for each animal, expressed as C) ambulations (adolescent amphetamine group, n=8; adult amphetamine group, n=6; adolescent vehicle group, n=9; adult vehicle group, n=9) and D) fine movements (adolescent amphetamine group, n=8; adult amphetamine group, n=6; adolescent vehicle group, n=9; adult vehicle group, n=9). In each graph, baseline dopamine efflux and motor behavior was measured prior to injection, represented by the first three time points displayed. Black arrows denote time of injection. Asterisk(s) denotes significant difference between amphetamine-treated age groups at a given time point (* p<0.05, ** p<0.01, ***p<0.001).

2.4 DISCUSSION

In the present study we found that, compared to adults, adolescent rats are less sensitive to the motor effects of amphetamine, and that dopamine efflux in the DS of adolescent rats is lower than adults. In contrast to the DS, we find very similar effects of amphetamine on dopamine efflux in the NAc in adolescence and adulthood. Our finding that there is a region-specific reduction in amphetamine-activated dopamine efflux as a function of age suggests that there are different mechanisms modulating the availability of dopamine in adolescent rats than in adults.

Our finding of age-related differences in sensitivity to amphetamine is consistent with previous behavior studies showing that, in general, adolescent rats are less sensitive than adults to the motor activating effects of psychostimulants such as amphetamine and cocaine (Bolanos et al., 1998; Laviola et al., 1999). The unconditioned behavioral responses to psychostimulants depend on dopamine function (Beninger, 1983). Decreasing or depleting dopamine, by blocking dopamine receptors or through lesions of dopamine cells with 6-OHDA produces hypolocomotion, while drugs that enhance dopamine neurotransmission, such as amphetamine and cocaine, produce hyperlocomotion and stereotypy (Beninger, 1983). There is a dorsal-ventral relationship in the striatum related to this effect in that stereotypy and fine movements are attributed to DS dopamine activation, whereas hyperlocomotion is attributed to dopamine activation in the NAc (Beninger, 1983; Delfs et al., 1990). Despite the observation that both amphetamine-induced hyperlocomotion and stereotypy were reduced in adolescents, we found a reduction in dopamine efflux that was selective to the DS. A lack of difference in dopamine efflux in the NAc suggests that dopamine-mediated behaviors such as locomotion that are

dependent on the functional integrity of this region in adults may be differently regulated in adolescents. This finding is similar to that observed by Frantz and colleagues, in that the extracellular levels of dopamine in the NAc were similar between adolescent and adult rats under basal conditions and after an acute systemic (i.p.) injection of cocaine (Frantz et al., 2007). However, this group found that adolescents have lower locomotor activity compared to adults, and argued that these age differences in locomotor effects are not mediated by NAc dopamine (Frantz et al., 2007). Our findings suggest that a low dose of amphetamine has similar behavioral and dopamine efflux effects in both adolescents and adults. These differences in findings may be due to differences in the drugs used (cocaine versus amphetamine), the rat strains used (Wistar versus Sprague-Dawley), or the time of adolescence studied (early adolescence, postnatal days 29-31 versus middle adolescence, postnatal days 34-38).

Our data indicate that at least some of the observed reduced behavioral effects of stimulants in adolescents may be attributed to hypoactive dopamine neurotransmission in the DS. The NAc and the DS are functionally and structurally distinct. The DS is involved in voluntary motor control, instrumental learning and habit formation (Joel and Weiner, 2000), while the NAc is involved in reward and reinforced behaviors (Mogenson et al., 1980; Kelley, 2004b). Infusing selective dopamine D1 or D2 receptor antagonists into the NAc has been shown to potently suppress locomotor responses associated with the motivational state of hunger (Baldo et al., 2002). Depleting dopamine using 6-OHDA lesions in the NAc has been shown to suppress instrumental responding reinforced with food (Heffner and Seiden, 1983). Inactivating the DS using lidocaine, on the other hand, has been shown to block the ability of animals to shift behavioral control from goal-directed actions to habits (Packard and McGaugh, 1996). Furthermore, local infusions of amphetamine into the NAc, but not the DS, have been shown to

potentiate conditioned reinforcement (Taylor and Robbins, 1984). At the structural level, the NAc and the DS are largely innervated by midbrain dopamine neurons originating from the ventral tegmental area and the substantia nigra pars compacta, respectively (Gerfen et al., 1987). In addition to the different dopaminergic inputs, there are also different cortical inputs to these regions. Cortical glutamatergic afferents innervate the striatum in a topographic gradient, where the dorsolateral striatum predominantly receives inputs from sensory and motor cortical areas, and the ventromedial striatum (including the NAc) largely receives inputs from the medial prefrontal cortex, amygdala and hippocampus (Groenewegen and Berendse, 1994; Voorn et al., 2004). The different cortical and midbrain influence over the DS compared to the NAc may contribute to the difference in dopamine neurotransmission observed in adolescent and adult rats in response to amphetamine.

The age-specific behavioral differences observed in response to pharmacological dopamine releasers such as amphetamine may be the result of underlying developmental neural changes in the dopamine circuitry during adolescence. Several lines of evidence indicate that the mesolimbic and nigrostriatal dopamine systems undergo major remodeling during adolescence. Studies have shown that adolescent rodents maintain lower basal extracellular levels of dopamine in the striatum than adults (though we do not observe this in our findings), accompanied by dopamine receptor overproduction and pruning in striatal regions (Gelbard et al., 1989; Andersen et al., 1997; Tarazi et al., 1998a; Badanich et al., 2006; Cao et al., 2007). Dopamine receptor binding shifts from being mildly inhibitory to strongly excitatory during adolescence into early adulthood (O'Donnell, 2010). Firing rates of midbrain dopamine neurons have also been shown to peak during adolescence before decreasing over time into adulthood (McCutcheon and Marinelli, 2009). The developmental remodeling of the striatum is of

particular interest because of its role in motor behaviors and decision-making processes. Previous models had implicated the limbic system, in particular the amygdala and the NAc, in functional immaturities of the adolescent brain. However, our present data with the mesolimbic and nigrostriatal dopamine systems, as well as a recent electrophysiology study from our laboratory, did not find functional differences in the NAc of adolescents as compared to adults. Instead, we find reduced dopamine neurotransmission in the DS, a region that is critically involved in learning, habit formation and action selections (Jog et al., 1999).

Dopamine availability relies on reuptake by DAT, but also on synthesis mediated by tyrosine hydroxylase (TH), and packaging through the vesicular monoamine transporter-2 (VMAT2). Based on our current findings, it appears that the dopamine availability in the DS is different in adolescents than in adults, and amphetamine may be affecting one or more of the mechanisms involved in dopamine availability throughout development. Based on previous studies, rat extracellular dopamine concentrations are lower in adolescence than adulthood in the NAc and the DS (Andersen and Gazzara, 1993), and DAT expression in rats has been shown to be at adult levels by adolescence in the NAc and at adult levels prior to adolescence in the DS (Coulter et al., 1996). Furthermore, studies also show that young adolescent rats (P30) have decreased accumbal and striatal dopamine synthesis compared to adults (Teicher et al., 1993; Andersen et al., 1997). However, together these individual factors do not explain why amphetamine causes a lower percentage increase in striatal dopamine from baseline in adolescent rats. Amphetamine has been shown to inhibit the reuptake of dopamine and cause release of dopamine from presynaptic terminals, through both vesicular and cystolic stores of dopamine (Sulzer et al., 1995; Jones et al., 1998). Our finding that there are region-specific differences in striatal dopamine transmission in response to amphetamine could indicate that DAT-mediated

dopamine availability in the adolescent DS may contribute to its reduced behavioral effects. It is also possible that amphetamine may disturb the protein ratios involved in synthesis, packaging, and reuptake, thereby causing greater increases in dopamine release in the DS of adults compared to adolescents.

Together, these data suggest that dopamine availability in the DS of adolescent rats may be differentially regulated than adults. In **Chapters 3 and 4** we attempt to identify possible mechanisms that are involved in the reduced dopamine neurotransmission in the adolescent DS.

3.0 THE EFFECTS OF STRIATAL TRANSPORTERS IN ADOLESCENT AND ADULT RATS

3.1 INTRODUCTION

Adolescence is considered a transitional period from childhood to adulthood, which overlaps with puberty, a time of sexual maturity (Pickles et al., 1998). It is during adolescence that the developing brain undergoes many neural alterations, including a massive loss of axons and synapses throughout the neocortex (Huttenlocher, 1984; Arnsten et al., 1994; Andersen et al., 2000). Brain rates of glucose metabolism plateau in adolescence before declining to adult levels (Chugani et al., 1987). Moreover, the adolescent brain undergoes neurogenesis (He and Crews, 2007), axonal growth (Benes et al., 2000), apoptosis (Markham et al., 2007) and myelination (Benes et al., 1994). Collectively, this remodeling is thought to be a form of developmental plasticity that allows adolescents to mature into adulthood (Crews et al., 2007). At the same time, these changes may make the brain more vulnerable to risky behavior and disease (Spear, 2000).

The mesocorticolimbic and nigrostriatal dopamine systems, in particular, have been shown to undergo major remodeling throughout adolescence (Kelley et al., 2004; Fareri et al., 2008). Neural alterations in these systems include dopamine receptor overproduction and pruning, changes in dopamine turnover and synthesis, and changes in dopamine reuptake (Teicher et al., 1993; Baldessarini and Tarazi, 1996; Coulter et al., 1996; Andersen et al., 1997).

This remodeling may lead to dynamic changes in the responsivity of the mesocorticolimbic and nigrostriatal dopamine systems to external stimuli that may be responsible for some of the behavioral changes of adolescents. The developmental dysfunction of these dopamine systems, which plays a key role in stress reactivity, cognitive flexibility, goal-directed behaviors, and reward processing (Robbins, 1997; Goldman-Rakic, 1998; Kelley, 2004a; Wise, 2005), has been implicated in various psychiatric disorders such as schizophrenia (Carlsson and Carlsson, 2006), mood (Schildkraut, 1965) and addictive disorders (Koob, 1992).

We have previously shown that, compared to adults, dopamine efflux in the DS of adolescent rats is lower in response to amphetamine. However, there were no changes in dopamine efflux in the NAc between age groups. The purpose of this study was to examine the underlying mechanisms involved in these region-specific differences as a function of age. Amphetamine is known to disrupt the function of DAT, causing efflux of both vesicular dopamine and the newly synthesized, “releasable” pool of dopamine, and has also been shown to alter VMAT2 activity (Sulzer et al., 1995; Jones et al., 1998; Riddle et al., 2005). Therefore, we compared the protein expression of these monoamine transporters in the NAc and DS of adolescent and adult rats. Furthermore, we compared the baseline and amphetamine-induced dopamine effects of blocking the function of DAT using the uptake blocker nomifensine.

3.2 METHODS

3.2.1 Subjects

Male Sprague-Dawley rats (Harlan, Frederick, MD) were used for this experiment. Pre-adolescent juvenile rats (postnatal days 21) and adults (postnatal days 60-70) were received at least two weeks prior to experimentation. This two week period allowed animals to habituate to the housing, and juvenile rats reached adolescence. By the time of experimentation, adolescent rats were between postnatal days 34-38 and the adults were between postnatal days 70-80. All subjects were pair housed in a 12 hr light/dark cycle (lights on at 19:00). All experiments and dissections were conducted during the animals' dark (active) phase. Animals had ad libitum access to water and rat chow food. Experiments were performed in accordance with and approval by the ethical guidelines of the Institutional Animal Care and Use Committee at the University of Pittsburgh.

3.2.2 Western blot

Tissue dissection. All tissue dissections were conducted between 13:00 and 15:00. Adolescent (n=4) and adult (n=4) rats were anesthetized with chloral hydrate (400 mg/kg), decapitated, and their brains removed rapidly and immediately placed in an iced metal brain slicer matrix (Ted Pella, Inc). Using the metal matrix as a guide, 1 mm slices were made using razor blades. From these 1 mm slices, the NAc and DS tissue were free-hand dissected using a scalpel (see Figure 3-1). Dissected tissue for each region of each animal were stored separately in Eppendorf tubes and immediately frozen and stored at -80°C, until used. The dissected tissue was homogenized with a

Polytron homogenizer in buffer D (20 mM HEPES, 125 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, pH 7.6), containing protease inhibitors (Pierce). Triton X-100 was added to a final concentration of 1%, and the samples were incubated with rotation for 1 hr at 4°C. Samples were centrifuged twice at 4°C at 10,000 × g for 10 min. The supernatant was collected and measured for protein concentration using a spectrophotometer using a BCA reagent (Pierce).

Western blot analysis. Western blot analysis was performed as described previously (Egana et al., 2009; Cartier et al., 2010). Samples containing 20% sample buffer were separated by SDS-PAGE on 10% Tris-glycine polyacrylamide gels by loading 100 µg of protein per lane and transferred to nitrocellulose membranes using the Bio-Rad system. Nitrocellulose membranes were first blocked for 1 hr in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, 0.2% Tween 20, pH 7.5) containing 5% dry milk and then incubated with the indicated primary antibody for 1 hr in blocking buffer, washed three times for 10 min each, and incubated with a horseradish peroxidase-conjugated secondary antibody. Following all antibody incubations, membranes were washed three times with TBS buffer, and protein bands were visualized using the West Pico SuperSignal system (Pierce) and LabWorks software. Densitometric analyses of protein bands were accomplished using ImageJ NIH software. The protein contents of DAT and VMAT2 were expressed as a ratio of tubulin band densities to ensure that changes in protein levels were not due to sample loading variances. Primary antibodies against DAT MAB369 and VMAT2 AB1598P were from Millipore and primary antibody against the control protein α -tubulin was from Sigma-Aldrich. Secondary antibodies conjugated with horseradish peroxidase were Anti-Rat IgG (Jackson) for DAT, Protein A (GE Healthcare UK Limited) for VMAT2, and Goat Anti-Mouse IgG (H + L) for tubulin (Bio-Rad Labs). Primary antibody concentrations

included anti-DAT MAB369 (1:1,000), anti-VMAT2 AB1598P (1:500), and anti-tubulin T6074 (1:1,000). Secondary antibody concentrations were 1:5,000 for DAT, 1:4,000 for VMAT2, and 1:10,000 for tubulin.

Tissue samples from the same adolescent and adult animals were used for measuring DAT and VMAT2 protein levels. Due to the low levels of DAT protein in adolescent animals (NAc and DS), data were replicated multiple times using different primary antibodies in order to provide a quantitatively measurable signal from adolescent tissue. The data reported here used the primary antibody listed above. However, due to a loss of sample from multiple replications, an $n = 3$ for adults is reported.

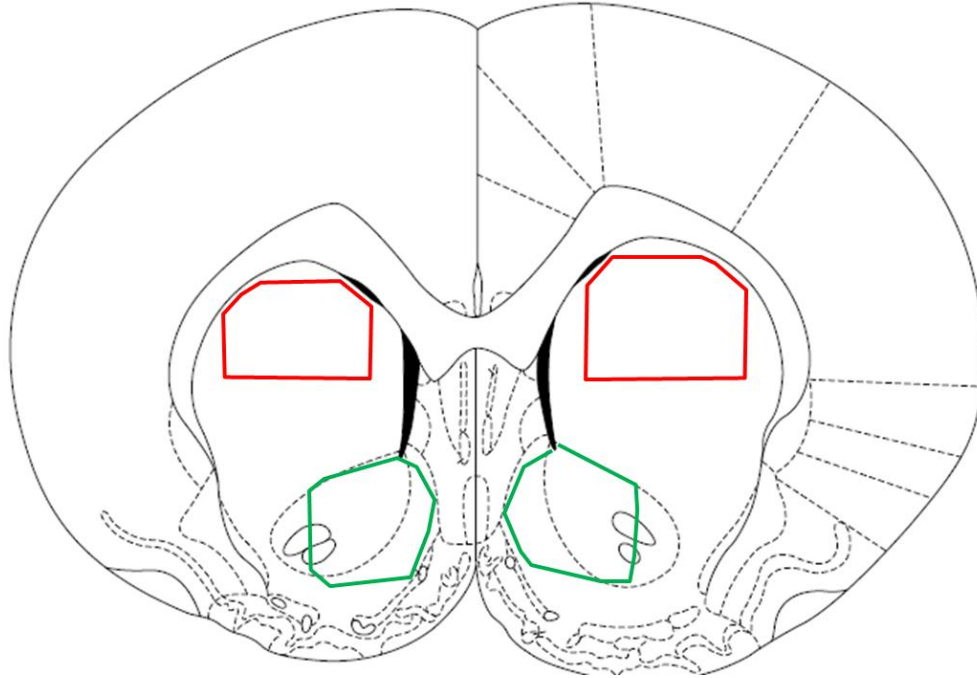


Figure 3-1 Regions for tissue dissection

Western blot analysis of DAT and VMAT2 used tissue that was free-hand dissected bilaterally from both the NAC (outlined in green) and the DS (outlined in red) of adolescent and adult rats. General regions of dissection in this diagram are indicated on a standard adult rat atlas image (Paxinos and Watson, 1998).

3.2.3 Surgical Procedure

Surgeries were performed between the hours 08:00 and 10:00. To implant microdialysis probes, rats were anesthetized with isoflurane, placed on a heating pad, and their heads secured in a stereotaxic apparatus with blunt ear bars. A small incision was made in the skin over the skull and the area irrigated with lidocaine. Dialysis probes were bilaterally implanted, with one probe in the DS (for adolescents: AP +0.7 from bregma, ML +2.0 from bregma, and DV -5.0 from skull; and for adults: AP +1.6, ML +2.2, DV -6.0 from skull) and one probe in the NAc (for adolescents: AP +1.0 from bregma, ML +1.2 from bregma, and DV -7.0 from skull; and for adults: AP +1.2, ML +1.1, DV -8.4 from skull). Coordinates for the dialysis probes were according to the atlas of Paxinos and Watson (Paxinos and Watson, 1998). Dialysis probes were constructed of Hospal AN69 polyacrylonitrile dialysis tubing (Renal Care, Lakewood, CO), with an outer diameter of 330 μm and an exposed tip of 1.0 mm for adolescent rats and 2.0 mm for adult rats. To account for the smaller size of the adolescent brain, dialysis probes with a smaller exposed tip were used. Dialysis probes were secured in a head cap consisting of dental acrylic and were fastened to the skull using two skull screws. Immediately following surgery, dialysis probes were connected to a liquid swivel balance arm assembly, and animals were singly housed in a clear polycarbonate cage with fresh bedding. Animals were housed in a temperature and humidity controlled room on the same light/dark schedule used in the vivarium (lights on at 19:00). The dialysis probes were perfused at a rate of 1.5 $\mu\text{L}/\text{min}$ with a Ringer's solution (in mM: 145 NaCl, 2.7 KCl, 1.0 MgCl₂, and 1.2 CaCl₂). Animals received rat chow softened with an acetaminophen solution. Rats were allowed approximately 24 hr to recover from the surgery

prior to the collection of microdialysis samples, which is accepted as a sufficient period of time for brain tissue to normalize after probe implantation (Krebs-Kraft et al., 2007). Our laboratory has previously shown previously that a 24 hr recovery period is also sufficient for the overall health of the animals to return to normal (Moghaddam and Gruen, 1991; Moghaddam and Bunney, 1993; Jedema and Moghaddam, 1994; Moghaddam and Bolinao, 1994). Thus, animals in this study did not show obvious signs of post-surgery distress; rather, the animals groomed normally, ate food and drank water, and had no signs of bleeding or infection around the wound or head cap. Rats had ad libitum access to food and water during the recovery period, but not during the experiment.

3.2.4 Microdialysis

On the day of the experiment, rats remained in their home-cage for collection of samples and behavioral testing. Dialysis experiments began between 09:00 and 11:00. Probes were perfused with Ringer's solution only (as above) at a flow rate of 2.0 $\mu\text{L}/\text{min}$ during sample collection. Dialysate samples were collected every 20 min for the duration of each experiment and immediately injected into a high-performance liquid chromatography system with electrochemical detection for the analysis of dopamine, as described previously (Adams and Moghaddam, 1998). One hour of baseline samples were analyzed before the systemic or local administration of drug. For local drug administration, nomifensine (dissolved in Ringer's solution) was infused directly through the microdialysis probes, at a flow rate of 2.0 $\mu\text{L}/\text{min}$. For analysis, dialysate samples were injected immediately onto an HPLC system with electrochemical detection for analysis of dopamine, with a detection limit of approximately 5 fmol.

3.2.5 Drugs

Amphetamine (1.0 mg/kg; Sigma-Aldrich) and nomifensine (20 μ M; Sigma-Aldrich) were dissolved in 0.9% saline and frozen for a maximum of 1 week prior to use. Nomifensine dissolved in saline at a concentration of 20 μ M was further diluted to a concentration of 5 μ M in the Ringer's solution prior to each infusion experiment and was used immediately. Once a stable, resting dopamine baseline was reached, dialysis probes were infused with nomifensine (5 μ M). Once a stable dopamine baseline was reached during nomifensine infusion, amphetamine (1.0 mg/kg) was administered systemically (i.p.).

The dose of nomifensine used in this study was chosen based on previous studies that show low doses of nomifensine infusion caused sustained increases in striatal dopamine (Adams et al., 2002; Ukai et al., 2004). The dose of amphetamine used in this study was consistent with our previous study (**Chapter 2**), showing that in adolescence and adulthood this dose caused increases in striatal dopamine and associated behaviors.

3.2.6 Histology

After completion of microdialysis experiments, rats were anesthetized with chloral hydrate (400 mg/kg) and perfused intracardially with 0.9% NaCl. Brains were removed and stored in a 10% Formalin solution. Serial coronal sections of 250 μ m thickness were taken through the regions of interest and then mounted on glass slides and stained with cresyl violet. Stained sections were

then evaluated for accuracy of probe placement. Only data from placements within the brain region of interest were used for further analysis (Figure 3-2).



Figure 3-2 Microdialysis probe placements

Microdialysis probe placement for rats included in the study. Probes were placed bilaterally in the NAC and DS of adolescent and adult rats. Black bars represent the approximate location of probe placements, overlaid on standard adult rat atlas images (Paxinos and Watson, 1998). The size of black bars represents the approximate size of the exposed dialysis tip: 1.0 mm for adolescents, 2.0 mm for adults. Numbers represent distance (in millimeters) anterior to bregma for adult animals.

3.2.7 Data analysis

For Western blot analysis, results are presented as optical density ratios (mean \pm sem). Statistical analysis for western blot data was conducted using student t-tests. Significance was set at $p < 0.05$.

Microdialysis data were analyzed as described previously (Adams and Moghaddam, 1998; Pehrson and Moghaddam, 2010). The microdialysis data were expressed as a percentage of baseline dopamine efflux (mean \pm sem), where baseline was defined as mean of the three baseline values obtained immediately before a pharmacological manipulation. Baseline means for each group, reported as fmol/ μ l, were analyzed using student t-tests. Statistical analysis of these dependent measures were conducted using two-factor repeated measures ANOVAs with age or drug treatment as the between-subjects factors and time as the within-subjects factor. Significance was set at $p < 0.05$. For microdialysis, five-eight subjects per group were required based on an estimated effect size (considering previous work in our laboratory).

3.3 RESULTS

3.3.1 Protein expression

Given the region specific differences in amphetamine-induced dopamine efflux between adolescent and adult rats (Section 2.4), we sought to investigate the molecular mechanisms

responsible for these differences. We used Western blot analysis to measure levels of proteins involved in dopamine availability and the effects of amphetamine, including DAT and the monoamine vesicular transporter-2 (VMAT-2) of adolescent and adult rats. Protein analyses showed that adolescents displayed reduced DAT protein level expression in both the NAc ($t_5 = 3.7$, $p < 0.05$) (Figure 3-3A) and the DS ($t_6 = 4.05$, $p < 0.01$) (Figure 3-3B) compared to adults. There were no significant differences between either age group in VMAT-2 levels in the NAc ($t_6 = 1.14$, n.s.) (Figure 3-3A) or the DS ($t_6 = -0.26$, n.s.) (Figure 3-3B).

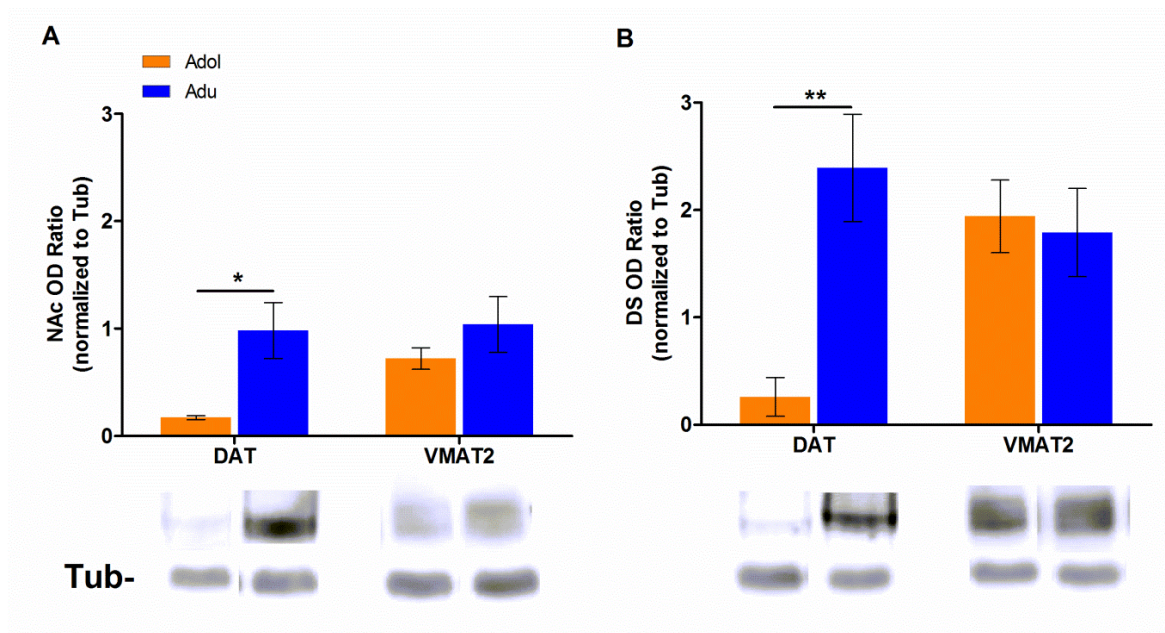


Figure 3-3 Protein expression of DAT and VMAT2 in adolescents and adults

Adolescent rats have decreased levels of DAT in both the NAc and the DS. Protein expression levels of DAT (adolescents, n=4; adults, n=3) and VMAT2 (n=4 for each group) were measured using Western blot analysis in the A) NAc and B) DS of adolescent (orange bars) and adult (blue bars) rats. The band images shown are from the same gel of the representative of adolescent and adult rats used to measure each protein. All protein optical densities were normalized to tubulin, shown along the bottom of the figure. Asterisk(s) denotes significant differences between age groups (* p<0.05, **p<0.01).

3.3.2 Dopamine Efflux

Although reduced DAT levels were observed in adolescents compared to adults in both the NAc and DS, the functional differences may be different in these regions, suggesting that reduced DAT function in the DS may account for reduced effects of amphetamine on dopamine efflux in adolescent DS (Figure 3-3). To test the validity of this mechanism, we compared the effect of locally applied DAT inhibitor nomifensine on basal and amphetamine-activated extracellular dopamine levels. The baseline means (in fmol/ μ l) for the NAc are 0.26 ± 0.09 in adolescents ($n = 11$) and 0.48 ± 0.22 in adults ($n = 11$), with no significant difference between age groups ($t_{20} = -0.955$, n.s.). The baseline mean (in fmol/ μ l) for the DS is 0.92 ± 0.37 in adolescents ($n = 13$) and 0.37 ± 0.14 in adults ($n = 11$), with no significant difference between age groups ($t_{22} = 1.32$, n.s.). Application of the DAT inhibitor nomifensine produced a near 6-fold increase in dopamine efflux that was sustained throughout drug infusion in the NAc (Figure 3-4A) and DS (Figure 3-4B) of both adolescents and adults (time main effect, $F(11, 220) = 34.82$, $p < 0.0001$). There were no significant age differences in dopamine efflux in the NAc (time \times age interaction, $F(11, 220) = 1.03$, n.s.). There was, however, a trend toward significance in the DS (time \times age interaction, $F(11, 253) = 1.82$, $p = 0.052$), such that adolescents demonstrated lower dopamine efflux over time compared to adults.

After nomifensine infusion caused a plateau in increased dopamine levels, systemic administration of amphetamine (1.0 mg/kg) caused significant decreases in dopamine efflux in the adolescent NAc (treatment \times age interaction, $F(10, 80) = 2.38$, $p < 0.05$) and DS (treatment \times age interaction, $F(10, 80) = 2.38$, $p < 0.05$), compared to saline controls. Amphetamine had no

effect on dopamine efflux in the adult NAc (treatment \times age interaction, $F(10, 90) = 0.54$, n.s.) or the DS (treatment \times age interaction, $F(10, 80) = 0.87$, n.s.), compared to saline controls (Figure 3-4C). However, amphetamine did not cause any significant age dependent differences in dopamine efflux during nomifensine infusion in the NAc (time \times age interaction, $F(10, 99) = 0.47$, n.s.) (Figure 3-4C) or in the DS (time \times age interaction, $F(10, 60) = 0.53$, n.s.) (Figure 3-4D). There were also no age differences in dopamine efflux in response to the saline vehicle administration during nomifensine infusion in the DS (time \times age interaction, $F(10, 100) = 0.64$, n.s.) (Figure 3-4D), though there was a significant age difference in the NAc (time \times age interaction, $F(10, 80) = 2.43$, $p < 0.05$).

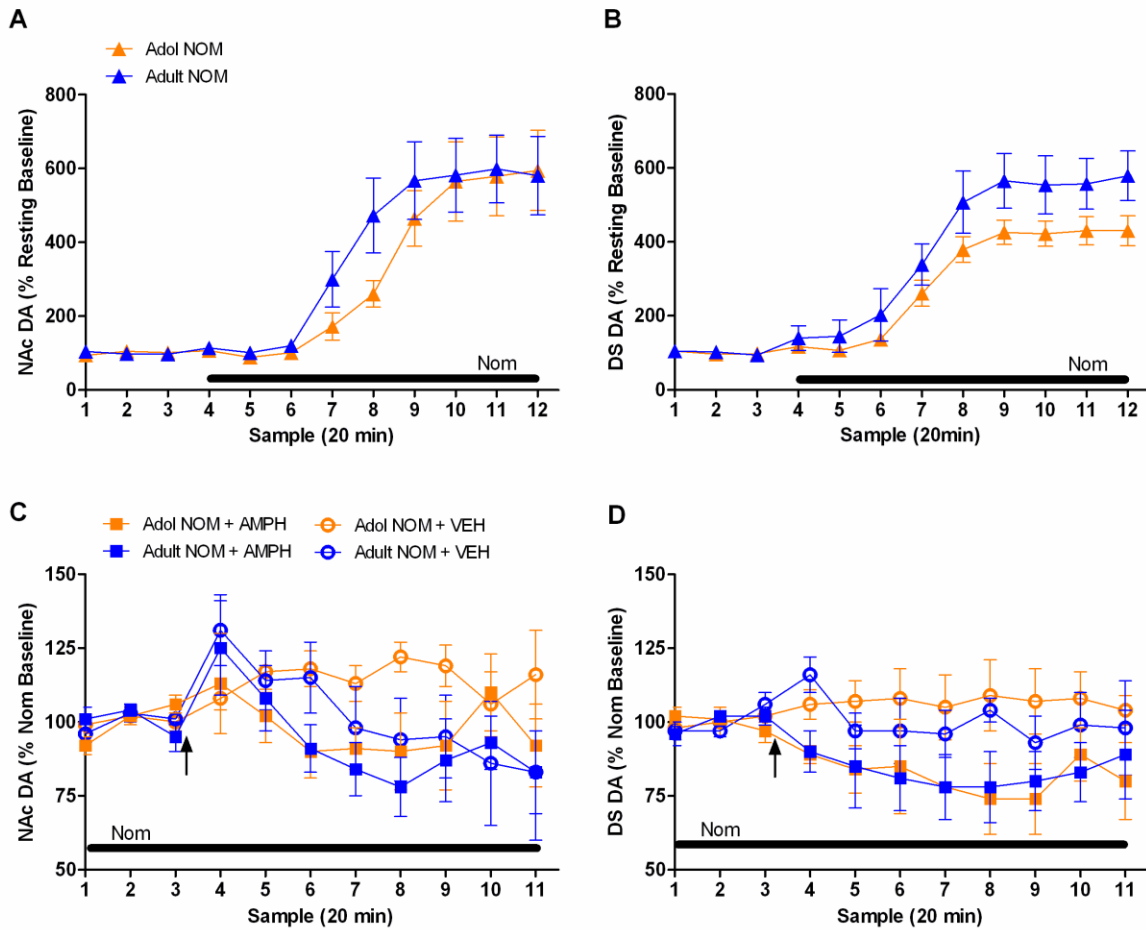


Figure 3-4 Dopamine efflux in response to nomifensine and amphetamine

Blockade of DAT produced similar regional effects on dopamine efflux in both adolescents and adults. The DAT inhibitor nomifensine was infused directly into the NAc and DS of adolescent (orange) and adult (blue) rats. Dopamine efflux was measured in the A) NAc (adolescent group, n=11; adult group, n=11) and B) DS (adolescent group, n=14; adult group, n=11). When maximum dopamine efflux was reached for at least 3 consistent time points during nomifensine infusion, animals were given an i.p. injection of 1.0mg/kg amphetamine (squares) or 0.9% saline vehicle (circles). Dopamine efflux continued to be measured in the C) NAc (adolescent amphetamine group, n=6; adult amphetamine group, n=6; adolescent vehicle group, n=5; adult vehicle group, n=5) and D) DS (adolescent amphetamine group, n=6; adult amphetamine group, n=5; adolescent vehicle group, n=8; adult vehicle group, n=6) following injection. In graphs A) and B), baseline dopamine efflux was measured prior to infusion, represented by the first three time points displayed. In graphs C) and D), baseline dopamine efflux was measured after nomifensine infusion caused a plateau in dopamine, represented by the first three time points displayed. Black bar denotes nomifensine infusion. Black arrow denotes time of injection.

3.4 DISCUSSION

In the present study we found that dopamine's hypoactive response to amphetamine in adolescent rats observed in **Chapter 2** is likely not due to mechanisms of dopamine packaging or reuptake. Though our findings showed that the density of DAT is lower in the adolescent NAc and DS compared to adults, blocking DAT locally in these regions produced similar effects on dopamine efflux in these regions regardless of age, suggesting that the differences in the density of DAT alone is not responsible for the region-specific effects of amphetamine on dopamine efflux seen previously. Furthermore, our findings indicate that there is no difference in the density of the dopamine packaging protein, VMAT2. Thus, other mechanisms besides dopamine packaging and reuptake, such as the rate of dopamine synthesis, may be responsible for the reduced dopamine neurotransmission observed in the adolescent DS.

Dopamine in the striatum relies heavily upon the function of DAT and VMAT2. DAT is responsible for transporting dopamine that is released into the synapse back into the presynaptic terminal of the dopamine neuron. VMAT2 is then responsible for transporting cytosolic, newly synthesized dopamine into synaptic vesicles, making dopamine available for synaptic release. Psychostimulants such as amphetamine are known to be modulators of dopamine neurotransmission, as amphetamine has been shown to reverse the reuptake of dopamine through DAT and cause increases in extracellular dopamine (Sulzer et al., 1995; Jones et al., 1998). Therefore, our previous finding that amphetamine caused reduced increases in dopamine efflux

in the DS of adolescent rats compared to adolescents indicated that DAT-mediated dopamine availability in the adolescent DS contributed to the observed reduced effects.

However, the data in this study suggest that though adolescents express less DAT in both the NAc and DS than adults, the functional consequences of DAT inhibition are similar in both regions. First, we will focus on the developmental pattern of expression of DAT in the striatum. Our data demonstrated that DAT is lower in both the NAc and DS of adolescent than adult rats. This finding is consistent with previous studies that observed higher DAT levels in adulthood compared to adolescence (Tarazi et al., 1998b; Volz et al., 2009). Conversely, there are other conflicting reports that have observed either DAT levels peak in adolescence and gradually decline with age (Meng et al., 1999; Moll et al., 2000), or that DAT expression reaches adult levels prior to or during adolescence in the NAc and DS (Coulter et al., 1996). The differences in striatal DAT levels may be due to the different experimental paradigms, techniques and ages used (e.g. early versus middle versus late adolescent ages) may contribute to these varying and conflicting results.

Age differences in the striatal expression of DAT might suggest functional differences in dopamine reuptake as a consequence. Yet, our findings show that there are no age-related differences in dopamine efflux when blocking dopamine reuptake through local infusion of a DAT inhibitor, nomifensine, in the NAc and DS. Our results do show, however, a trend towards significance in the DS, with adolescent rats having smaller increases in dopamine efflux than adults. Since nomifensine blocks dopamine reuptake and the dopamine released is impulse dependent, our findings might suggest that adolescent animals have reduced levels of dopamine in the DS compared to adults. Though our measures of basal dopamine level concentrations in the DS do not show a significant age difference. Previous reports, however, indicate that basal

dopamine levels are lower in both the DS and NAc of adolescent rats, using either in vivo microdialysis to measure dopamine levels or dissected striatal tissue measured on HPLC (Badanich et al., 2006; Cao et al., 2007). Further studies should be conducted to assess basal levels of dopamine in these striatal subregions across adolescence (early, middle, late) and compared to adulthood.

Our results also indicated that the blockade of DAT similarly prevented any further effects of amphetamine administration on dopamine efflux in the NAc and the DS. This suggests that DAT blockade is functionally similar in both striatal subregions of adolescents and adults, regardless of age-related differences in DAT expression. Consistent with these data, Volz and colleagues have previously shown that while adolescent rats have lower DAT expression in striatal tissue, there are increased levels of functionally active DAT within this region as measured by dopamine transport velocities and DAT immunoreactivity (Volz et al., 2009). Although this study did not distinguish between dorsal and ventral striatum, the results suggest that although adolescent rats express less DAT, these DATs are functionally more efficacious at dopamine clearance than those expressed in adults. Our findings of a similar pattern of dopamine increase in both striatal subregions during adolescence and adulthood in response to DAT blockade support this equivalent reuptake hypothesis.

Our previous finding that there are region-specific differences in striatal dopamine efflux in response to amphetamine suggested that DAT-mediated dopamine availability in the adolescent DS may contribute to its reduced behavioral effects. However, together with the findings of this study, the differences in the density of DAT does not explain why amphetamine causes a greater percentage increase from baseline in DS dopamine of adult rats, but similar

increases in NAc dopamine. Other mechanisms involved in dopamine availability, such as the synthesis rate limiting enzyme, tyrosine hydroxylase, may be involved.

Though DAT may not explain the hypoactive dopamine response in the DS of adolescence to amphetamine, DAT plays an important role in dopaminergic transmission that can lead to dysfunction in disorders such as ADHD. The dysfunction of the mesocorticolimbic and nigrostriatal dopamine systems has been implicated in the pathophysiology of ADHD through studies that suggest there is an overexpression of the DAT gene as well as imaging studies that show the efficiency of treatment with dopamine reuptake inhibitors such as methylphenidate (Faraone et al., 2005). There have been other groups that have found that striatal DAT density is significantly higher in patients with ADHD compared to normal controls (Cheon et al., 2003; Krause, 2008). Furthermore, amphetamine-like stimulants that target DAT are commonly prescribed to human youths with ADHD for primary therapeutic treatment (Kutcher et al., 2004; Zhu and Reith, 2008), though little is known about the impact of these drugs on dopamine neurotransmission at this age or the longer term consequences. Therefore, a greater understanding of the normal development of DAT in the adolescent striatum is important for determining the underlying mechanisms involved in the disruption of DAT function in psychiatric illness and provide insight into prevention.

In summary, similar patterns of dopamine reuptake in both the NAc and DS suggested that there are other mechanisms that govern dopamine availability in adolescents that selectively target the DS, making it hyporesponsive to stimulants such as amphetamine. Our data strongly indicate that this regionally selective mechanism does not involve changes in dopamine reuptake or packaging, but instead may involve differences in dopamine synthesis in the DS of adolescents. We address this possibility in **Chapter 4**.

4.0 THE EFFECTS OF DOPAMINE SYNTHESIS IN ADOLESCENT AND ADULT RATS

4.1 INTRODUCTION

Dopamine is a neuromodulator involved in regulating various behaviors such as learning (Wise, 2004), cognitive flexibility (Stefani and Moghaddam, 2006; Robbins and Roberts, 2007), reward perception (Schultz et al., 1997; Berridge and Robinson, 1998), and motor movements (Beninger, 1983; LeMoal and Simon, 1991). There are anatomically and functionally distinct subgroups of dopamine neurons in the ventral midbrain that innervate forebrain structures such as the nucleus accumbens (NAc), and dorsal striatum (DS) (Carlsson et al., 1962). The DS, which plays an important role in voluntary motor control (Joel and Weiner, 2000), as well as instrumental learning (Balleine et al., 2009), is largely innervated by dopamine neurons from the substantia nigra pars compacta (Gerfen et al., 1987). The NAc, on the other hand, which is thought to play a key role in adolescent risk taking and sensation seeking (Ernst et al., 2006; Casey et al., 2008; Steinberg, 2008), is innervated by dopamine neurons from the ventral tegmental area (Gerfen et al., 1987).

It is during adolescence that the mesolimbic and nigrostriatal dopamine systems undergo major remodeling (Kelley et al., 2004; Fareri et al., 2008). Neural alterations in these systems include dopamine receptor overproduction and pruning, changes in dopamine turnover and

synthesis, and changes in dopamine reuptake (Teicher et al., 1993; Baldessarini and Tarazi, 1996; Coulter et al., 1996; Andersen et al., 1997). This remodeling may lead to dynamic changes in the responsivity of the dopamine system to external stimuli that may be responsible for some of the behavioral changes of adolescents. For example, changes in reward sensitivity in adolescence (Spear, 2000; Kelley et al., 2004) may be due to dopamine mediated neuronal remodeling in striatal, limbic and frontal cortical regions in preparation for adulthood (LeMoal and Simon, 1991). The developmental dysfunction of the mesocorticolimbic and nigrostriatal dopamine systems has been implicated in various psychiatric disorders such as schizophrenia (Carlsson and Carlsson, 2006), mood (Schildkraut, 1965) and addictive disorders (Koob, 1992).

Our previous studies have shown that dopamine neurotransmission in the DS of adolescent rats is hypoactive in response to acute amphetamine compared to adults. Since amphetamine is known to effect dopamine release via the dopamine transporter (DAT), we have also shown that this hypoactive response is likely not due to age-related differences of the protein density of DAT alone. The purpose of this study was to examine other underlying mechanisms involved in the hypoactive function of dopamine in the adolescent DS. We measured the expression of the rate-limiting enzyme in dopamine synthesis, tyrosine hydroxylase (TH) in the NAc and DS of adolescent and adult rats. Further, we examined the behavioral effects of blocking dopamine synthesis by systemically administering a TH inhibitor.

4.2 METHODS

4.2.1 Subjects

Adolescent (PND 34-38, ~130-150 g) and adult (PND 70-80, ~330-400 g) male Sprague-Dawley rats (Harlan) were used. Pre-adolescent juvenile rats (postnatal day 21) and adults were received at least two weeks prior to experimentation, allowing animals to habituate to the housing. During this time, juvenile rats reached adolescence (PND 34-38). All subjects were pair housed in a 12 hr light/dark cycle (lights on at 19:00). Experiments were conducted during the animals' dark (active) phase. All animals had ad libitum access to water and rat chow food. All animal experiments were performed in accordance with and approval by the ethical guidelines of the Institutional Animal Care and Use Committee at the University of Pittsburgh.

4.2.2 Western Blot

Tissue samples from the same adolescent and adult animals used to measure DAT and VMAT2 in **Chapter 3** were used for measuring TH protein levels. There was an n of 4 for each group.

Tissue dissections and Western blot analysis were conducted as described previously in **Chapter 3** (Section 3.2.2). TH protein levels were expressed as a ratio of tubulin band densities to ensure that changes in protein levels were not due to sample loading variances. Primary antibody against TH AB152 was from Millipore and primary antibody against the control protein α -tubulin was from Sigma-Aldrich. Secondary antibodies conjugated with horseradish peroxidase were Protein A for TH (GE Healthcare UK Limited) and Goat Anti-Mouse IgG (H + L) for tubulin (Bio-Rad Labs). Primary antibody concentration for anti-TH AB152 and anti-

tubulin T6074 was 1:1,000. Secondary antibody concentrations were 1:4,000 for TH and 1:10,000 for tubulin.

4.2.3 Behavior

Behavioral experiments began between 09:00 and 11:00. Adolescent and adult rats were individually placed in a clear polycarbonate cage with fresh bedding, with no access to food or water, in a temperature and humidity controlled room on the same light/dark schedule used in the vivarium (lights on at 19:00). Animals were given 1 hr to habituate to the experimental cage before receiving pharmacological manipulation. Motor behavior was quantified as described in **Chapter 2** (Section 2.2.4).

4.2.4 Drugs

Alpha-methyl-DL-tyrosine (50 mg/kg; TCI America) and amphetamine (1.0 mg/kg; Sigma-Aldrich) were dissolved in 0.9% saline. Alpha-methyl-DL-tyrosine was used immediately, while amphetamine was frozen for a maximum of 1 week prior to use. Both alpha-methyl-DL-tyrosine and amphetamine were both administered systemically (i.p.).

The dose of alpha-methyl-DL-tyrosine used in this study was chosen based on previous studies demonstrating that pre-treatment with 50 mg/kg (i.p.) alpha-methyl-DL-tyrosine attenuates the stimulation of locomotor activity induced by amphetamine (Finn et al., 1990; Di Lullo and Martin-Iverson, 1991). The dose of amphetamine used in this study was consistent with our use in previous studies (**Chapters 2 and 3**), showing that this dose (1.0 mg/kg) leads to increased dopamine efflux and associated motor behaviors in adolescent and adult rats.

4.2.5 Behavior experimental design

Animals were given one hour to habituate to their novel cage environment. Behavior was immediately recorded once the animal was placed in its cage. After one hour of habituation, animals were pre-treated with alpha-methyl-DL-tyrosine (50 mg/kg), administered systemically (i.p.). After 2 hr of pre-treatment, animals were given another systemic (i.p.) injection of either 0.9% saline (1 ml/kg) or amphetamine (1.0 mg/kg). The animals' behavior was recorded for another 2 hr.

4.2.6 Data analysis

For Western blot analysis, results are presented as optical density ratios (mean \pm sem). Statistical analysis for western blot data was conducted using student t-tests. Significance was set at $p < 0.05$.

Behavior data were separately expressed as the number of infrared beam breaks (for either XY ambulations or fine movements) within a 5 min bin (mean \pm sem). Statistical analysis of these dependent measures was conducted using two-factor repeated measures ANOVAs, with age or drug treatment as the between-subjects factors and time as the within-subjects factor. Significance was set at $p < 0.05$. Five-eight subjects per group were required based on an estimated effect size (considering previous work in our laboratory).

4.3 RESULTS

4.3.1 Protein expression

We used Western blot analysis to measure levels of protein in the NAc and DS of adolescent and adult rats. Protein analyses showed that TH protein expression levels, relative to the housekeeping protein tubulin, were selectively lower in DS of adolescents compared to adults ($t_6 = 3.45$, $p < 0.05$) (Figure 4-1B) while levels were comparable in NAc of both age groups ($t_6 = 0.45$, n.s.) (Figure 4-1A).

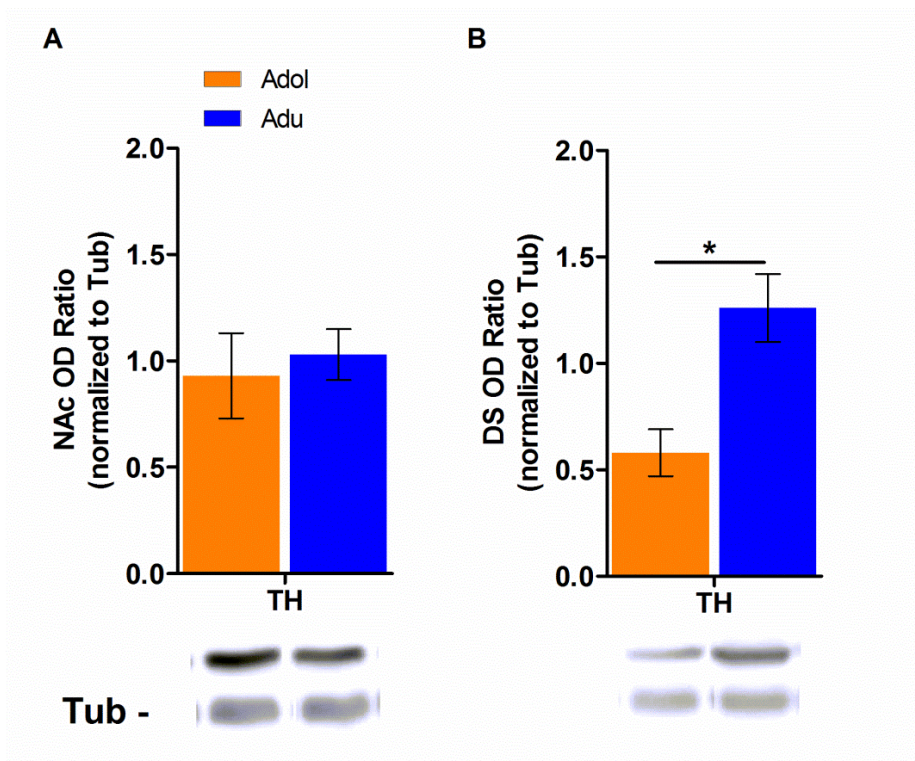


Figure 4-1 Protein expression of TH in adolescents and adults

Adolescent rats have decreased levels of TH in the DS. Protein expression levels TH (n=4 for each group) were measured using Western blot analysis in the (A) NAc and (B) DS of adolescent (orange bars) and adult (blue bars) rats. The band images shown are from the same gel of the representative of adolescent and adult rats used to measure the protein. All protein optical densities were normalized to tubulin, shown along the bottom of the figure. Asterisk(s) denotes significant differences between age groups (* p<0.05).

4.3.2 Behavior

To examine the role of TH regulation on dopamine-mediated motor behaviors, animals were pretreated with the TH inhibitor alpha-methyl-DL-tyrosine (50 mg/kg, i.p.) for 2 hrs prior to amphetamine (1.0 mg/kg, i.p.) administration. Amphetamine exposure after alpha-methyl-DL-tyrosine pretreatment significantly increased locomotor activity in adolescents (treatment \times age interaction, $F(29, 464)=4.26$, $p<0.0001$) and adults (treatment \times age interaction, $F(29, 406) = 3.52$, $p < 0.0001$), as well as stereotypy in adolescents (treatment \times age interaction, $F(29, 406) = 3.52$, $p < 0.0001$) and adults (treatment \times age interaction, $F(29, 464) = 2.89$, $p < 0.0001$), compared to saline controls. Adolescents and adults responded similarly to amphetamine induced locomotor ambulations (time \times age interaction, $F(29, 406) = 0.93$, n.s.) (Figure 4-2A), whereas amphetamine significantly reduced stereotyped movements in adolescents (time \times age interaction, $F(29, 406) = 1.74$, $p < 0.01$) (Figure 4-2B) compared to adults. Vehicle administration showed no effect on locomotion (time \times age interaction, $F(29, 406) = 1.21$, n.s.) (Figure 4-2A) or fine movements between groups (time \times age interaction, $F(29, 406) = 1.26$, n.s.) (Figure 4-2B).

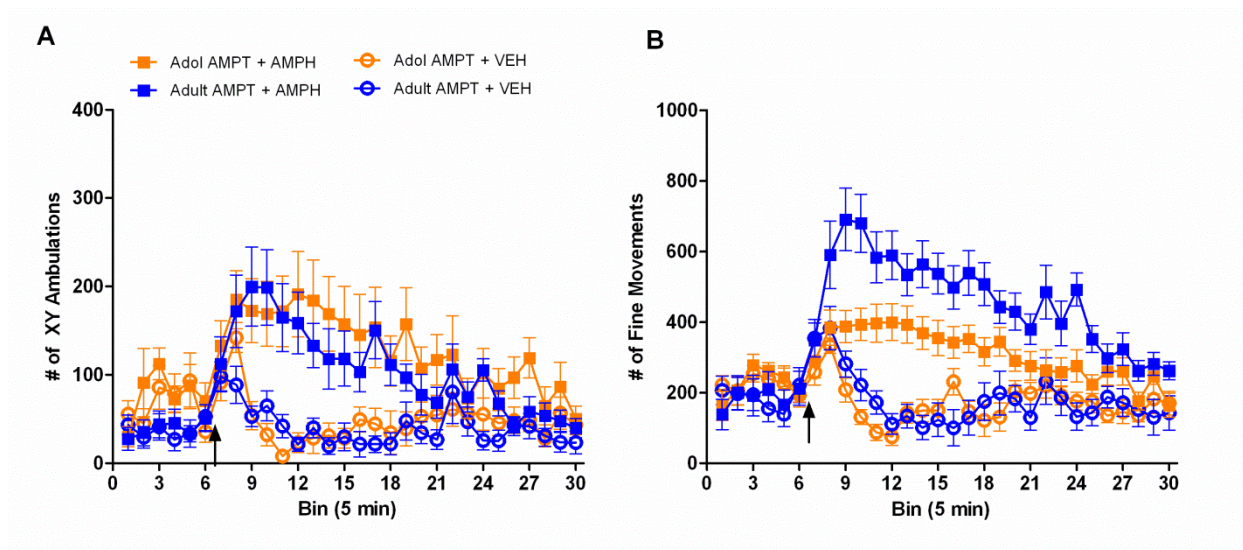


Figure 4-2 Behavior in response to alpha-methyl-DL-tyrosine and amphetamine

TH inhibition caused lower increases in fine movements in response to amphetamine exposure in adolescent rats. Adolescent (orange) and adult (blue) rats were pretreated with alpha-methyl-DL-tyrosine before given an i.p. injection of 1.0mg/kg amphetamine (squares) or 0.9% saline vehicle (circles). Motor behavior was measured for each animal, expressed as (A) ambulations (n=10 for each group) and (B) fine movements (n=10 for each group). In each graph, baseline motor behavior during pretreatment was measured prior to amphetamine injection, represented by the first six time points displayed. Black arrow denotes time of amphetamine injection.

4.4 DISCUSSION

In the present study we found that TH expression is reduced in the DS of adolescent rats compared to adults. Similar levels of TH expression were seen in both age groups in the NAc. Our data further indicate that blocking dopamine synthesis with a TH inhibitor attenuates amphetamine-induced stereotypy (mediated by dopamine in the DS) in adolescents to a greater degree than adults. These findings support the idea that reduced protein expression of TH in the DS of adolescents leads to diminished dopamine synthesis in this region, and therefore reduced dopamine availability, which likely contributes to the mechanism for reduced amphetamine-induced dopamine activity in the DS during adolescence.

Little is known about the developmental trajectory of TH or dopamine synthesis throughout development. Dopamine synthesis is mediated by TH, a rate limiting enzyme that converts tyrosine to L-DOPA (Molinoff and Axelrod, 1971). Dopamine synthesis and availability are activity dependent. TH is regulated long-term through gene expression and short-term through feedback inhibition by catecholamines, allosteric regulation, and phosphorylation (Kumer and Vrana, 1996). We observed that there are region-specific differences in TH expression between adolescents and adults. Lower levels of TH in the adolescent DS would lead to reduced dopamine synthesis, potentially leading to reduced dopamine transmission in this region. Our finding that amphetamine-induced stereotypy, a motor behavior dependent on dopamine neurotransmission in the DS (Beninger, 1983), was selectively attenuated in adolescents by a TH inhibitor further suggests adolescent DS may also have a lower functional

state of TH activity. However, we did not measure extracellular dopamine levels in response to alpha-methyl-DL-tyrosine, and it is unclear if the dose administered, and the time allotted for pretreatment prior to amphetamine administration was sufficient to significantly deplete dopamine stores in the NAc or the DS. Therefore, we cannot directly correlate changes in behavior in response to TH inhibition with changes in extracellular dopamine levels. Further studies are needed to establish which of these mechanisms governing TH activity contribute to reduced TH protein expression and the functional consequences of reduced TH in the adolescent DS.

Our data strongly indicate that the regionally selective mechanism for reduced amphetamine-induced dopamine activity in the adolescent DS involves expression of TH. This study provides further data to support the claim that dopamine neurotransmission is selectively hypofunctional in the DS but not the NAc of adolescents. The NAc and the DS receive dopaminergic midbrain projections from the ventral tegmental area and the substantia nigra pars compacta, respectively (Joel and Weiner, 2000). The distinct anatomical and structural connectivity of these striatal regions may explain the different functional roles these regions play in various behaviors. The DS is involved in voluntary motor control, instrumental learning and habit formation (Joel and Weiner, 2000), while the NAc is involved in reward and reinforced behaviors (Mogenson et al., 1980; Kelley, 2004b). Blocking dopamine receptors in the NAc has been shown to potently suppress locomotor responses associated with the motivational state of hunger (Baldo et al., 2002). Depleting dopamine in the NAc has been shown to suppress instrumental responding reinforced with food (Heffner and Seiden, 1983), while inactivating the DS, on the other hand, has been shown to block the ability of animals to shift behavioral control from goal-directed actions to habits (Packard and McGaugh, 1996). Furthermore, administering

amphetamine locally into the NAc, but not the DS, has been shown to potentiate conditioned reinforcement (Taylor and Robbins, 1984).

Understanding the remodeling of the striatum throughout adolescent development is important because of the striatum's role in motor behaviors and decision-making processes. Previous models have implicated the limbic system, in particular the amygdala and the NAc, in functional immaturities of the adolescent brain. However, our present data with the dopamine system, as well as recent electrophysiology study from our laboratory (Sturman and Moghaddam, 2012), do not find functional differences in the NAc of adolescent rats as compared to adults. Instead, we find reduced dopamine neurotransmission in the DS, a region that is critically involved in learning, habit formation and action selection (Jog et al., 1999). Dopamine's action in the striatum is primarily inhibitory (Bamford et al., 2004). Furthermore, dopamine is considered to be involved in strengthening synaptic corticostriatal connections by modulating striatal interneurons (Wickens et al., 2007a; Surmeier et al., 2011), and reduced dopamine activity might suggest that the normal inhibitory control of dopamine is reduced in the DS of adolescents. This notion is consistent with hyper-responsiveness of adolescent DS neurons to salient events such as reward expectancy (Sturman and Moghaddam, 2012). Reduced dopamine neurotransmission in the DS during this developmental phase may influence DS-dependent behavioral functions, such as response selection and habit formation, and may serve as a mechanism for increased vulnerability to dopamine related illnesses like schizophrenia and addiction. Thus, reduced dopamine activity in this region during adolescence may enhance the function of afferent input that mediates learning and habit formation.

5.0 GENERAL DISCUSSION

5.1 SUMMARY AND INTERPRETATION OF FINDINGS

This dissertation investigates one aspect of the developmental dynamics of dopamine in adolescent rats, compared to adults, in two functionally distinct and dopamine innervated striatal subregions, the NAc and the DS. Using the systemic administration of amphetamine to activate dopamine release in behaving rats, we demonstrated that adolescents are less sensitive to amphetamine-induced hyperlocomotion than adults. This is likely a result of lower levels of amphetamine-induced dopamine efflux in the DS, but not in the NAc, of adolescent rats compared to adults, since these behaviors are mediated by striatal dopamine. We demonstrated that the reduced effects of amphetamine in the DS were likely not due to developmental differences in the functional capacity of DAT. Though adults show greater expression of DAT protein in both NAc and DS tissue, there were no region-specific differences in DAT-mediated clearance of dopamine by age group. In both regions, blocking DAT with the inhibitor nomifensine produced similar levels of basal and amphetamine-induced dopamine efflux in adults and adolescents. We further showed that reduced amphetamine-induced dopamine in the DS of adolescent rats by amphetamine is likely due to region-specific reductions in TH levels in the DS. We also provided evidence that adolescent rats are more sensitive to the effects of a TH inhibitor when evaluating stereotypies, but not locomotion. Together, these data suggest that

dopamine neurotransmission in the DS of adolescents is hypofunctional compared to adults, and this is likely the result, in part, of reduced TH expression and activity. Further, this age-related difference is restricted to the dorsal subregion of the striatum, suggesting regional specificity in the development of the dopamine system.

5.2 NEURAL MECHANISMS OF ADOLESCENT BEHAVIOR

5.2.1 Neurobehavioral models of adolescence

The striatum is essential in motor planning and decision making, and therefore the developmental remodeling of this region throughout adolescence is important to study. Previous models have implicated the limbic system, in particular the amygdala and the NAc, in the functional immaturities of the adolescent brain (Spear, 2000). For example, the triadic node model, a systems-based model of adolescent decision-making, asserts that neurodevelopment and function of the key regions involved in the control of motivated behavior (such as the amygdala, NAc, and medial-ventral prefrontal cortex) are underdeveloped, as well as the balance between these regions (Ernst et al., 2006). Ernst suggests that the risk-taking propensity of adolescents can be explained by a strong reward system (NAc), a weak harm-avoidant system (amygdala), and/or an inefficient supervisory system (medial-ventral prefrontal cortex). Disruptions in the balance of this triadic system may contribute to the expression of psychopathology. Another neurobiological model of adolescence, proposed by Casey and colleagues, asserts that differences in the developmental trajectory of the limbic reward system (ventral striatum and the amygdala) relative to top-down control systems (prefrontal cortex) and their circuit connections

may account for the behavioral changes observed during adolescence, including impulsive choices and risky decision making (Casey et al., 2008). This model, therefore suggests that immature prefrontal function alone does not account for adolescent behavior, as children's less developed prefrontal cortex and cognitive abilities do not look similar or worse than adolescents. In further support of Casey's model, human imaging studies have shown that in emotionally salient situations, adolescent NAc activity was similar to adults, while activity in the orbitofrontal cortex was more like that of children than of adults (Galvan et al., 2006; Galvan et al., 2007).

As such, both of the aforementioned models embody the notion that there is an imbalance of neurodevelopmental maturity in cortical regions relative to subcortical regions during adolescence, where each of these regions are all still immature in childhood but all mature in adulthood (Somerville et al., 2010). The data presented in this dissertation, as well as data from a recent electrophysiology study from our laboratory (Sturman and Moghaddam, 2012), are partially consistent with the interpretation of there being no differences in the NAc of adolescents as compared to adults. However, our findings implicate the reduced dopamine neurotransmission in the DS, a region that is involved critically in learning, habit formation and action selections (Jog et al., 1999; Yin et al., 2008), may be a potential source for adolescent behavioral differences and associated vulnerabilities.

5.2.2 A proposed model for hypodopaminergia in the DS of adolescents

As mentioned in **Section 1.3**, the DS is heavily innervated by dopamine neurons from the substantia nigra pars compacta, and dopamine signaling in the DS plays an important role in voluntary motor control, instrumental learning, and habitual behavior. In addition to

dopaminergic inputs, the DS is heavily innervated by the cortex. Cortical glutamatergic afferents that innervate the DS are predominantly from sensory-motor cortical areas, the anterior cingulate cortex, and prelimbic cortex (Groenewegen and Berendse, 1994; Voorn et al., 2004). Dopamine is involved in modulating striatal neurons primarily by modulating the impact of excitatory inputs onto these neurons (Wickens et al., 2007b; Surmeier et al., 2011). Several anatomical and functional studies have shown that dopamine directly regulates glutamate release from corticostriatal terminals by stimulating D2 receptors on a subset of non-dopaminergic cortical afferents, which serve as a mechanism for dampening critical cortical signals (Calabresi et al., 1993; O'Donnell and Grace, 1994; Sesack et al., 1994; Hsu et al., 1995; Cepeda et al., 2001; West and Grace, 2002). Thus, reduced dopamine activity on corticostriatal afferents in the DS during adolescence may lead to enhanced function of the cortical input, particularly prefrontal input that mediates learning and habit formation. The differential cortical and midbrain influence over the DS compared to the NAc may contribute to differences in dopamine neurotransmission in these areas throughout adolescence, and may serve as a mechanism for increased vulnerability to dopamine related illnesses like schizophrenia and addiction.

The findings in this dissertation may support the notion that the modulatory role of dopamine is reduced in the DS of adolescents. Therefore, we propose that the reduced levels of TH in the DS of adolescent rats compared to adults are due to weaker cortical input to the DS or DS-projecting dopamine cells in SNc (Figure 5-1). We suggest that these cortical inputs likely originate from the anterior cingulate cortex, due to its role in impulsivity, a behavior that is characteristic to adolescents. Though it should be noted that the DS receives input from areas other than the cortical regions, such as thalamic nuclei (Voorn et al., 2004), for simplicity we have only included the striatum, cortex, and midbrain structures in this model. We believe that

corticostriatal connections involving portions of the medial prefrontal cortex, in particular the anterior cingulate cortex, may be an important yet overlooked circuit involved in adolescent development and behavior. The anterior cingulate cortex has projections to the DS and SNc and has been implicated in inhibitory control and conflict resolution, disruption of which results in impulsivity (Volkow et al., 2004). Additionally, the anterior cingulate cortex has been shown to receive dopaminergic input from the substantia nigra pars compacta (Berger et al., 1991). We propose that projections from the anterior cingulate cortex are weaker, or less developed in adolescents compared to adults. In contrast, we suggest that cortical inputs from the infralimbic portion of the medial prefrontal cortex projecting to the NAc are mature and fully developed in adolescent rats and are similar to those corticostriatal connections of adults. There is little overlap between projections from these cortical areas to the dorsal and ventral striatum and therefore these cortical regions may be differentially innervating their striatal targets. It should also be noted that very little is known about the developmental trajectories of these connections between the cortex, striatum and midbrain. The anatomical and functional connectivity of these areas is understudied, and require further attention to gain a better understanding of how these circuits develop.

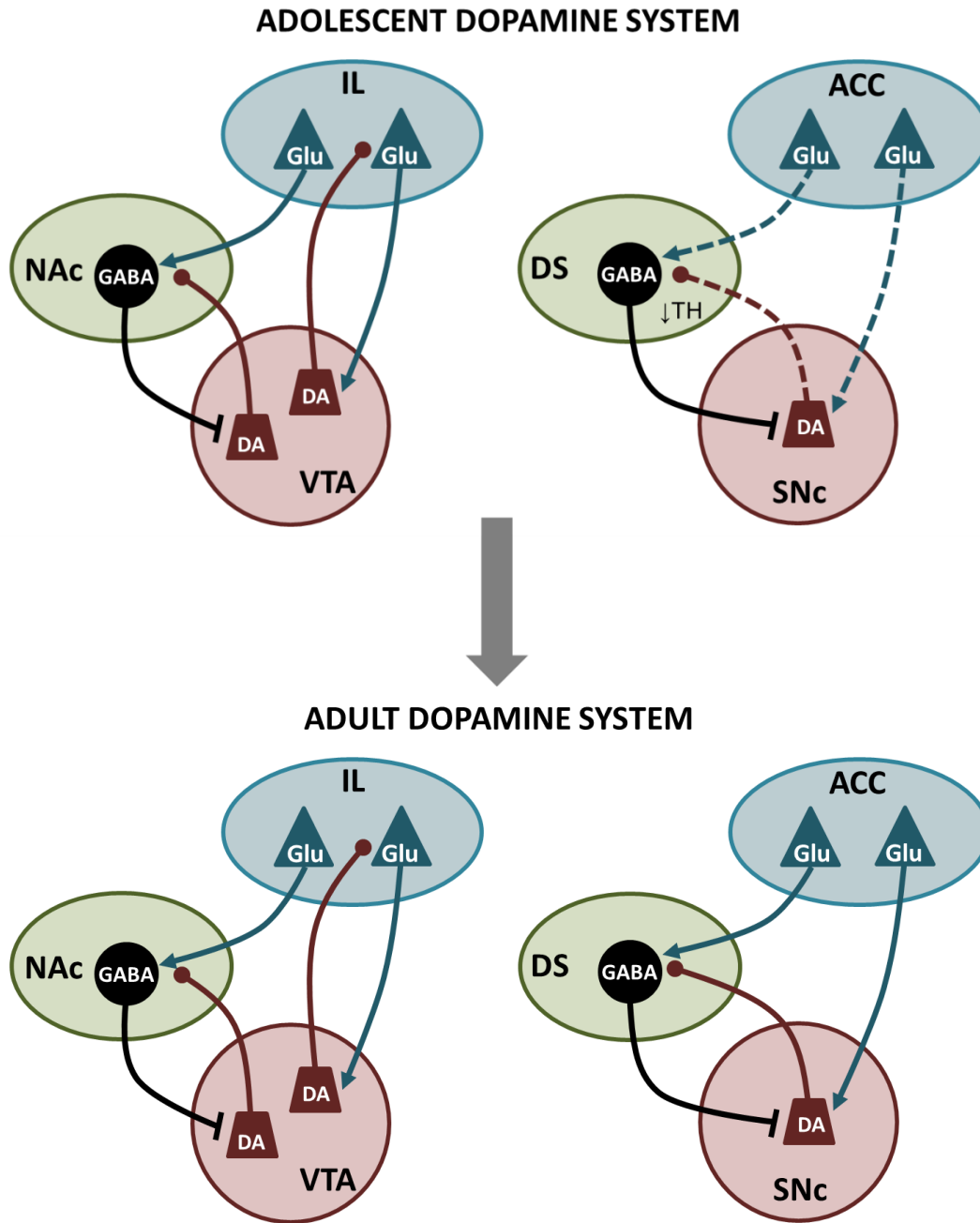


Figure 5-1 A model of hypodopaminergic neurotransmission in the adolescent DS

A simplistic diagram of corticostriatal circuits and dopamine modulation in adolescents and adults. Dopamine neurotransmission in the DS (right hand side) of adolescents is hypofunctional compared to adults, likely due to the reduced levels of TH protein found in adolescent DS. Dopamine synthesis and availability is activity dependent, and TH activity may be influenced by cortical projections to the DS or the SNc, or both. The dashed lines indicate reduced activity or connectivity from ACC to the DS and/or SNc. In contrast, dopaminergic neurotransmission is similar in the NAc of adolescent and adult rats. This may be a result of adult-like cortical projections to the NAc and VTA during adolescence. Abbreviations: ACC, anterior cingulate cortex; DA, dopamine; DS, dorsal striatum; Glu, glutamate; IL, infralimbic cortex; NAc, nucleus accumbens; SNc, substantia nigra pars compacta; VTA, ventral tegmental area.

Dopamine synthesis and availability is activity dependent, and therefore synthesis is determined by TH activity, which is regulated through gene expression (long term regulation) and through feedback inhibition by catecholamines, allosteric regulation, and phosphorylation (short term regulation) (Kumer and Vrana, 1996). It is unlikely that the reduced expression of TH in the DS of adolescents is due to maturing midbrain dopamine structures. Dopamine neuron migration to the midbrain and innervation of their targets has reached maturation before the start of adolescence, and these dopamine neurons have acquired adult morphology and functionality by the third postnatal week in rats (Voorn et al., 1988). However, firing rates of ventral tegmental area dopamine neurons have been shown to peak during late adolescence before decreasing over time into adulthood (McCutcheon and Marinelli, 2009), suggesting that there are other factors that influence and regulate dopamine discharge and availability in the adolescent striatum. Yet, there is no published work on dopamine firing in the substantia nigra throughout development. Further studies are necessary to establish basal midbrain dopamine neuron activity in adolescence, as well as characterizing adolescent midbrain dopamine neurons response to external stimuli and pharmacological manipulation. Also, it would be worth examining the molecular mechanisms that influence dopamine activity.

To further understand the region specific differences in the DS of adolescent and adult rats, it is necessary to identify the neural mechanisms involved in adolescent neurodevelopment and associated vulnerabilities, as well as determine the functional connectivity of cortical and striatal regions. What are the mechanisms influencing reduced dopamine in the DS in response to amphetamine? Are prefrontal cortical regions regulating striatal function differently as a function of age? Is there immature corticostriatal connectivity involving the DS during adolescence, compared to more mature corticostriatal connectivity involving the NAc? Is this functional

immaturity correlated with impulsivity or risky decision making? As our lab has reported recently, there are neural processing differences in both the orbital frontal cortex as well as the DS of adolescent and adult rats during reward-related tasks (Sturman and Moghaddam, 2011b, 2012). There may also be processing differences found in other prefrontal cortical structures. It would be beneficial to further examine corticostriatal interactions by using electrophysiology recordings in behaving adolescent and adult rats. For example, simultaneously recording from various areas in the prefrontal cortex and striatal subregions (further divided based on its dorsal-ventral functional and connectivity gradient) would reveal whether there are age-related differences in functional connectivity between the anterior cingulate cortex and the DS, versus similarities between the infralimbic cortex and the NAc.

5.2.3 The importance of the DS in models of adolescent vulnerabilities

The data presented in this dissertation suggest that the DS is an important region involved in adolescent neurodevelopment, and may be regulated differently in adolescents versus adults. In contrast, our findings further support the notion that the NAc is similar in both adolescents and adults. Though our studies have directly implicated differences only in dopamine neurotransmission in the DS of adolescents, we have proposed (in **Section 5.2.2**) that cortical inputs to the DS may be less mature in adolescents than adults, whereas cortical inputs to the NAc are similar in both age groups. Therefore, this proposal may provide new insights for examining the role of dopamine in cortico-dorsal-striatal circuits in adolescents. Based on previous studies, it had been established that thalamo-cortico-striatal loops are modulated by dopamine afferents from the ventral tegmental area and substantia nigra pars compacta, and this is thought to promote the learning of action sequences, selecting relevant behavioral patterns

while inhibiting irrelevant ones, and performing motivated behavior (Graybiel et al., 1994; Packard and Knowlton, 2002; Graybiel, 2005; Costa, 2007).

As mentioned previously, the DS, along with the NAc and various prefrontal cortical areas, are involved in extreme habit formation such as drug addiction (Everitt & Robbins 2005, Kalivas et al. 2005, Kalivas & Volkow 2005). In addition, dopamine function in the DS has been implicated in the pathophysiology of schizophrenia (Howes et al., 2012). This is important in the context of adolescent neural and behavioral changes, as late adolescence is a period of conversion to psychosis in individuals at high risk to develop schizophrenia (Hafner et al., 1994). In addition, adolescents are more prone to risk taking and drug addiction than adults (as reviewed in **Section 1.1**). Though the NAc has received close attention for its role in addiction, the DS is also thought to be important in the craving stage of addiction (Koob and Volkow, 2010). Studies have shown that the DS circuitry is involved in habitual compulsive cocaine use, where it was observed that there was an increase in dopamine release in the DS of prolonged cocaine-seeking, but not in the ventral striatum of adult rats (Ito et al., 2002). Disconnecting the NAc from the DS in rats self-administering cocaine also has been shown to result in decreased drug intake in animals with well-established compulsive use (Belin and Everitt, 2008). Furthermore, dopamine and glutamate neurotransmission in the DS is involved in cue-context dependent craving (Volkow et al., 2006). Together, these studies identify the DS as a fundamental component of drug addiction. More studies are needed to identify differences in the DS of adolescent and adult rats in the context of both normal habit formation and habitual drug use. Currently, it is unknown whether adolescents are more prone to forming habits than adults, or if there are age-related functional differences in neural activity of regions involved in habit formation, impulsivity and risky behavior – namely the DS and associated prefrontal cortical areas.

5.3 CONCLUSIONS

Though the proposed model above is a hypothesis for adolescent development, our data provides a platform to conduct future studies of functional connectivity of developing corticostriatal circuits. Our data suggest that under some circumstances dopamine neurotransmission in the DS of adolescents is hypofunctional compared to adults, and this reduced dopaminergic activity may be due to reduced dopamine synthesis via reduced TH, the function of which we hypothesize is modulated by cortical inputs. This notion is consistent with exaggerated responsiveness of adolescent DS neurons to salient events such as reward expectancy compared to adults, whereas NAc neurons responded similarly in adolescents and adults (Sturman and Moghaddam, 2012). Furthermore, these findings support the idea that developmental changes of the mesocorticolimbic and nigrostriatal dopamine systems may contribute to adolescent vulnerability to illness (Chambers et al., 2003; O'Donnell, 2011), but more specifically indicates that a locus of this dopaminergic involvement in adolescent vulnerability is the DS. As part of normal development, reduced DS dopamine activity may in turn reduce inhibitory modulation of DS neurons by dopamine and facilitate learning. The same mechanism also may contribute to increased vulnerability to habit formation, impulsive decision making, and development of cognitive and affective illnesses that are associated with dysregulated dopamine neurotransmission.

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