METAPLASTICITY: HOW EXPERIENCE DURING BRAIN DEVELOPMENT INFLUENCES THE SUBSEQUENT EXPOSURE TO A DRUG OF ABUSE

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ABSTRACT

The influence of experience during brain development was investigated on juvenile behavior, adult amphetamine sensitization, and neuronal structural plasticity in rats. Two experiential factors (i.e., tactile stimulation and stress) were studied either before or soon after birth. Early experience feminized social behavior in males; however, only stress enhanced anxiety-like behavior in males. Repeated amphetamine administration resulted in the development and persistence of behavioral sensitization. However, tactile stimulation attenuated the drug-induced behavioral sensitization whereas stress failed to influence the degree of sensitization. Neuroanatomical findings revealed that early experience altered the cortical and subcortical structures. Furthermore, drug exposure reorganized the brain structures involved in addiction but early experience prevented the drug-associated changes. Early adverse experience influences the subsequent exposure to a drug of abuse at anatomical level whereas a favorable experience has an effect both at behavioral and anatomical levels and thus may play a protective role against drug-induced sensitization and addiction.

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Arif Muhammad

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ACTH	adrenocorticotropic hormone
AID	dorsal agranular insular cortex
AMPH	amphetamine
BDNF	brain-derived neurotrophic factor
Cg3	cingulate area 3
DA	dopamine
EE	environmental enrichment
EPM	elevated plus maze
FGF-2	basic fibroblast growth factor
GR	glucocorticoid receptor
HPA	hypothalamic-pituitary-adrenal axis
LG	licking and grooming
LTD	long-term depression
LTP	long-term potentiation
mPFC	medial prefrontal cortex
MS	maternal separation
NAc	nucleus accumbens
NOR	novel object recognition
OFC	orbital frontal cortex
P	postnatal
PFC	prefrontal cortex
PS	prenatal stress
TS	tactile stimulation
VTA	ventral tegmental area

CHAPTER 1

General Introduction

The brain goes through various stages of development including neurogenesis, cell migration, differentiation, synaptogenesis, neuronal pruning, and myelination. Although genetic predetermination sets the stage for brain development, various experiential factors play a contributory role in offsetting some of the developmental processes (e.g., Hubel & Wiesel, 1970). Malnutrition, toxins, non-drug chemicals, drugs, and alcohol are some of the examples that have been shown to influence brain development. Experience during development, either beneficial or adverse, substantially reorganizes brain circuits with profound and long-term impact on the brain and subsequently behavior. Studies in rodents, for instance, have demonstrated that early adverse experience (e.g., stress) resulted in impaired learning and memory and altered structural plasticity (e.g., decreased spine density) in various brain regions (e.g., hippocampus) (reviewed by Weinstock, 2008). In contrast, a favorable experience (e.g., environmental enrichment) during development has been related to improved learning and memory (Rosenzweig & Bennett, 1996) and reorganization of brain architecture (van Praag, Kempermann, & Gage, 2000).

Experience during development has an enduring impact via programming the brain circuits. Consequently, the experience-induced reorganization of brain circuits modulates the response (e.g., at neuronal level) to a subsequent experience (e.g., drug exposure). The idea of experience-dependent plasticity influenced by prior experience(s), termed metaplasticity, was first coined by Abraham and Bear (1996). Metaplasticity, manifested in the brain at various levels (e.g., neuronal level), can be studied employing

different techniques (e.g., neuroanatomical) (Abraham, 2008; Kolb, Muhammad, & Gibb). Previous reports indicated that prior experience altered the response to subsequent experience that was manifested at the structural level in individual neurons (Hamilton & Kolb, 2005; Kolb, Gorny, Li, Samaha, & Robinson, 2003; Zehle, Bock, Jezierski, Gruss, & Braun, 2007). Studies from the Kolb lab concluded that psychostimulant drugs (e.g., amphetamine) increased the spine density in the nucleus accumbens in a manner similar to rearing in an enriched environment (Kolb, Gorny, et al., 2003; Norrholm et al., 2003; Robinson & Kolb, 1997). However, prior drug exposure followed by rearing in an enriched environment prevented the increase in spine density associated with the enrichment experience (Hamilton & Kolb, 2005; Kolb, Gorny, et al., 2003).

Drug exposure in adult rats was followed by rearing experience in an enriched environment in the studies reported by Kolb and associates. We wondered about the outcome if the order of experiential factors (i.e. drug and rearing environment) is switched such that a rearing environment, either a favorable or an adverse, is followed by exposure to a psychostimulant drug. Moreover, the metaplasticity studied in the previously reported experiments was limited to neuroanatomical findings where only adult rats were exposed to both prior and subsequent experiences. In the present thesis, we studied the metaplasticity at behavioral, in addition to neuroanatomical, levels. Furthermore, we exposed rats to favorable or an adverse experience during development followed by drug exposure in adulthood.

More specifically, we exposed the rats to either tactile stimulation (TS) or stress experience during development to study the long term influence of early experience on drug exposure later in life. TS, a sensory stimulation, was used as a favorable experience

whereas stress was used as an adverse experience. We hypothesized that a favorable experience during development will play a protective role whereas an adverse experience will act as a risk factor in drug-induced behavioral sensitization, an augmented locomotor response to repeated drug administration. In addition, early experience, either TS or stress, would interact with the subsequent experience (i.e., amphetamine exposure) to alter the structural architecture of the brain regions implicated in drug addiction.

The present research project, therefore, investigated the effect of prior experience (i.e. TS or stress) on subsequent exposure to amphetamine (AMPH). The metaplasticity was studied at both behavioral (i.e. AMPH-induced behavioral sensitization) and neuroanatomical levels (i.e. cortical and subcortical architecture, dendritic morphology and spine density) in brain regions known to be modulated by experience and drugs (Kolb, Gorny, et al., 2003; Robinson & Kolb, 2004).

1.1. Drug addiction

Drug addiction, characterized by persistent behavioral and neuronal adaptation, is believed to have its roots in brain development (Felitti, 2002). Although genetic predisposition cannot be underestimated (Gelernter & Kranzler, 2010), experience during pre- or postnatal brain development plays a contributory role in the development of drug addiction (Caprioli, Celentano, Paolone, & Badiani, 2007).

Most drugs of abuse exert their reinforcing effect by acting on various brain circuits associated with reward, motivation, and executive functions (Figure 1.1). Prolonged exposure to drugs in experimental animals and humans, for instance, resulted in enhancement of motivation for drugs while devaluing natural rewards (e.g., food and

sex) (reviewed by Kelley & Berridge, 2002). In addition, drug administration resulted in impaired goal-directed behavior, decision-making, behavioral inhibition, reversal learning, and working memory (Dalley et al., 2005; George, Mandyam, Wee, & Koob, 2007; Jentsch & Taylor, 1999; Schoenbaum, Saddoris, Ramus, Shaham, & Setlow, 2004). Similar cognitive behavioral deficits associated with chronic drug abuse have been reported in humans (Monterosso, Ehrman, Napier, O'Brien, & Childress, 2001; Rogers et al., 1999).

The behavioral deficits associated with repeated drug administration are the result of disrupted homeostasis due to extra physiological release of neurotransmitters (e.g., glutamate and dopamine) in the reward system. Consequently, a cascade of events takes place leading to structural (Robinson & Kolb, 2004), functional (Kalivas, Volkow, & Seamans, 2005), and epigenetic neuroadaptation (Renthal & Nestler, 2008) in various brain circuits (e.g., reward system). The underlying mechanisms behind the drug-induced neuroadaptation include alteration in neurotransmitters, membrane receptors, cell signaling pathways, transcription factors, gene expression, and protein synthesis (Koob, 2006). The long term transformation in brain circuits associated with drug abuse thus results in maladaptive behaviors (e.g., impulsivity).

1.2. Regions implicated in addiction

The drug-induced behavioral deficits reported in animals and humans have been associated with brain regions important for survival (i.e. food and sex). Although chronic drug administration results in neuroadaptation in various brain regions (Koob, 2006), the nucleus accumbens (NAc) and prefrontal cortex (PFC) are the regions of interest in

addiction research because of their role in relapse to drug abuse (Figure 1.1) (Kalivas, et al., 2005). Relapse is a challenging phenomenon in the field of addiction research that hinders drug addiction treatment (O'Brien, 2005).

1.2.1. Striatum including nucleus accumbens

The striatum is divided into a dorsal (i.e. caudate nucleus and putamen) and ventral region (i.e. nucleus accumbens). Both the dorsal and ventral striatum have been implicated in drug addiction in experimental animals including rodents and monkeys (Gerdeman, Partridge, Lupica, & Lovinger, 2003; Ito, Dalley, Robbins, & Everitt, 2002; Li, Kolb, & Robinson, 2003). Similarly, human imaging studies also confirmed the involvement of the striatum in drug addiction (Volkow, Fowler, & Wang, 2003).

NAc, a part of the ventral striatum, receives inputs from the limbic regions (e.g., the PFC) and projects to the motor system (e.g., the ventral pallidum). The circuitry provides the NAc with a central position to translate motivation received from the limbic system into a goal-directed behavior (Figure 1.1) (Groenewegen, Wright, Beijer, & Voorn, 1999; Mogenson, Jones, & Yim, 1980). The NAc is critical for the development of drug addiction as experimental animals self-administer psychostimulant drugs in the NAc, whereas a lesion in this area abolishes the drug seeking behavior (reviewed by Ikemoto & Panksepp, 1999). Most addictive drugs (e.g., cocaine and amphetamine) modulate neurotransmitter (e.g., dopamine and glutamate) levels, gene and protein expression, and neurotrophic and transcription factor levels (reviewed by Koob & Volkow, 2010). Drugs of abuse modulate long-term potentiation and depression (LTP and LTD), two principle forms of synaptic plasticity, that play a major role in drug

associated neuroadaptation in the NAc (Russo et al., 2010). The resulting altered neuronal physiology with repeated drug administration (e.g., of synaptic connectivity) is thus reflected in individual neurons through structural modification of dendritic growth and spine density. For instance, repeated psychostimulants (cocaine, amphetamine, and nicotine), either self- or experimenter- administered, altered neuronal morphology by increasing dendritic arborization, length, and spine density in the NAc (Norrholm, et al., 2003; Robinson & Kolb, 2004). The maladaptation as a result of repeated drug administration may be associated with impaired NAc-dependent behaviors (e.g., reward processing).

1.2.2. Prefrontal cortex

Prefrontal cortex, the most anterior region of the frontal lobe, is involved in so called 'executive' functions. The PFC has been associated with the execution of functions such as planning, decision making, behavioral inhibition, and working memory to name few. The PFC, divided in the medial PFC and the orbital frontal cortex (OFC) in rodents, receives dopaminergic inputs from ventral tegmental area (VTA) and projects to the NAc in addition to other regions of the limbic system (e.g., hippocampus) (Figure 1.1).

The PFC, similar to the NAc, plays a contributory role in the development of drug addiction. For example, experimental animals will self-administer most addictive drugs in the various subregions of the PFC (Goeders & Smith, 1993). Similarly, human imaging has shown activation of the PFC in response to intravenous administration of methylphenidate in cocaine abusers (Volkow et al., 2005). Furthermore, chronic drug exposure produced neuroadaptation studied at genetic, molecular, functional, and

structural levels in the subregions of the PFC (Castner, Vosler, & Goldman-Rakic, 2005; Freeman et al., 2010; Robinson & Kolb, 2004). For example, Robinson and Kolb reported alteration in neuronal morphology in the subregions of the PFC as a result of repeated psychostimulant administration in rats (1997; 2001; 2004; 2005; 2009) . Furthermore, the drug-induced structural alterations (i.e. dendritic arborization, length, and spine density) persisted for months in rats (Kolb, Gorny, et al., 2003).

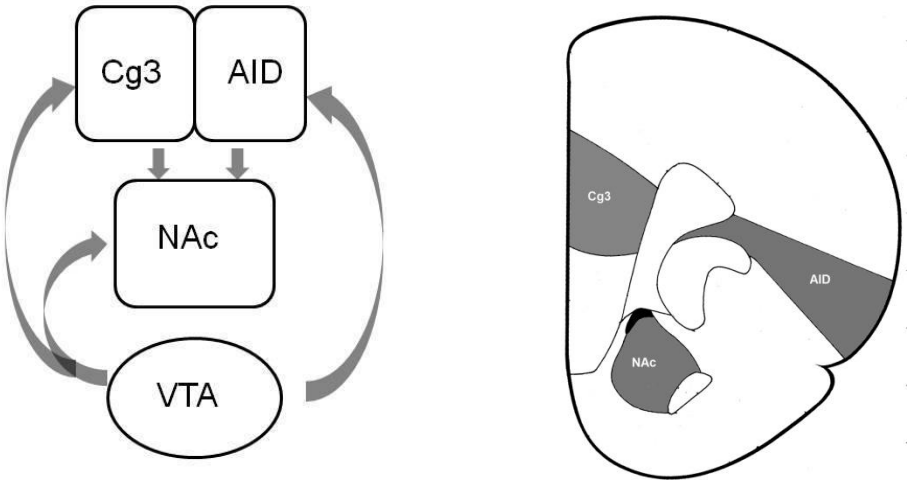

Figure 1.1. Brain regions implicated in drug addiction. Left: a simplified diagram of the addiction circuit showing the nucleus accumbens (NAc), and Cg3 region of the medial prefrontal cortex, and the dorsal agranular insular cortex (AID) of the orbital frontal cortex. Right: a coronal section showing the NAc, Cg3 and AID regions studied for the structural plasticity associated with early experience, drug exposure, and an interaction between the two.

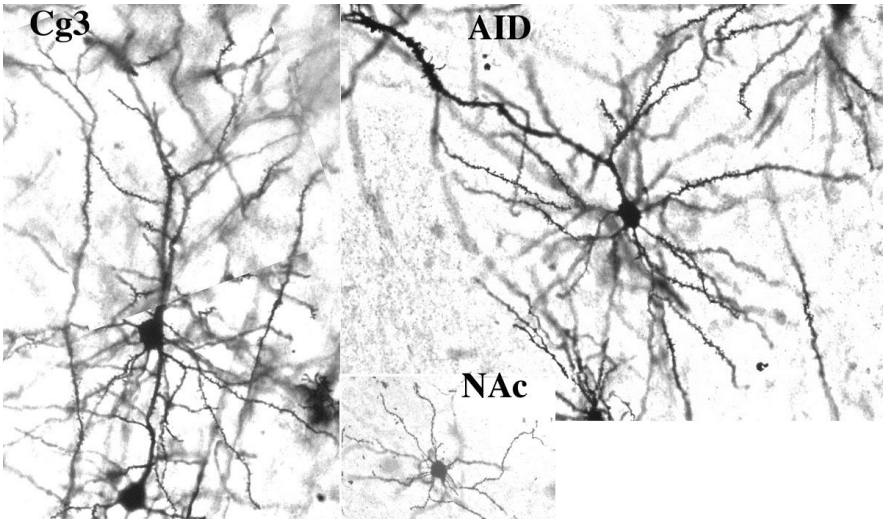

Figure 1.2. Photomicrographic (200X) examples of Golgi stained dendritic segments of neurons in Cg3, AID, and NAc.

Drug-induced structural modulation could be the result, for instance, of altered neurotransmitter levels (e.g., dopamine and glutamate), neurotrophic factor levels (e.g., BDNF), gene expression (e.g., Cdk5), and neurophysiology (e.g., LTP) of the PFC in experimental animals (Dietz, Dietz, Nestler, & Russo, 2009; Homayoun & Moghaddam, 2006; Niwa, Yan, & Nabeshima, 2008). Similar to experimental animals, drug abuse in humans produced structural and functional alterations in the PFC. For example, human imaging studies showed that stimulant abuse produced cortical thickness abnormalities (Makris et al., 2008) and increased activation of the PFC (Volkow, et al., 2003).

1.3. Experiential factors

Drug-induced neuroadaptation in the PFC-NAc circuit is believed to be the core of drug seeking and taking behavior in addition to relapse to drug addiction. However, a predisposition to abused drugs is strongly associated with the degree of neuroadaptation as a result of drug administration (Nader & Czoty, 2005; Volkow et al., 1999). The role

of genetic predisposition should not be down played but environment (e.g., experience during development) is a major contributor in the development and relapse to drug addiction. For example, retrospective studies in humans reported an increased propensity to abuse drugs and alcohol in individuals with adverse childhood experiences (Felitti, 2002). Environmental manipulation in lab animals through exposure to various experiential factors (e.g., stress) corroborated the findings of addiction vulnerability in humans (Goeders, 1998). It is therefore hypothesized that early experience has a programming effect on the brain and subsequently behavior (Kaffman & Meaney, 2007; Lupien, McEwen, Gunnar, & Heim, 2009; Pryce & Feldon, 2003). The idea of the 'preprogrammed' brain led to the present project where I investigated the role of various experiential factors (e.g. tactile stimulation and stress) in modulating behavioral sensitization in rats.

1.3.1. Tactile stimulation

Sensory stimulation during development is an example of a favorable experience in rodents that has been associated with long-term beneficial influence on brain and behavior. Sensory stimulation such as environmental enrichment and maternal licking and grooming has been studied extensively in rodents. Licking and grooming, for instance, provides sensory stimulation to newborn pups through physical interaction. High, compared to low licking and grooming, has been associated with positively modulating emotionality, improving learning and memory, and enhancing maternal bonding and attachment (Kaffman & Meaney, 2007). Furthermore, the positive outcome associated with sensory stimulation during development has been associated with a

dampened stress response, through upregulation of glucocorticoid receptors in the brain, in facing challenging situations later in life (Meaney, 2001).

Postnatal tactile stimulation (TS), a form of sensory stimulation, is closely related to maternal licking and grooming in rats (Gonzalez, Lovic, Ward, Wainwright, & Fleming, 2001; Lovic & Fleming, 2004; Lovic, Fleming, & Fletcher, 2006). Rats exposed to TS experience receive sensory stimulation by gentle stroking either through a human hand or mechanical means (e.g., a soft hair brush) (Figure 1.3). Such stimulation works as 'enrichment' for the developing brain and leaves a favorable impact to face challenging experiences later in life. Previous works in rodents suggest that early sensory stimulation improves learning and memory (Rosenzweig & Bennett, 1996), positively regulates emotionality (Fernandez-Teruel, Escorihuela, Castellano, Gonzalez, & Tobena, 1997), and dampened the hypothalamic-pituitary-adrenal (HPA) axis response (Pauk, Kuhn, Field, & Schanberg, 1986). Furthermore, Kolb and Gibb (2010) investigated the effect of stimulation on neonatal brain injury and demonstrated that TS during development improved recovery from early brain injuries and altered neuronal morphology in rats.

Similarly in humans, massage therapy, a form of tactile stimulation during pregnancy in women, has produced positive outcomes for children such as alleviating slow sensory-motor development associated with preterm birth, and enhancing mother-child bonding (Bellieni et al., 2007). Moreover, Cuba's system of maternal health program also supports the benefits of early sensory stimulation on children's learning abilities in addition to a positive health outcome for mother and child (Keon, 2009).

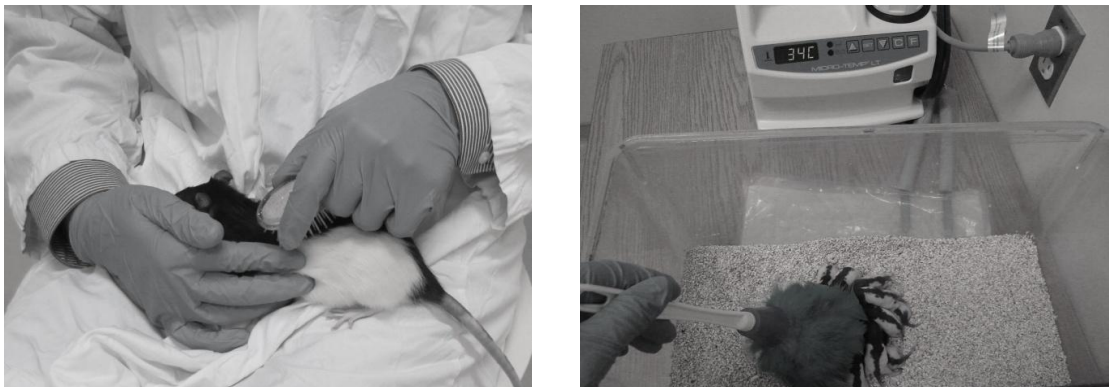

Figure 1.3. The rats received tactile stimulation during gestational development through stroking a pregnant rat with a small hair brush (left) or stroking newborn pups with a feather duster (right).

1.3.2. Stress

Stress has been employed as an adverse early life experience in experimental animals by either exposing the pregnant dam to stress [prenatal stress (PS)] or removing pups from the dam [maternal separation (MS)] during the critical period of brain development (Figure 1.4). Experimental animals have been studied for the last few decades to categorize the long term impact of stress during development on brain and behavior. Previous reports indicate that stress during development, in rodents as well as non-human primates, produced behavioral abnormalities such as an elevated and prolonged stress response, impaired learning and memory, deficits in attention, and altered exploratory behavior (reviewed by Weinstock, 2008). Similarly in humans, stress during development predisposes individuals to maladaptive behaviors and psychopathologies. Gestational stress has not only been associated with cognitive impairments (Brouwers, van Baar, & Pop, 2001; King & Laplante, 2005; O'Connor, Heron, Golding, Beveridge, & Glover, 2002) but also to mental disorders. For instance, prenatal stress is a risk factor in the development of schizophrenia, attention-deficit

hyperactivity disorder, depression, and drug addiction (Felitti, 2002). Nonetheless, most of the human findings, inferred from retrospective studies, are confounded by intrinsic and extrinsic factors that are difficult to control. Even so in controlled human studies, the study of gestational stress in relation to brain development is mostly limited to the early years of development (O'Connor, et al., 2002; Van den Bergh & Marcoen, 2004).

Similarly, the postnatal stress model of MS, for instance, has been associated with a number of behavioral abnormalities and alterations in the brain anatomy and physiology. For example, MS in rats influenced a number of behaviors such as exploration, emotionality, social behavior, and learning and memory (Lehmann & Feldon, 2000). In addition, increased alcohol preference and intake has been reported in maternally separated rats (Huot, Thrivikraman, Meaney, & Plotsky, 2001). The enhanced alcohol preference could be the result, for example, of altered key neurotransmitters in the brain (Matthews, Dalley, Matthews, Tsai, & Robbins, 2001) in addition to resetting the HPA-axis (Plotsky & Meaney, 1993). Furthermore, prolonged MS produced persistent structural alteration in various brain regions (e.g., hippocampus) (reviewed by McEwen, 2000) that could be associated with some of the reported impaired behaviors (e.g., learning and memory).

Stress has been developed as an adverse experience in animals although the findings among studies are not conclusive. Depending on the severity of the stress in addition to other factors (e.g., test paradigm), stress might not always be detrimental for brain and behavior. For example, studies related to mild stress during development reported either no persistent maladaptation or even indicated a beneficial influence on the developing brain and subsequently the behavior.

Figure 1.4. Rats were exposed to prenatal stress (PS) where a pregnant rat was allowed to stay on an elevated platform (left) or the newborn pups were deprived of maternal care for a certain period of time (MS (right).

1.4. Procedural considerations

Studies related to the long-term impact of early experience on brain and behavior is conducted employing a variety of behavioral and neuroanatomical procedures. Therefore, the comparison among studies is hindered by procedural differences in addition to other factors (e.g., animal species, strain, sex, and age). This might account for some of the reasons for discrepancy in the findings among different studies. Hence, one should be aware of such factors while interpreting and generalizing the results related to the influence of early experience on brain and behavior. Although a discussion in detail about every possible factor is beyond the scope of the present thesis, the following are some of the major procedural caveats that need to be addressed.

1.4.1. Juvenile behavior

To study the effect of early experience on juvenile behavior, the rats were tested for exploratory, anxiety-like, cognitive and social behaviors. The tasks included open field locomotion, elevated plus maze, novel object recognition, and play fighting

behavior. Previous work has shown the effect of early experience on the above mentioned behaviors (e.g., see Weinstock, 2008). In addition, behavioral impairment (e.g., altered social behavior) has a correlation with later drug sensitization or enhanced self-administration (e.g., see Miczek, 2008). The brain regions that are implicated in drug addiction (e.g., the medial and orbital frontal cortices) are also mediate behaviors such as temporal order memory and social behaviors (Bell, Pellis, & Kolb, 2010; Hannesson, Howland, & Phillips, 2004). Therefore, juvenile behavior could potentially play an important role to be investigated as a possible link between early behavior and later drug abuse susceptibility or resiliency. The objective of testing rats for various behaviors is to find out how early experience affects such behaviors and a possible association between juvenile behavior and drug sensitization as adults.

1.4.2. Addiction model

Addiction compared to other neurological disorders has some excellent experimental models including drug self-administration, conditioned place preference and behavioral sensitization. The experimenter-administered behavioral sensitization model (Robinson & Berridge, 1993) was employed in the present studies. Behavioral sensitization, usually measured as locomotor activity in rats, is an enhanced response to drugs with repeated administration. The non-contingent drug-induced behavioral sensitization might not have face validity for human drug abuse that involves voluntary intake. However, experimenter-administered and self-administration studies in laboratory animals show similarities at the behavioral level and in associated neurobiological alterations (but see McFarland, Lapish, & Kalivas, 2003). For example, prior

experimenter-administered drug treatment in rats showed enhanced drug self-administration (Piazza, Deminiere, le Moal, & Simon, 1990) or the animals worked harder to receive the drug (Vezina, Lorrain, Arnold, Austin, & Suto, 2002). Drugs that are experimenter-administered, similar to self-administration, have rewarding properties assessed in drug-associated enhanced conditioned place preference (Lett, 1989).

Comparable neuroplastic adaptation (i.e. dendritic growth and spine density) was observed in the NAc and the PFC of both behavioral sensitization and self-administration rat models (Robinson & Kolb, 2004). Repeated drug administration slowly up regulates the ΔFosB, a transcription factor associated with drug sensitization, in brain regions associated with addiction (e.g., NAc). ΔFosB targets, in addition to other genes, Cdk5, which is associated with alteration in dendritic growth and spine density. Interestingly, the ΔFosB phenotype, a mouse model of drug addiction, shows increased drug conditioned place preference and enhanced drug self-administration in addition to expressing behavioral sensitization to repeated drug administration (reviewed by McClung et al., 2004). Humans, similar to laboratory animals, also show drug-induced behavioral sensitization with repeated use. The locomotor effects of drug-sensitization include, for instance, increased speech and eye-blink response (reviewed by Robinson & Berridge, 2008).

Owing to the similarities between behavioral sensitization and self-administration models of drug addiction, the former has been extensively studied to characterize drug-induced neural plasticity. In addition, the behavioral sensitization model has the advantage of ease of drug delivery because it does not involve surgical manipulation and extensive animal training to self-administer drugs.

1.4.3. Stress paradigm

MS has been employed as a model of early adverse experience although there are major procedural differences within MS paradigms (reviewed by Gutman & Nemeroff, 2002; Pryce & Feldon, 2003; Sanchez, Ladd, & Plotsky, 2001). For example, there is a wide range in the duration and frequency of separation (e.g., 30-180 minutes for 1-3 weeks) in addition to other extraneous factors (e.g. thermoregulation during separation). Similarly, the prenatal stress paradigm (i.e. elevated platform) we employed is different from the few previous reports of prenatal stress (e.g., restraint stress) to study drug addiction. The elevated platform stress is believed to be more of a psychological stress in nature compared to restraint stress, which additionally involves physical discomfort. The elevated platform model is an established stress paradigm in which Wong, et al., (2007) reported elevated blood corticosterone level immediately after a 30-minutes stress procedure. However, we are the first lab, to the best of our knowledge, to employ the elevated platform stress paradigm to study the long-term effect of prenatal stress.

1.4.4. The choice of control group

The choice of control group to compare the effects of pup-dam isolation (e.g., during MS) has been a point of discussion (see Pryce & Feldon, 2003); however, there is no clear consensus among researchers. The commonly employed control groups in experiments where separation of pups from dam include handled, non-handled, and animal-facility reared groups. The handled group is separated from the dam, depending on the test paradigm, mostly for 5-15 minutes. In addition, depending on the practices in a specific laboratory, twice a week the handled group receives routine animal husbandry

of cage change by the animal care staff. In contrast, the non-handled group is neither separated from the dam nor goes through the regular animal care procedure of cage change. An additional group, called animal-facility reared, has been extensively utilized as a control group. Although not separated from the dam, the animal-facility reared group receives the routine cage change by animal care staff, usually twice a week in most laboratories. Each group has its own limitations, for instance, the non-handled group is not considered the 'normal' control group in a lab setting because it does not go through the routine husbandry practices.

Following animal welfare guidelines to reduce the number of animals, where possible, we decided to employ the animal-facility reared group as the control for two reasons. First, the inclusion of every possible control group was beyond the scope of the present research project. Second, animal-facility reared was the only possible control group for comparison to groups exposed to both pre- and postnatal experiences.

1.4.5. Maternal care

The quality of maternal care has been shown to play a role in mediating pup behavior in addition to modulating brain physiology (reviewed by Cameron et al., 2008). The limited literature available, regarding maternal care in studies that involved dam-pup separation, suggests no general agreement on the role of maternal care in mediating the effects of separation. Whereas some authors suggest there is a disruption of maternal care, still others fail to find any role of maternal care in mediating the effects of separation. For example, the effects of maternal separation were proposed to be the result of alterations in the quality of maternal care rather than from direct effects of the

separation (Huot, Gonzalez, Ladd, Thrivikraman, & Plotsky, 2004). In contrast, several studies did not find maternal care to play a role in mediating the effects of separation (Macri, Mason, & Wurbel, 2004; Pryce, Bettschen, & Feldon, 2001). Whatever the case may be, the maternal factors cannot be neglected while studying the influence of developmental factors on pups. The investigation of the maternal care hypothesis needs exhaustive investigation and was beyond the scope of the present study.

1.5. Objectives of the thesis

The present study was designed with a main objective to investigate metaplasticity, at behavioral and neuroanatomical levels, associated with prior favorable or adverse experience and subsequent drug exposure.

The following objectives were achieved through the present thesis:

- To investigate the influence of a favorable or an adverse experience, before or soon after birth, on juvenile exploratory emotional, cognitive, and social behaviors;
- To study the long-term effect of an early favorable or adverse experience on psychostimulant-induced behavioral sensitization; and,
- To investigate the structural plasticity associated with early experience, drug, or an interaction between the two, in brain regions implicated in drug addiction.

1.6. Hypotheses

The following hypotheses guided the research:

- An early favorable experience of tactile stimulation, before or soon after birth, will attenuate the amphetamine-induced behavioral sensitization in adults;
- An early adverse experience of stress, before or soon after birth, will augment the amphetamine-induced behavioral sensitization in adults; and,
- Prior experience, either tactile stimulation or stress, will neuroanatomically interact with subsequent (i.e. amphetamine) exposure (experience).

1.7. Organization of the thesis

The thesis includes four experiments, two investigating tactile stimulation before or soon after birth (i.e. pre- or postnatal TS) and two looking at stress before or soon after birth (PS or MS). The experiments are presented as individual papers, each of which has been submitted to a journal for review and publication. As a result, many of the methods are repeated from study to study. The early environmental manipulation was different in each study but for the most part the behavioral and anatomical procedures were identical. One exception is that owing to poor staining, it was not possible to obtain dendritic measures in the TS experiments. Instead, we availed ourselves of the opportunity to study prefrontal cortical thickness and striatum size. We examined the PS- and MS-associated spine density and dendritic morphology and reported this with the behavioral findings. Due to the far-reaching implications, beside ease of understanding, of metaplasticity at neuroanatomical level, the dendritic morphology (i.e. branching and length) findings for both prenatal stress and maternal separation experiments were reported in a separate chapter.

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CHAPTER 2

Prenatal tactile stimulation attenuates drug-induced behavioral sensitization, modifies behavior, and alters brain architecture*

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Abstract

Based on the findings of postnatal tactile stimulation (TS), a favorable experience in rats, the present study examined the influence of prenatal TS on juvenile behavior, adult amphetamine (AMPH) sensitization, and structural alteration in the prefrontal cortex (PFC) and the striatum. Female rats received TS with a baby hair brush throughout pregnancy, and the pups born were tested for open field locomotion, elevated plus maze (EPM), novel object recognition (NOR), and play fighting behaviors. Development and persistence of drug-induced behavioral sensitization in adults was tested by repeated AMPH administration and a challenge, respectively. Structural plasticity in the brain was assessed from the prefrontal cortical thickness and striatum size from serial coronal sections. The results indicate that TS females showed enhanced exploration in the open field. TS resulted in feminization of play in males exhibited by decreased frequency of playful attacks whereas the response to face or evade an attack was not affected. Anxiety-like behavior and cognitive performance were not influenced by TS. AMPH administration resulted in gradual increase in locomotor activity (i.e. behavioral sensitization) that persisted at least for 2 weeks. However, both male and female TS rats exhibited attenuated AMPH sensitization compared to sex-matched controls. Furthermore, the drug-associated alteration in the prefrontal cortical thickness and striatum size observed in controls were prevented by TS experience. In summary, TS during prenatal development modified juvenile behavior, attenuated drug-induced behavioral sensitization in adulthood, and reorganized brain regions implicated in drug addiction.

Keywords: behavioral sensitization, addiction, plasticity, tactile stimulation, massage, rough and tumble play

2.2. Introduction

Massage therapy, a form of tactile stimulation, during pregnancy in women is gaining recognition for its anxiolytic and pain relieving properties in addition to mitigating other pregnancy-related problems (Davidson, Jacoby, & Brown, 2000; Field et al., 1999; Hart et al., 2001; Wang et al., 2005). However, despite beneficial impact of massage on women's health, the long term effects of prenatal stimulation on a child's brain and behavior is understudied and generally limited to the first few months of life. Nevertheless, the findings of studies related to massage therapy are promising. Massage during pregnancy has a positive outcome for children, alleviating slow sensory-motor development associated with preterm birth, and enhancing mother-child bonding (Bellieni, et al., 2007). Moreover, Cuba's system of maternal health program also corroborates the benefits of early tactile stimulation intervention on children's learning abilities in addition to a positive health outcome for mother and child (Keon, 2009).

During the last few decades, researchers have manipulated the environment during brain development, by exposing laboratory animals to a variety of experiences in order to study the long term impact of early intervention on brain and behavior (reviewed by Fone & Porkess, 2008; Weinstock, 2008; Zhang & Meaney, 2010). However, most of the early interventions, either favorable (e.g., maternal licking and grooming) or adverse (e.g., social isolation), are studied during postnatal brain development (reviewed by Kaffman & Meaney, 2007; Kikusui & Mori, 2009). On the other hand, the influence of prenatal experiential manipulation on brain and behavior is not well studied and if investigated is mostly related to adverse experiences, such as infectious agents (Watson, Mednick, Huttunen, & Wang, 1999), malnutrition (Brown et al., 1996), toxins (Tamaru,

Hirata, & Matsutani, 1988), drugs and alcohol (Eriksson, Ankarberg, & Fredriksson, 2000; Noble & Ritchie, 1989; Sobotka, 1989; Streissguth, Sampson, & Barr, 1989), and stress (Clarke & Schneider, 1997).

Recently, researchers started taking interest in the beneficial impact of favorable experiences during prenatal period. For example, prenatal environmental enrichment accelerated structural maturation of the retina in rat fetus (Sale et al., 2007) and helped in recovery from neonatal brain injuries (R. Gibb and B. Kolb, unpublished observations). Similarly, tactile stimulation (TS), a form of sensory stimulation, during prenatal development improved recovery from neonatal brain injuries and facilitated motor activity (R. Gibb and B. Kolb, unpublished observations). Additionally, TS altered dendritic morphometry and spine density in various brain regions in rats (Kolb & Gibb, 2010).

We recently reported the favorable long term effect of *postnatal* TS in rats on amphetamine-induced behavioral sensitization, and structural plasticity in brain regions implicated in drug addiction (Muhammad, Hossain, Pellis, & Kolb, 2011). The promising results of postnatal TS in rats laid the foundation for the present study to investigate the long-term effect of TS during prenatal period on drug-induced behavioral sensitization. Juvenile exploratory, emotional, cognitive, and social behaviors were also studied for the potential modulation by prenatal TS. Previous reports indicated that experience (e.g., environmental enrichment) interacted with psychomotor stimulants (e.g., nicotine) in structural alteration of brain regions (e.g. nucleus accumbens, prefrontal cortex) (Hamilton & Kolb, 2005). Previous reports indicated that prior experience dramatically reduced the response to subsequent experience, which was manifested at the structural

level in the brain (Hamilton & Kolb, 2005; Kolb, et al., 2003b; Zehle, et al., 2007). For example, psychostimulants (e.g., amphetamine), similar to rearing in an enriched environment, increased the spine density in the nucleus accumbens (Kolb, et al., 2003b; Norrholm, et al., 2003; Robinson & Kolb, 1997). However, prior drug exposure followed by rearing in an enriched environment blocked the increase in spine density associated with the enrichment experience (Hamilton & Kolb, 2005; Kolb, et al., 2003b). In the present study we switched the order of experiential factors such that an experience (i.e., TS) was followed by drug exposure, unlike previous studies where drug exposure was followed by rearing experience in complex housing. We examined brain regions, implicated in drug addiction (i.e., medial prefrontal cortex, orbital frontal cortex, and nucleus accumbens) for structural alterations in cortical thickness and neuronal morphology to investigate the interaction of prenatal TS experience and AMPH-induced behavioral sensitization.

2.3. Material and methods

2.3.1. Animals

Long Evans dams received tactile stimulation while pregnant. The pups born were randomly selected with not more than two pups of each sex from the same litter. Male (control: 16; TS: 19) and female (control: 16; TS: 21) rats, were used in the experiment. The number of rats run in the behavioral tasks varies owing to technical problems, such as, video filming or inadvertent moving of one of the objects from its original position in the NOR task. The control rats were also used in another experiment (Muhammad, et al., 2011). The pups were housed in the breeding colony with their respective dams at the

Canadian Centre for Behavioral Neuroscience (CCBN), University of Lethbridge, Alberta, Canada. At weaning, prenatal TS rats were housed in pairs in standard shoe-box cages with another animal of the same sex in temperature- and humidity-controlled room. Rat chow food and water were provided *ad lib*. The rats were left undisturbed except for regular cage cleaning, running behavioral tasks, and amphetamine (AMPH) administration.

2.3.2. Tactile stimulation

Long Evans female rats, raised in the breeding colony at our facility, received tactile stimulation (TS) throughout pregnancy starting a week before being paired with males. Briefly, female rats were transported in a box with new bedding to a separate room. Individual rats were placed in the experimenter's lap and gently stroked with a baby hairbrush by stroking from the neck to the lower back. The procedure was carried out for 15 minutes three times a day, approximately the same time and by the same experimenter. The control dams were transported along the TS dam to the separate room but were not subjected to TS procedure. Once born, the mother and pups were left undisturbed (aside from animal care maintenance) until the commencement of behavioral testing.

2.3.3. Behavior

The effect of early TS on juvenile behavior was investigated by testing the rats between postnatal (P) 30-40 in a battery of behavioral tasks. The tests included open field

locomotion, elevated plus maze (EPM), novel object recognition (NOR)-recency discrimination version, and play fighting behavior.

2.3.3.1. Open field locomotion

Exploratory behavior of the rats was evaluated as open field locomotion, recorded for ten minutes using Accuscan activity monitoring Plexiglas boxes (L 42cm, W 42cm, H 30cm). The activity was recorded as the number of sensors beam breaks in the boxes attached to a computer. The horizontal beam breaks, used as an index of locomotor activity, were recorded on the computer with VersaMax^{TM} program and converted to spreadsheet using VersaDat^{TM} software (AccuScan Instruments, Inc., Columbus, OH).

2.3.3.2. Elevated plus maze

The EPM, a '+' shape maze with two closed and two open arms, was used to test anxiety-like behavior. The length of each arm of the maze measured 113 cm with a width of 10 cm while the maze was elevated 88 cm above the ground. Rats were placed in the center of the maze facing a closed arm and were allowed to explore the maze for 5 minutes. Exploration behavior was videotaped with a camera. The time spent in closed arms and the number of entries in each open and closed arms were scored and analyzed to assess anxiety-like and exploratory behaviors, respectively.

2.3.3.3. Novel object recognition

Novel object recognition was carried out to evaluate exploration of a novel object as well as exploration of objects in temporal order. Rats were habituated to a Plexiglas

box for 15-20 minutes for four days prior to the commencement of the testing sessions. The NOR task was comprised of three trials following the procedure with minor modification described elsewhere (Hannesson, Howland, & Phillips, 2004; Mitchell & Laiacona, 1998). Briefly, rats were allowed to explore objects and taped with a video camera. Glass candle holders, of similar size but different shape and color, were used as objects. During the first training trial, a rat was exposed to two novel but similar objects for a 4-minute period. After a 60-minute delay, the rat was again exposed to two new objects, again similar but different objects from the first trial. After an additional 60-minute delay, the rat was exposed to one object each from the first and second trial, termed as 'old' and 'recent' familiar objects, respectively. The objects and testing area were cleaned with 30% alcohol between each trial for disinfection and odor removal. Rats were transported back to their home cages in the 60-minute delay between the trials.

Exploration of each novel object in the first two trials and 'old' and 'recent' familiar objects in the third trial was scored. The ratio of time spent with 'old' familiar object was calculated as the difference between times spent with 'old' and 'recent' familiar object divided by the total time exploring both objects [i.e. (old – recent)/(old + recent)] (Hannesson, et al., 2004).

2.3.3.4. Play fighting

Juvenile rats were allowed to play in pairs to assess the effect of TS on social behavior. TS and control rats were housed in a pair as playmates for a period of about two weeks. The playmates, as juveniles, were habituated to a play box (50 cm X 50 cm X 50 cm) for about 30 minutes for 3 days. For play deprivation before testing, rats were

housed individually in an isolation room for 24 hours at the end of habituation period. The play behavior was recorded for 10 minutes with a night shot camera. On testing day both playmates were color marked on the tail with two separate paints to make them identifiable in the video recording, which was filmed in the dark. Rats were transported to their home cage after the play session and pair-housed for the rest of the experiment. The video recording, analyzed frame by frame, was scored for number of attacks, and complete rotation defense or evasion generated in response to an attack (Pellis & Pellis, 1990). The defense was scored as 'complete rotation' when a rat had both fore- and hind limbs in the air while lying in a supine position. If the rat turned away from the attacker instead of facing an attack, it was scored as evasion. The probability of complete rotation or evasion was calculated as the number of complete rotation or evasion divided by the total number of attacks carried out by the playmate (Pellis & Pellis, 1990).

2.3.4. Amphetamine sensitization

2.3.4.1. Amphetamine administration

To see the effects of prenatal TS on psychomotor stimulant behavioral sensitization, adult rats (P80) were administered with D-amphetamine sulfate (Sigma Aldrich, St. Louis, MO, USA) dissolved in sterile 0.9% saline. Locomotor activity, used as an index of behavioral sensitization (Wise & Bozarth, 1987), was recorded using Accuscan activity monitoring system, comprised of Plexiglas boxes (L 42cm, W 42cm, H 30cm) connected to a computer. The rats were habituated to the activity boxes for 30 minutes followed by AMPH (1 mg/kg body weight, i.p.) or 0.9% saline administration, both at a volume of 1 ml/kg. Rats were immediately placed back in the activity boxes

post injections and the activity was recorded for 90 minutes. The drug was administered once a day for 14 consecutive days, at approximately the same time every day. Locomotor activity recorded on a computer with VersaMax^{TM} program was converted to spreadsheet using VersaDat^{TM} software (AccuScan Instruments, Inc., Columbus, OH). The rats were returned back to their home cages each day after the end of AMPH testing session. The development of sensitization was determined by analyzing activity recorded over a 14-day period using mixed ANOVA with experience (TS or control), drug (AMPH or saline) as independent factors, and day (day 1-14 days) as a repeated measure factor, followed by Bonferroni's post hoc test for multiple comparisons.

2.3.4.2. Challenge

The rats were given a withdrawal period of 2 weeks after AMPH administration period, followed by a challenge with AMPH (1mg/kg, i.p.) given to both prior AMPH- and prior saline-treated rats. Locomotor activity was recorded in activity monitoring boxes similar to the development of AMPH sensitization procedure described above. All rats were challenged with AMPH to see the persistence of behavioral sensitization in prior AMPH-treated rats compared to prior saline-treated rats. Please refer to Figure 2.1 for the timeline of the experiment.

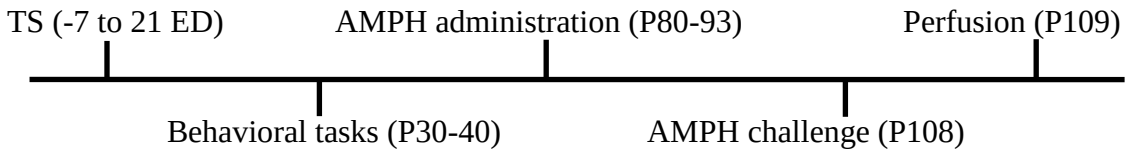

Figure 2.1. A timeline of the experiment. Rats received tactile stimulation during embryonic development (ED) starting a week before conception till birth. As juveniles, rats were run in a battery of behavioral tasks followed by chronic amphetamine (AMPH) administration. The rats were challenged with AMPH after a withdrawal period of two weeks and perfused the next day.

2.3.5. Anatomy

2.3.5.1. Perfusion and staining

Approximately 24 hours post AMPH challenge, the rats were given an overdose of sodium pentobarbital solution i.p. and perfused with 0.9% saline solution intracardially. The brains removed from the skull were trimmed by cutting the olfactory bulb, optic nerves and spinal cord. The brains were then weighed and preserved in Golgi-Cox solution for 14 days followed by transfer to 30% sucrose solution at least for 3 days. The brains were sliced at a thickness of 200 μm on a Vibratome and fixed on gelatinized slides. The slides mounted with brain sections were processed for Golgi-Cox staining, following the protocol described by Gibb and Kolb (1998). Unfortunately, owing to unforeseen technical problems, the Golgi staining proved unreliable so the tissue was used for gross measures of cortical change, namely cortical thickness and striatal cross-sectional area.

2.3.5.2. Prefrontal cortical thickness

Golgi-Cox stained coronal sections were used to measure prefrontal cortical thickness following the procedure, with minor modification, described by Stewart and Kolb (1988). Briefly, the coronal sections containing slides were mounted on a magnifying projector at a magnification of 17.5 X. The measurement was carried out in three regions; Cg 1 and Cg 3 regions of anterior cingulate of the medial prefrontal cortex, and the dorsal agranular insular cortex (AID) region of the orbital frontal cortex as described by Zilles (1985). The cortical thickness, measured with a metric ruler on a petrographic projector, was analyzed for hemispheric difference and the data were collapsed in the absence of any difference.

2.3.5.3. Striatum size

The striatal area was measured following the procedure described elsewhere (Kolb, Sutherland, & Whishaw, 1983) with minor modification. Briefly, digital images were taken from Golgi-Cox stained coronal brain sections from the anterior (Bregma ~ 1.7 mm) and posterior (Bregma ~ 0.2 mm) regions of the striatum. The total striatal area was measured using NIH Image software in both anterior and posterior planes.

2.4. Results

The behavioral data were analyzed using experience (TS or control) and sex as independent factors. However, both sexes were analyzed independently after the introduction of drug (AMPH or saline) as a factor either because of sex-dependent differences (e.g., response to AMPH administration) or for the clarity of results description (e.g. in cortical thickness). Furthermore, all ANOVAs were followed by Bonferroni's post hoc test for multiple comparisons.

2.4.1. Behavior

2.4.1.1. Open field locomotion

Prenatal TS experience led to increased open field exploratory behavior in females but did not alter the open field locomotion in males. A two-way ANOVA with experience (TS or control) and sex as independent factors revealed a main effect of experience [F (1, 68) = 4.91, p = 0.030], sex [F (1, 68) = 7.34, p = 0.009], and an interaction between the two [F (1, 68) = 4.37, p = 0.040]. Pairwise comparison revealed that only prenatal TS females exhibited enhanced activity compared to sex-matched

controls (p = 0.003). Furthermore, TS females were more active in the open field compared to experience-matched males (p = 0.001) (Figure 2.2).

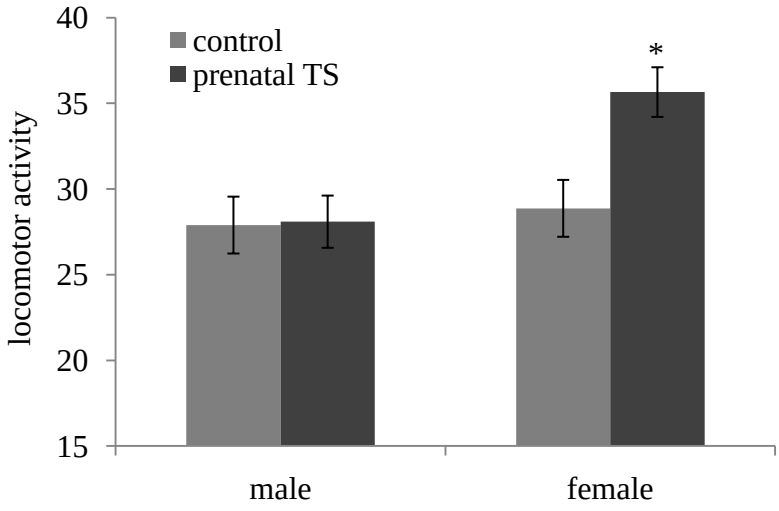

Figure 2.2. Mean (± SEM) locomotor activity showing open field locomotion for 10 minutes. Prenatal TS females compared to sex-matched controls (*, p = 0.003) and TS males (*, p = 0.001) exhibited increased activity in the open field. TS did not affect exploratory behaviour in males.

2.4.1.2. Elevated plus maze

The time spent and the frequency of entry into closed arms were used as indicators of anxiety-like and exploratory behavior, respectively. The time spent in closed arms was not affected by early experience but TS females exhibited diminished activity in the maze.

A two-way ANOVA (Experience x Sex) of the time spent in closed arms revealed no main effect of experience [F (1, 67) = 0.19, p = 0.661], sex [F (1, 67) < 0.001, p = 0.996], nor an interaction between the two [F (1, 67) = 0.03, p = 0.855]. When subjected to a two-way ANOVA, the number of entries in closed arms revealed a main effect of experience [F (1, 67) = 9.01, p = 0.004] and a marginal effect of sex [F (1, 67) = 3.79, p

= 0.057] with no interaction between the two [F (1, 67) = 0.20, p = 0.658]. Pairwise comparisons revealed that the TS female group entered closed arms less frequently than the control group (p = 0.017). Similar to females, TS males appeared to enter in the closed arms less compared to the respective controls, however there was no significant difference between the two (p = 0.077) (Figure 2.3).

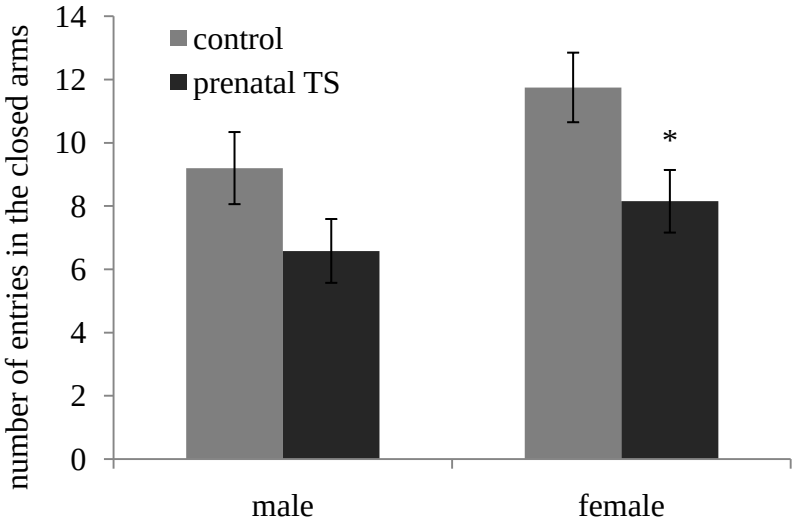

Figure 2.3. Means (±SEM) of the total number of entries in closed arms of EPM. Prenatal TS females entered the closed arms less compared to their sex-matched controls (p = 0.017). TS males also appeared to be less active however, the effect was marginal (p = 0.07).

2.4.1.3. Novel object recognition

The rats tended to spend more time with the old compared to recent familiar object, although not significantly. Prenatal TS did not influence the object temporal order memory. The ratio of time spent with old compared to recent familiar object in the third test trial, an index of object temporal order memory, when subjected to a two-way ANOVA (Experience x Sex) revealed no main effect of experience [F (1, 55) = 0.57, p =

0.45], sex [F (1, 55) = 0.005, p = 0.944], nor an interaction between the two [F (1, 55) = 1.31, p = 1.31].

2.4.1.4. Play fighting

Early TS tended to feminize play fighting in males, but had little effect in females. When subjected to a two-way ANOVA (Experience x Sex), the frequency of play attacks revealed a main effect of experience [F (1, 49) = 4.4, p = 0.040], no main effect of sex [F (1, 49) = 2.78, p = 0.102], nor an interaction between the two [F (1, 49) = 0.99, p = 0.324]. Pairwise comparison revealed that prenatal TS males compared to sex-matched controls showed reduction in the frequency of play attacks (p = 0.031). The frequency of play attacks in females, however, was not affected by prenatal TS. There was a sex difference in the frequency of play attacks in the control group where males compared to experience-matched females showed an increase in the frequency of play attacks (p = 0.039). Moreover, there was no sex difference within TS group (Figure 2.4).

When subjected to a two-way ANOVA (Experience x Sex), the probability of complete rotation defense revealed no main effect of experience [F (1, 49) = 0.01, p = 0.924], sex [F (1, 49) = 0.01, p = 0.755], nor an interaction between the two [F (1, 49) = 0.63, p = 0.431]. The probability of evasion in response to an attack when subjected to a two-way ANOVA (Experience x Sex) revealed no main effect of experience [F (1, 49) = 0.21, p = 0.650], sex [F (1, 49) = 0.05, p = 0.825], nor an interaction between the two [F (1, 49) = 0.71, p = 0.402].

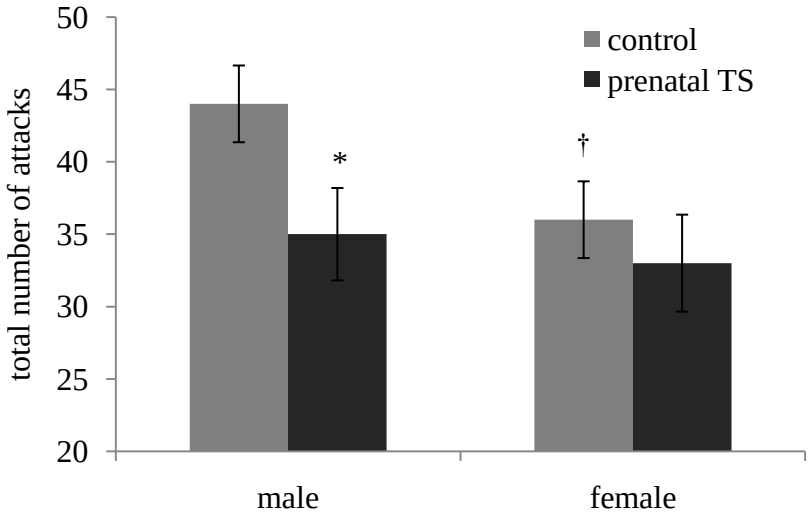

Figure 2.4. Mean (±SEM) of play attacks in 10 minutes of play fighting behavior. The playful attacks were sexually dimorphic where control males attacked more frequently compared to experience-matched females (†, p = 0.039). TS, however, resulted in the feminization of play in males by preventing the sex differences. Furthermore, prenatal TS decreased the frequency of play attacks in males (*, p = 0.031).

2.4.2. Amphetamine sensitization

2.4.2.1. Acute administration

The locomotor activity on the first day of 14-day drug administration period was analyzed to evaluate the effect of TS to an acute AMPH injection. Because there are known sex differences in both open field activity and the effects of amphetamine on activity (e.g., Forgie & Stewart, 1993), the two sexes were analyzed separately. Amphetamine acutely increased activity in both sexes, although the effect was larger in females.

A two-way ANOVA (Experience X Drug) of activity in the male group revealed a main effect of drug (AMPH or saline) [F (1, 31) = 56.83, $p < 0.001$], and no main effect

of experience (TS or control) [F (1, 31) = 0.95, p = 0.336], nor an interaction between the two [F (1, 31) = 0.003, p = 0.955]. AMPH-treated groups (both TS and control) exhibited augmented activity compared to saline-treated groups (see Figure 2.5A for activity on the first day).

A two-way ANOVA of locomotor activity in response to acute AMPH-administration in females showed a main effect of drug [F (1, 33) =107.76, p < 0.001], a marginal main effect of experience [F (1, 33) = 3.90, p = 0.057], and no interaction between the two [F (1, 33) = 0.871, p = 0.357]. Pairwise comparisons revealed that TS in females resulted in diminished locomotor activity response to acute AMPH administration compared to control group (p = 0.037). AMPH administration, in both TS and control groups, resulted in increased activity compared to their respective saline-treated control groups (see Figure 2.5B for activity on the first day).

2.4.2.2. Development of sensitization

Locomotor activity, a standard measure of behavioral sensitization in rats, was gradually increased with repeated AMPH administration. The activity recorded on the first and the last day of drug administration period was compared to determine the development of sensitization in the 14-day AMPH administration period. This comparison revealed that AMPH administration resulted in the development of behavioral sensitization and, in addition, prenatal TS resulted in an attenuated sensitization response compared to control AMPH-administered rats.

Mixed ANOVA on the first and the last day activity in males revealed a main effect of drug (AMPH or saline) [F (1, 31) = 252.10, p < 0.001]. AMPH compared to

saline administration resulted in augmented activity (i.e., behavioral sensitization) on the last day compared to the first day in control and TS groups. In contrast, saline administration in both control and TS groups, although not significant, tended to lead to decreased activity on the last day compared to the first day (see Figure 2.5A for the activity on days 1 and 14).

Mixed ANOVA of the 14-day activity in males with experience and drug as independent factors and days as repeated measure factor revealed a main effect of drug [F (1, 31) = 175.86, $p < 0.001$], and experience [F (1, 31) = 3.95, $p = 0.056$], with no interaction between the two [F (1,31) = 2.87, $p = 0.10$]. AMPH compared to saline treatment resulted in enhanced activity. However, TS compared to controls males revealed an attenuated AMPH-induced behavioral sensitization for most of the days (except day 1, 2, 5, 7, and 8). In contrast, saline administration did not result in any significant differences between TS and control groups in any of the days (Figure 2.5A).

Similar to males, repeated AMPH administration in females resulted in a gradual increase in the locomotor activity (i.e., behavioral sensitization). The first and the last day activity comparison revealed a main effect of drug (AMPH or saline) [F (1, 33) = 190.07, $p < 0.001$].AMPH administration in females resulted in enhanced activity on the last day compared to the first day (see Figure 2.5B for the activity on days 1 and 14).

Mixed ANOVA of 14-day AMPH administration in females revealed a main effect of drug [F (1, 33) = 195.65, $p < 0.001$] and no main effect of experience [F (1, 33) = 1.16, $p = 0.289$], nor an interaction between the two [F (1, 33) = 0.02, $p = 0.891$]. Repeated AMPH administration resulted in increased activity in both control and TS groups. When we looked at the effect of TS on behavioral sensitization, most of the

female rats exhibited a blunted sensitization response; however, there was no significant effect of TS on drug-induced sensitization. Upon close assessment of the data, it was noticed that 3 out of 13 rats in the AMPH-treated TS group exhibited hyperactivity even as juveniles, and which was unrelated to drug sensitization. That is, these 3 rats were outliers throughout the testing. The 14-day activity data were reanalyzed after excluding the hyperactive rats. As anticipated, the analysis revealed a main effect of experience [F (1, 30) = 4.84, p = 0.036]. TS in females, similar to males, resulted in attenuated sensitization (Figure 2.5B).

2.4.2.3. Persistence of sensitization

The persistence of drug-induced sensitization, after 2-week withdrawal period, was determined by challenging both prior saline- and prior AMPH-treated rats with AMPH administration. Both sexes showed a persistent enhancement in activity with prior AMPH exposure.

A two-way ANOVA (Experience X Prior drug treatment) of the activity recorded on the challenge day in males revealed a main effect of prior drug treatment [F (1, 31) = 57.78, $p < 0.001$], and no main effect of experience [F (1, 31) = 1.74, p = 0.20], nor an interaction between the two [F (1, 31) = 1.21, p = 0.280]. AMPH challenge resulted in enhanced locomotor activity, exhibiting persistence of behavioral sensitization in prior AMPH-treated compared to prior saline-treated rats. These data appear to show that prior AMPH-treated TS compared to control rats exhibited an diminished activity in response to a challenge but pairwise comparison revealed only a trend (p = 0.07) (see Figure 2.5A for the activity on the challenge day).

The females rats, in response to AMPH challenge after a withdrawal period, revealed a main effect of prior drug treatment [F (1, 30) = 34.38, $p < 0.001$], and no main effect of experience [F (1, 30) = 0.01, $p = 0.938$], nor an interaction between the two [F (1, 30) = 0.12, $p = 0.730$]. Like males, AMPH challenge in females resulted in augmented locomotor activity exhibiting persistence of behavioral sensitization in prior AMPH-treated compared to prior saline-treated rats (see Figure 2.5B for the activity on the challenge day).

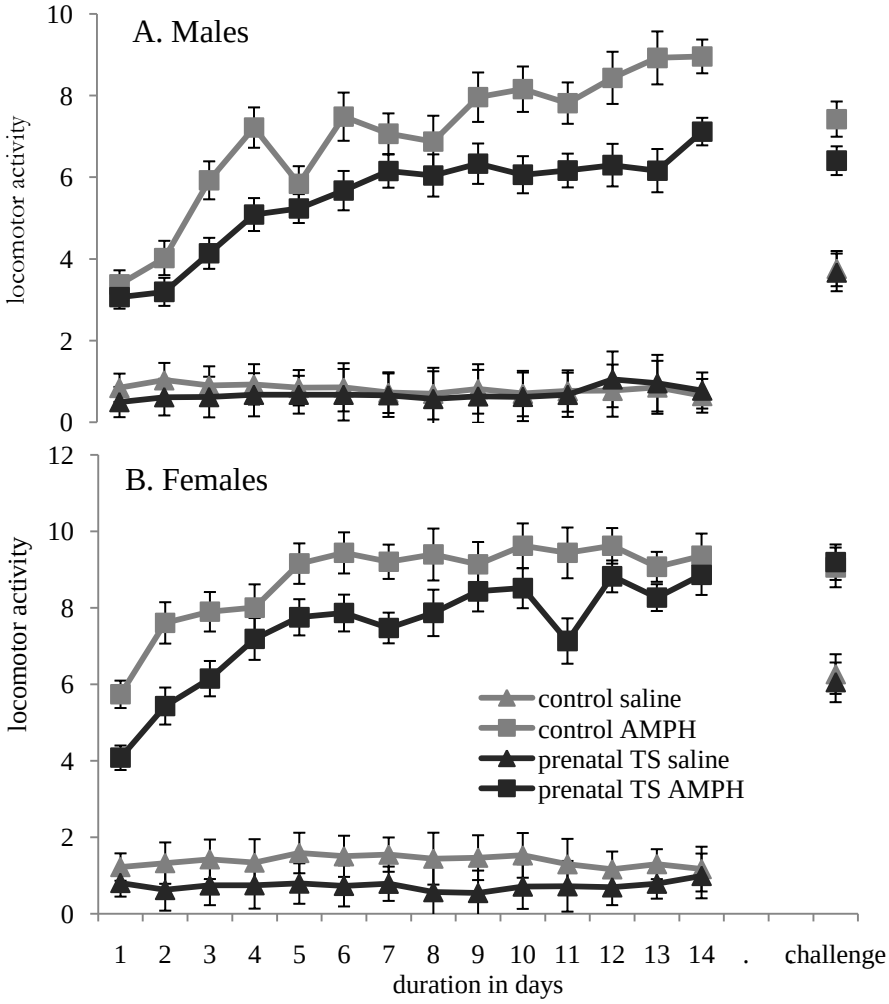

Figure 2.5. Mean (± SEM) locomotor activity (beam crossing x 10^3) recorded for 90 minutes after saline or AMPH (1 mg/kg) administration over 14-day period followed by AMPH challenge in both prior saline- and AMPH-treated rats. Prior AMPH treated rats both males (*, $p < 0.001$) (A) and females (*, $p < 0.001$) (B) compared to their respective saline-treated groups exhibited enhanced activity showing persistence of behavioral sensitization. TS attenuated AMPH-induced behavioral sensitization (all *p*s <0.05) except for days 1, 2, 5, 7, and 8 in males (A). See the text for the effect of TS on AMPH sensitization in females (B).

2.4.3. Anatomy

2.4.3.1. Brain weight

A two-way ANOVA (Experience X Drug) of brain weights in males revealed a main effect of experience (TS or control) [F (1, 31) = 10.84, p = 0.002], and no main effect of drug (AMPH or saline) [F (1, 31) = 0.04, p = 0.836], nor an interaction between the two [F (1, 31) = 0.004, p = 0.952]. TS resulted in lighter brain weights in male rats (Figure 2.6).

In contrast to males, neither TS nor AMPH administration affected brain weights in females. A two-way ANOVA revealed no main effect of experience [F (1, 33) = 1.88, p = 0.179], drug [F (1, 33) = 0.93, p = 0.343], nor an interaction between the two [F (1, 33) = 0.036, p = 0.851].

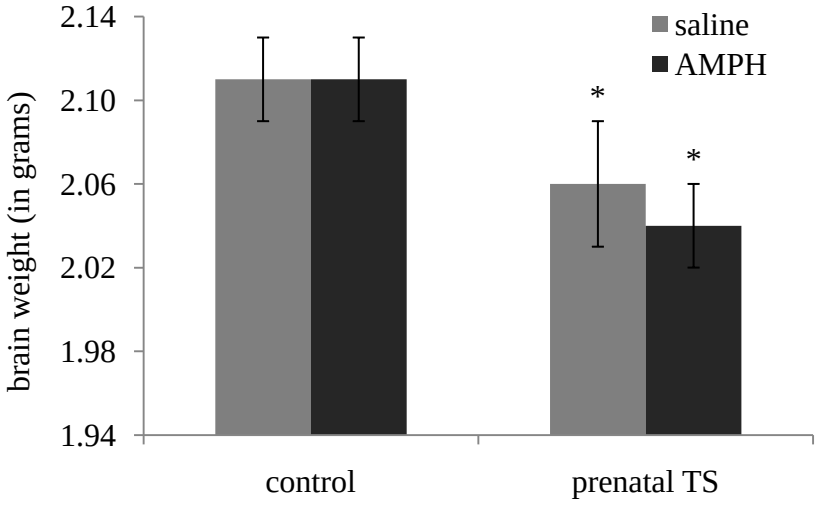

Figure 2.6. Mean (±SEM) brain weight (in grams). Prenatal TS resulted in lighter brain weights in both saline- (*, p = 0.033) and AMPH-treated male groups (*, p = 0.020).

2.4.3.2. Cortical thickness in Cg 1

AMPH administration in females, but not males, reduced the Cg 1 thickness in the control group and this effect was blocked by TS. In addition, TS interacted with AMPH administration resulting in increased Cg 1 thickness in both sexes whereas no such effect was observed in the saline-treated group.

A two-way ANOVA (Experience X Drug) on the Cg 1 thickness in females showed no main effect of experience [F (1, 70) = 3.12, p = 0.082], but a main effect of drug [F (1, 70) = 3.93, p = 0.051] with an interaction between the two [F (1, 70) = 9.64, p = 0.003]. The interaction reflected the decreased cortical thickness in the control group but not TS group (Figure 2.7A). A two-way ANOVA (Experience X Drug) in males revealed no main effect of experience [F (1, 66) = 1.51, p = 0.224], drug [F (1, 66) = 0.44, p = 0.507], nor an interaction between the two [F (1, 66) = 2.95, p = 0.091]. Inspection of the data did suggest that AMPH increased cortical thickness in the TS group, which was confirmed by a pairwise comparison (p = 0.029).

2.4.3.3. Cortical thickness in Cg 3

As in Cg 1, prenatal TS in females interacted with AMPH administration: AMPH reduced Cg3 thickness and this was prevented by TS. Unlike females, neither early experience nor drug influenced Cg 3 thickness in males.

A two-way ANOVA (Experience X Drug) of Cg 3 thickness in females showed a main effect of experience [F (1, 70) = 5.03, p = 0.028], drug [F (1, 70) = 16.58, p < 0.001], and an interaction between the two [F (1, 70) = 7.74, p = 0.007]. The interaction reflected the AMPH-induced decrease in thickness that was prevented by the prenatal TS

(Figure 2.7B). A two-way ANOVA (Experience X Drug) in males revealed no main effect of experience [F (1, 66) = 1.19, p = 0.280], drug [F (1, 66) = 1.61, p = 0.209], nor an interaction between the two [F (1, 66) = 0.711, p = 0.402].

2.4.3.4. Cortical thickness in AID

As in the medial regions, AID thickness was reduced in females by AMPH, and this effect was blocked by TS. In contrast to females, males did not show a substantial alteration in the AID thickness associated with early experience or drug.

The AID thickness in females, when subjected to a two-way ANOVA (Experience X Drug), revealed no main effect of experience [F (1, 70) = 0.18, p = 0.675] but there was a main effect of drug [F (1, 70) = 6.72, p = 0.012] with an interaction between the two [F (1, 70) = 12.69, p = 0.001]. Once again, the interaction reflected the TS induced blockade of the AMPH-related drop in thickness (Figure 2.7C). Dorsal agranular insular cortical thickness data in males, when subjected to a two-way ANOVA (Experience X Drug), revealed no main effect of experience [F (1, 66) = 0.02, p = 0.875], drug [F (1, 66) = 1.50, p = 0.225], nor an interaction between the two [F (1, 66) = 0.47, p = 0.497].

2.4.3.5. Striatum size

Prenatal TS resulted in a larger anterior striatum in both saline- and AMPH-treated male and female groups. TS increased posterior striatal size in saline-treated males and AMPH-treated females. Repeated AMPH administration enlarged the posterior striatum in male controls, a result that was prevented by TS.

A two-way ANOVA (Experience X Drug) of the anterior striatum in males revealed a main effect of experience [F (1, 66) = 24.61, $p < 0.001$] with no main effect of drug [F (1, 66) = 0.76, $p = 0.385$], nor an interaction between the two [F (1, 66) = 0.005, $p = 0.944$]. Prenatal TS resulted in larger anterior striatum in males (Figure 2.8A). When subjected to a two-way ANOVA (Experience X Drug), the posterior striatum in males revealed a main effect of experience [F (1, 66) = 4.05, $p = 0.048$], and no main effect of drug [F (1, 66) = 1.45, $p = 0.233$], nor an interaction between the two [F (1, 66) = 3.43, $p = 0.068$]. TS in saline-treated rats resulted in larger posterior striatum ($p = 0.012$) but AMPH treatment prevented the TS-dependent increase in size. Furthermore, AMPH compared to saline administration in controls resulted in enlarged striatum ($p = 0.039$) and TS alleviated the AMPH-induced striatal enlargement effect in controls (Figure 2.8B).

A two-way ANOVA (Experience X Drug) of the anterior striatum in females revealed a main effect of experience [F (1, 70) = 19.99, $p < 0.001$] with no main effect of drug [F (1, 70) = 3.13, $p = 0.081$], nor an interaction between the two [F (1, 70) = 0.25, $p = 0.616$]. Prenatal TS compared to the respective control group resulted in an enlarged striatum in females (Figure 2.8A).When subjected to a two-way ANOVA (Experience X Drug), the posterior striatum in females revealed no main effect of experience [F (1, 68) = 0.43, $p = 0.511$] nor drug [F (1, 68) = 0.32, $p = 0.570$] but there was an interaction between the two [F (1, 68) = 5.00, $p = 0.029$]. TS compared to controls resulted in larger posterior striatum in AMPH-treated group ($p = 0.035$) without affecting the saline-treated females. Furthermore, AMPH compared to saline administration in TS group resulted in larger posterior striatum ($p = 0.041$), with no effect on the control group (Figure 2.8B).

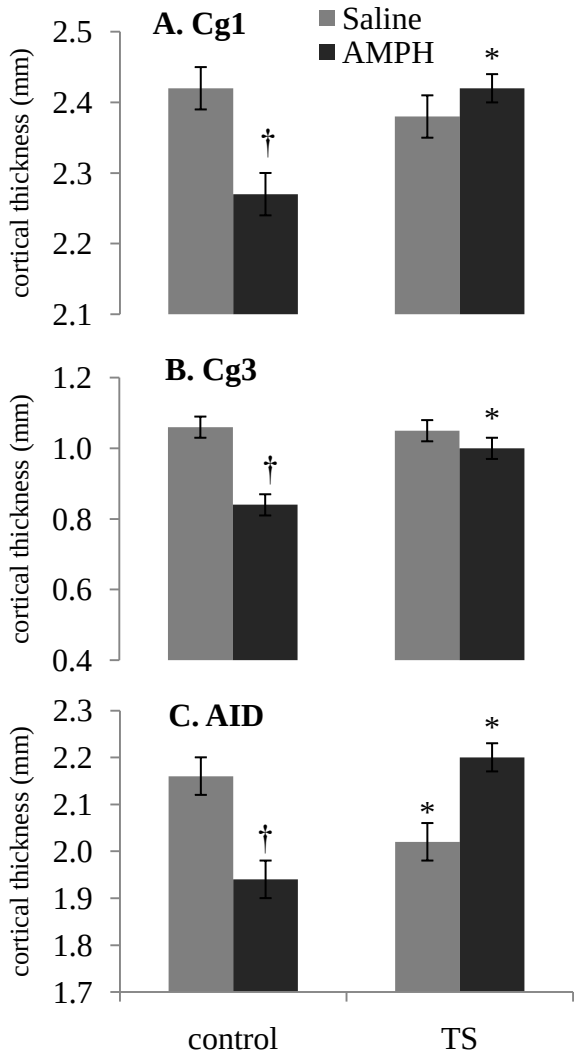

Figure 2.7. Mean (± SEM) of the cortical thickness in (A) Cg 1 and (B) Cg 3 regions of medial PFC and (C) AID region of OFC in females. TS increased the cortical thickness in the Cg 1 (*, $p = 0.001$), Cg 3 (*, $p < 0.001$), and AID regions (*, $p = 0.009$) in AMPH-treated group without any substantial effect on saline-treated group. In addition, a reduction in thickness was observed in AMPH-treated controls in Cg 1 (†, $p = 0.001$), Cg 3 (†, $p < 0.001$), and AID regions (†, $p < 0.001$) however, TS prevented the reduction in medial prefrontal cortical regions. In AID, TS itself caused a reduction in thickness that was reversed with AMPH administration.

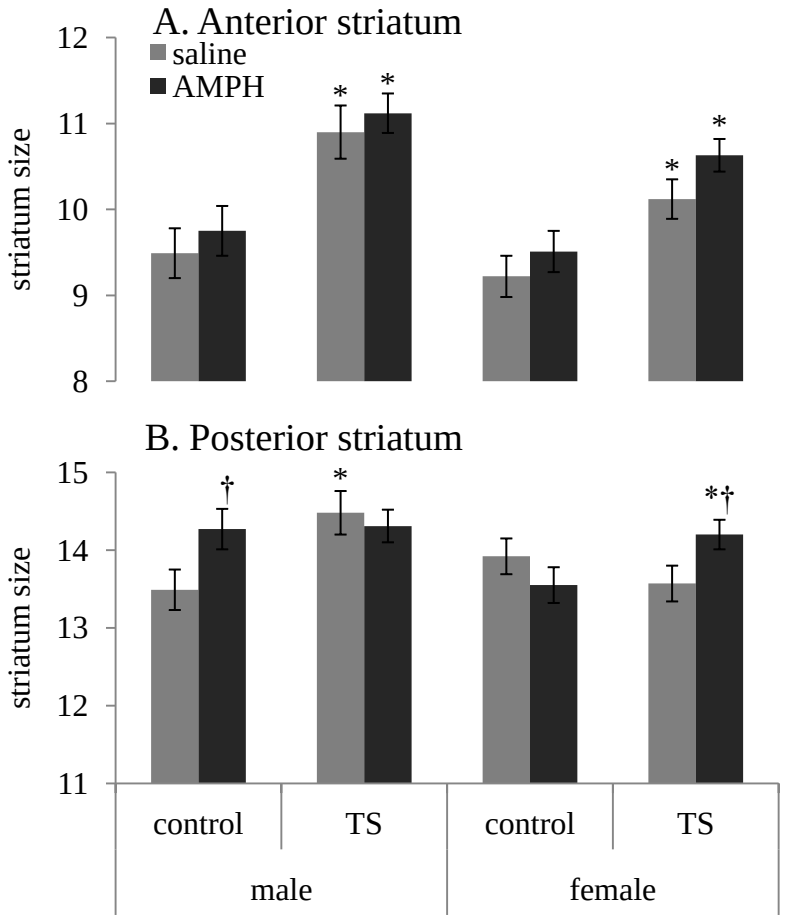

Figure 2.8. Mean (± SEM) of anterior (Bregma, ~ 1.7 mm) and posterior striatum (Bregma, ~ 0.2 mm). Prenatal TS resulted in larger anterior striatum in the saline- and AMPH-treated TS groups in males (*, p = 0. 043 and 0.015, respectively) and females (*, p = 0. 009 and *, $p < 0.001$, respectively). In contrast, the posterior striatum was enlarged by TS only in saline-treated males (*, $p = 0.012$). TS in females increased the posterior striatum size in AMPH-treated females (*, $p = 0.035$). Furthermore, AMPH compared to saline administration in male controls resulted in enlarged posterior striatum (†, $p = 0.039$) but TS prevented the AMPH-induced striatal enlargement.

2.5. Discussion

Tactile stimulation during prenatal brain development altered brain and behavior in rats. TS effectively modulated juvenile exploratory, cognitive, and social behaviors, and attenuated adult AMPH-induced behavioral sensitization. In addition, TS in interaction with AMPH altered prefrontal cortical thickness and striatum size; brain regions implicated in drug addiction. The structural alterations in the brain however, were sex-dependent.

2.5.1 Behavior

Juvenile rats that received prenatal TS were tested in a number of behavioral tasks to investigate the effect of early TS experience on exploratory, emotional, cognitive, and social behaviors. The tasks included open field locomotion, elevated plus maze, novel object recognition, and play fighting behavior. Prenatal TS increased exploratory behavior, tested as open field locomotion, only in females without affecting males (Maruoka, Kodomari, Yamauchi, Wada, & Wada, 2009). However, reduced exploration was observed in the EPM, tested as the number of entries in closed arms, in both male and females. The contrary results for exploratory behavior in open field and EPM points to the importance of context of testing paradigm (e.g., EPM is comparatively more *anxiogenic*) that may have an adaptive role in exploratory behavior. The time spent in closed arms of the EPM, used as a measure of anxiety-like behavior, was not affected by TS in both male and female groups (Cirulli et al., 2010). However, the findings for females are not consistent with that of Maruoka et al., (2009) who reported reduced anxiety-like behavior in mice raised in enriched environment and tested in open field.

The difference could be due to procedural differences such as the experience paradigm, animal species, and age at testing.

Play fighting behavior, comprised of a series of attacks and defense generated in response to an attack, was modified differentially by prenatal TS. Prenatal TS modulated juvenile play differently in male and female rats. There was a sex difference in the frequency of play attacks with control males showing enhanced playfulness compared to experience-matched females (Pellis & Pellis, 1990). However, prenatal TS in males resulted in female-like play behavior with reduced number of play attacks. Previous studies related to early experience manipulations (e.g., prenatal stress or maternal licking and grooming) reported sex-dependent alterations in social behavior with pronounced influence on males (Parent & Meaney, 2008; Ward & Stehm, 1991). The strategy to face (i.e. complete rotation defense) or evade a play attack was not altered by early TS experience. Arnold and Siviy (2002) also reported negligible influence of early handling experience on defense strategy.

The effects of prenatal TS or enrichment experience on brain development are little studied to date. Consequently, it is hard to speculate about possible mechanisms involved in modulating the exploratory, cognitive, and social behaviors examined in the current study. But previous reports related to prenatal experience in general associate alterations in brain and behavior with the modulation of neurotrophic factors, crucial in early brain development (Riva & Mocchetti, 1991), such as BDNF (Maier, Cramer, West, & Sohrabji, 1999), and NGF (Fiore et al., 2009). In addition, the PFC is altered structurally and functionally (Lu, Lim, & Poo, 2009) by early experiences, and is a key

player in modulating behaviors such as NOR (Hannesson, et al., 2004) and play behavior (Bell, Pellis, & Kolb, 2010).

There is an obvious dissociation between male and female groups in juvenile behavior, which points to the likelihood of some sort of early experience-dependent differences. It is hypothesized that experience during development has differential sex-specific influence in rodents that could possibly be linked to gonadal hormones and associated receptors. For example, previous reports described that only prenatally stressed females exhibited enhanced anxiety-like behavior whereas only prenatally stressed males were impaired in spatial learning (Zagron & Weinstock, 2006). Similarly, the sex-specific differences in behavior associated with early experience have been correlated with epigenetic alteration in the brain (e.g., estrogen receptors methylation) (McCarthy et al., 2009).

2.5.2. Amphetamine sensitization

Repeated amphetamine administration in rats produces a gradual increase in locomotor activity (i.e., behavioral sensitization) in experimental animals (Kalivas & Stewart, 1991). In contrast, saline administration over time in rats reduces activity possibly as a result of habituation to the testing environment. In addition, behavioral sensitization also persists after months and even years of withdrawal in rodents (Kolb, et al., 2003b) and monkeys (Castner & Goldman-Rakic, 1999). We manipulated prenatal experience by exposing rats to TS, which resulted in an attenuated sensitization response to acute as well as chronic AMPH administration. The AMPH-induced attenuated sensitization in males was very conspicuous; however, TS females did not exhibit a

marked attenuation because of three very hyperactive rats. The hyperactivity was unrelated to drug-induced sensitization because these rats were hyperactive from the first day of drug administration period and even as juveniles (Figure 2.2). When the hyperactive rats were excluded from the analysis, as anticipated the females showed an attenuated AMPH-induced behavioral sensitization.

There is no report to our knowledge that has studied the influence of prenatal stimulation or enrichment on drug-induced behavioral sensitization. However, we previously reported comparable findings, where postnatal TS resulted in attenuated AMPH-induced sensitization in both male and female rats (Muhammad, et al., 2011). The attenuation of drug-induced behavioral sensitization exhibited by pre- and postnatal TS rats demonstrates the enrichment effect of early stimulation experience.

Repeated stimulant administration results in drug-induced behavioral sensitization but the degree of sensitization is experience-dependent. For example, stress during development has been correlated with augmented psychomotor stimulant sensitization later in life (Deminiere et al., 1992). Although there are published studies on the influence of prenatal adverse experience on drug sensitization, we are unaware of any published reports that have investigated the relationship of prenatal enrichment or stimulation and drug-induced behavioral sensitization. Based upon earlier 'enrichment' studies of postnatal brain development such as early environmental enrichment (Bardo et al., 1995) or maternal licking and grooming (Francis & Kuhar, 2008), which resulted in blunted drug-induced sensitization, we may conjecture that the effects of prenatal TS are similar in nature.

The brain during the prenatal period goes through different stages of development such as neurogenesis, proliferation, synaptogenesis, and apoptosis. Exposure to any experience could potentially alter the rate or degree of brain development, which may be reflected at the behavioral level later in life. The possible mechanisms of how experience might alter brain and behavior could be via modulation of neurotrophic factors and neurotransmitters. For example, maternal stimulation through exercise during pregnancy was associated with enhanced hippocampal neurogenesis and increased BDNF production in rat (Lee et al., 2006). Similarly, prenatal stress modifies catecholamine transmission in the PFC, which might serve as a contributing factor in vulnerability to drug addiction (Carboni et al., 2010). The attenuated drug-induced sensitization resulting from prenatal TS might be the result of epigenetic alterations in brain regions associated with drug sensitization. For instance, TS (Bear, Connors, & Paradiso, 2006) upregulates glucocorticoid receptors in the hippocampus and the PFC, key regions in the HPA axis feedback mechanism (Sapolsky, Krey, & McEwen, 1984). Intracerebroventricular administration of corticotropin releasing factor in rodents on the other hand is positively correlated with increased sensitivity to the effects of amphetamine (Cador, Cole, Koob, Stinus, & Le Moal, 1993). Nevertheless the modulation of behavior and drug-induced behavioral sensitization need further investigation to pin point the possible mechanism(s).

2.5.3. Anatomy

2.5.3.1. Prefrontal cortex

We investigated the interaction of experience (i.e., TS) and drug (i.e., AMPH) on the structural plasticity of the PFC. Kolb and associates previously reported that

psychostimulant administration followed by rearing in an enriched environment blocked the structural alterations associated with environmental enrichment (Hamilton & Kolb, 2005; Kolb, et al., 2003b). We used cortical thickness measurements to investigate structural alteration in the PFC subregions (i.e., mPFC and OFC) associated with early TS experience and later AMPH exposure.

Our findings suggest that prenatal TS experience interacted with later AMPH exposure to modulate cortical thickness in the PFC subregions. However, the structural alteration was more pronounced in females. Whereas AMPH reduced the prefrontal cortical thickness in control females, prenatal TS prevented the drug-induced reduction in the cortical thickness. We did not observe any substantial alteration in prefrontal cortical thickness in control males, which is consistent with the findings of the Bartzokis (2000) study of frontal lobe volume in human male addicts and controls.

The prefrontal cortex of the rat is divided in various regions based on cytoarchitectonic features. Following the terminology described by Zilles (1985), Cg 1 and Cg 3 regions of anterior cingulate cortex, form part of the medial PFC whereas AID, a region of the insular cortex, forms part of the OFC in rats. Both the PFC subregions (i.e. medial and OFC) play a vital role in the development of drug addiction in experimental animals (reviewed by Porrino & Lyons, 2000) and humans (reviewed by Volkow, et al., 2003). In addition to drug-induced impairment of PFC functions (Baron, Wright, & Wenger, 1998), altered structural plasticity (e.g., spine density) has also been reported in experimental animals (Robinson & Kolb, 2004; Selemon, Begovic, Goldman-Rakic, & Castner, 2007). Although there is no, or at best limited, work related to the prefrontal cortical thickness in experimental animals, structural alterations have been extensively

studied in human patients through the application of imaging technology (Bartzokis, et al., 2000; Kim et al., 2006; Lawyer et al., 2010).

We observed that repeated AMPH administration reduced the cortical thickness in the mPFC and the OFC regions but only in control females. Structural alterations in these cortical regions in human studied through imaging techniques support our findings. A decrease in the cortical gray matter density or volume in the anterior prefrontal cortex (Kim, et al., 2006; Schwartz et al., 2010) and medial orbital cortex (Tanabe et al., 2009) was reported in chronic AMPH or methamphetamine abusers. Other PFC-related neurological disorders, such as schizophrenia, in a manner similar to drug addiction, also reduce the frontal cortical thickness (Kuperberg et al., 2003; Rimol et al., 2010).

The drug-induced reduction in prefrontal cortical thickness observed in control rats, however, was prevented by prenatal TS experience: AMPH compared to saline administration did not alter the cortical thickness. In addition, the medial prefrontal cortical thickness was not affected in the saline-treated TS rats but AMPH administration increased the thickness in this region. We observed interesting findings in the AID region, where a reduction in the cortical thickness was observed in the saline-treated TS females. In contrast, AMPH administration in the same group resulted in increased AID thickness. Sensory stimulation (e.g., environmental enrichment) appears to prevent the decrease in cortical thickness associated with an insult, for instance, neonatal brain injury (Comeau, Gibb, Hastings, Cioe, & Kolb, 2008) or early social isolation stress (Hellemans, Benge, & Olmstead, 2004). We believe that the prevention of drug-induced reduction in the prefrontal cortical thickness by TS could be the protective mechanism that resulted in attenuated AMPH-induced behavioral sensitization observed in our study.

Further studies are needed to expand the mechanistic (e.g., epigenetic or molecular) details of the enrichment experience and drug associated structural alterations in the PFC.

2.5.3.2. Striatum

The striatum has been associated with drug addiction in laboratory animals and human patients (Gerdeman, et al., 2003; Ito, et al., 2002; Li, Kolb, et al., 2003; Volkow, et al., 2003). Similar to the prefrontal cortical alteration, we observed a sex-dependent modulation of the striatum size. But unlike the PFC, where the reduction in the cortical thickness was observed only in control females, AMPH administration increased the size of the posterior striatum but only in control males. The change in the striatum size has also been reported in human drug addicts. For example, MRI studies related to the alteration in the striatal morphology in chronic cocaine abusers showed an increase in the striatal volume (Jacobsen, Giedd, Gottschalk, Kosten, & Krystal, 2001). The drug-induced enlargement in striatum size could be attributed to the augmented dopamine release in the striatum (Robinson & Becker, 1982).

Interestingly, TS prevented the drug-induced increase in the posterior striatum observed in control rats. In addition, TS increased the size of anterior striatum regardless of sex or drug exposure. In contrast, the increase in the posterior striatum was sex- and drug-dependent. A TS-induced increase was observed in saline-treated males with no effect in sex-matched AMPH-treated rats. TS followed by AMPH-administration increased the posterior striatum size in females with no effect in saline-treated females. Similar to our investigation of experience- and drug-associated metaplasticity at structural levels, researchers also studied the same phenomenon at molecular levels.

Levels of the basic fibroblast factor (FGF-2), a protein associated with neuronal plasticity, have been increased in stress (Riva, Fumagalli, & Racagni, 1995) and psychostimulants administration (Fumagalli, Pasquale, Racagni, & Riva, 2006) in various brain regions. However, chronic stress exposure interacted with cocaine administration in region-dependent modulation of FGF-2 in the PFC and the striatum (Fumagalli, Di Pasquale, Caffino, Racagni, & Riva, 2008).

In summary, prenatal tactile stimulation in rats modulated social behavior with diminished playful attacks resulting in the feminization of male play fighting behavior. Exploratory or anxiety-like behavior and cognitive performance were not affected. Repeated amphetamine administration gradually increased the locomotor activity resulting in the development of behavioral sensitization that persisted at least for two weeks. Prenatal tactile stimulation, however, blunted the drug-induced behavioral sensitization. Furthermore, prenatal TS modulated the cortical thickness and striatum size in a sexually dimorphic manner. TS increased the cortical thickness in the subregions of the prefrontal cortex in the AMPH-treated females without any substantial effect on males. In addition, an overall reduction in the prefrontal cortical thickness was observed in AMPH-treated controls but TS prevented the reduction in cortical thickness. Prenatal TS resulted in a larger anterior striatum regardless of drug exposure and sex. But the posterior striatum showed enlargement depending on the experience, drug, and sex. TS increased the posterior striatal size only in saline-treated males but, in contrast, an increase was observed only in AMPH-treated females. Furthermore, repeated AMPH administration in control males resulted in enlarged posterior striatum but TS prevented the AMPH-induced striatal enlargement.

The present study highlighted the role of a favorable experience during prenatal brain development in modulating the subsequent response to drug-induced behavioral sensitization and associated anatomical changes in the brain. The attenuated behavioral sensitization as a result of early favorable experience (i.e., TS) might be related to the prevention of drug-induced structural reorganization of the PFC and the striatum and therefore, may play a protective role against stimulant-induced behavioral sensitization. The present findings have practical implications for child development practices as they underpin the importance of early intervention in brain development that starts with a successful conception (or even earlier than that). Massage in women during pregnancy, therefore, could potentially play a protective role in the prevention of drug abuse, which is harder to treat later in life because of limited plasticity in the brain.

2.6. References

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CHAPTER 3

Tactile stimulation during development attenuates amphetamine sensitization and structurally reorganizes prefrontal cortex and striatum in a sex-dependent manner*

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Abstract

This study investigated the effect of postnatal tactile stimulation (TS) on juvenile behavior, adult amphetamine (AMPH) sensitization, and the interaction of TS and AMPH on prefrontal cortical (PFC) thickness and striatum size. Pups received TS by stroking daily with a feather duster from birth till weaning and were tested, as juveniles, in behavioral tasks including open field locomotion, elevated plus maze (EPM), novel object recognition, and play fighting behavior. Development and persistence of drug-induced behavioral sensitization was tested by chronic AMPH administration and challenge, respectively. PFC thickness and striatum size were assessed from serial brain sections. The findings showed that TS rats spent less time with novel objects on first exposure but open field locomotion and EPM tasks were not affected substantially. TS reduced the frequency of play fighting and enhanced evasion in response to a playful attack, but only in males. The probability of complete rotation defense, leading to a supine posture during play, was reduced in both sexes. AMPH administration resulted in gradual increase in behavioral sensitization that persisted at least for 2 weeks. However, TS rats exhibited attenuated AMPH sensitization compared to sex-matched controls. Neuroanatomically, AMPH reduced the PFC thickness in control females but enlarged the posterior striatum in control males. TS experience blocked these effects. In sum, TS during development modulated the response to novel objects and altered social behaviors and attenuated AMPH-induced behavioral sensitization by preventing drug-induced structural alteration in the PFC and the striatum, brain regions implicated in drug abuse.
Keywords: behavioral sensitization, addiction, plasticity, tactile stimulation, rough and tumble play

3.2. Introduction

Stimulant drug addiction is a life devastating brain disorder characterized by chronic drug taking and drug seeking behavior with high chances of relapse in 'susceptible' individuals. A substantial amount of research has been focused on investigation of the addiction mechanism whereby drugs of abuse 'hijack' brain circuits involved in decision making, behavioral inhibition, and reward and motivation. Despite a wealth of knowledge about addiction neurobiology ranging from structural morphology (Robinson & Kolb, 1997; Singer, et al., 2009) to molecular biology (Graybiel, Moratalla, & Robertson, 1990; Maze et al., 2010), there has been only modest success in finding an effective treatment strategy for drug addiction both at behavioral and pharmacological level.

A number of theories explains the transition from recreational drug use to compulsive drug abuse (Koob, 2006), but none of the theories are mutually exclusive. Nevertheless there is a growing consensus that drug abuse has its roots in brain development (Felitti, 2002). This led to the hypothesis that the brain of an addict is already programmed for potential abuse (Volkow, et al., 1999), and the reason not every individual becomes addicted despite recreational drug use. The idea is further strengthened by the notion that experience during development plays a fundamental role in the organization of brain and subsequently influence behavior. The footprints of such an experience, either 'positive' or 'negative', remain throughout life with profound impact on later experiences. For instance, childhood adverse experiences not only affect general health but also act as predisposing factors for psychopathologies such as schizophrenia, depression, and attention deficit hyperactivity disorder (Felitti, 2002).

Likewise in rodents, sensory stimulation, such as maternal licking and grooming (LG) and environmental enrichment (EE), during development reorganizes the brain and modulates behavior that persists throughout life.

Tactile stimulation (TS), a form of sensory stimulation, is closely related to maternal LG in rats (Gonzalez, et al., 2001; Lovic & Fleming, 2004; Lovic, et al., 2006). Rat exposed to TS experience receive sensory stimulation by gentle stroking either through a human hand or a mechanical mean (e.g., a soft hair brush). Such stimulation works as 'enrichment' for the developing brain and leaves a favorable impact to face challenging experiences later in life. Previous works in rodents suggest that early sensory stimulation has improved learning and memory (Rosenzweig & Bennett, 1996), positively regulated emotionality (Fernandez-Teruel, et al., 1997), and dampened hypothalamic-pituitary-adrenal (HPA) axis response (Pauk, et al., 1986). Furthermore, Kolb and Gibb (2010) investigated the effect of stimulation on brain injury and demonstrated that TS during development improved recovery from early brain injuries and altered neuronal morphology in the rat brain. However, ventral hippocampal lesions in rats during infancy with simultaneous exposure to high maternal tactile stimulation resulted in marked behavioral deficits (e.g., impaired working memory) (Wood, Quirion, & Srivastava, 2003).

Similar to TS experience, psychomotor stimulant (e.g. cocaine, amphetamine, nicotine) drug administration has been shown to alter neuronal morphology by increasing dendritic growth in various brain regions (Brown & Kolb, 2001; Robinson & Kolb, 1997, 1999). However, when rats were administered stimulant drugs followed by enrichment rearing experience two weeks later in complex housing, drugs interfered with structural

changes normally associated with enrichment (Hamilton & Kolb, 2005; Kolb, Gorny, et al., 2003). The interference of drugs with later experience raised the question of how early experience followed by stimulant drugs would interact to alter brain regions.

The present study was conducted with the objectives to determine the effect of postnatal TS on juvenile behavior, adult psychomotor stimulant sensitization, and interaction of early experience (i.e., TS) with later stimulant (i.e., AMPH) sensitization on structural alteration in brain regions (i.e. PFC and striatum) associated with stimulant sensitization. The findings showed that although TS modulated novelty-seeking and play behaviors during juvenile period, the overall affect was not robust in less conflicting behaviors such as exploration. Chronic AMPH exposure during adulthood though resulted in the development and persistence of behavioral sensitization, but the response was substantially attenuated in both male and female TS groups. TS during development followed by drug exposure in adulthood produced sex-specific structural alterations in the PFC and the striatum, brain regions implicated in drug addiction.

3.3. Materials and methods

3.3.1. Animals

Long-Evans male (n=28) and female (n=28) rats were used in the experiment. Rat pups were randomly selected with not more than two pups from the same litter and were housed with their respective dams in the breeding colony at the Canadian Centre for Behavioural Neuroscience (CCBN), University of Lethbridge, Alberta, Canada. Both control (i.e. animal facility reared) and TS were housed in standard shoe-box cages with the same sex in a group of two after weaning. The room temperature and humidity was

maintained at 74% and 22^{o}C, respectively. Standard rat diet and water were provided *ad lib*. The rats were left undisturbed till the commencement of behavioral tests and AMPH administration.

3.3.2. Tactile stimulation

Tactile stimulation was carried out between postnatal days (P) 2-21 following the procedure described by Kolb and Gibb (2010). Briefly, rat pups were transported in a box with new bedding to a separate room. The transport box was placed on warming pad having a temperature maintained around 34^{o}C. The huddled rat pups were gently stroked with a soft feather duster for 15 minutes three times a day for 20 consecutive days, approximately the same time and by the same experimenter. Control animals were treated the same except they received no TS.

3.3.3. Behavior

The effect of early TS on juvenile behavior was investigated by testing the rats between P30-40 in a battery of behavioral tasks. The tests included open field locomotion, elevated plus maze (EPM), novel object recognition (NOR)-recency discrimination version, and play fighting behavior.

3.3.3.1. Open field locomotion

Exploratory behavior of the rats was evaluated as open field locomotion, recorded for ten minutes using Accuscan activity monitoring Plexiglas boxes (L 42cm, W 42cm, H 30cm). The activity was recorded as the number of sensors beam breaks in the boxes

attached to a computer. The horizontal beam breaks, used as an index of locomotor activity, were recorded on the computer with VersaMax^{TM} program and converted to spreadsheet using VersaDat^{TM} software (AccuScan Instruments, Inc., Columbus, OH).

3.3.3.2. Elevated plus maze

The EPM, a '+' shape maze with two closed and two open arms, was used to test anxiety-like behavior exhibited by the rats. Rats were placed in the centre of the maze facing a closed arm and were allowed to explore the maze for 5 minutes. Exploration behavior was videotaped with a camera installed in such a way to spot both open arms. The time spent in the closed arms and number of entries in each of the open and closed arms were scored and analyzed to assess anxiety-like and exploratory behaviors, respectively.

3.3.3.3. Novel object recognition

Novel object recognition was carried out to evaluate exploration of novel object as well as exploration of objects in temporal order. Rats were habituated to a Plexiglas box for 15-20 minutes for four days prior to the commencement of the testing sessions. The NOR task was comprised of three trials following the procedure with minor modification described elsewhere (Hannesson, et al., 2004; Mitchell & Laiacona, 1998). Briefly, rats were allowed to explore objects and taped with a video camera. Glass candle holders, with similar sizes but different shapes and colors, were used as objects. During the first training trial, a rat was exposed to two novel but similar objects for a 4-minute period. Followed by a 60-minute delay, the rat was again exposed to two new objects,

again similar but different objects from the first trial. After an additional 60-minutes delay, the rat was exposed to one object each from the first and second trial, termed as 'old' and 'recent' familiar objects, respectively. The objects and testing area were cleaned with 30% alcohol between each trial for disinfection and odor removal. Rats were transported back to their home cages in the 60-minute delay between the trials.

Exploration of each novel object in the first two trials and 'old' and 'recent' familiar objects in the third trial was scored. The time spent with 'old' familiar object was calculated as the difference between times spent with 'old' and 'recent' familiar object divided by total time exploring both objects (Hannesson, et al., 2004). In addition the total time spent with both objects in each trial was also analyzed.

3.3.3.4. Play fighting

Juvenile rats were allowed to play in pairs to assess the effect of TS on social behavior. TS and control rats were housed as pairs for a period of about two weeks. The pair mates, as juveniles, were habituated to a testing enclosure (50 cm X 50 cm X 50 cm) for about 30 minutes per day for 3 days. To increase the frequency of play on the day of testing, rats were housed individually for 24 hours at the end of habituation period (Pellis & Pellis, 1990). The play behavior was recorded in the dark for 10 minutes with a night shot camera. On the testing day both pair mates were color marked on the tail with two separate paints to make them identifiable in the video recording. Rats were transported to their home cage after the play session and pair-housed for the rest of the experiment. The video recording, analyzed frame by frame, was scored for attacks, and complete rotation defense or evasion generated in response to an attack (Pellis, Pellis, & Whishaw, 1992).

The defense was scored as 'complete rotation' when a rat had both fore- and hind limbs in the air while lying in a supine position. If the rat turned away from the attacker instead of facing an attack, it was scored as evasion. The probability of complete rotation or evasion was calculated as the number of complete rotation or evasion divided by the total number of attacks carried out by the playmate (Pellis & Pellis, 1990).

3.3.4. Amphetamine sensitization

3.3.4.1. Amphetamine administration

To see the effects of postnatal TS on psychomotor stimulant behavioral sensitization, adult rats (P80) were administered with D-amphetamine sulfate (Sigma Aldrich, St. Louis, MO, USA) dissolved in sterile 0.9% saline. Locomotor activity, used as an index of behavioral sensitization (Wise & Bozarth, 1987), was recorded using an Accuscan activity monitoring system, comprised of Plexiglas boxes (L 42cm, W 42cm, H 30cm) connected to a computer. The rats were habituated to the activity boxes for 30 minutes followed by AMPH (1 mg/kg body weight, i.p.) or 0.9% saline administration, both at a volume of 1 ml/kg. Rats were immediately placed back in the activity boxes post injections and the activity was recorded for 90 minutes. The drug was administered once a day for 14 consecutive days, approximately the same time every day. Locomotor activity recorded on a computer with VersaMax^{TM} program was converted to spreadsheet using VersaDat^{TM} software (AccuScan Instruments, Inc., Columbus, OH). The rats were returned back to their home cages each day after the end of AMPH testing session. The development of sensitization was determined by analyzing activity recorded over 14-day period using mixed ANOVA with experience (TS or control), drug (AMPH or saline) as

independent factors, and day (day 1-14 days) as a repeated measure factor, followed by Bonferroni's post hoc test for multiple comparisons.

3.3.4.2. Challenge

The rats were given a withdrawal period of 2 weeks after AMPH administration period, followed by a challenge with AMPH (1mg/kg, i.p.) given to both prior AMPH- and prior saline-treated rats. Locomotor activity was recorded in activity monitoring boxes similar to the development of AMPH sensitization procedure described above. All rats were challenged with AMPH to see the persistence of behavioral sensitization in prior AMPH-treated rats compared to prior saline-treated rats.

3.3.5. Anatomy

3.3.5.1. Perfusion and staining

Approximately 24 hours post AMPH challenge, the rats were given an overdose of sodium pentobarbital solution i.p. and perfused with 0.9% saline solution intracardially. The brains removed from the skull were trimmed by cutting the olfactory bulb, optic nerves and spinal cord. The brains were then weighed and preserved in Golgi-Cox solution for 14 days followed by transfer to 30% sucrose solution at least for 3 days. The brains were sliced at a thickness of 200 μm on a Vibratome and fixed on gelatinized slides. The slides mounted with brain sections were processed for Golgi-Cox staining, following the protocol described by Gibb and Kolb (1998). Unfortunately, owing to unforeseen technical problems, the Golgi staining proved unreliable so the tissue was

used for gross measures of cortical change, namely cortical thickness and striatal cross-sectional area.

3.3.5.2. Prefrontal cortical thickness

Golgi-Cox stained coronal sections were used to measure prefrontal cortical thickness following the procedure, with minor modification, described by Stewart and Kolb (1988). Briefly, the coronal sections containing slides were mounted on a magnifying projector at a magnification of 17.5 X. The measurement was carried out in three regions; Cg 1 and Cg 3 regions of anterior cingulate of the medial prefrontal cortex, and dorsal agranular insular cortex (AID) region of the orbital frontal cortex described by Zilles (1985) (see Figure 3.1A). The cortical thickness, measured with a metric ruler on a petrographic projector, was analyzed for hemispheric difference and the data were collapsed in the absence of any difference.

3.3.5.3. Striatum size

The striatal size was measured following the procedure described elsewhere (Kolb, et al., 1983) with minor modification. Briefly, digital images were taken from Golgi-Cox stained coronal brain sections from the anterior (Bregma ~ 1.7 mm) and posterior (Bregma ~ 0.2 mm) of the striatum (see Figure 3.1B). The total striatal area was measured using NIH Image software in both anterior and posterior planes.

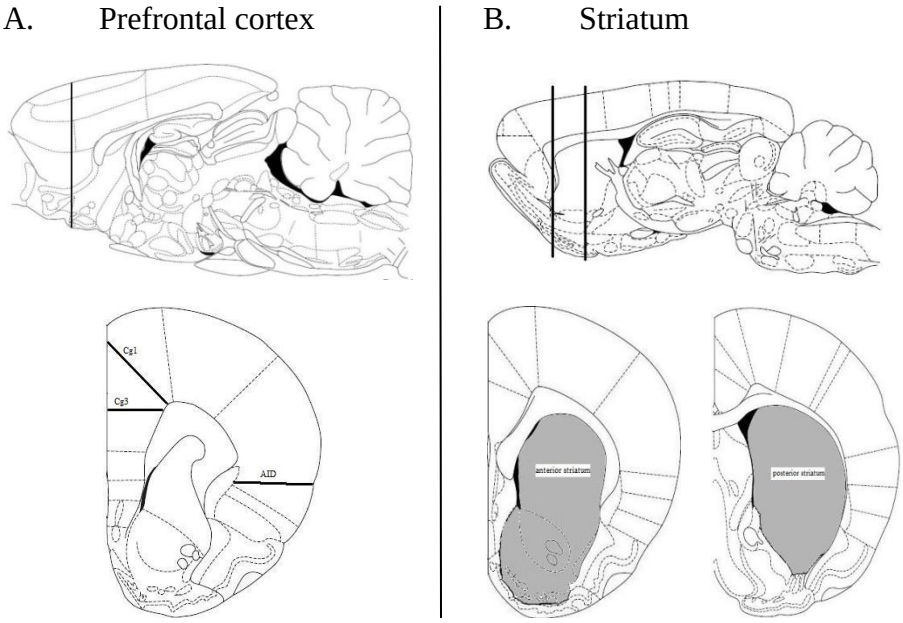

Figure 3.1. Schematic diagrams of the sagittal and coronal sections showing the rat brain. (A) A sagittal section depicting the Cg1, Cg3, and AID regions (B) Sagittal sections showing the anterior and posterior planes of the striatum.

3.4. Results

3.4.1. Behavior

The behavioral data were analyzed using experience (TS or control) and sex as independent factors. However, both sexes were analyzed independently after the introduction of drug (AMPH or saline) as a factor because of a large sex-dependent difference in the response to AMPH administration. Furthermore, all ANOVAs were followed by Bonferroni's post hoc test for multiple comparisons.

3.4.1.1. Open field locomotion

Exploratory behavior tested as open field locomotion was not influenced by postnatal TS in either male or female groups. When subjected to a two-way ANOVA

with experience (TS or control) and sex as independent factors, the activity data revealed no main effect of experience [$F(1, 52) = 0.79$, $p = 0.38$], sex [$F(1, 52) = 0.23$, $p = 0.64$] nor an interaction between the two [$F(1, 52) < 0.001$, $p = 0.10$].

3.4.1.2. Elevated plus maze

The time spent and the frequency of entry into a closed arm were used as indicators of anxiety-like and exploratory behavior, respectively. The time spent in closed arms was not affected by early experience although TS females showed diminished exploratory behavior in the maze. The time spent in closed arms when subjected to a two-way ANOVA revealed no effect of experience [F (1, 52) = 3.17, $p = 0.081$], no effect of sex [F (1, 52) < 0.001, $p = 0.991$] nor an interaction between the two [F (1, 52) = 0.04, $p = 0.837$]. When subjected to a two-way ANOVA, the number of entries into closed arms revealed a main effect of experience [F (1, 51) = 6.25, $p = 0.016$], but no effect of sex [F (1, 51) = 0.92, $p = 0.342$] nor an interaction between the two [F (1, 51) = 1.55, $p = 0.218$]. Pairwise comparison revealed that TS females compared to sex-matched controls entered less frequently into the closed arms ($p = 0.010$) but males were not affected by early experience. Furthermore, no significant sex difference was observed within control or TS groups (see Figure 3.2).

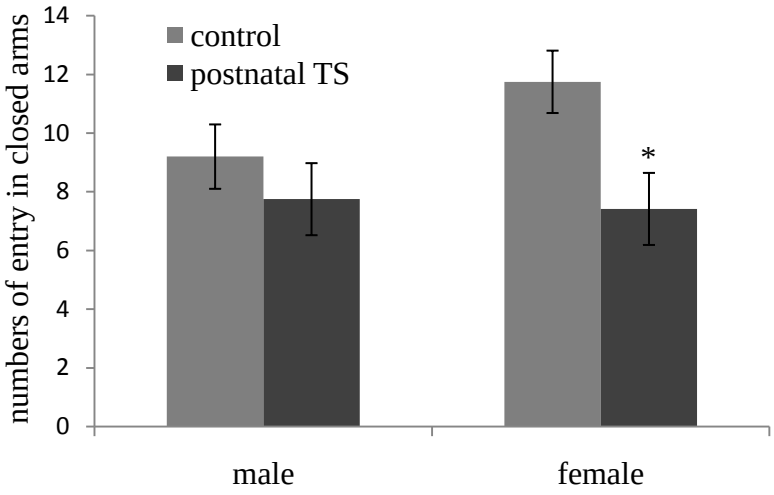

Figure 3.2. Frequency of entry (mean ± SEM) in the closed arms of EPM. Postnatal TS reduced the frequency of entry in females only (*, $p = 0.010$).

3.4.1.3. Novel object recognition

The time spent with 'old' and 'recent' familiar objects, an index of object memory in temporal order, was not affected by early experience but the time spent with both similar objects in trial 1, showing response to novelty, was greatly reduced in both TS males and females.

A two-way ANOVA (Experience x Sex) of the ratio of time spent with old *vs.* recent familiar object revealed no main effect of experience [F (1, 41) = 1.66, $p = 0.205$], sex [F (1, 41) = 1.66, $p = 0.205$] nor an interaction between the two [F (1, 41) = 0.008, $p = 0.930$]. The time spent with both similar objects in trial 1 when subjected to a two-way ANOVA revealed a main effect of experience [F (1, 47) = 10.06, $p = 0.003$] with no main effect of sex [F (1, 47) = 2.01, $p = 0.163$] nor an interaction between the two [F (1, 47) = 0.135, $p = 0.715$]. Pairwise comparisons revealed that both TS males ($p = 0.041$) and females ($p = 0.022$) compared to sex-matched controls spent less time exploring the novel

objects. Furthermore, there were no significant sex differences in object exploration time within TS or control group (see Figure 3.3).

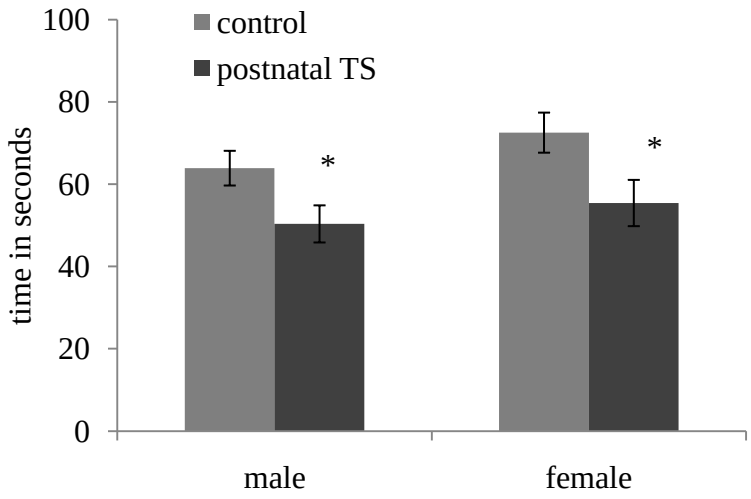

Figure 3.3. Mean (± SEM) of the total time spent with objects on first exposure in trial 1 of NOR, an index of response to novelty. TS in both males (*, $p = 0.041$) and females (*, $p = 0.022$) reduced exploration time of novel objects. There were no significant sex differences within TS or control groups.

3.4.1.4. Play fighting

Play fighting behavior was scored as the frequency of attacks and the probability of complete rotation defense or evasion generated in response to an attack. Postnatal TS decreased the frequency of play fighting in males. The probability of complete rotation defense was significantly reduced in both male and females. Evasion instead of facing an attack was significantly increased in TS males only (see Table 3.1).

The frequency of play attacks when subjected to a two-way ANOVA (Experience x Sex) revealed no main effect of experience [F (1, 52) = 0.85, p = 0.364] nor sex [F (1, 52) = 0.13, p = 0.717], but there was an interaction between the two [F (1, 52) = 4.76, p = 0.034]. Pairwise comparison revealed that postnatal TS males compared to sex-matched controls reduced the frequency of play attacks (p = 0.033) (see Table 3.1). Play attacks in females, however, were not affected by early experience. Control males compared to experience-matched females appeared to have increased frequency of play attacks, though the analysis revealed a marginal effect (p = 0.057). There was no sex difference within the TS group, however (see Table 3.1).

When subjected to a two-way ANOVA (Experience x Sex), the probability of complete rotation defense revealed a main effect of experience [F (1, 52) = 13.08, p = 0.001], no main effect of sex [F (1, 52) = 0.10, p = 0.755], nor an interaction between the two [F (1, 52) = 0.08, p = 0.777]. Pairwise comparison revealed that postnatal TS decreased the probability of complete rotation in both males (p = 0.022) and females (p = 0.008). Furthermore, there was no sex difference within the TS or control group (see Table 3.1).

The probability of evasion in response to an attack when subjected to a two-way ANOVA (Experience x Sex) revealed a main effect of experience [F (1, 52) = 8.41, p = 0.005], no main effect of sex [F (1, 52) = 0.35, p = 0.555], nor an interaction between the two [F (1, 52) = 1.56, p = 0.217]. Pairwise comparison revealed that TS males compared to sex-matched controls responded more frequently with evasion (p = 0.005) whereas, females were not affected by early experience. Furthermore, TS males compared to

experience-matched females showed enhanced evasion response ($p = 0.008$) but there was no sex differences within the control group (see Table 3.1).

Table 3.1

Mean (±SEM) of play attacks and probability of complete rotation defense and evasion

	Male		Female	
	Control	Postnatal TS	Control	Postnatal TS
Attacks	44 ± 2.70	31 ± 3.11*	37 ± 2.70	43 ± 3.11
Complete rotation	0.52 ± 0.03	0.42 ± 0.03*	0.54 ± 0.03	0.42 ± 0.03*
Evasion	0.022 ± 0.008	0.058 ± 0.009*	0.028 ± 0.008	0.042 ± 0.009

Play fighting behavior was scored as the total number of attacks and either facing an attack as complete rotation defense or evasion. Postnatal TS decreased the frequency of play attacks in males (*, $p = 0.033$). The probability of complete rotation defense was significantly reduced in both male (*, $p = 0.022$) and females (*, $p = 0.008$). Evasion instead of facing an attack was significantly increased in males only (*, $p = 0.005$).

3.4.2. Amphetamine sensitization

The effect of early postnatal TS on AMPH administration was investigated by analyzing the locomotor response to acute administration, and on the development and persistence of drug-induced behavioral sensitization in rats.

3.4.2.1. Acute administration

Early tactile stimulation attenuated the locomotor response to amphetamine (see Table 3.2 for activity on the first day). The first day of drug administration was analyzed to evaluate the effect of an acute injection of AMPH on TS rats compared to controls. A two-way ANOVA on the male group revealed a main effect of experience [F (1, 24) =4.68, p = 0.04] and drug [F (1.24) = 48.04, $p < 0.001$] with no interaction between the two [F (1, 24) = 1.99, p = 0.17]. Pairwise comparison revealed a significant diminished activity response to an acute injection of AMPH by TS rats compared to the sex-matched control rats (p = 0.009). Furthermore, AMPH administration compared to saline resulted in augmented locomotor activity in both TS and control groups (both ps <0.001) (see Table 3.2A for activity on the first day).

Similarly, female groups also showed a main effect of experience [F (1, 24) =7.59, p = 0.011] and drug [F (1, 24) = 104.08, $p < 0.001$] with an interaction between the two [F (1, 24) = 4.75, p = 0.039]. Pairwise comparison revealed that TS resulted in decreased activity response to acute AMPH administration in females (p = 0.001). Similar to males, AMPH administration in females resulted in enhanced activity in TS and control groups (both ps < 0.001) (see Table 3.2B for activity on the 'First day').

Table 3.2

Mean (±SEM) locomotor activity on the first and last day of 14-day AMPH/saline administration period

	Control		Postnatal TS	
	First day (acute)	Last day	First day (acute)	Last day
A. Male				
Saline	8425 ± 2714	6452 ± 4290	6143 ± 3838	3078 ± 6066
AMPH	33740 ± 2714	89596 ± 4290*	22890 ± 2714	70448 ± 4290*
B. Female				
Saline	12202 ± 3264	11667 ± 5419	10099 ± 4615	7518 ± 7664
AMPH	57376 ± 3264	93578 ± 5419*	39375 ± 3264	84376 ± 5419*

Postnatal TS resulted in diminished activity response to acute AMPH administration in male (A) and female groups (B) (*, p = 0.009 and 0.001, respectively). In response to repeated administration both male (A) and female rats (B) developed AMPH-induced behavioral sensitization by exhibiting enhanced activity on the last compared to the first day of drug administration (all ps <0.001). Saline treatment although not significant resulted in diminished activity on the last day compared to the first day in both male (A) and female group (B) showing habituation to the testing conditions.

3.4.2.2. Development of sensitization

Behavioral sensitization, usually measured as locomotor activity in rats, is a gradual increase in response to similar doses of repeated drug administration over a

certain period. The activity, recorded over consecutive 14-day period, was analyzed to determine the development of behavioral sensitization in both TS and control groups. Additionally, the first and the last days of AMPH administration were also compared to see the development of sensitization. Postnatal TS in both male and female rats resulted in an attenuated sensitization response compared to control AMPH-administered rats (see Table 3.2).

The activity recorded on the first and last day of 14-day period in males when compared revealed a main effect of drug [F (1, 24) = 195.6, $p < 0.001$] in both AMPH-administered TS and control groups. Pair-wise comparisons revealed that AMPH administration resulted in significantly enhanced activity on the last day in both control and TS groups compared to the first day (both ps < 0.001), showing behavioral sensitization in AMPH-administered compared to saline-administered rats (see Table 3.2A). In contrast, saline administration in both control and TS groups, although not significant, resulted in decreased activity on the last day compared to the first day.

Repeated administration in both control and TS groups resulted in a gradual increase in locomotor activity over a 2-week period exhibiting development of behavioral sensitization (see Figure 3.4). Mixed ANOVA with experience (TS or no TS) and drug (AMPH or saline) as independent factors and days as repeated measure factor in male rats showed a main effect of experience [F(1, 24) = 6.4, p = 0.018], and drug [F(1, 24) = 206.21, $p < 0.001$], with no significant interaction among the two [F(1, 24) = 2.93, p = 0.10]. Pairwise comparison showed that TS resulted in attenuated behavioral sensitization in response to AMPH administration compared to their respective control rats (see Figure 3.4A). Moreover, the individual days of 14-day drug administration period when

subjected to pairwise comparison revealed that TS group exhibited attenuated behavioral sensitization response 11 out 14 days (all *p*s =< 0.05) where day 2, 5, and 8 were not significantly different from the control group (see Figure 3.4A).

In female rats, comparison of the first and the last day activity revealed a main effect of drug [$F(1, 24) = 240.57$, $p < 0.001$] and when subjected to pairwise comparison showed that AMPH-treated female rats exhibited increased behavioral sensitization on the last day compared to the first day in both control and TS groups (both *p*s < 0.001) (see Table 3.2B). Chronic 14-day AMPH administration in female rats resulted in the development of behavioral sensitization and when analyzed showed a main effect of experience [$F(1, 24) = 5.42$, $p = 0.029$] and drug [$F(1, 24) = 356.19$, $p < 0.001$] with no interaction between the two [$F(1, 24) = 1.25$, $p = 0.28$]. In addition, , when individual days of the drug administration period when subjected to pairwise comparison revealed that TS attenuated behavioral sensitization in response to AMPH administration 9 out of 14 days (all *p*s =< 0.05) where day 3, 4, 7, 9, and 14 were not significantly different from the control group (see Figure 3.4B).

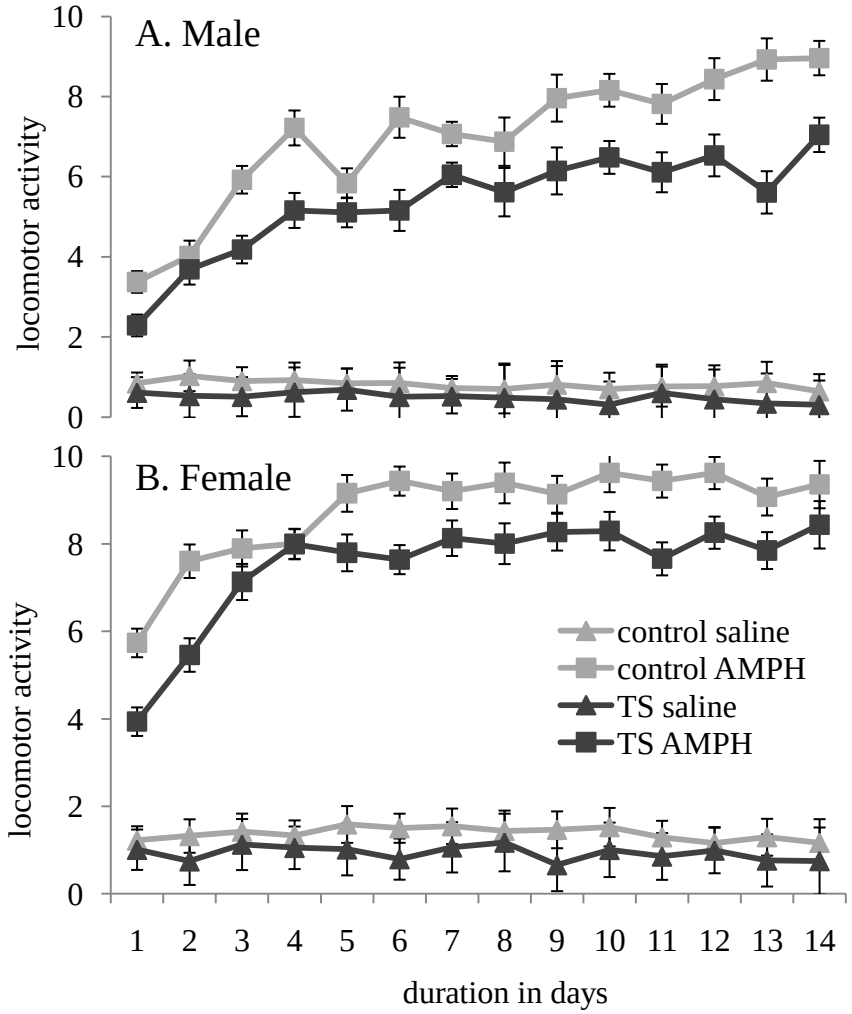

Figure 3.4. Mean (± SEM) locomotor activity (beam crossing x 10^3) recorded for 90 minutes after saline or AMPH (1 mg/kg) administration over 14-day period. TS attenuated AMPH-induced behavioral sensitization (all *p*s <0.05) except day 2, 5, and 8 in males (A) and in females (all *p*s < 0.05) except day 3, 4, 7, 9, and 14 (B).

3.4.2.3. Persistence of sensitization

To determine if drug-induced sensitization persisted after 2-week withdrawal period, both prior saline- and prior AMPH-treated male rats were administered with AMPH. A two-way ANOVA of the locomotor activity revealed a main effect of

experience [$F(1, 24) = 11.91$, $p = 0.002$], and prior drug treatment [$F(1, 24) = 50.09$, $p < 0.001$], with no interaction between the two [$F(1, 24) = 1.69$, $p = 0.206$]. Pair-wise comparison showed that prior AMPH-treated rats were more sensitized compared to prior saline-treated rats in both control and TS groups ($p =<0.001$), demonstrating persistence of sensitization in prior AMPH-treated rats. There was no significant difference between control and TS groups in prior saline-treated rats; however, prior AMPH-treated TS rats exhibited attenuated sensitization to drug challenge compared to their respective control group ($p = 0.001$) (see Table 3.3A).

In response to a challenge injection, female rats revealed a main effect of experience [$F(1, 24) = 4.92$, $p = 0.036$], and prior drug treatment [$F(1, 24) = 20.28$, $p < 0.001$] with no interaction between the two [$F(1, 24) =0.07$, $p = 0.791$]. Similar to males, prior AMPH-treated female rats were more sensitized compared to prior saline-treated rats in both control and TS groups ($p =<0.012$). There was no significant difference between control and TS groups in prior saline-treated as well as in prior AMPH-treated rats. TS rats appeared to exhibit an attenuated behavioral sensitization compared to their respective control group but the data when statistically analyzed showed only a trend ($p = 0.061$) (see Table 3.3B).

Table 3.3

Mean (±SEM) locomotor activity of male (A) and female rats (B) in response to an AMPH challenge injection

	Prior saline-treated	Prior AMPH-treated
A. Male		
Control	37618 ± 3910	74242 ± 3910*
Postnatal TS	28221 ± 5529	53471 ± 3910*
B. Female		
Control	62676 ± 5230	90576 ± 5230*
Postnatal TS	51280 ± 7396	76045 ± 5230*

Prior saline- and AMPH treated rats were given AMPH challenge to investigate persistence of behavioral sensitization after a 14-day withdrawal period. Prior AMPH treated male (A) and female rats (B) compared to sex-matched saline-treated groups exhibited enhanced activity showing persistence of behavioral sensitization (*, all *p*s =< 0.01).

3.4.3. Anatomy

3.4.3.1. Brain weight

Postnatal TS in males produced lighter brains than controls. When subjected to a two-way ANOVA, brain weights in male groups revealed a main effect of experience [$F(1, 24) = 5.60$, $p = 0.026$], no main effect of drug [$F(1, 24) = 0.12$, $p = 0.73$], nor an interaction between the two [$F(1, 24) = 0.23$, $p = 0.63$]. Pairwise comparison revealed that TS compared to controls did not significantly reduce brain weights in the saline-treated

group although it is clear from Table 3.4 that the trend was in that direction. AMPH administration in TS compared to controls resulted in lighter brains ($p = 0.034$) (see Table 3.4A).

As in males, TS experience in females appeared to reduce brain weight but the drug (AMPH and saline) did not influence the weights (see Table 3.4B). The brain weight data in females when subjected to a two-way ANOVA revealed a marginal effect of experience [$F(1, 24) = 3.73$, $p = 0.06$] and no main effect of drug [$F(1, 24) = 1.68$, $p = 0.21$] nor an interaction between the two [$F(1, 24) = 0.10$, $p = 0.76$].

Table 3.4

Mean (±SEM) brain weight (in grams)

	Control	Postnatal TS
A. Male		
Saline	2.11 ± 0.02	2.06 ± 0.03
AMPH	2.11 ± 0.02	2.04 ± 0.02*
B. Female		
Saline	1.93 ± 0.03	1.86 ± 0.04
AMPH	1.96 ± 0.03	1.91 ± 0.03

AMPH administration in TS group resulted in lighter brains (*, $p = 0.034$) compared to AMPH-treated control group in males (A) however, there was no effect of experience or drug on brain weight in females (B).

3.4.3.2. Cortical thickness in Cg 1

TS in females interacted with AMPH administration resulting in increased Cg1 thickness whereas no such effect was observed in saline-treated group. Furthermore, AMPH administration reduced the Cg 1 thickness in the control group whereas TS prevented the AMPH-induced decrease in thickness. In contrast, neither early experience nor drug substantially altered Cg 1 thickness in male groups (see Table 3.5).

A two-way ANOVA (Experience x Drug) on female Cg 1 cortical thickness revealed a main effect of experience [$F(1, 52) = 4.71$, $p = 0.035$], drug [$F(1, 52) = 4.22$, $p = 0.045$], and an interaction between the two [$F(1, 52) = 4.35$, $p = 0.042$]. Pairwise comparisons revealed that AMPH administration significantly increased cortical thickness in postnatal TS compared to the sex-matched control group ($p = 0.001$) (see Table 3.5B). Furthermore, AMPH compared to saline administration decreased Cg 1 thickness in the control group ($p = 0.002$). However, TS mitigated the AMPH-induced reduction in cortical thickness as there was no difference between AMPH- and saline-treated female rats (see Table 3.5B). In contrast to females, a two-way ANOVA of the Cg 1 thickness in males revealed no main effect of experience [$F(1, 52) = 0.28$, $p = 0.594$], drug [$F(1, 52) = 0.003$, $p = 0.955$] nor an interaction between the two [$F(1, 52) = 0.90$, $p = 0.347$] (see Table 3.5A).

3.4.3.3. Cortical thickness in Cg 3

Postnatal TS in females interacted with drug exposure to increase the Cg 3 thickness in AMPH treated group without any effect on saline-treated group. In addition, a reduction in Cg 3 thickness was observed in AMPH-treated controls but TS prevented

the reduction in cortical thickness. Unlike females, neither early experience nor drug influenced Cg 3 thickness in males.

A two-way ANOVA (Experience x Drug) of the Cg 3 thickness in females revealed a main effect of experience [F (1, 52) = 6.11, p = 0.017], drug [F(1, 52) = 6.70, p =0.011], and an interaction between the two [F(1, 52) = 18.04, p < 0.001]. Pairwise comparison revealed that AMPH administration increased cortical thickness in the postnatal TS group compared to the sex-matched control group (p < 0.001) (see Table 3.5B). However, no significant difference was found in saline-treated control and TS group. In addition, AMPH compared to saline administration decreased cortical thickness in the control female group (p < 0.001) however, postnatal TS abated the decrease in cortical thickness exhibiting no significant difference between AMPH- and saline-treated females (see Table 3.5B). In contrast to females, males revealed no main effect of experience [F(1, 52) = 0.001, p = 0.971], drug [F(1, 52) = 1.09, p = 0.302], nor an interaction between the two [F(1, 52) = 0.50, p = 0.483] (see Table 3.5A).

3.4.3.4. Cortical thickness in AID

AID cortical thickness in females was increased by postnatal TS experience in AMPH-treated group without a significant effect on saline-treated group. In addition, AMPH administration decreased cortical thickness in the control group whereas an increase was recorded in the TS alone group. In contrast, males did not show a substantial alteration in the AID thickness related to early experience or drug.

When subjected to a two-way ANOVA (Experience x Drug), AID thickness in females revealed a main effect of experience [F(1, 52) = 4.61, p = 0.037], no main effect

of drug [$F(1, 52) = 1.03$, $p = 0.314$] and an interaction between the two [$F(1, 52) = 17.10$, $p < 0.001$]. Pairwise comparison revealed that AMPH administration increased cortical thickness in the AID region of the TS compared to sex-matched controls ($p < 0.001$) (see Table 3.5B). However, no significant difference in thickness was observed between saline-treated TS and control groups. In addition, AMPH administration produced an opposite effect on AID thickness in TS when compared to the control group. AMPH compared to saline administration decreased cortical thickness in the control group ($p < 0.001$) but a significant increase was observed in the TS group ($p = 0.049$) (see Table 3.5A). Unlike females, AID thickness in males when subjected to a two-way ANOVA (Experience x Drug) revealed no main effect of experience [$F(1, 52) = 0.50$, $p = 0.481$], drug [$F(1, 52) = 3.25$, $p = 0.077$], nor an interaction between the two [$F(1, 52) = 0.18$, $p = 0.672$] (see Table 3.5A).

Table 3.5

Mean (± SEM) of the cortical thickness in Cg 1 and Cg 3 regions of medial PFC and AID region of OFC

	Control		Postnatal TS	
	Saline	AMPH	Saline	AMPH
A. Male				
Cg 1	2.42 ± 0.02	2.40 ± 0.02	2.38 ± 0.02	2.38 ± 0.02
Cg 3	0.97 ± 0.04	0.95 ± 0.04	1.00 ± 0.05	0.93 ± 0.04
AID	2.12 ± 0.04	2.06 ± 0.04	2.17 ± 0.05	2.07 ± 0.04
B. Female				
Cg 1	2.42 ± 0.03	2.27 ± 0.03†	2.43 ± 0.05	2.43 ± 0.03*
Cg 3	1.06 ± 0.03	0.84 ± 0.03†	1.01 ± 0.04	1.06 ± 0.03*
AID	2.16 ± 0.04	1.94 ± 0.04†	2.08 ± 0.05	2.21 ± 0.04*†

TS in females increased the cortical thickness in the Cg 1, Cg 3, and AID regions (*, all *p*s =< 0.001) in the AMPH-treated group. In addition, a reduction in thickness was observed in AMPH-treated controls in Cg 1 and Cg 3 regions (†, both *p*s =< 0.001); however, TS prevented the reduction in both regions. Moreover, AMPH administration decreased AID thickness in the control group whereas it increased thickness (†, both *p*s=< 0.05) in TS rats. Unlike females, neither early experience nor drug significantly influenced prefrontal cortical thickness in males.

3.4.3.5. Striatum size

Postnatal TS in males influenced the anterior striatum resulting in a larger striatum in both saline- and AMPH-treated TS groups. In contrast, the posterior striatum

was influenced by AMPH administration resulting in an enlarged posterior striatum in male controls. TS prevented the AMPH-induced striatal enlargement, however. Neither experience nor drug influenced the striatum at either the anterior and posterior planes in females.

A two-way ANOVA (Experience x Drug) of the anterior striatum in males revealed a main effect of experience [$F(1, 52) = 10.19$, $p = 0.002$] with no main effect of drug [$F(1, 52) = 0.43$, $p = 0.513$] nor an interaction between the two [$F(1, 52) = 0.001$, $p = 0.988$]. Pairwise comparison revealed that postnatal TS resulted in bigger striatum in both saline- and AMPH-treated males ($p = 0.043$ and 0.015, respectively) (see Table 3.6A). However, AMPH compared to saline administration did not influence the anterior striatum (see Table 3.6A). When subjected to a two-way ANOVA (Experience x Drug), the posterior striatum in males revealed a main effect of experience [$F(1, 52) = 5.05$, $p = 0.029$] and drug [$F(1, 52) = 6.16$, $p = 0.016$] with no interaction between the two [$F(1, 52) = 0.22$, $p = 0.639$]. Pairwise comparison revealed that TS compared to the controls in the saline-treated group appeared to have larger striatum, however, the effect was not significant ($p = 0.085$). Furthermore, AMPH compared to saline administration in controls resulted in enlarged striatum ($p = 0.023$) (see Table 3.6A). TS prevented the AMPH-induced striatal enlargement effect in controls because there was no difference between saline and AMPH administration in TS group (see Table 3.6A).

A two-way ANOVA (Experience x Drug) of the anterior striatum in females revealed a marginal effect of experience [$F(1, 52) = 3.49$, $p = 0.067$] with no effect of drug [$F(1, 52) = 0.05$, $p = 0.828$] nor an interaction between the two [$F(1, 52) = 0.65$, $p = 0.424$]. Pairwise comparison revealed that saline-treated TS compared to the respective

control group appeared to result in enlarged striatum, however the difference was not significant (p = 0.090) (see Table 3.6B). Similarly, AMPH administration did not influence striatum in either the control or TS group. In addition, early experience or drug did not influence the posterior striatum in females. When subjected to a two-way ANOVA (Experience x Drug), the posterior striatum revealed no main effect of experience [$F(1, 52) = 0.03$, $p = 0.851$], drug [$F(1, 52) = 0.56$, $p = 0.457$], nor an interaction between the two [$F(1, 52) = 0.261$, $p = 0.612$] (see Table 3.6B).

Table 3.6

Mean (± SEM) of anterior (~ Bregma 1.7 mm) and posterior striatum (~ Bregma 0.2 mm) area measured with NIH Image

	Control		Postnatal TS	
	Saline	AMPH	Saline	AMPH
A. Male				
Anterior striatum	9.49 ± 0.35	9.75 ± 0.35	10.75 ± 0.50*	11.00 ± 0.35*
Posterior striatum	13.49 ± 0.23	14.27 ± 0.23*	14.21 ± 0.33	14.74 ± 0.23
B. Female				
Anterior striatum	9.22 ± 0.25	9.51 ± 0.25	9.97 ± 0.35	9.81 ± 0.25
Posterior striatum	13.92 ± 0.23	13.55 ± 0.26	13.83 ± 0.37	13.76 ± 0.26

Postnatal TS in males resulted in larger anterior striatum in both saline- and AMPH-treated TS groups (*, p = 0. 043 and 0.015, respectively). In contrast, posterior striatum was enlarged by AMPH administration in control males (*, p = 0.023). However, TS prevented the AMPH-induced striatal enlargement. Experience or drug did not influence the striatum substantially both at the anterior and posterior planes in females.

3.5. Discussion

Stimulation of neonatal rats through gentle stroking provides sensory sensation that is believed to be similar to maternal licking and grooming. For instance, TS and LG have been reported to modulate rat behavior and alter brain anatomy and physiology in a similar fashion. For example, both LG (Diorio, Viau, & Meaney, 1993; Liu et al., 1997; Meaney et al., 1985), and TS (Bear, et al., 2006) up regulated glucocorticoid receptors in the hippocampus and the PFC, key regions in the HPA axis feedback mechanism (Sapolsky, et al., 1984). In addition, sensory stimulation during development has successfully ameliorated or even reversed some of the deleterious effects of early adverse experiences (Escorihuela, Tobena, & Fernandez-Teruel, 1994; Morley-Fletcher, Rea, Maccari, & Laviola, 2003; Wakshlak & Weinstock, 1990) (but see de Medeiros, Fleming, Johnston, & Walker, 2009; Wood, et al., 2003). Studies have also supported the beneficial effect of TS (i.e. massage) during development on human health and brain development (Field, 2001; Guzzetta et al., 2009).

The exact mechanism of how sensory stimulation translates into a 'positive' experience for the brain and behavior is not well characterized. However some of the possible mechanisms involved could be the modulation of the HPA axis through suppressed stress-induced elevations of ACTH secretion (Suchecki, Rosenfeld, & Levine, 1993), and up regulation of glucocorticoid receptors in the hippocampus and the PFC (Meaney, et al., 1985). Being part of the negative feedback mechanism, the higher density of glucocorticoid receptors in these regions helps in shutting down HPA-axis response upon exposure to a stressful stimulus. Furthermore, sensory stimulation modulates key molecules such as dopamine (DA; including the DA transporter and

receptors) (Del Arco et al., 2007; Lovic, et al., 2006), and growth factors such as brain derived neurotrophic factor (BDNF) and fibroblast growth factor 2 (Gibb & Kolb, unpublished observations) in various brain regions. Sensory stimulation would possibly alter brain and behavior either directly through modulating the above mentioned molecules in the brain or indirectly through a cascade of other events that take place in response to such modulation.

3.5.1. Behavior

Juvenile rats were tested on a number of behavioral tasks to investigate the effect of early TS on exploratory, emotional, cognitive, and social behavior. The behavioral tasks included open field locomotion, elevated plus maze, novel object recognition, and play fighting behavior. Exploratory behavior, tested as open field locomotion, was not modified substantially by early TS. The behavior, partly mediated by dopamine, might not be affected by early stimulation as previous work has shown that neonatal handling in rats did not result in alteration in the dopamine transporter in the nucleus accumbens region (Brake, Zhang, Diorio, Meaney, & Gratton, 2004). TS also did not produce any differences in anxiety-like behavior in EPM compared to control rats (Silveira, Portella, Clemente, Gamaro, & Dalmaz, 2005; Wakshlak & Weinstock, 1990); however, Imanaka et al., (2008) reported lower anxiety-like behavior exhibited by TS rats in EPM. The possible reason for such inconsistency could be differences between experimental protocols such as the time, duration and procedure of TS, and age of the rats at the time of testing. Age has been shown as one of the modulating factor, as the beneficial effects of stimulation were more observable at later age (Meaney, Aitken, Bhatnagar,

VanBerkel, & & Sapolsky, 1988). Age as a factor is also confirmed in the present study, where attenuated response to amphetamine sensitization was observed in adult rats. Another possibility of not so visible changes in behavior because of TS could be the nature of the tasks, e.g., handled rats perform well in tasks involving conflicting situations as compared to 'simpler' tasks (e.g. open field locomotion or EPM) (Nunez, Ferre, Escorihuela, Tobena, & Fernandez-Teruel, 1996).

In contrast to open field locomotion and EPM, NOR and play behavior were significantly modified by early stimulation. Although TS did not affect the exploration of objects in temporal order as there was no difference between exploration times of 'old' and 'recent' objects. The overall time spent with novel objects in the first trial was greatly reduced, reflecting attenuated novelty-seeking in TS rats. The moderated response to novelty-seeking could be a preventive measure against drug abuse propensity, as novelty-seeking and risk taking behaviors have been strongly implicated as predisposing factors for drug abuse (Hooks, Jones, Smith, Neill, & Jr., 1991).

Play behavior is comprised of a series of play attacks, generally targeting the nape. The attack is faced by the play partner, either as complete or partial rotation defense. However, sometimes the rat, instead of facing, evades the attack (Pellis & Pellis, 1990). The features of play behavior are different in juveniles compared to adults. For example, juveniles compared to infants or adults exhibit increased frequency of complete rotation defense (Pellis & Pellis, 1997). In addition, juvenile play fighting is sexually dimorphic with males engaging in play more frequently than females (Pellis & Pellis, 1990). Previous studies related to the influence of early experience (e.g. neonatal handling) on play reported altered social behavior in rat (Aguilar, Carames, & Espinet,

2009). Our findings showed that TS substantially modulated play attacks and defense strategy in males, whereas females exhibited only an altered defense response to a play attack. TS males showed decreased frequency of play attacks as well as the probability of complete rotation defense. Furthermore, TS males did not face the attack but instead showed an enhanced frequency of evasions. Only a decrease in the probability of complete rotation defense was observed without alteration in the frequency of play in TS females. Our findings are consistent with previous reports related to the effect of enhanced maternal licking and grooming, which decreased the frequency of play behavior (Birke & Sadler, 1987; Parent & Meaney, 2008). In addition, the influence of stimulation was more obvious in males, similar to our findings, whereas females were not affected substantially (Birke & Sadler, 1987; Parent & Meaney, 2008). In contrast to our finding, the frequency of play behavior and the probability of defense was not altered by neonatal handing in the study reported by Arnold and Siviy (2002). However, Arnold and Siviy reported female-like play patterns in males exposed to neonatal handling. The differences could be attributed to differences in stimulation procedure (TS *vs.* handling) and duration (i.e. number of days) and rat strain in addition to other procedural differences. Indeed, a subsequent study by the same laboratory did show an effect of early handling on juvenile play, further supporting the possibility that procedural and strain differences could be important factors (Siviy & Harrison, 2008).

Modifications of play behavior by TS experience may play an important role in brain development. For example, Bell, Pellis & Kolb (2010) showed that the structure of pyramidal neurons in the medial and orbital prefrontal regions is significantly modified by manipulating play behavior. Amphetamine acts to alter neuronal structure in these

regions (Robinson & Kolb, 2004). It is thus possible that part of the reason for the attenuated response to amphetamine in the TS animals is related to structural modifications in the prefrontal regions.

3.5.2. Amphetamine sensitization

To determine the effect of early 'positive' experience on later amphetamine sensitization, adult rats stroked as pups were chronically administered with AMPH. The dose, duration and route of AMPH were selected based on previous experiments conducted in Kolb and Robinson labs (Robinson & Kolb, 2004; Singer, et al., 2009) that resulted in the development and persistence of behavioral sensitization.

Stimulant drugs in rats not only develop behavioral sensitization with repeated exposure but the sensitization also persists after a withdrawal period for months in rodents (Kolb, Gorny, et al., 2003) to years in monkeys (Castner & Goldman-Rakic, 1999). Chronic AMPH administration in the present study resulted in the development of behavioral sensitization, measured as locomotor activity, in both male and female control and TS groups. Early TS experience, however, resulted in attenuated AMPH sensitization in both male and female groups compared to their respective control groups. Sensitization also persisted in AMPH-treated rats at least for two weeks, when prior AMPH-treated rats exhibited augmented behavioral sensitization to AMPH challenge compared to prior saline-treated rats (Peleg-Raibstein & Feldon, 2008). However, similar to the development of sensitization, prior AMPH-treated TS groups (both male and female) exhibited an attenuated response to APMH challenge.

Our study demonstrated that postnatal TS in rats attenuated the AMPH-induced acute locomotor response as well as chronic AMPH-induced behavioral sensitization. Similarly, Lovic, Fleming, and Fletcher (2006) reported the attenuation of AMPH-induced sensitization by tactile stimulation provided to artificially reared rats that were both mother and social deprived. The increased locomotor activity in response to AMPH administration exhibited by artificially reared rats was reversed by TS. Sensory stimulation experiences, similar to TS, during development, have favorable outcomes related to stimulant-induced sensitization. For instance, previous studies have shown that handling (Campbell & Spear, 1999), maternal licking and grooming (Francis & Kuhar, 2008), and environmental enrichment (Bardo, et al., 1995; Bardo, Klebaur, Valone, & Deaton, 2001) resulted in attenuated sensitization response to AMPH. The attenuated drug-induced sensitization, reported in the literature, has been observed across all drug abuse models including self-administration, conditioned place preference, and behavioral sensitization. Sensory stimulation in human and non-human primates, like rodents, showed a similar tendency in terms of stimulant sensitization (Morgan et al., 2002; Ussher, Taylor, West, & McEwen, 2000).

The possible reasons for attenuated AMPH sensitization in TS rats could be the result of alterations in interlinked molecular processes in the brain (i.e. regulation of dopamine and growth factors, and modulation of HPA axis). For example, dopamine (DA) release in the mesolimbic circuit plays a major role in drug abuse (Hooks & Kalivas, 1995), in addition to modulating locomotor activity (Koob, Stinus, & Le Moal, 1981). Nevertheless to mediate its effect, DA relies on other related molecules such as DA transporter and the D1- and D2-like receptors. Stimulation (e.g. environmental

enrichment) during development, has been reported to attenuate locomotor activity; an effect mediated by down regulation of dopamine transporters (Bezard et al., 2003). Moreover, TS has been shown to up-regulate growth factors such as BDNF (Bezard, et al., 2003) and fibroblast growth factor-2 (Gibb & Kolb, in submission) expression in various brain regions. Growth factors have been implicated in the growth and survival of neurons especially during development. Recent reports showed a relationship between growth factors and drug abuse. For example, BDNF overexpression in the ventral tegmental area and NAc facilitated, but in contrast over expression in medial PFC attenuated drug self-administration (Berglind et al., 2007). In contrast, exposure to stress, both in rats and non-human primates, resulted in enhanced sensitization to stimulants drugs (reviewed by Corominas, Roncero, Ribases, Castells, & Casas, 2007). The increased drug-induced sensitization has a negative correlation with glucocorticoid receptors (GR) density. One of the beneficial effects of early stimulation on the brain is the increase in GR in the PFC and hippocampus. These regions are involved in the negative feedback mechanism of HPA-axis to help shut down the stress reaction (Meaney, et al., 1985). The higher receptor density might be one of the factors directly or indirectly contributing to attenuated AMPH-induced behavioral sensitization of TS rats.

3.5.3. Anatomy

3.5.3.1. Prefrontal cortex

The present study investigated structural plasticity of the PFC regions (i.e. mPFC and OFC) associated with early experience (i.e. TS) and later drug (AMPH) exposure. Cortical thickness was used as a measure to investigate structural alteration in the PFC.

The findings suggest that early experience interacted with later AMPH exposure in a sex-dependent fashion to alter cortical thickness in the PFC subregions. Whereas AMPH reduced the prefrontal cortical thickness in control females, postnatal TS mitigated the drug-induced reduction in thickness. However, we did not observe any substantial alteration in prefrontal cortical thickness in males, which is consistent with a study of frontal lobe volume in human male addicts and controls (Bartzokis, et al., 2000).

Cg 1 and Cg 3, regions of anterior cingulate cortex, form part of the medial PFC whereas AID, a region of the insular cortex, forms part of the OFC in rats. The role of the PFC subregions (i.e. medial and OFC) is well established in drug addiction in experimental animals (reviewed by Porrino & Lyons, 2000) and humans (reviewed by Volkow, et al., 2003). In addition to drug-induced modulation in the functions of the PFC region, structural alteration (e.g., spine density) has also been reported in rodents (Robinson & Kolb, 2004) and monkeys (Selemon, et al., 2007). We are unaware of any published study that investigated drug-induced alteration in the prefrontal cortical thickness in rodents or in monkeys. However, human imaging studies reported drug-induced structural alteration in the frontal lobe (Kim, et al., 2006).

Our study suggests that the prefrontal cortical thickness in both medial and orbital subregions was substantially altered by TS, AMPH exposure, and/or the interaction between the two in females. AMPH administration for two weeks resulted in a reduction in cortical thickness in both mPFC and OFC subregions in the control group. Our findings corroborate the studies related to drug-induced structural alteration in humans. Imaging studies in patients with a history of AMPH or methamphetamine abuse reported a decrease in cortical gray matter density or volume in the anterior prefrontal cortex

(Kim, et al., 2006; Schwartz, et al., 2010) and medial orbital cortex (Tanabe, et al., 2009). Furthermore, structural abnormalities in the frontal lobe with AMPH or methamphetamine abuse were correlated with impaired cognitive performance (e.g. Wisconsin Card Sorting Test) (Kim, et al., 2006; Rogers, et al., 1999; Schwartz, et al., 2010). Similar to drug addiction, other PFC-related neurological disorders e.g. schizophrenia also resulted in reduced cortical thickness of the frontal region (Kuperberg, et al., 2003; Rimol, et al., 2010).

While AMPH exposure decreased prefrontal cortical thickness in the control female group, postnatal TS experience prevented the thinner cortex effect as there was no difference between saline- and AMPH-treated TS group. Furthermore, there was an interaction between early experience and later drug exposure. Whereas TS in the saline group did not alter the thickness, AMPH administration increased the prefrontal cortical thickness. Similar to our findings, environmental enrichment ameliorated the decrease in cortical thickness resulting from neonatal frontal cortical injury (Comeau, et al., 2008) or early social isolation stress (Hellemans, et al., 2004). The drug-induced reduction in the prefrontal cortical thickness and the alleviation by TS needs further investigations, for instance at immunohistological levels as the findings may not necessarily reflect an increase in neuropil but could be due to an increase in glial population. Epigenetic studies could also be helpful to examine experience-dependent gene expression profile.

3.5.3.2. Striatum

The striatum is divided in dorsal (i.e., caudate nucleus and putamen) and ventral (i.e., nucleus accumbens) regions and both the dorsal and ventral striatum have been

implicated in drug addiction in experimental animals (Gerdeman, et al., 2003; Ito, et al., 2002; Li, Kolb, et al., 2003). Similarly, human imaging studies also confirmed the involvement of striatum in addiction (Volkow, et al., 2003).

Our findings suggest the influence of early experience or drug was robust only in males. TS but not AMPH administration structurally altered the anterior striatum. In contrast, the posterior striatum was modified in the control group by AMPH administration but not by early experience. Postnatal TS in males resulted in enlarged striatum in both saline- and AMPH-treated groups. Conversely, the posterior striatum became larger as result of AMPH administration in male controls however, postnatal TS experience prevented the enlarged striatal effect. The findings for male rats are consistent with the imaging reports of human amphetamine abusers (Chang et al., 2005; Jernigan et al., 2005). Interestingly, Jernigan et al., (2005), similar to our findings in males, failed to find an effect of methamphetamine on cerebral cortical thickness. Similarly an increase in striatal volume has also been reported in cocaine abusers (Jacobsen, et al., 2001). The enlarged striatum could be the result of enhanced dopamine release in the striatum observed with repeated AMPH administration (Robinson & Becker, 1982).

There is sex-specific dissociation between prefrontal cortical thickness and striatum size in our study. We found an AMPH-induced decrease in cortical thickness in control female rats that was prevented by postnatal TS whereas there was no effect of experience or drug on the striatum. In contrast to females, AMPH administration in males enlarged the posterior striatum in controls that was prevented by TS. Furthermore, TS in males enlarged anterior striatum in both saline- and AMPH-treated groups. However, there was no influence of early experience or drug on the prefrontal cortical thickness in

males. The sex-dependent cortical and subcortical structural alteration could be related, for instance, to differential epigenetic modulation (e.g., of ERα promoter region) in male and female rats associated with somatosensory stimulation during early brain development (reviewed by McCarthy, et al., 2009). In addition, TS during development might modulate key molecules (e.g., MeCP2) in the brain that are expressed differentially in both sexes and are linked with neuronal plasticity. MeCP2 was not only associated with structural alterations in the brain (Zhou et al., 2006) but also modulated AMPH-induced conditioned place preference (Deng et al., 2010). Interestingly, MeCP2 alteration in the brain reorganized juvenile play behavior in a sex-dependent manner such that males showed female-like play, similar to our finding (Kurian, Bychowski, Forbes-Lorman, Auger, & Auger, 2008).

The areal difference could be related to different neuronal population in the PFC and the striatum. Pyramidal cells represent the majority of the neuronal population in the PFC, whereas the striatum has medium spiny neurons in abundance. However, the opposite results for both sexes add to the complexity of the experience and drug interaction. Previous reports indicated sex-specific structural differences with repeated drug abuse in humans. For example, Chang et al. (2005) reported enlargement of the posterior corpus callosum in female methamphetamine abusers but not in males. Likewise, a study related to methamphetamine-induced dopamine depletion in the striatum reported that female compared to male mice demonstrated less depletion(Dluzen, Tweed, Anderson, & Laping, 2003). The sex-specific structural alteration could be related to gonadal hormones. For example, similar to psychostimulants (Robinson & Kolb, 2004), stress exposure resulted in impaired

functions and altered dendritic organization in the PFC (Liston et al., 2006), although in this case there was a reversed effect, namely increased dendritic length in OFC and decreased length in mPFC. Previous reports indicated that females exhibited enhanced sensitivity to stress-induced PFC dysfunction that was dependent on high levels of estrogens (Shansky et al., 2003). Likewise, stress (Riva, et al., 1995) and psychostimulants (e.g. cocaine) (Fumagalli, et al., 2006) modulated FGF-2 expression in various brain regions. Stress exposure, however, interacted with cocaine administration in region-dependent modulation of FGF-2 in the PFC and the striatum (Fumagalli, et al., 2008).

In sum, based on the findings it might be concluded that TS during development modulated the response to novel objects and social behaviors without influencing simple and less conflicting behaviors. Repeated AMPH administration resulted in the development of behavioral sensitization and a challenge after a withdrawal period showed the persistence of sensitization in rats. However, postnatal TS resulted in attenuated drug-induced behavioral sensitization in male and female rats. The attenuated behavioral sensitization might be related to a reduction in the drug-induced structural reorganization of the PFC and the striatum, regions implicated in drug addiction.

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CHAPTER 4

Mild prenatal stress modulated behavior and neuronal spine density without affecting amphetamine sensitization*

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* Muhammad, A., & Kolb, B. (2011). Mild Prenatal Stress-Modulated Behavior and Neuronal Spine Density without Affecting Amphetamine Sensitization. *Developmental Neuroscience, In Press, Accepted Manuscript*.

Abstract

The present study investigated the effect of prenatal stress (PS) on juvenile behavior, adult amphetamine (AMPH) sensitization, and the interaction of experience (i.e. PS) and drug (i.e. AMPH) on neuronal morphology in corticolimbic regions in rats. Juvenile male and female rats, exposed to gestational stress, were tested in behavioral tasks that included open field locomotion, elevated plus maze (EPM), novel object recognition (NOR), and play fighting behavior. The development and persistence of drug-induced behavioral sensitization in adults were tested by chronic AMPH administration and challenge, respectively. Spine density in corticolimbic regions was examined for structural plasticity. The findings showed that PS produced anxiety-like behavior in males. Furthermore, PS in males resulted in female-like play and enhanced partial rotation defense whereas in females PS increased the probability of evasion in response to an attack. AMPH administration resulted in gradual increase in behavioral sensitization that persisted at least for 2 weeks however, PS did not influence AMPH-induced behavioral sensitization in either male or female rats. Moreover, PS increased the spine density in the nucleus accumbens (NAc) and decreased it in the medial prefrontal cortex (mPFC) without any alteration in the orbital frontal cortex (OFC). Similarly, AMPH administration increased spine density in the NAc and mPFC whereas a decrease was observed in the OFC. However, PS prevented the drug-induced alterations in the spine density observed in controls. In sum, PS modulated juvenile behavior and altered brain morphology without influencing AMPH-induced behavioral sensitization substantially.

Key words: behavioral sensitization, addiction, psychostimulant, amphetamine, dendritic spine density, plasticity, prenatal stress, rough and tumble play, brain development, novel object recognition

4.2. Introduction

The brain shows marked plasticity during development, and is generally thought to be most responsive to environmental manipulation at this time (Hubel & Wiesel, 1970). Research related to experience based brain development was traditionally centered on postnatal life (e.g., Hubel & Wiesel, 1970) however, recently the focus has been shifted to prenatal period (Lupien, et al., 2009; Weinstock, 2001). The brain goes through a series of critical developmental stages before birth and any experience (e.g., stress) during the prenatal period has a programming effect on brain circuitry (reviewed by Seckl, 2004; Weinstock, 2008). For example, prenatal alcohol exposure produces enduring changes in the neurotransmitter levels (e.g. corticotropin-releasing factor) in the brain (reviewed by Lee, Choi, Kang, & Rivier, 2008) and subsequently behavior (reviewed by Riley, 1990). Similarly, exposure to adverse experiences during prenatal brain development acts as a predisposing factor for psychopathologies in addition to behavioral abnormalities (reviewed by Kofman, 2002). Infectious agents (Watson, et al., 1999), licit and illicit drugs and alcohol (Eriksson, et al., 2000; Lu, et al., 2009; Noble & Ritchie, 1989), malnutrition (Brown, et al., 1996), and non-drug chemicals (Tamaru, et al., 1988) are examples of adverse experiences that have resulted in maladaptive behavior in addition to altering the structural and chemical composition of the brain in experimental animals.

Similarly in humans, stress during development predisposes individuals to abnormal behavior and psychopathologies later in life. Children exposed to gestational stress exhibit cognitive impairment, (e.g. impaired language development) (Brouwers, et al., 2001; King & Laplante, 2005; O'Connor, et al., 2002). Furthermore, several mental

disorders have been associated with abnormal brain development. For instance, prenatal stress has a strong correlation with the development of schizophrenia, attention-deficit hyperactivity disorder, depression, and drug addiction (Felitti, 2002). The correlation in human studies, mostly inferred from retrospective studies, is confounded by intrinsic and extrinsic factors. Nevertheless, even in controlled human studies, the study of gestational stress in relation to brain development is mostly limited to early years of development (O'Connor, et al., 2002; Van den Bergh & Marcoen, 2004).

Experimental animals have been studied for the last few decades to categorize the long term impact of developmental stress on brain and behavior. Overall, the literature indicates that stress during development in rodents and monkeys produces behavioral abnormalities such as elevated and prolonged stress response, impaired learning and memory, deficits in attention, and altered exploratory behavior (reviewed by Weinstock, 2008).

We previously reported that tactile stimulation, a form of sensory stimulation, during the prenatal period attenuated AMPH sensitization (Muhammad, et al., 2011). Having the favorable impact of the tactile stimulation in mind, we hypothesized that stress during prenatal brain development would negatively impact juvenile behavior and increase drug-induced sensitization later in life. Furthermore, we previously reported that drugs (e.g. nicotine) and experience (e.g. enriched environment) altered neuronal morphology (e.g. dendritic growth and spine density) in key brain regions, such as nucleus accumbens, and prefrontal cortex (Kolb, Gorny, et al., 2003; Norrholm, et al., 2003; Robinson & Kolb, 1997). However, the drug-experience interaction showed that drug exposure followed by the experience of rearing in an enriched environment

interfered with structural plasticity associated with the enrichment experience (Hamilton & Kolb, 2005). In addition to studying behavior and drug-induced sensitization, we investigated the effect of experience (i.e. PS) and drug (i.e. AMPH) interaction on structural plasticity (i.e. spine density) in brain regions implicated in drug addiction (i.e. prefrontal cortex, striatum).

4.3. Materials and methods

4.3.1. Animals

Long Evans pups [male n = 28 (control = 8, PS = 6) and female n = 28 (control = 8, PS = 6)], stressed during gestation, were randomly allotted with not more than two pups of each sex from the same litter. The control rats, animal facility reared, were also used in another experiment (Muhammad, et al., 2011). The pups along their respective dams were housed in the breeding colony at the Canadian Centre for Behavioural Neuroscience, University of Lethbridge, Alberta, Canada. After weaning, the rats were housed in standard shoe-box cages with the same sex in a group of two in temperature- and humidity-controlled room. Rat chow food and water were provided *ad lib*. The rats were left undisturbed except regular cage cleaning and when tested for behavioral tasks and AMPH administration.

4.3.2. Prenatal stress

Pregnant rats were subjected to stress during the second week of gestation from day 12 to 16 twice a day for 10 minutes. The stress procedure was adopted from Wong et al. (2007). Briefly, pregnant rats, transported to a separate room for stress procedure,

were placed on an elevated Plexiglas platform (1 m tall, 21 x 21 cm). The rats that occasionally jumped from the platform were placed back immediately. At the end of stress procedure, rats were transported back to their home cages. The pups born of the stressed and unstressed dams, used in the experiment, were left undisturbed till the commencement of behavioral testing.

4.3.3. Behavior

The effect of early PS on juvenile behavior was investigated by testing the rats between postnatal (P) 30-40 in a battery of behavioral tasks. The tests included open field locomotion, elevated plus maze (EPM), novel object recognition (NOR)-recency discrimination version, and play fighting behavior.

4.3.3.1. Open field locomotion

Exploratory behavior of the rats was evaluated as open field locomotion, recorded for ten minutes using Accuscan activity monitoring Plexiglas boxes (L 42cm, W 42cm, H 30cm). The activity was recorded as the number of sensors beam breaks in the boxes attached to a computer. The horizontal beam breaks, used as an index of locomotor activity, were recorded on the computer with VersaMax^{TM} program and converted to spreadsheet using VersaDat^{TM} software (AccuScan Instruments, Inc., Columbus, OH).

4.3.3.2. Elevated plus maze

The EPM, a '+' shape maze with two closed and two open arms, was used to test anxiety-like behavior exhibited by the rats. The length of each arm of the maze measured

113 cm with a width of 10 cm while the maze was elevated 88 cm above the ground. Rats were placed in the centre of the maze facing a closed arm and were allowed to explore the maze for 5 minutes. Exploration behavior was videotaped with a camera installed in such a way to spot both open arms. The time spent in closed arms and number of entries in each open and closed were scored and analyzed to assess anxiety-like and exploratory behaviors, respectively.

4.3.3.3. Novel object recognition

Novel object recognition was carried out to evaluate exploration of novel object as well as exploration of objects in temporal order. Rats were habituated to a Plexiglas box for 15-20 minutes for four days prior to the commencement of the testing sessions. The NOR task was comprised of three trials following the procedure with minor modification described elsewhere (Hannesson, et al., 2004; Mitchell & Laiacona, 1998). Briefly, rats were allowed to explore objects and taped with a video camera. Glass candle holders, with similar sizes but different shapes and colors, were used as objects. During the first training trial, a rat was exposed to two novel but similar objects for a 4-minute period. Followed by 60 minutes delay, the rat was again exposed to two new objects, again similar but different objects from the first trial. After an additional 60-minutes delay, the rat was exposed to one object each from the first and second trial, termed as ‘old’ and ‘recent’ familiar objects, respectively. The objects and testing area were cleaned with 30% alcohol between each trial for disinfection and odor removal. Rats were transported back to their home cages in the 60-minute delay between the trials.

Exploration of each novel object in the first two trials and 'old' and 'recent' familiar objects in the third trial was scored. The time spent with 'old' familiar object was calculated as the difference between times spent with 'old' and 'recent' familiar object divided by total time exploring both objects (Hannesson, et al., 2004). In addition the total time spent with both objects in each trial was also analyzed.

4.3.3.4. Play fighting

Juvenile rats were allowed to play in a pair to assess the effect of PS on social behavior. PS and control rats were housed in pairs as playmates for a period of about two weeks. The playmates, as juveniles, were habituated to a play box (50 cm x 50 cm x 50 cm) for about 30 minutes for 3 days. For play deprivation before testing, rats were housed individually in an isolation room for 24 hours at the end of habituation period. The play behavior was recorded for 10 minutes with a night shot camera. On testing day both playmates were color marked on the tail with two separate paints to make them identifiable in the video recording, which was filmed in the dark. Rats were transported to their home cage after the play session and pair-housed for the rest of the experiment. The video recording, analyzed frame by frame, was scored for attacks, and complete/partial rotation defense or evasion generated in response to an attack (Pellis & Pellis, 1990). The defense was scored as 'complete rotation' when a rat had both fore- and hind limbs in the air while lying in a supine position. The defense was considered 'partial rotation' when only forelimbs were in the air with hind limbs touching the ground. If the rat, turned away from the attacker instead of facing an attack, it was scored as evasion. The probability of complete/partial rotation defense or evasion was calculated as the number

of complete/partial rotation or evasion divided by the total number of attacks carried out by the playmate (Pellis & Pellis, 1990).

4.3.4. Amphetamine sensitization

4.3.4.1. Amphetamine administration

To see the effects of postnatal PS on psychomotor stimulant behavioral sensitization, adult rats (P80) were administered with D-amphetamine sulfate (Sigma Aldrich, St. Louis, MO, USA) dissolved in sterile 0.9% saline. Locomotor activity, used as an index of behavioral sensitization (Wise & Bozarth, 1987), was recorded using Accuscan activity monitoring system, comprised of Plexiglas boxes (L 42cm, W 42cm, H 30cm) connected to a computer. The rats were habituated to the activity boxes for 30 minutes followed by AMPH (1 mg/kg body weight, i.p.) or 0.9% saline administration, both at a volume of 1 ml/kg. Rats were immediately placed back in the activity boxes post injections and the activity was recorded for 90 minutes. The drug was administered once a day for 14 consecutive days, approximately the same time every day. Locomotor activity recorded on a computer with VersaMax^{TM} program was converted to spreadsheet using VersaDat^{TM} software (AccuScan Instruments, Inc., Columbus, OH). The rats were returned back to their home cages each day after the end of AMPH testing session. The development of sensitization was determined by analyzing activity recorded over 14-day period using mixed ANOVA with experience (PS or control), drug (AMPH or saline) as independent factors, and day (day 1-14 days) as a repeated measure factor, followed by Bonferroni's post hoc test for multiple comparisons.

4.3.4.2. Challenge

The rats were given a withdrawal period of 2 weeks after AMPH administration period, followed by a challenge with AMPH (1mg/kg, i.p.) given to both prior AMPH- and prior saline-treated rats. Locomotor activity was recorded in activity monitoring boxes similar to the development of AMPH sensitization procedure described above. All rats were challenged with AMPH to see the persistence of behavioral sensitization in prior AMPH-treated rats compared to prior saline-treated rats.

4.3.5. Anatomy

4.3.5.1. Perfusion and staining

Approximately 24 hours post AMPH challenge, the rats were given an overdose of sodium pentobarbital solution i.p. and perfused with 0.9% saline solution intracardially. The brains removed from the skull were trimmed by cutting the olfactory bulb, optic nerves and spinal cord. The brains were then weighed and preserved in Golgi-Cox solution for 14 days followed by transfer to 30% sucrose solution at least for 3 days. The brains were sliced at a thickness of 200 μm on a Vibratome and fixed on gelatinized slides. The slides mounted with brain sections were processed for Golgi-Cox staining, following the protocol described by Gibb and Kolb (1998).

4.3.5.2. Spine density

The distal dendrites of individual neurons were traced from Golgi-Cox stained brain sections using a camera Lucida mounted on a microscope. Brain regions selected for neuron tracing were nucleus accumbens shell region (NAc), Cg3 (layer III) region of

anterior cingulate of the medial prefrontal cortex (mPFC), and dorsal agranular insular cortex (AID, layer III) of the orbital frontal cortex (OFC) described by Zilles (1985). The dendritic segments traced met the criteria of being thoroughly stained and without overlapping another dendrite or blood vessel. Spine density was measured at 1000X and calculated by counting the number of spines on a length of distal dendrite that was at least 50 microns in length. The exact length of the dendrite segment was calculated and density expressed per 10 microns. As with the dendritic length, 5 segments were drawn per hemisphere and a mean value calculated to use as the unit of measurement.

4.4. Results

The behavioral data was analyzed using experience (PS or control) and sex as independent factors. However, both sexes were analyzed independently after the introduction of drug (AMPH or saline) as a factor either because of sex-dependent difference (e.g., response to AMPH administration) or for the clarity of results description (e.g., in spine density). Furthermore, all ANOVAs were followed by Bonferroni's post hoc test for multiple comparisons.

4.4.1. Behavior

4.4.1.1. Open field locomotion

Prenatal stress did not modulate the exploratory behavior tested as open field locomotion in either male or female groups. The horizontal activity, used as an index of open field locomotion, when subjected to a two-way ANOVA with experience (PS or control) and sex as independent factors revealed no main effect of experience [F (1, 52) =

0.12, $p = 0.730$], sex [F (1, 52) = 0.29, $p = 0.59$] nor an interaction between the two [F (1, 52) = 0.001, $p = 0.973$].

4.4.1.2. Elevated plus maze

The anxiety-like behavior, tested as the time spent in the closed arms of EPM, was influenced by PS only in males. However, maze exploration was not significantly affected by early experience in either sex. A two-way ANOVA (Experience X Sex) of the time spent in the closed arms revealed no main effect of experience [F (1, 51) = 2.41, $p = 0.127$] with marginal effect of sex [F (1, 51) = 3.17, $p = 0.081$] and an interaction between the two [F (1, 51) = 3.84, $p = 0.055$]. Pairwise comparisons revealed that PS males spent more time in the closed arms of the maze compared to sex-matched controls ($p = 0.018$) as well as PS females ($p = 0.018$). However, early experience did not influence anxiety-like behavior in females. Furthermore, there was no significant sex difference within the control group.

The frequency of entry in the closed arms when subjected to a two-way ANOVA (Experience X Sex) revealed a main effect of experience [F (1, 50) = 4.82, $p = 0.033$] with no effect of sex [F (1, 50) = 2.34, $p = 0.133$] nor an interaction between the two [F (1, 50) = 0.19, $p = 0.667$]. PS females appeared to enter the closed arms less frequently, although pairwise comparison revealed only a trend ($p = 0.064$). Males were not affected by early experience. Similarly, there was no significant sex difference within the control or PS group (see Figure 4.1).

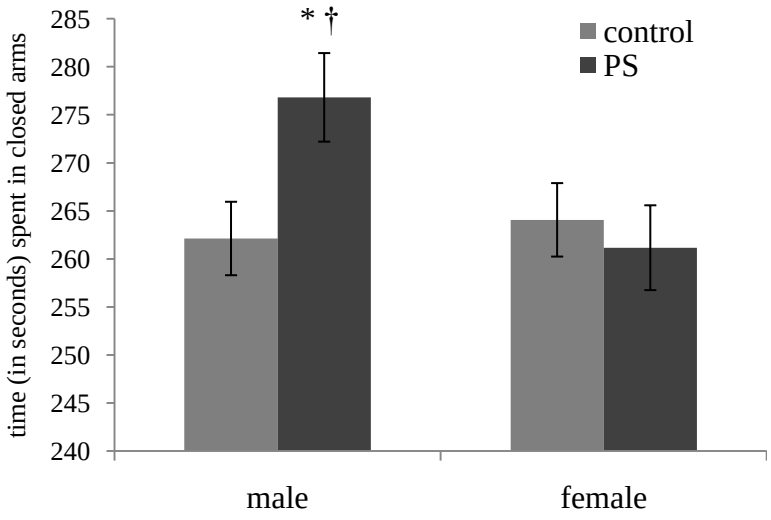

Figure 4.1. Mean (± SEM) of total time spent in closed arms of EPM, an index of anxiety-like behavior. PS males spent more time in closed arms of EPM compared to sex-matched controls (*, $p = 0.018$) and PS females (†, ps = 0.018).

4.4.1.3. Novel object recognition

The cognitive performance, tested as the object temporal order memory, was not influenced by PS experience. The ratio of time exploring old vs. recent familiar object in the third test trial of the NOR task, an index of object temporal order memory, when subjected to a two-way ANOVA (Experience X Sex) revealed no main effect of experience [F (1, 47) = 0.26, $p = 0.874$], sex [F (1, 47) = 1.34, $p = 0.253$] nor an interaction between the two [F (1, 47) <0.001, $p = 0.993$].

4.4.1.4. Play fighting

When subjected to a two-way ANOVA (Experience X Sex), the frequency of playful attacks revealed no main effect of experience [F (1, 46) = 0.001, $p = 0.987$], sex [F (1, 46) = 1.05, $p = 0.310$] nor an interaction between the two [F (1, 46) = 1.79, p =

0.187]. Pairwise comparison revealed that PS did not influence the frequency of playful attacks in male and female rats compared to sex-matched controls. There was a sex difference in the frequency of playful attacks in controls where males compared to experience-matched females showed an increase in the frequency of playful attacks (p = 0.05). Moreover, there was no sex difference within PS group (see Table 4.1).

When subjected to a two-way ANOVA (Experience X Sex), the probability of complete rotation defense revealed no main effect of experience [F (1, 46) = 2.38, p = 0.130], sex [F (1, 46) = 0.61, p = 0.436] nor an interaction between the two [F (1, 46) = 0.09, p = 0.763]. However, the probability of partial rotation defense when subjected to a two-way ANOVA (Experience X Sex) revealed a main effect of experience [F (1, 46) = 4.45, p = 0.040] with no main effect of sex [F (1, 46) = 1.65, p = 0.205] nor an interaction between the two [F (1, 46) = 1.71, p = 0.198]. Pairwise comparison revealed that PS males responded more with a partial rotation defense to an attack compared to sex-matched controls (p = 0.016). Partial rotation defense in females however, was not modulated by PS experience. Furthermore, there was no sex difference with the control or PS groups (see Table 4.1).

The probability of evasion in response to an attack when subjected to a two-way ANOVA (Experience X Sex) revealed a main effect of experience [F (1, 46) = 5.52, p = 0.023] and a marginal effect of sex [F (1, 46) = 3.19, p = 0.080] with no interaction between the two [F (1, 46) = 1.77, p = 0.284]. Pairwise comparison revealed that PS experience in females compared to sex-matched controls resulted in enhanced probability of evasion (p = 0.023) without affecting males. Females compared to males in the PS

group appeared to have increased number of evasions however, the result was not significant ($p = 0.08$) (see Table 4.1).

Table 4.1

Mean (±SEM) of playful attacks, probability of complete rotation defense and evasion

	Male		Female	
	Control	PS	Control	PS
Attacks	44 ± 2.83	39 ± 3.58	36 ± 2.83†	40 ± 4.01
Complete rotation	0.52 ± 0.03	0.45 ± 0.04	0.54 ± 0.03	0.49 ± 0.04
Partial rotation	0.39 ± 0.02	0.49 ± 0.03*	0.39 ± 0.02	0.41 ± 0.03
Evasion	0.022 ± 0.007	0.032 ± 0.009	0.028 ± 0.007	0.055 ± 0.010*

Play fighting behavior was scored as the total number of attacks and either facing an attack as complete rotation defense or evasion. PS did not influence the frequency of playful attacks and the probability of complete rotation defense in males and females. There was a sex difference where control males attacked more compared to experience-matched females (†, $p = 0.05$). However, PS abolished the sex difference in the number of attacks. The probability of partial rotation in males (*, $p = 0.016$) and evasion in females (*, $p = 0.023$) response to an attack was increased in PS rats.

4.4.2. Amphetamine sensitization

4.4.2.1. Acute administration

PS did not influence locomotor activity in response to an acute dose of AMPH (i.e. first day of drug administration period). A two-way ANOVA (Experience X Drug) of the activity recorded on the first day of 14-day drug administration period in male groups revealed a main effect of drug (AMPH or saline) [F (1, 24) = 90.54, $p < 0.001$]

and no main effect of experience (PS or control) [F (1, 24) = 0.48, p = 0.495], nor an interaction between the two [F (1, 24) = 0.013, p = 0.910]. AMPH compared to saline administration in rats resulted in enhanced activity in males regardless of early experience (see Table 4.2A for the activity on the 'First day').

Similarly, a two-way ANOVA of the first day activity in female groups showed a main effect of drug [F (1, 24) =161.16, p < 0.001] with no main effect of experience [F (1, 24) = 0.04, p = 0.841] nor an interaction between the two [F (1, 24) = 0.27, p = 0.610]. Like males, AMPH compared to saline administration in females resulted in increased locomotor activity (see Table 4.2 B for the activity on the 'First day').

4.4.2.2. Development of sensitization

Repeated AMPH administration in rats resulted in a gradual increase in locomotor activity (i.e. behavioral sensitization). The activity, recorded consecutively over a 14-day period, was analyzed to determine the development of behavioral sensitization. Additionally, the last day activity was also compared to the first day of AMPH administration period to confirm the development of sensitization.

The activity recorded on the first and the last day when compared in male groups revealed a main effect of drug [F (1, 24) = 379.65, p < 0.001]. AMPH administration resulted in enhanced the activity on the last day compared to the first day (see Table 4.2A), showing behavioral sensitization as a result of AMPH compared to saline administration. In contrast, saline administration, although not significant, resulted in decreased activity on the last day compared to the first day (see Table 4.2A).

Mixed ANOVA of the activity recorded over 14-day drug administration period in males, with experience (PS or control) and drug (AMPH or saline) as independent factors and days as repeated measure factor, showed a main effect of drug [$F(1, 24)= 525.90$, $p < 0.001$] and no main effect of experience [$F(1, 24)= 2.90$, $p = 0.102$] nor an interaction between the two [$F(1, 24) = 0.93$, $p = 0.344$]. AMPH- compared to saline-treated rats exhibited augmented locomotor activity regardless of early experience (see Figure 4.2A).

In female rats, comparison of the first and the last day activity revealed a main effect of drug [$F(1, 24) = 536.54$, $p < 0.001$]. AMPH-treated females exhibited augmented activity (i.e. behavioral sensitization) on the last day compared to the first day (see Table 4.2B). Saline administration, on the other hand, although not significant resulted in decreased activity on the last compared to the first day (see Table 4.2A).

When subjected to mixed ANOVA with experience (PS or control) and drug (AMPH or saline) as independent factors and days as repeated measure factor, the 14-day AMPH-induced activity in females revealed a main effect of drug [$F(1, 24)= 434.09$, $p < 0.001$] with no main effect of experience [$F(1, 24)=0.90$, $p = 0.767$] nor an interaction between the two [$F(1, 24)= 1.58$, $p = 0.227$]. Repeated AMPH compared to saline administration resulted in behavioral sensitization. However, PS compared to control did not influence AMPH-induced behavioral sensitization in female rats (see Figure 4.2B).

Table 4.2

Mean (±SEM) locomotor activity on the first and last day of 14-day AMPH/saline administration period

	Control		PS	
	First day (acute)	Last day	First day (acute)	Last day
A. Male				
Saline	8425 ± 2493	6452 ± 3678	6252 ± 2879	5070 ± 4247
AMPH	33740 ± 2493	89596 ± 4134*	32180 ± 2879	71619 ± 4247*
B. Female				
Saline	12202 ± 3434	11667 ± 5729	9535 ± 3965	9544 ± 6616
AMPH	57376 ± 3434	93578 ± 5729*	58539 ± 3965	98894 ± 6616*

PS in males (A) and females (B) did not influence the locomotor activity response to acute AMPH administration (i.e. first day). AMPH treated male (A) and female groups (B) developed behavioral sensitization (last day vs. first day) regardless of experience (i.e. control and PS) by exhibiting enhanced activity on the last compared to the first day (*, both *p*s < 0.001). The saline treated groups although not significant showed diminished activity on the last day compared to the first day in both male (A) and female group (B).

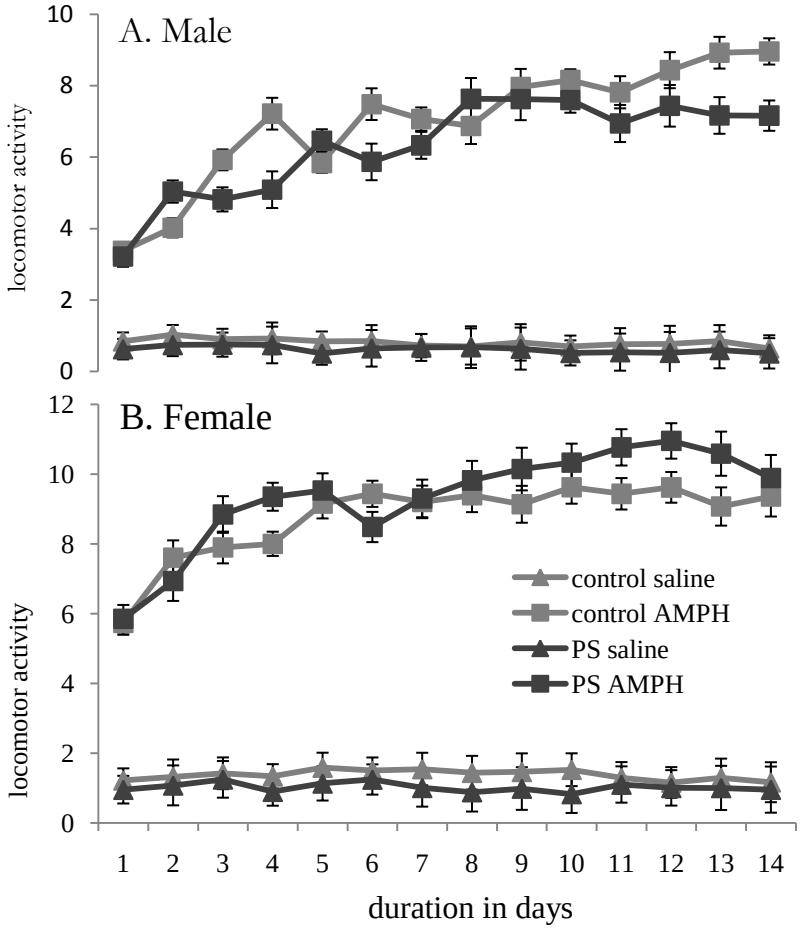

Figure 4.2. Mean (± SEM) locomotor activity (beam crossing x 10^3)recorded for 90 minutes after saline or AMPH (1 mg/kg) administration over 14-day period. PS did not influence AMPH-induced behavioral sensitization in males (A) and in females (B).

4.4.2.3. Persistence of sensitization

Behavioral sensitization persisted in AMPH- compared to saline-treated male and female rats. The persistence of drug-induced behavioral sensitization was determined, after 2-week withdrawal period, by giving an AMPH challenge injection to both prior saline- and prior AMPH-treated rats. The activity recorded on the challenge day in male groups when subjected to a two-way ANOVA (Experience X Prior drug treatment)

revealed a main effect of prior drug treatment [$F(1, 24) = 51.73$, $p < 0.001$], and no main effect of experience [$F(1, 24) = 2.14$, $p = 0.157$] nor an interaction between the two [$F(1, 24) = 0.004$, $p = 0.950$]. Prior AMPH- compared to prior saline-treated rats exhibited enhanced activity, demonstrating persistence of sensitization in prior AMPH-treated rats (see Table 4.3A).

When subjected to a two-way ANOVA (Experience X Prior drug treatment), the challenge day activity in females revealed a main effect of prior drug treatment [F (1, 24) = 17.57, $p < 0.001$], no main effect of experience [F (1, 24) = 1.08, $p = 0.310$] nor an interaction between the two [F (1, 24) = 0.08, $p = 0.775$]. Similar to males, prior AMPH-treated females were more sensitized compared to prior saline-treated rats (see Table 4.3B).

Table 4.3

Mean (±SEM) locomotor activity of male (A) and female groups (B) in response to AMPH (1mg/kg) challenge injection

	Control	PS
A. Male		
prior saline-treated	37618 ± 4674	30555 ± 5397
prior AMPH-treated	74242 ± 4674*	66545 ± 5397*
B. Female		
prior saline-treated	62676 ± 6618	68027 ± 7642
prior AMPH-treated	90576 ± 6618*	100064 ± 7642*

Prior saline- and AMPH treated rats were given AMPH challenge to see persistence of behavioral sensitization after a 14-day withdrawal period. Prior AMPH- compared to saline-treated exhibited enhanced activity in PS and control groups in males (A) (*, $p < 0.001$) and females (B) (*, $p = 0.007$), showing persistence of behavioral sensitization.

4.4.2.4. AMPH-dependent sex differences

To determine sex differences to an acute AMPH administration and in the development and persistence of behavioral sensitization, sex was used as factor in addition to experience and drug. A three-way ANOVA (Sex X Experience X Drug) of the activity on the first day of drug administration period revealed a main effect of sex [F (1, 48) = 38.74, $p < 0.001$] where AMPH-treated females in both control and PS groups were more active compared to the drug and experience-matched males (both ps < 0.001) (see Table 3.2). Similarly, a mixed ANOVA (Sex X Experience X Drug X Day) of the 14-day activity data showed a main effect of sex [F (1, 48) = 39.61, $p < 0.001$] where AMPH-treated control and PS females compared to the drug and experience-matched males showed enhanced activity (both ps < 0.001) (see Figure 4.2). Similar results were obtained when the challenge day activity was subjected to a three-way ANOVA (Sex X Experience X Drug). There was a main effect of sex [F (1, 48) = 41.23, $p < 0.001$] where both prior saline- and AMPH-treated females in both control and PS groups compared to the drug- and experience-matched males showed enhanced activity in response to the AMPH challenge (all ps < 0.05) (see Table 3).

4.4.3. Anatomy

4.4.3.1. Brain weight

The PS experience resulted in lighter brain weight regardless of drug exposure but only in males. When subjected to a two-way ANOVA (Experience X Drug), brain weights in males revealed a main effect of experience [F (1, 24) = 10.23, $p = 0.004$], no main effect of drug [F (1, 24) = 0.10, $p = 0.756$], nor an interaction between the two [F

(1, 24) = 0.04, p = 0.838]. PS males compared to controls resulted in significantly lighter brains. However, neither AMPH nor saline administration affected brain weights in PS or control group (see Table 4.4A). Experience or drug administration in females, in contrast to males, did not affect brain weights. A two-way ANOVA (Experience X Drug) of the brain weights revealed no main effect of experience [F (1, 24) = 1.29, p = 0.268], drug [F (1, 24) =0.84, p = 0.368], nor an interaction between the two [F (1, 24) = 0.03, p = 0.869] (see Table 4.4B).

Table 4.4

Mean (±SEM) brain weight (in grams)

	Control	PS
A. Male		
Saline	2.11 ± 0.03	2.01 ± 0.03*
AMPH	2.11 ± 0.03	2.02 ± 0.03*
B. Female		
Saline	1.93 ± 0.03	1.90 ± 0.03
AMPH	1.96 ± 0.03	1.93 ± 0.03

AMPH administration in PS in males (A) resulted in lighter brains in saline- (*, p = 0.024) and AMPH-treated rats (*, p = 0.045) without affecting females (B). AMPH administration did not affect brain weights in both male and female groups.

4.4.3.2. Spine density

The spine density, calculated from 5 dendritic segments drawn per hemisphere, was analyzed using hemisphere as a factor, in addition to experience and drug. However, the data were collapsed in the absence of hemispheric differences.

4.4.3.2.1. Nucleus accumbens

The PS experience resulted in an increase in the spine density on the medium spiny neurons of the NAc shell region. In addition, repeated AMPH administration resulted in increased spine density in the NAc of control rats, which was prevented by PS experience.

When subjected to a two-way ANOVA (Experience X Drug), the spine density on the medium spiny neurons in the shell region of the NAc in males revealed a main effect of experience [F (1, 38) = 26.63, $p < 0.001$] and drug [F (1, 38) = 5.60, $p = 0.023$] with no interaction between the two [F (1, 38) = 1.10, $p = 0.301$]. The PS experience in males compared to sex-matched controls resulted in increased spine density. Moreover, AMPH compared to saline administration increased the spine density in the NAc in control males ($p = 0.013$). However, PS prevented the increased spine density as no difference was observed between saline- and AMPH-treated males (see Figure 4.3A).

Similarly in females, the spine density when subjected to a two-way ANOVA (Experience X Drug) revealed a main effect of experience [F (1, 40) = 25.63, $p < 0.001$] and drug [F (1, 40) = 3.96, $p = 0.05$] with no interaction between the two [F (1, 40) = 2.37, $p = 0.132$]. PS in females compared to sex-matched controls increased the spine density in the NAc. In addition, AMPH compared to saline administration resulted in

increased spine density in control females ($p = 0.009$). However, the drug-induced increase in the spine density was prevented by PS as there was no difference between saline- and AMPH-administered females (see Figure 4.3B).

4.4.3.2.2. Medial prefrontal cortex

The spine density on the apical and the basilar dendrites of the pyramidal neurons in the Cg3 region of the mPFC was significantly reduced as a result of PS experience. Furthermore, PS prevented the drug-induced increase in the spine density observed in control rats.

The spine density on apical dendrites in the Cg3 in males when subjected to a two-way ANOVA (Experience X Drug) revealed a main effect experience [F (1, 50) = 11.23, $p = 0.002$], a marginal effect of drug [F (1, 50) = 3.29, $p = 0.076$] with no interaction between the two [F (1, 50) = 0.683, $p = 0.413$]. Pairwise comparison revealed that PS in males compared to sex-matched controls decreased the spine density in AMPH-treated group ($p = 0.004$). The saline-treated rats appeared to have decreased spine density however, pairwise comparison showed only a trend ($p = 0.088$). Moreover, control males showed AMPH-induced increase in the spine density on the Cg3 apical dendrites ($p = 0.043$). However, the drug-induced increase in the spine density was prevented by PS experience in males (see Figure 4.3A).

The spine density on the basilar dendrites of the Cg3 in males when subjected to a two-way ANOVA (Experience X Drug) revealed a main effect of experience [F (1, 44) = 21.55, $p < 0.001$] and drug [F (1, 44) = 4.58, $p = 0.038$] with no interaction between the two [F (1, 44) = 1.70, $p = 0.199$]. PS in males compared to sex-matched controls

decreased the spine density. Moreover, AMPH administration in controls increased the spine density ($p = 0.014$). However, PS prevented the drug-induced increase as there was no significant difference between saline- and AMPH-treated males (Figure 4.3A).

When subjected to a two-way ANOVA (Experience X Drug), the spine density on Cg3 apical dendrites in females revealed a main effect of experience [F (1, 42) = 32.39, $p < 0.001$] and no main effect of drug [F (1, 42) = 0.24, $p = 0.622$] with an interaction between the two [F (1, 42) = 8.83, $p = 0.005$]. PS in females compared to sex-matched controls decreased the spine density. Furthermore, AMPH compared to saline administration resulted in increased spine density on the apical dendrites of the Cg 3 region of the control females ($p = 0.016$). PS experience however, interfered with the drug-induced increase in the spine density observed in control females (see Figure 4.3B).

When subjected to a two-way ANOVA (Experience X Drug), the Cg3 basilar spine density in females revealed a main effect of experience [F (1, 44) = 22.70, $p < 0.001$] and no main effect of drug [F (1, 44) = 0.512, $p = 0.478$] with no interaction between the two [F (1, 44) = 2.13, $p = 0.152$]. Similar to males, PS in females compared to sex-matched controls decreased the spine density. In addition, AMPH compared to saline administration did not influence the spine density on the basilar dendrites in the Cg3 region in both the control and PS females (see Figure 4.3B).

4.4.3.2.3. Orbital frontal cortex

PS experience did not influence the spine density on the basilar dendrites of the pyramidal neurons in the AID region of the OFC. However, AMPH-induced reduction in the spine density observed in control rats was blocked by PS.

The spine density on the basilar dendrites of the AID in males when subjected to a two-way ANOVA (Experience X Drug) revealed no main effect of experience [F (1, 46) = 0.012, p = 0.914], drug [F (1, 46) = 1.79, p = 0.187] nor an interaction between the two [F (1, 46) = 3.21, p = 0.080]. Spine density on the basilar dendrites of the AID region was not influenced by PS experience in both saline- and AMPH-treated males. Furthermore, AMPH compared to saline administration decreased the spine density in the AID region in control males (p = 0.023). However, PS experience blocked the drug-induced decrease in the spine density as there was no difference between saline- and AMPH-treated rats (see Figure 4.3A).

When subjected to a two-way ANOVA (Experience X Drug), the spine density on the basilar dendrites in the AID in females revealed no main effect of experience [F (1, 44) = 0.09, p = 0.764] and a main effect of drug [F (1, 44) = 4.38, p = 0.042] with no interaction between the two [F (1, 44) = 2.13, p = 0.151]. Similar to males, PS experience in females compared to sex-matched controls did not influence the spine density in the AID regions of both saline- and AMPH-treated rats. In addition, pairwise comparison revealed that AMPH compared to saline administration resulted in decreased spine density in the AID region of control females (p = 0.012) however, PS experience prevented the drug-induced decrease in the spine density (see Figure 4.3B).

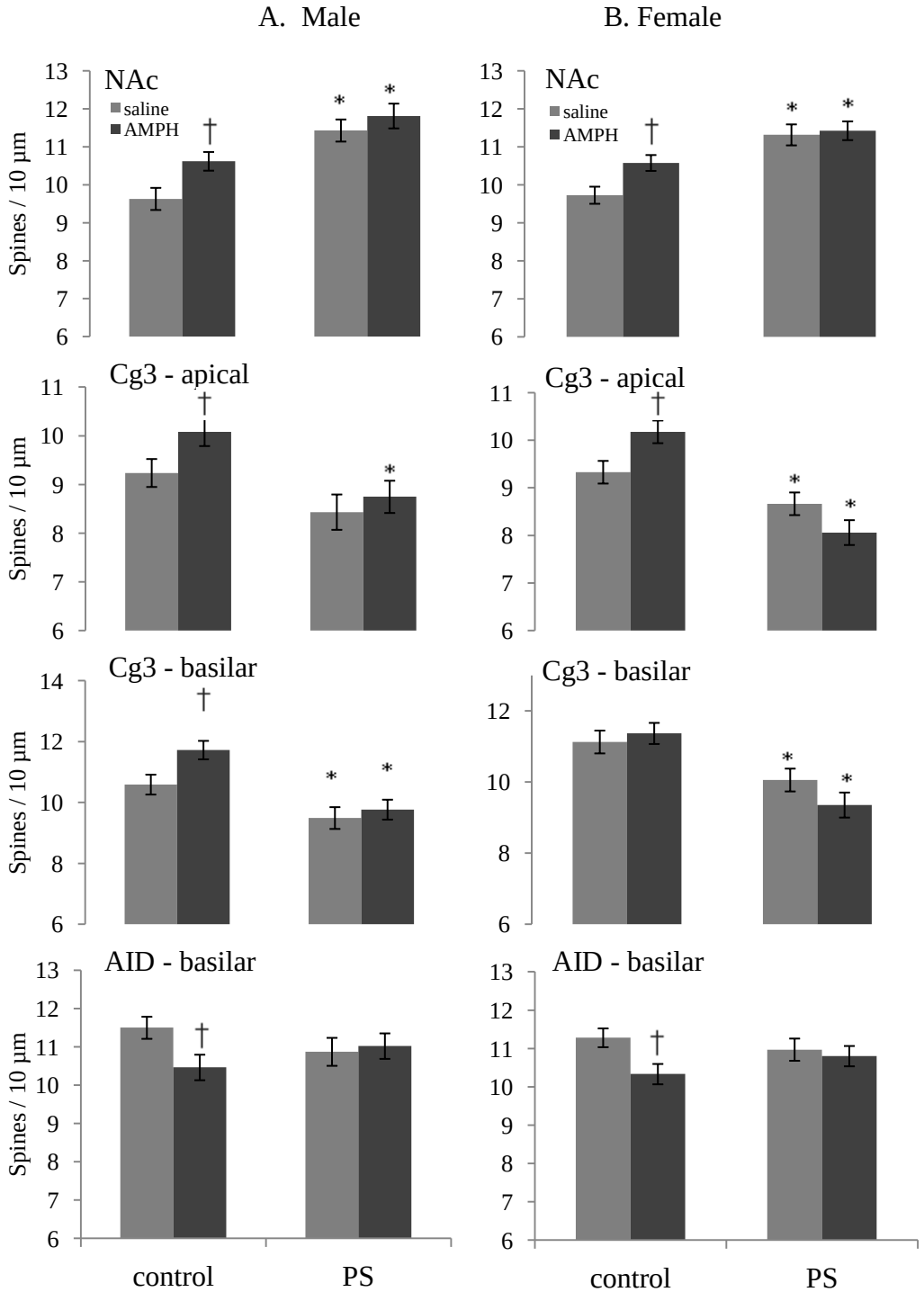

Figure 4.3. Mean (± SEM) of the spine density per 10 µm on the distal dendrites in the shell of the NAc, Cg3 of the mPFC, and AID of the OFC. PS increased the spine density in the NAc and decreased it in Cg3 (*, all *p*s =< 0.05). In addition, repeated AMPH administration increased the spine density in the NAc, and Cg3 whereas, decreased the same in the AID (†, all *p*s =< 0.05). However, PS experience followed by drug exposure prevented the drug-induced alteration in the spine density in the NAc and subregions of the PFC.

4.5. Discussion

The present study investigated the effect of prenatal stress on juvenile behavior, adult AMPH sensitization, and neuronal spine density in Long Evans rats. The findings showed that PS resulted in enhanced anxiety-like behavior in males. Furthermore, PS modulated the social behavior without affecting exploration in rats. In addition, PS presumably reorganized the brain circuits through alterations in the spine density of the NAc and the PFC, key brain regions implicated in drug addiction. However, PS failed to influence AMPH-induced behavioral sensitization.

The influence of PS experience on brain and behavior in rats has been studied by employing a number of stress paradigms with variable frequency and duration. For example, previous studies employed restraint stress that lasted for as short as 20 minutes (Gomez-Gonzalez & Escobar, 2010) to as long as six hours (Chung et al., 2005) with a variable frequency of one day (Cannizzaro et al., 2006) to throughout gestation (Weinstock, Matlina, Maor, Rosen, & McEwen, 1992). In addition to procedural differences, the effect of PS on behavioral modulation, and neuronal anatomy and physiology depends on a variety of other factors, such as stage of brain development, sex, age of animal tested, test protocol, etc. (reviewed by Weinstock, 2008). The review of literature pointed to inconsistencies in the findings but the overall emerging picture indicated that severe prenatal stress has detrimental effects (reviewed by Kofman, 2002) whereas, mild stress has a beneficial role for the brain and behavior (reviewed by Lyons, Parker, & Schatzberg, 2010). We employed an elevated platform paradigm as it leads to psychological stress compared to restraint stress, which also involves physical discomfort. Exposure to elevated platform stress for 30 minutes in rats has been shown to

elevate blood corticosterone level immediately after the procedure (Wong, et al., 2007). Our initial plan was to expose pregnant dams to stress for 30 minutes twice a day during gestational day 12 to 21. However, the stress procedure for unknown and non investigated reasons resulted in spontaneous abortion in rats. We believed that the severity of stress might be playing a role in inducing abortions and, therefore, decided to reduce the frequency and duration of stress to 10 minutes twice a day from gestation day 12 to 16. The dams were clearly affected by the procedure, for example, there was about a 5% body weight loss at the end of the 5-day stress duration. A reduction instead of gain in weight during the late stages of pregnancy clearly shows the effect of stress procedure. In addition, hair loss and irritability was also observed in rats exposed to stress.

4.5.1. Behavior

Male and female juvenile rats, exposed to 'mild' prenatal stress, were given behavioral tasks to investigate the effect of PS on the exploratory, emotional, cognitive, and social behaviors. The tasks included open field locomotion, elevated plus maze, novel object recognition, and play fighting behavior.

Exploratory behavior, tested as open field locomotion, was not influenced by PS in either male or female rats (Lee, Brady, Shapiro, Dorsa, & Koenig, 2007). In contrast, Vallee, et al. (1997) reported PS-induced augmented open field locomotion for the first five minutes without significant effect on the total activity for 15 minutes. The discrepancy in exploratory behavior in both studies could be related to age of the rats tested (juvenile vs. adults), in addition to other procedural differences (e.g. restraint stress for 45 minutes). Prenatally stressed males exhibited enhanced anxiety-like behavior by

spending more time in the closed arms of EPM (Vallee, et al., 1997). However, females were not substantially influenced by PS which was consistent with the findings of Zargon and Weinstock (2006) although they also reported no effect of restraint stress on anxiety-like behavior in males.

The cognitive performance (object temporal order memory) revealed no significant effect by PS experience (Vallee, et al., 1997). Previous studies related to the effect of PS on cognitive performance reported mixed findings (reviewed by Weinstock, 2008). For example, 45-minute daily stress during the last week of gestation resulted in impaired spatial memory in male rats (Lemaire, Koehl, Le Moal, & Abrous, 2000) whereas, others reported no impairment (Vallee, et al., 1997).

Similar to other mammals, play fighting is a form of social interaction in rodents. Although play can be observed during the nursing period, rats engage in play behavior more frequently during the juvenile period (Thor & Holloway, 1984). Play fighting behavior in rats is comprised of a series of attacks, generally targeting the nape. In response to an attack, the play mate either faces (e.g., complete rotation defense) or evades the attack, instead of facing (Pellis & Pellis, 1990). The characteristics of play behavior are different in juveniles compared to adults in addition to sex difference (Pellis & Pellis, 1990). Our findings revealed that play behavior was sexually dimorphic with enhanced frequency of playful attacks in males (Pellis & Pellis, 1990). PS in males however, abolished the sex difference resulting in female-like play behavior (Ward & Stehm, 1991). The strategy to face a playful attack (i.e. complete rotation defense) was not altered in either sex. However, PS rats in response to an attack increased the probability of partial rotation defense in males and evasion in females. Enhanced partial

rotation defense usually reflects a mild aggressive behavior with less interest in social interaction (Pellis & Pellis, 1990). The experience-dependent modulation of social behavior could be attributed to underlying altered neurocircuits. For example, PS reorganized the mPFC by decreasing the spine density described below in the anatomical findings. Similarly, PFC exhibited play-dependent structural reorganization in rats (Bell, et al., 2010). In addition, PS experience differentially influenced the play behavior in male and female rats, that is, increased partial rotation vs. evasion, respectively. Previous studies also reported sex differences in juvenile and adult behavior in rats exposed to PS (reviewed by Weinstock, 2007). The sex differences observed in play behavior could be attributed to altered neurochemistry of the associated brain regions in addition to the difference in the levels of gonadal hormones. For example, Dalla et al. (2008) reported sex differences in dopaminergic activity of the PFC and hippocampus in response to two different stress paradigms.

Play, a rewarding behavior may be dependent on the levels of dopamine in the PFC, a region associated with the processing of rewards (Li, Huang, Sui, Han, & Chung, 2010). In addition, the excitation of metabotropic dopamine receptors alters the spine density through the activation of several cell signaling pathways (reviewed by Dietz, et al., 2009). Interestingly, the manipulations of play behavior in rats structurally alter the mPFC and the OFC subregions of the PFC (Bell, et al., 2010). The PS experience might have reorganized the PFC (see the Anatomy section below) in such a way that might have influenced the social behavior (but see the following Amphetamine sensitization section).

4.5.2. Amphetamine sensitization

Repeated AMPH administration results in behavioral sensitization, an increase in drug response with repeated exposure, expressed as a gradual increase in locomotor activity in rats (Doremus-Fitzwater & Spear, 2010; Robinson & Becker, 1982). In addition, sensitization persists even after months of drug-free period (Kolb, Gorny, et al., 2003). The dose, duration, and route of the AMPH were chosen based on previous reports that indicated the development and persistence of behavioral sensitization with the similar dose and route (Mattson et al., 2007; Singer, et al., 2009). We investigated the influence of PS experience on AMPH-induced behavioral sensitization in adult male and female rats. AMPH administration consecutively for 14-day period, resulted in the development and persistence of behavioral sensitization in both PS and control groups. However, we failed to find the influence of PS on acute locomotor response (Henry et al., 1995) as well as chronic AMPH-induced sensitization in males (Thomas, Hu, Lee, Bhatnagar, & Becker, 2009) or females. Our finding for the female group was not in accordance with Thomas, et al. (2009) who reported augmented cocaine-induced behavioral sensitization in PS females. The discrepancy between the results for the females could be attributed to the escalating doses used in the latter study in addition to drug (AMPH vs. cocaine) and test paradigm. Similarly, Van Waes et al. (Van Waes et al., 2010) also reported that PS did not affect spontaneous preference and motivation for alcohol consumption in adolescent and adult male rats. The limited literature available related to the influence of PS on psychostimulant administration in rodents indicates that PS increased AMPH-induced behavioral sensitization (Deminiere, et al., 1992; Henry, et al., 1995), as well as cocaine and AMPH self-administration (Deminiere, et al., 1992;

Kippin, Szumlinski, Kapasova, Rezner, & See, 2008). The PS-dependent augmented drug response reported, unlike our study, might be due to severe restraint stress paradigm employed, which lasted for 45 minutes, three times a day throughout the last week of gestation. Besides the difference in the stress paradigm several other factors might have played a contributory role in drug-induced behavioral sensitization reported in the studies mentioned above. For example, the dose of the drug administered could influence the reinforcing effects of addictive drugs. Low compared to a higher drug dose in PS rats, for instance, did not influence drug taking behavior in self-administration paradigm (Thomas, et al., 2009). The role of stress during adulthood has an established role in the relapse to drugs of abuse in humans and experimental animals (reviewed by Shalev, Erb, & Shaham, 2010; Stewart, 2000). However, for an effective clinical application, the long term influence of pre-clinical PS on drug-induced behavioral sensitization needs further investigation to rule out procedural differences reported in the literature.

4.5.3. Anatomy

The present study investigated the influence of exposure to PS and AMPH on the spine density, a measure of structural plasticity, in regions (i.e. the NAc and the PFC) known to be reorganized structurally in response to both experience and drug. Previously, we reported that exposure to either stimulant drugs (i.e. cocaine, amphetamine, and nicotine) or environmental enrichment altered spine density in various brain regions in rats (Kolb, Gorny, et al., 2003; Robinson & Kolb, 2004). However, when drug administration was followed by environmental enrichment, early drug exposure interfered with later experience, that is, enrichment-associated dendritic growth was not observed

(Kolb, Gorny, et al., 2003). We wondered if switching the order of drug and experience, especially during brain development, would interact to prevent drug-associated structural alteration in the NAc and the PFC regions. Our findings suggest that spine density was altered by both PS and AMPH administration in the NAc and the PFC. In addition, early experience prevented the drug-associated modification of spine density observed in the control rats.

4.5.3.1. Nucleus accumbens

PS increased the spine density in the NAc. The only study available, to the best of our knowledge, related to the influence of PS on the spine density in the NAc, in contrast to our findings, reported a decrease in the spine density in Sprague-Dawley male rats sacrificed at postnatal day 65 (Martínez-Téllez, Hernández-Torres, Gamboa, & Flores, 2009). However, the decrease in the spine density was not observed at postnatal day 35 (Martínez-Téllez, et al., 2009). In addition to other procedural differences, the severity of stress in terms of the duration, frequency, and paradigm, could be a possible factor in the reported discrepancy.

Consistent with previous reports, repeated AMPH administration resulted in increased spine density on the medium spiny neurons of the NAc in control rats (Robinson & Kolb, 2004). However, PS experience prevented the drug-induced increase in the spine density, even though the animals still showed behavioral sensitization. The anatomical results are in accordance with previous reports related to the interaction of psychostimulant exposure and experience (i.e. environmental enrichment) on structural alteration in the NAc. When amphetamine, cocaine, or nicotine administration was

followed by rearing experience in complex housing, prior drug exposure prevented experience-associated increase in the spine density in the NAc (Hamilton & Kolb, 2005; Kolb, Gorny, et al., 2003). This was not a simple ceiling effect on spine density, however, because giving additional drug could still increase spine density (Kolb, Gorny, et al., 2003).

4.5.3.2. Prefrontal cortex

The PFC subregions assessed for alteration in the spine density included Cg3 (layer III) region of mPFC and dorsal agranular insular cortex (AID, layer III) of the OFC described by Zilles (1985). The PS experience resulted in a significant reduction in the spine density on the apical and the basilar dendrites of the pyramidal neurons in the Cg3 region of the mPFC. The PS-dependent decreased spine density was observed in both male and female rats regardless of drug exposure. Similarly, previous studies reported significant spine density reduction in the mPFC as a result of stress exposure both during prenatal development (Murmu et al., 2006) and adulthood (Radley et al., 2008). In addition to spine density, previous studies also reported an overall stress-induced reduction in the length and dendritic complexity in the mPFC pyramidal neurons (Cook & Wellman, 2004; Liston, et al., 2006). In contrast to the mPFC, PS failed to influence the spine density in the AID region of the OFC, which is not in accordance with the findings reported by Murmu et al. (2006) who observed a decreased spine density in the OFC. However, the decreased spine density reported by Murmu et al. was observed in the ventral and lateral orbital cortex of the OFC without any distinction between the two regions compared to the AID region in our study.

Repeated AMPH administration increased the spine density on the apical dendrites of the Cg3 region in controls of both sexes and on the basilar dendrites in only male controls (Robinson & Kolb, 2004). However, the PS experience prevented the drug-induced increase in the spine density observed in control rats. Similarly, a previous study related to stress and drug interaction indicated that cocaine increased the levels of basic fibroblast growth factor (FGF-2), a protein associated with neuronal plasticity, in the PFC. However, prior stress interfered with the drug-induced modulation of FGF-2 expression in the PFC (Fumagalli, et al., 2008).

There are some paradoxical spine density findings in our study. Specifically, we observed a PS-dependent dissociation between the NAc and the PFC, such that, there was an increase in the NAc and a decrease in the spine density in the mPFC. In our experience, the spine changes in NAc and mPFC are always in the same direction (e.g., Robinson & Kolb, 2004). For example, spine density was consistently increased in response to psychostimulant administration and decreased after opiate exposure in both regions (Robinson & Kolb, 2004). It is quite possible that the mild PS stress, as opposed to psychostimulants or severe stress, might have modulated both the PFC and the NAc through activation of different systems (e.g., neurotransmission, gene expression or cell signaling pathways) that could have resulted in the spine density modulation in opposite directions. There is no published report in our knowledge that studied the effect of mild PS on the spine density in the NAc, although severe PS resulted in decreased spine density in the NAc (Martínez-Téllez, et al., 2009) and the PFC (Murmu, et al., 2006).

In summary, prenatal stress in rats produced anxiety-like behavior in males. Furthermore, social behavior was modulated by PS experience with resultant

feminization of play fighting in males. Exploration and cognitive performance were not affected. Repeated amphetamine administration resulted in the development of behavioral sensitization that persisted at least for two weeks. The degree of sensitization, however, was not influenced by early stress in male or female rats. In addition, prenatal stress altered the dendritic morphology through increased spine density in the nucleus accumbens and decreased in the medial prefrontal cortex without any alteration in the orbital frontal cortex. In contrast, repeated amphetamine administration increased the spine density in the nucleus accumbens and medial prefrontal cortex whereas a decrease was observed in the orbital frontal cortex in control rats. Prenatal stress interacted with the amphetamine exposure thus preventing the drug-induced spine density alterations observed in control rats.

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CHAPTER 5

Maternal separation altered behavior and neuronal spine density without influencing amphetamine sensitization*

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Abstract

We studied the long term influence of maternal separation (MS) on periadolescent behavior, adult amphetamine (AMPH) sensitization, and structural plasticity in the corticolimbic regions in rats. Male and female pups, separated daily for 3 hours from the dam during postnatal day 3-21, were tested for periadolescent exploratory, emotional, cognitive, and social behaviors. The development and persistence of drug-induced behavioral sensitization was tested by repeated AMPH administration and a challenge, respectively. The spine density was examined in the nucleus accumbens (NAc), the medial prefrontal cortex (mPFC), and the orbital frontal cortex (OFC) from Golgi-Cox stained neurons. The results showed that MS enhanced anxiety-like behavior in males. MS abolished the sex difference in playful attacks observed in controls with resultant feminization of male play behavior. Furthermore, the probability of complete rotation defense to face an attack was decreased in females. AMPH administration resulted in the development of behavioral sensitization that persisted at least for two weeks. Sensitization was not influenced by MS. MS increased the spine density in the NAc, the mPFC, and the OFC. Repeated AMPH administration increased the spine density in the NAc and the mPFC, and decreased it in the OFC. MS blocked the drug-induced alteration in these regions. In sum, MS during development influenced periadolescent behavior in males, and structurally reorganized cortical and subcortical brain regions without affecting AMPH-induced behavioral sensitization.

Key words: behavioral sensitization, addiction, amphetamine, plasticity, maternal separation stress, rough and tumble play

5.2. Introduction

Repeated psychostimulant (e.g., cocaine, amphetamine, and nicotine) administration results in the development of behavioral sensitization (Robinson & Becker, 1982) that persists beyond drug use for months in rodents and years in monkeys (Castner & Goldman-Rakic, 1999; Kolb, Gorny, et al., 2003). In addition to behavioral modulation, chronic drug use produces neuroadaptation in key brain regions (e.g., reward system) implicated in drug addiction (Kalivas & Volkow, 2005; Koob & Nestler, 1997; Robinson & Kolb, 2004). The neuroadaptation as a result of drug exposure, especially in the mesocorticolimbic regions, is believed to be responsible for the addictive behavior (Kalivas & Volkow, 2005). Indeed the study of drug-induced alteration at behavioral (Shuster, Yu, & Bates, 1977), epigenetics (Kumar et al., 2005), molecular (Brami-Cherrier et al., 2005), and morphological levels (Robinson & Kolb, 1997, 2004) generated a wealth of knowledge regarding brain-behavior relationships in drug abuse. However, despite endless efforts to reverse the neuroadaptation developed as a result of repeated drug administration, a very modest success has been achieved so far (Herin, Rush, & Grabowski, 2010; Schmidt & Pierce, 2010).

A majority of the human population consumes licit and illicit drugs for pleasure but very few of such recreational users become addicted to drugs. There is a growing consensus that not only drug-induced neuroadaptation but the vulnerability to abuse drugs plays a major role in the development and relapse to addiction (Morgan, et al., 2002). We recently reported that tactile stimulation (TS), a form of sensory stimulation, during early postnatal brain development in rats attenuated drug-induced behavioral sensitization (Muhammad, et al., 2011). The promising results for the TS during the

postnatal brain development emphasizes the importance of prior experience that can have a long-term programming effect in altering the response to subsequent experience. Similar to the beneficial effect of TS, we hypothesized that an adverse experience of postnatal stress (i.e. MS) would enhance drug-induced behavioral sensitization in adult rats and modulate synaptic changes in the brain.

Maternal separation in lab animals is a well-studied model of an early adverse experience that has been associated with maladaptive behavior and altered brain morphology. For example, enhanced alcohol preference and intake has been reported in maternally-separated rats (Huot, et al., 2001). The altered response to drugs of abuse could be the result of MS-induced structural alterations in various brain regions. For instance, MS reorganized the dendritic spine density in the prefrontal cortex, a region implicated in drug addiction (Bock, Gruss, Becker, & Braun, 2005). Similarly, the negative influence of MS on brain and behavior has also been reported in both human and non-human primates (Chapman et al., 2004; Higley, Hasert, Suomi, & Linnoila, 1991).

Previous reports have indicated that repeated psychostimulant administration results in behavioral sensitization that is correlated with an alteration in neuronal morphology in regions implicated in drug addiction (i.e., NAc and PFC) (Kiraly et al., 2010; Robinson & Kolb, 1997, 2004). Similarly, experiences (e.g., environmental enrichment) during development also produce enduring morphological alteration in different brain regions (Leggio et al., 2005). However, when AMPH (or other psychostimulant) administration is followed by environmental enrichment experience, the prior psychostimulant exposure blocks the effect of later experience (Hamilton & Kolb,

2005; Kolb, Gorny, et al., 2003). The current study examined the influence of MS on periadolescent behavior, AMPH-induced behavioral sensitization in adulthood, and the effects of these treatments on neuronal spine density in the subregions of the PFC and the NAc.

5.3. Materials and methods

5.3.1. Animals

Pup litters of Long-Evans females were randomly selected for MS or control group. The rats were housed in the breeding colony with their respective dams at the Centre for Behavioural Neuroscience, University of Lethbridge, Alberta, Canada. After weaning, pups from each group were randomly selected with not more than two pups of each sex from the same litter. The rats were housed in standard shoe-box cages with the same sex in a group of two in a temperature- and humidity-controlled room. Standard laboratory rat food and water were provided *ad lib*. The rats were left undisturbed until the commencement of behavioral tests and AMPH administration.

5.3.2. Maternal separation

The MS procedure, carried out between postnatal (P) 3-21, was conducted following the protocol described earlier (Plotsky & Meaney, 1993). Briefly, the rat pups were transported in a box with new bedding to a separate room and placed on a warming pad with a temperature of ~ 34˚C. The rat pups were separated daily for 3 hours, approximately the same time of the day. The experimenter remained with the pups during

the separation period to ensure the pups were comfortable and the temperature was properly maintained. The pups were returned to their home cage after carrying out the MS procedure. The control pups, which were not given MS, were also used in another experiment (Muhammad, et al., 2011).

5.3.3. Behavior

The effect of early MS on periadolescent behavior was investigated by testing the rats between P30-40 in a battery of behavioral tasks. The tests included open field locomotion, elevated plus maze (EPM), novel object recognition (NOR), and play fighting behavior.

5.3.3.1. Open field locomotion

Exploratory behavior of the rats was evaluated as open field locomotion, recorded for ten minutes using Accuscan activity monitoring Plexiglas boxes (L 42cm, W 42cm, H 30cm). The activity was recorded as the number of sensors beam breaks in the boxes attached to a computer. The horizontal beam breaks, used as an index of locomotor activity, were recorded on the computer with VersaMax^{TM} program and converted to spreadsheet using VersaDat^{TM} software (AccuScan Instruments, Inc., Columbus, OH).

5.3.3.2. Elevated plus maze

The EPM, a '+' shape maze with two closed and two open arms, was used to test anxiety-like behavior exhibited by the rats. The length of each arm of the maze measured

113 cm with a width of 10 cm while the maze was elevated 88 cm above the ground. Rats were placed in the centre of the maze facing a closed arm and were allowed to explore the maze for 5 minutes. Exploration behavior was videotaped with a camera installed in such a way to spot both open arms. The time spent in closed arms and number of entries in each open and closed were scored and analyzed to assess anxiety-like and exploratory behaviors, respectively.

5.3.3.3. Novel object recognition

Novel object recognition task was carried out to evaluate exploration of novel object as well as exploration of objects in temporal order. Rats were habituated to a Plexiglas box for 15-20 minutes for four days prior to the commencement of the testing sessions. The NOR task was comprised of three trials following the procedure with minor modification described elsewhere (Hannesson, et al., 2004; Mitchell & Laiacona, 1998). Briefly, rats were allowed to explore objects and taped with a video camera. Glass candle holders, with similar sizes but different shapes and colors, were used as objects. During the first training trial, a rat was exposed to two novel but similar objects for a 4-minute period. Followed by 60 minutes delay, the rat was again exposed to two new objects, again similar but different objects from the first trial. After an additional 60-minutes delay, the rat was exposed to one object each from the first and second trial, termed as ‘old’ and ‘recent’ familiar objects, respectively. The objects and testing area were cleaned with 30% alcohol between each trial for disinfection and odor removal. Rats were transported back to their home cages in the 60-minute delay between the trials.

Exploration of each novel object in the first two trials and 'old' and 'recent' familiar objects in the third trial was scored. The time spent with 'old' familiar object was calculated as the difference between times spent with 'old' and 'recent' familiar object divided by total time exploring both objects (Hannesson, et al., 2004). In addition the total time spent with both objects in each trial was also analyzed.

5.3.3.4. Play fighting

Periadolescent rats were allowed to play in a pair to assess the effect of MS on social behavior. MS and control rats were housed in a pair as playmates for a period of about two weeks. The playmates, as periadolescents, were habituated to a play box (50 cm X 50 cm X 50 cm) for about 30 minutes for 3 days. For play deprivation before testing, rats were housed individually in an isolation room for 24 hours at the end of habituation period. The play behavior was recorded for 10 minutes with a night shot camera. On testing day both playmates were color marked on the tail with two separate paints to make them identifiable in the video recording, which was filmed in the dark. Rats were transported to their home cage after the play session and pair-housed for the rest of the experiment. The video recording, analyzed frame by frame, was scored for attacks, and complete rotation defense or evasion generated in response to an attack (Pellis & Pellis, 1990). The defense was scored as 'complete rotation' when a rat had both fore- and hind limbs in the air while lying in a supine position. If the rat turned away from the attacker instead of facing an attack, it was scored as evasion. The probability of complete rotation or evasion was calculated as the number of complete rotation or

evasion divided by the total number of attacks carried out by the playmate (Pellis & Pellis, 1990).

5.3.4. Amphetamine sensitization

5.3.4.1. Amphetamine administration

To see the effects of postnatal MS on psychomotor stimulant behavioral sensitization, adult rats (P80) were administered with D-amphetamine sulfate (Sigma Aldrich, St. Louis, MO, USA) dissolved in sterile 0.9% saline. Locomotor activity, used as an index of behavioral sensitization (Wise & Bozarth, 1987), was recorded using Accuscan activity monitoring system, comprised of Plexiglas boxes (L 42cm, W 42cm, H 30cm) connected to a computer. The rats were habituated to the activity boxes for 30 minutes followed by AMPH (1 mg/kg body weight, i.p.) or 0.9% saline administration, both at a volume of 1 ml/kg. Rats were immediately placed in the activity boxes post injections and the activity was recorded for 90 minutes. The drug was administered once a day for 14 consecutive days, approximately the same time every day. Locomotor activity recorded on a computer with VersaMax^{TM} program was converted to spreadsheet using VersaDat^{TM} software (AccuScan Instruments, Inc., Columbus, OH). The rats were returned back to their home cages each day after the end of AMPH testing session. The development of sensitization was determined by analyzing activity recorded over 14-day period using mixed ANOVA with experience (MS or control), drug (AMPH or saline) as independent factors, and day (day 1-14 days) as a repeated measure factor, followed by Bonferroni's post hoc test for multiple comparisons.

5.3.4.2. Challenge

The rats were given a withdrawal period of 2 weeks after AMPH administration period, followed by a challenge with AMPH (1mg/kg, i.p.) given to both prior AMPH- and prior saline-treated rats. Locomotor activity was recorded in activity monitoring boxes similar to the development of AMPH sensitization procedure described above. All rats were challenged with AMPH to see the persistence of behavioral sensitization in prior AMPH-treated rats compared to prior saline-treated rats.

5.3.5. Anatomy

5.3.5.1. Perfusion and staining

Approximately 24 hours post AMPH challenge, the rats were given an overdose of sodium pentobarbital solution i.p. and perfused with 0.9% saline solution intracardially. The brains removed from the skull were trimmed by cutting the olfactory bulb, optic nerves and spinal cord. The brains were then weighed and preserved in Golgi-Cox solution for 14 days followed by transfer to 30% sucrose solution at least for 3 days. The brains were sliced at a thickness of 200 μm on a Vibratome and fixed on gelatinized slides. The slides mounted with brain sections were processed for Golgi-Cox staining, following the protocol described by Gibb and Kolb (1998).

5.3.5.2. Spine density

The distal dendrites of individual neurons were traced from Golgi-Cox stained brain sections using a camera lucida mounted on a microscope. Brain regions selected for neuron tracing were nucleus accumbens shell region (NAc), Cg3 (layer III) region of

anterior cingulate of the medial prefrontal cortex (mPFC), and dorsal agranular insular cortex (AID, layer III) of the orbital frontal cortex (OFC) described by Zilles (1985). The dendritic segments traced met the criteria of being thoroughly stained and without overlapping another dendrite or blood vessel (Figure 5. 1). Spine density was measured at 1000X and calculated by counting the number of spines on a length of distal dendrite that was at least 50 microns in length. The exact length of the dendrite segment was calculated and density expressed per 10 microns. Five segments, each from a different neuron, were drawn per hemisphere and a mean value calculated to use as the unit of measurement.

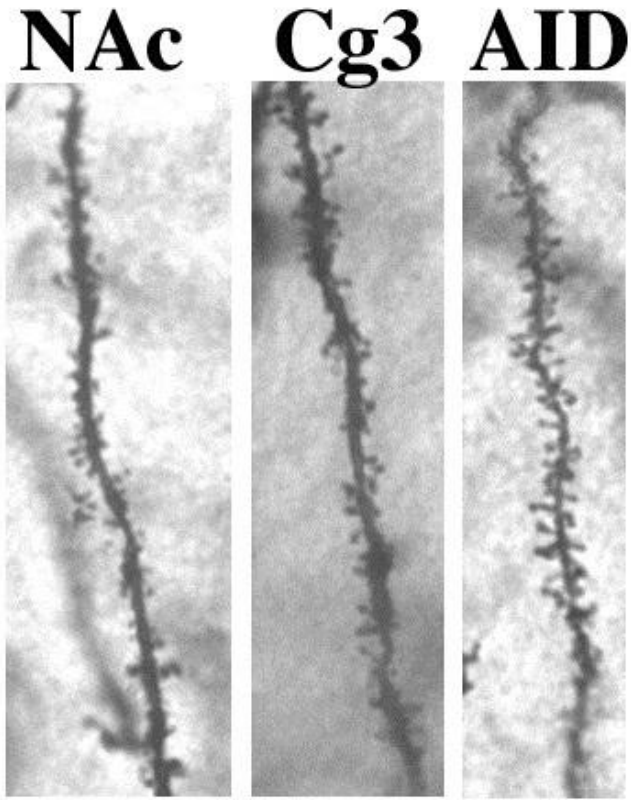

Figure 5.1. Photomicrographic (200X) examples of Golgi stained dendritic segments of neurons in Cg3, AID, and NAc.

5.4. Results

The behavioral data were analyzed using experience (MS or control) and sex as independent factors. However, both sexes were analyzed independently after the introduction of drug (AMPH or saline) as a factor either because of a sex-dependent difference (e.g., response to AMPH administration) or for the clarity of results description (e.g. in spine density). Furthermore, all ANOVAs were followed by Bonferroni's post hoc test for multiple comparisons.

5.4.1. Behavior

5.4.1.1. Open field locomotion

Open field locomotion was not influenced by early experience in either male or female rats. The horizontal activity in an open field, used as an index of exploratory behavior, when subjected to a two-way ANOVA with experience (MS or control) and sex as independent factors revealed no main effect of experience [F (1, 52) = 0.70, p = 0.41], sex [F (1, 52) = 2.60, p = 0.113] nor an interaction between the two [F (1, 52) = 0.86, p = 0.359].

5.4.1.2. Elevated plus maze

The anxiety-like behavior was measured as the time spent in the closed arms of the EPM. MS males, but not females, spent more time in the closed arms. A two-way ANOVA (Experience X Sex) on time spent in the closed arms revealed a marginal effect of experience [F (1, 51) = 3.33, p = 0.074] with no main effect of sex [F (1, 55) = 1.93, p

= 0.171] nor an interaction between the two [F (1, 55) = 2.53, p = 0.118]. Pairwise comparison revealed that MS males compared to sex-matched controls spent more time in the closed arms of the maze (p = 0.021) whereas females were not affected by the early experience. MS males compared to experience-matched females appeared to spend more time in the closed arms but pairwise comparison revealed only a marginal effect (p = 0.056). Similarly, there was no significant sex difference within the control group (Figure 5. 2).

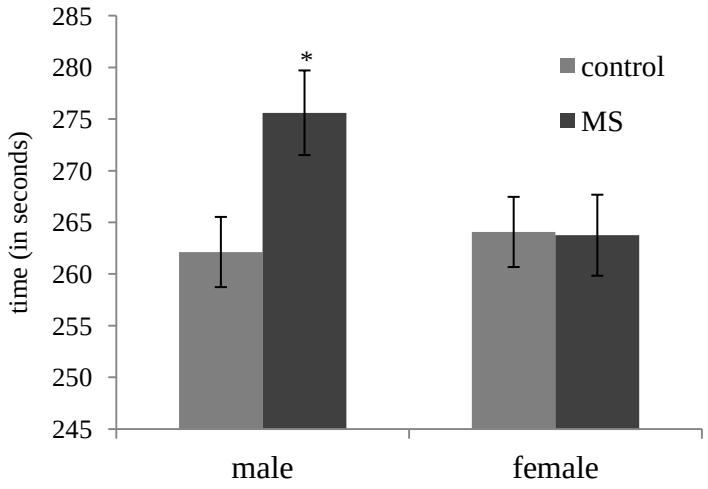

Figure 5.2. Mean (± SEM) of the total time spent in the closed arms of EPM. MS males spent more time in the closed arms of EPM (*, p = 0.021) compared to sex-matched controls but females were not affected by early experience.

5.4.1.3. Novel object recognition

The object novel object recognition, tested in the NOR task, was not influenced by MS experience. The ratio of time spent with old vs. recent familiar object was calculated as an index of object memory in temporal order. A two-way ANOVA (Experience X Sex) of the ratio of time spent with old *vs*. recent familiar object revealed

no main effect of experience [F (1, 44) = 0.39, p = 0.537], sex [F (1, 44) = 0.009, p = 0.926], nor an interaction between the two [F (1, 44) = 1.10, p = 0.300].

5.4.1.4. Play fighting

MS not only decreased the number of playful attacks but also feminized the male play behavior. When subjected to a two-way ANOVA (Experience X Sex), the frequency of play attacks revealed no main effect of experience [F (1, 44) = 0.68, p = 0.412] nor sex [F (1, 44) = 0.12, p = 0.726] but there was an interaction between the two [F (1, 44) = 4.26, p = 0.045]. Pairwise comparison revealed that MS in males compared to sex-matched controls decreased the frequency of play attacks (p = 0.047). However, MS in females did not influence the number of play attacks. There was a sex difference in the frequency of play attacks in the control group whereby males showed an increase in the frequency of play attacks compared to females (p = 0.042). In contrast, no sex difference was observed within the MS group (Table 5.1).

A two-way ANOVA (Experience X Sex) of the probability of complete rotation defense revealed a main effect of experience [F (1, 44) = 5.69, p = 0.021] but no main effect of sex [F (1, 44) = 0.543, p = 0.465] or an interaction between the two [F (1, 44) = 1.51, p = 0.226]. Pairwise comparison revealed that MS females compared to sex-matched controls responded less frequently with complete rotation defense (p = 0.014) whereas, this strategy of defense in response to a play attack was not affected in males. Furthermore, there was no sex-difference in the control or MS group related to the probability of facing an attack (Table 5.1).

When subjected to a two-way ANOVA (Experience X Sex) the probability of evasion in response to an attack revealed no main effect of experience [F (1, 44) = 2.99, p = 0.090], sex [F (1, 44) = 2.10, p = 0.154] nor an interaction between the two [F (1, 44) = 0.37, p = 0.546] (Table 5.1).

Table 5.1

Mean (±SEM) of playful attacks, probability of complete rotation defense and evasion

	Male		Female	
	Control	MS	Control	MS
Attacks	44 ± 2.68	34 ± 3.79*	36 ± 2.68†	40 ± 3.37
Complete rotation	0.52 ± 0.03	0.48 ± 0.04	0.54 ± 0.03	0.41 ± 0.04*
Evasion	0.022 ± 0.005	0.006 ± 0.008	0.028 ± 0.005	0.020 ± 0.008

Play fighting behavior was scored as the total number of attacks and either facing an attack as complete rotation defense or evasion. The playful attacks were sexually dimorphic where control males attacked more frequently compared to experience-matched females (†, p = 0.042). MS resulted in the feminization of male play behavior by preventing the sex difference. Moreover, MS reduced the frequency of play attacks in males (*, p = 0.047) and the complete rotation defense in females (*, p = 0.014).

5.4.2. Amphetamine sensitization

5.4.2.1. Acute administration

AMPH compared to saline administration resulted in enhanced locomotor activity on the first day of drug administration period regardless of experience and sex. However,

the activity in response to acute AMPH administration was not affected by MS. In order to simplify the presentation of the results, males and females were first analyzed separately.

A two-way ANOVA (Experience X Drug) of activity recorded on the first day of AMPH administration in males revealed a main effect of drug (AMPH or saline) [F (1, 24) = 56.65, $p < 0.001$], with no main effect of experience (MS or control) [F (1, 24) = 0.21, $p = 0.648$] nor an interaction between the two [F (1, 24) = 1.04, $p = 0.318$]. AMPH compared to saline administration resulted in enhanced activity in both MS and control groups (see Table 5.2A for the activity on the 'First day'). Similarly, a two-way ANOVA of the first day locomotor activity in females revealed a main effect of drug [F (1, 24) = 214.24, $p < 0.001$], with no main effect of experience [F (1, 24) = 1.46, $p = 0.238$] nor an interaction between the two [F (1, 24) = 0.51, $p = 0.483$]. AMPH compared to saline administration resulted in augmented activity in both MS and control groups (see Table 5.2B for the activity on the 'First day').

5.4.2.2. Development of sensitization

Chronic AMPH administration resulted in behavioral sensitization, shown by a gradual increase in the locomotor activity, in both MS and control groups regardless of sex. The development of sensitization was assessed by analyzing the activity recorded over a 14-day period with a steady enhancement in the activity. Moreover, when the first and the last day were compared, there was augmented activity on the last day of AMPH administration compared to the first day, confirming the development of sensitization (see Figure 5. 3 and Table 5.2).

Comparison of the first and the last day activity in males exhibited a main effect of drug [F (1, 24) = 236.56, $p < 0.001$] in both AMPH-administered MS and control groups. Repeated AMPH administration resulted in enhanced activity on the last day compared to the first day in both control and MS groups. In contrast, saline administration in both control and MS groups, although not significant, resulted in decreased activity on the last day compared to the first day (Table 5.2A).

Repeated administration over a 2-week period in males regardless of early experience produced a gradual increase in locomotor activity exhibiting development of behavioral sensitization (Figure 5. 3). Mixed ANOVA of the activity in males with experience (MS or control) and drug (AMPH or saline) as independent factors and days (day 1-14) as repeated measure factor showed a main effect of drug [F (1, 24) = 298.87, $p < 0.001$] with no main effect of experience [F (1, 24) = 0.88, $p = 0.358$] nor an interaction between the two [F (1, 24) = 1.28, $p = 0.269$]. Repeated AMPH compared to saline administration resulted in augmented activity throughout the drug administration period in both MS and control groups. However, MS compared to control group did not influence AMPH-induced behavioral sensitization (Figure 5. 3A).

In female rats, comparison of the first and the last day activity revealed a main effect of drug [F (1, 24) = 227.14, $p < 0.001$]. An augmented AMPH-induced locomotor activity was observed on the last day compared to the first day in both control and MS groups (Table 5.2B). Saline administration, although not significant, resulted in diminished activity on the last day compared to the first day in MS and control females. Similar to males, mixed ANOVA of the 14-day locomotor activity in females showed a main effect of drug [F (1, 24) = 310.99, $p < 0.001$] with no main effect of experience [F

(1, 24) = 0.64, p = 0.431] nor an interaction between the two [F (1, 24) = 1.27, p = 0.271]. Repeated AMPH compared to saline administration resulted in augmented activity over 14-day drug administration period. However, similar to males, MS in females compared to sex-matched controls did not affect the degree of behavioral sensitization (Figure 5. 3B).

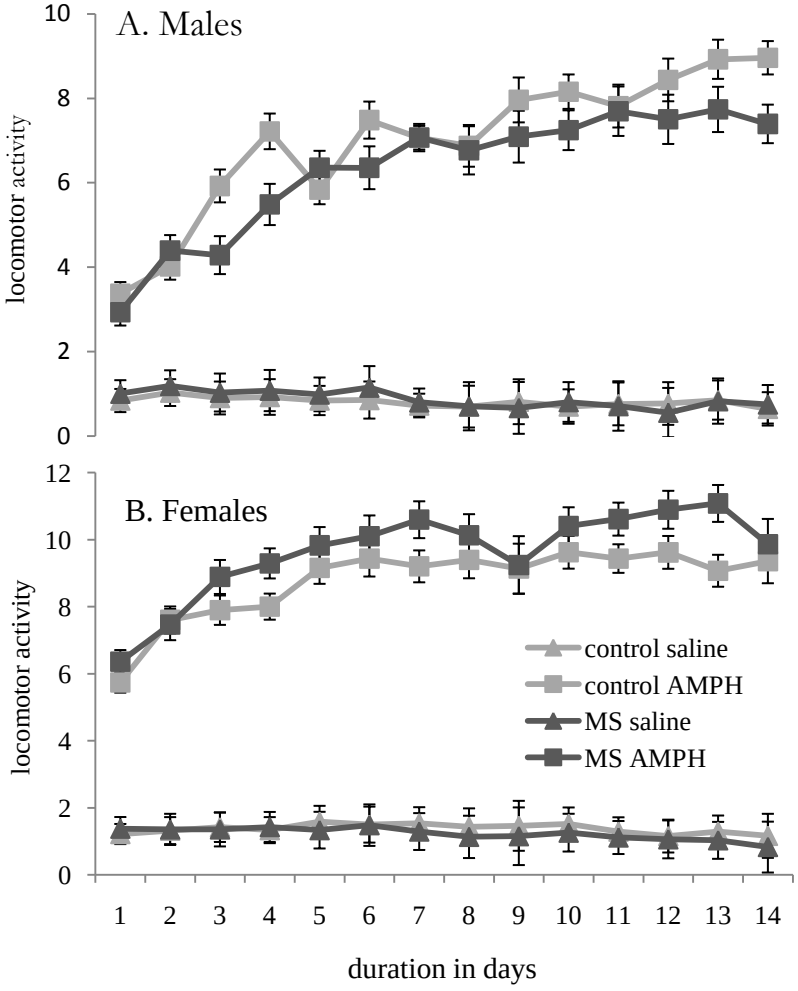

Figure 5.3. Mean (± SEM) locomotor activity (beam crossing x 10^3) recorded for 90 minutes after saline or AMPH (1 mg/kg) administration over 14-day period. AMPH administration resulted in the develpment of behavioural sensitziation regardless of experience however, MS did not influence AMPH-induced behavioral sensitization in males (A) and in females (B).

Table 5.2

Mean (±SEM) locomotor activity on the first and the last day of 14-day drug (AMPH/saline) administration period

	Control		MS	
	First day (acute)	Last day	First day (acute)	Last day
A. Male				
Saline	8425 ± 2742	6452 ± 3951	10079 ± 3166	7535 ± 4562
AMPH	33740 ± 2742	89596 ± 3951*	29348 ± 3166	73933 ± 4562*
B. Female				
Saline	12202 ± 3004	11667 ± 6571	13816 ± 3468	8312 ± 7588
AMPH	57376 ± 3004	93578 ± 6571*	63613 ± 3468	98605 ± 5788*

MS in males (A) and females (B) did not influence the locomotor activity response to acute AMPH administration (i.e. First day). AMPH treated male (A) and female groups (B) developed behavioral sensitization (last day *vs.* first day) regardless of early experience (i.e. control and MS) by exhibiting enhanced activity on the last compared to the first day (*, both *p*s < 0.001). Saline administration, although not significant, resulted in diminished activity on the last day compared to the first day in both male (A) and female group (B).

5.4.2.3. Persistence of sensitization

After a 2-week withdrawal period, the persistence of drug-induced behavioral sensitization was assessed by administering a challenge AMPH dose to prior saline- and prior AMPH-treated rats. The activity recorded on the challenge day in males when

subjected to a two-way ANOVA (Experience X Prior drug treatment) revealed a main effect of prior drug treatment [F (1, 24) = 81.26, $p < 0.001$], with no main effect of experience [F (1, 24) = 1.08, $p = 0.309$], nor an interaction between the two [F (1, 24) = 0.004, $p = 0.948$]. Prior AMPH compared to prior saline administration resulted in augmented activity in both control and MS groups showing persistence of sensitization in prior AMPH-treated rats (Figure 5. 4A).

The locomotor activity in response to an AMPH challenge injection in females when subjected to a two-way ANOVA (Experience X Prior drug treatment) revealed a main effect of prior drug treatment [F (1, 24) = 21.26, $p < 0.001$], with no main effect of experience [F (1, 24) = 0.75, $p = 0.394$] nor an interaction between the two [F (1, 24) = 0.14, $p = 0.714$]. Similar to males, prior AMPH- compared to prior saline-treated females showed enhanced locomotor activity, exhibiting persistence of sensitization, in both control and MS groups (Figure 5. 4B).

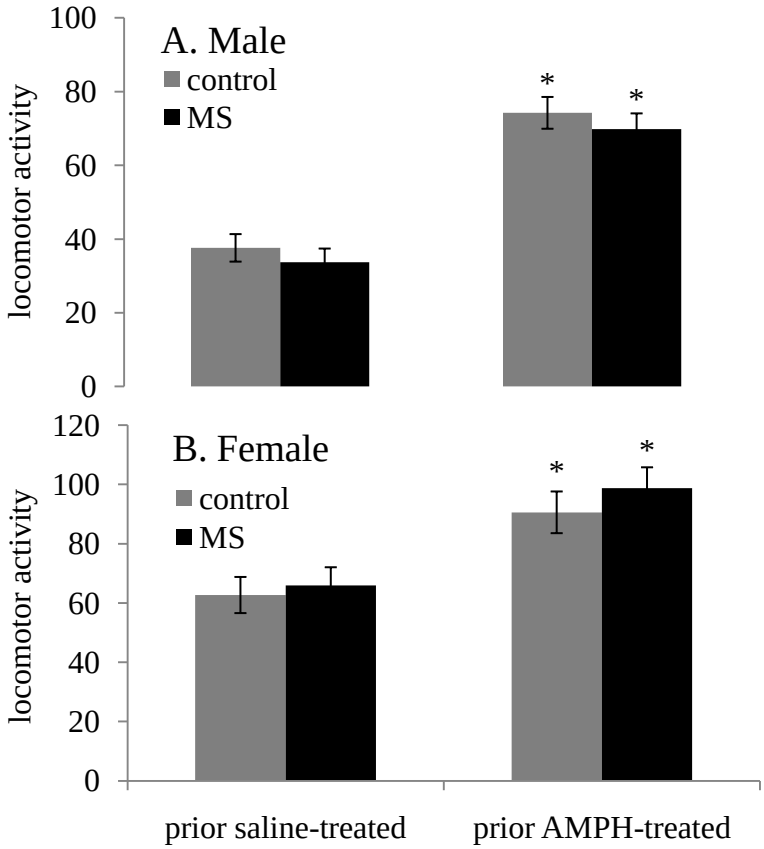

Figure 5.4. Mean (±SEM) locomotor activity (beam crossing x 10^3) in response to AMPH challenge injection in male (A) and female rats (B). Prior AMPH- compared to prior saline-treated males and females exhibited enhanced activity (*, all *p*s =< 0.05) regardless of experience (control or MS), showing persistence of behavioral sensitization.

5.4.2.4. AMPH-dependent sex differences

To examine sex differences to an acute AMPH administration and in the development and persistence of behavioral sensitization, sex was used as factor in addition to experience and drug. A three-way ANOVA (Sex X Experience X Drug) of the activity on the first day of drug administration period revealed a main effect of sex [*F*

(1, 48) = 55.43, $p < 0.001$] where AMPH-treated females in both control and MS groups were more active compared to the drug and experience-matched males (see Table 5.2). Similarly, a mixed ANOVA (Sex X Experience X Drug X Day) of the 14-day activity data showed a main effect of sex [F (1, 48) = 28.70, $p < 0.001$] where AMPH-treated control and MS females compared to the drug and experience-matched males showed enhanced activity (see Figure 5. 3). Similar results were obtained when the challenge day activity was subjected to a three-way ANOVA (Sex X Experience X Drug) . There was a main effect of sex [F (1, 48) = 44.17, $p < 0.001$] where both prior saline- and AMPH-treated females in both control and MS groups compared to the drug- and experience-matched males showed enhanced activity in response to the AMPH challenge (see Figure 5. 4).

5.4.3. Anatomy

5.4.3.1. Brain weight

MS resulted in lighter brains in saline-treated males and AMPH administration reduced this difference. When subjected to a two-way ANOVA (Experience X Drug), brain weights in males revealed a main effect of experience [F (1, 24) = 4.33, $p = 0.048$], with no main effect of drug [F (1, 24) = 1.05, $p = 0.315$] nor an interaction between the two [F (1, 24) = 0.77, $p = 0.389$]. Pairwise comparison revealed that MS compared to sex-matched controls resulted in significantly lighter brain weights in saline-treated groups ($p = 0.047$). Whereas, AMPH administration in males prevented the lighter brain effect as there was no difference between AMPH-treated MS and control groups (Table 5.3A).

The brain weight was not influenced by MS or drug administration in females. A two-way ANOVA (Experience X Drug) of the female brain weights revealed no main effect of experience [F (1, 24) = 0.31, p = 0.586], drug [F (1, 24) = 1.16, p = 0.292] nor an interaction between the two [F (1, 24) = 0.16, p = 0.691] (Table 5.3B).

Table 5.3

Mean (±SEM) brain weight (in grams)

	Control	MS
A. Male		
Saline	2.11 ± 0.02	2.05 ± 0.02*
AMPH	2.11 ± 0.02	2.09 ± 0.02
B. Female		
Saline	1.93 ± 0.02	1.93 ± 0.02
AMPH	1.96 ± 0.02	1.94 ± 0.02

MS in males (A) resulted in lighter brains in saline-treated rats (*, p = 0.047) however, AMPH administration blocked this difference. Experience or drug administration did not affect brain weights females (B).

5.4.3.2. Spine density

The spine density was examined in the shell region of the nucleus accumbens (NAc), Cg3 (layer III) region of the mPFC, and the agranular insular cortex (AID, layer

III) region of the OFC. The spine density, calculated from 5 dendritic segments from five different cells drawn per hemisphere, was analyzed using hemisphere as a factor, in addition to experience and drug. However, the data were collapsed in the absence of any significant hemispheric difference.

5.4.3.2.1. Nucleus accumbens

The spine density on the medium spiny neurons of the NAc shell region was increased in response to MS experience regardless of drug exposure, except in AMPH-administered males. Furthermore, repeated AMPH administration increased the spine density in the NAc however, MS experience blocked the drug-induced increase in the spine density in both sexes (Figure 5. 5).

When subjected to a two-way ANOVA (Experience X Drug), the spine density in the NAc region in males revealed a main effect of experience [F (1, 44) = 8.15, p = 0.007] and no main effect of drug [F (1, 44) = 0.245, p = 0.245] nor an interaction between the two [F (1, 44) = 3.06, p = 0.087]. Pairwise comparison revealed that MS experience in males compared to sex-matched controls increased the spine density in saline-treated rats (p = 0.003). However, there was no difference between MS and sex-matched control males in the AMPH-treated group. In addition, AMPH compared to saline administration resulted in increased spine density in the NAc in control males (p = 0.046). However, MS blocked the increased spine density as no difference between saline- and AMPH-treated males was observed (Figure 5. 5).

Similarly in females, the spine density when subjected to a two-way ANOVA (Experience X Drug) revealed a main effect of experience [F (1, 46) = 27.44, p < 0.001]

with no main effect of drug [F (1, 44) = 1.42, p = 0.239] nor an interaction between the two [F (1, 44) = 2.91, p = 0.095]. MS compared to controls in females increased the spine density in the NAc of saline and AMPH-treated groups. In addition, AMPH compared to saline administration resulted in increased spine density in the NAc in control females (p = 0.042). However, the drug-induced increase in the spine density was blocked by MS experience as there was no difference between saline- and AMPH-administered females (Figure 5. 5).

5.4.3.2.2. Medial prefrontal cortex

MS experience increased the spine density on the apical dendrites of the pyramidal neurons in the Cg3 region of the mPFC regardless of drug exposure or sex, except in APMH-treated males. However, MS did not influence the spine density on the basilar dendrites of the pyramidal neurons in the Cg3 region. In addition, AMPH administration increased the spine density on the apical dendrites in control male and female rats and on the basilar dendrites only in control males. However, MS blocked the drug-induced increase in the spine density observed in both male and female controls (Figure 5. 5).

The spine density on the apical dendrites in the Cg3 region in males when subjected to a two-way ANOVA (Experience X Drug) revealed a main effect experience [F (1, 52) = 14.38, p < 0.001] with no main effect of drug [F (1, 52) = 2.15, p = 0.149] nor an interaction between the two [F (1, 52) = 1.73, p = 0.194]. Pairwise comparison revealed that MS compared to sex-matched control rats increased the spine density in saline-treated males (p = 0.001). Whereas AMPH-treated rats appeared to have increased

spine density however pairwise comparison showed only a trend ($p = 0.086$). Moreover, control males showed increased spine density on the apical dendrites in response to AMPH compared to saline administration ($p = 0.038$). However, the drug-induced increased spine density observed in controls was blocked by MS experience in males because there was no difference between saline- and AMPH administered rats in this group (Figure 5. 5).

A two-way ANOVA (Experience X Drug) of the spine density on the Cg3 basilar dendrites in males revealed no main effect of experience [F (1, 46) = 0.08, $p = 0.773$] and a marginal effect of drug [F (1, 46) = 3.81, $p = 0.057$] with no interaction between the two [F (1, 46) = 1.68, $p = 0.202$]. MS did not influence the spine density in saline- or AMPH-treated males. In addition, AMPH compared to saline administration in controls increased the spine density ($p = 0.024$). However, MS blocked the drug-induced increase because pairwise comparison did not reveal a significant difference between saline- and AMPH-treated males (Figure 5. 5).

When subjected to a two-way ANOVA (Experience X Drug), the spine density on Cg3 apical dendrites in females revealed a main effect of experience [F (1, 44) = 32.10, $p < 0.001$], no main effect of drug [F (1, 44) = 1.45, $p = 0.235$] with an interaction between the two [F (1, 44) = 5.77, $p = 0.021$]. MS compared to sex-matched controls increased the spine density in both saline- and AMPH-treated females. Furthermore, AMPH compared to saline administration in control females increased the spine density on the Cg3 apical dendrites ($p = 0.014$). MS experience however, blocked the drug-induced increase in the spine density observed in controls as there was no difference between saline- and AMPH-administered females (Figure 5. 5).

Neither MS nor drug affected the Cg3 basilar spine density in females. When subjected to a two-way ANOVA (Experience X Drug), the Cg3 basilar spine density in females revealed no main effect of experience [F (1, 46) = 2.87, p = 0.178], drug [F (1, 44) = 0.03, p = 0.873] nor an interaction between the two [F (1, 44) = 0.274, p = 0.603] (Figure 5. 5).

5.4.3.2.3. Orbital frontal cortex

Similar to the mPFC, the MS experience increased the spine density on the basilar dendrites of the AID region of the OFC regardless of sex and drug exposure. In contrast to the mPFC, the spine density was decreased as a result of AMPH exposure, which was more pronounced in controls but MS experience blocked the drug-induced decrease in spine density (Figure 5. 5).

The spine density on the AID basilar dendrites in males when subjected to a two-way ANOVA (Experience X Drug) revealed a main effect of experience [F (1, 48) = 17.17, $p < 0.001$] and drug [F (1, 48) = 4.48, p = 0.039] with no interaction between the two [F (1, 48) = 0.58, p = 0.451]. MS compared to sex-matched controls increased the AID spine density in both saline- and AMPH-treated males. Furthermore, AMPH compared to saline administration decreased the spine density in the AID region in control males (p = 0.040). However, MS experience blocked the drug-induced decrease in the spine density as there was no difference between saline- and AMPH-treated males (Figure 5. 5).

When subjected to a two-way ANOVA (Experience X Drug), the AID spine density in females revealed a main effect of experience [F (1, 46) = 37.95, $p < 0.001$] and

drug [F (1, 46) = 4.71, p = 0.035] with no interaction between the two [F (1, 46) = 0.97, p = 0.330]. Similar to males, MS in females compared to sex-matched controls increased the AID spine density in both saline- and AMPH-treated rats. In addition, pairwise comparison revealed that AMPH compared to saline administration resulted in decreased spine density in the AID region of control females (p = 0.028). However, MS experience blocked the drug-induced decreased spine density observed in controls as there was no difference between saline- and AMPH-treated females (Figure 5.5).

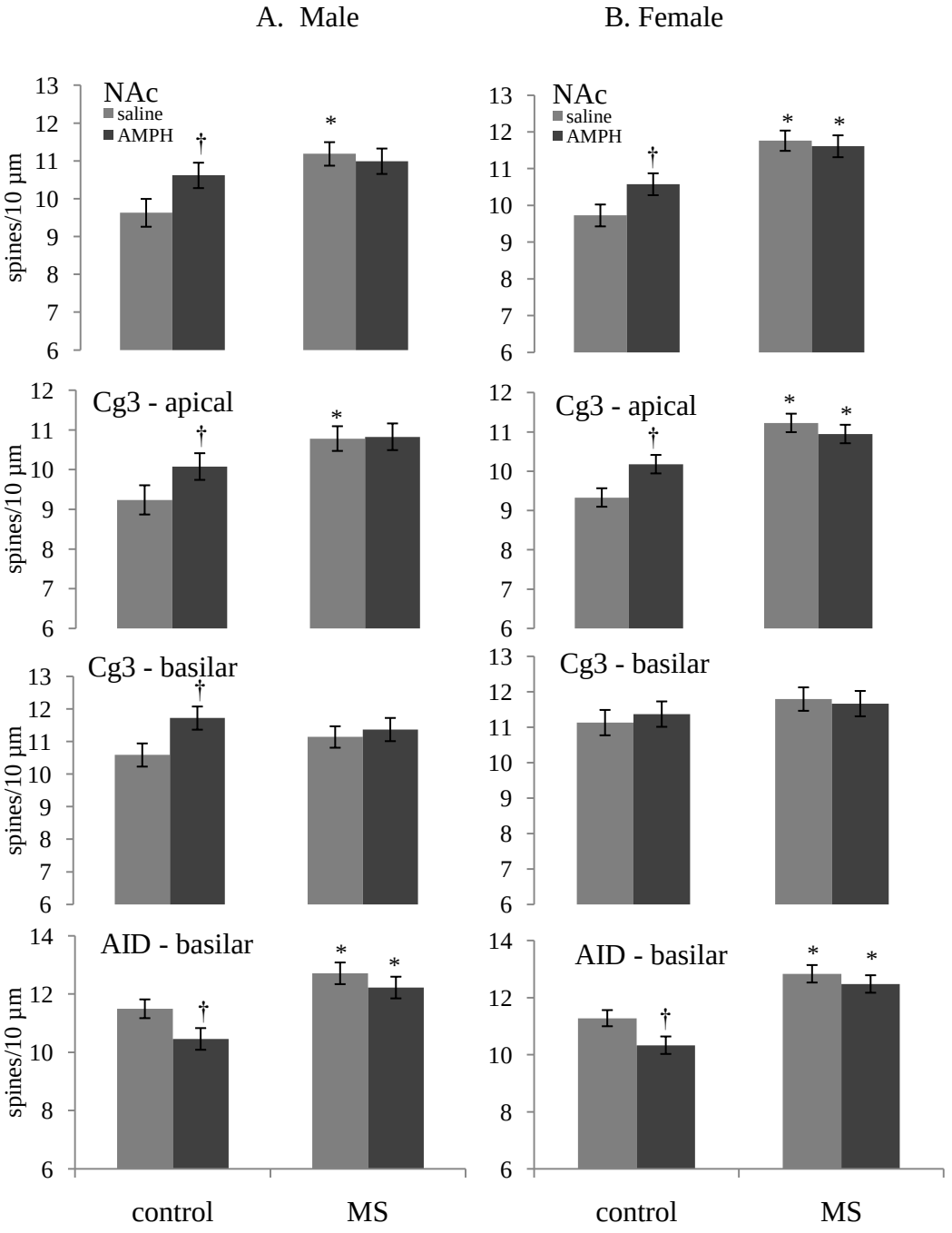

Figure 5.5. Mean (± SEM) of the spine density on the distal dendritic segment in the NAc, the Cg3, and the AID regions. MS increased the spine density in the NAc, Cg3 (apical dendrites only), and AID regions (*, all *p*s =< 0.05). Repeated AMPH administration increased the spine density in the NAc, Cg3, and decreased the same in the AID region (†, all *p*s =< 0.05). However, MS followed by drug exposure blocked the drug-induced alteration in the spine density in the NAc and subregions of the PFC.

5.5. Discussion

We investigated the effect of MS experience on periadolescent behavior, adult amphetamine sensitization, and interaction of early experience and later drug exposure on structural plasticity in brain regions implicated in addiction (i.e. NAc and PFC). MS increased anxiety-like behavior and feminized play fighting in periadolescent males, and increased spine density in the cortical and subcortical brain regions without any influence on the degree of AMPH-induced behavioral sensitization.

MS has been employed as a model of early adverse experience but the findings among studies are not conclusive. There are major procedural differences within MS paradigm in the studies reported (reviewed in Gutman & Nemeroff, 2002; Pryce & Feldon, 2003; Sanchez, et al., 2001). For example, there are differences in the duration and frequency of separation, and choice of comparison group (i.e. handled, animal facility reared or non-handled) in addition to other extraneous factors (e.g., thermoregulation during separation). Before comparing the long term impact of MS among studies on brain and behavior, the MS-specific procedural differences in addition to other factors (e.g., animal species, age of animals tested) should be kept in mind.

5.5.1. Behavior

Male and female periadolescent rats, subjected to MS during the nursing period, were run in a battery of behavioral tasks to investigate the effect of early experience on the exploratory, emotional, cognitive, and social behaviors. The tasks included open field locomotion, elevated plus maze, novel object recognition, and play fighting behavior.

MS did not affect exploratory behavior, tested as open field locomotion, in either male or female rats (Brake, et al., 2004; Caldji, Francis, Sharma, Plotsky, & Meaney, 2000; Li, Robinson, & Bhatnagar, 2003; Marmendal, Roman, Eriksson, Nylander, & Fahlke, 2004; Zimmerberg & Shartrand, 1992). MS male rats showed enhanced anxiety-like behavior by spending more time in the closed arms, a behavior that could be attributed to elevated stress reactivity (Aisa, Tordera, Lasheras, Del Río, & Ramírez, 2007; Huot, et al., 2001). However, we did not find an effect of MS in female rats on either exploratory or anxiety-like behavior. Wigger and Neumann (1999) also reported similar sex-dependent differences in emotional behavior, where their male rats were more affected by MS experience.

The object novel object recognition was tested in the NOR task. The NOR results showed that MS did not influence the cognitive performance. Conversely, our finding was not in agreement with previous reports that found cognitive impairment in MS rats (Aisa, et al., 2007). The possible reasons for such discrepancy in both studies might be related to the differences in, for example, the NOR paradigm (temporal order *vs*. object discrimination memory) and age of the rat tested (periadolescents *vs.* adults).

The play fighting behavior was scored as a series of attacks and the probability to either face (i.e. complete rotation defense) or evade an attack (Pellis & Pellis, 1990). Our findings showed a sex difference in the frequency of playful attacks where control males attacked more frequently compared to experience-matched females. However, MS resulted in the feminization of playful attacks by abolishing the sex difference with reduced number of attacks in males (Arnold & Siviy, 2002). Furthermore, the probability of complete rotation defense was reduced in MS females without any substantial

influence on males. The MS experience produced sexually dimorphic results with altered frequency of attacks in males and modulated defense strategy in females. Our finding for the complete rotation defense was in agreement with Veenema and Neumann (2009) although they also reported offensive play fighting with increased frequency of nape attacks. The possible reasons for the discrepancy regarding aggressive behavior, in addition to strain difference, could be related to the social play paradigms as they tested the play behavior in the rat home cage with an unknown partner.

5.5.2. Amphetamine sensitization

Chronic administration of amphetamine, like other stimulants (e.g., cocaine and nicotine), not only produces a gradual increase in the locomotor activity (i.e. behavioral sensitization) but the sensitization also persists for weeks beyond drug exposure (Li, Kolb, et al., 2003). We manipulated postnatal experience by exposing male and female pups to MS to investigate the effect of early adverse experience on later drug-induced behavioral sensitization. Chronic intermittent AMPH administration in both MS and control groups, regardless of sex, resulted in the development behavioral sensitization that persisted at least for 2 weeks. However, based on 14-day AMPH administration followed by 2-week withdrawal period, we failed to find an effect of MS on the development or persistence of behavioral sensitization in adult male and female rats.

Previous studies show discrepancies in experiments related to the effect of MS on drug-induced behavioral sensitization or self-administration in rodents and monkeys. There are reports of enhanced behavioral sensitization (Rots et al., 1996) or self-administration (Kikusui, Faccidomo, & Miczek, 2005), no effect (Meaney, Brake, &

Gratton, 2002; Weiss, Domeney, Heidbreder, Moreau, & Feldon, 2001), or even an attenuated behavioral sensitization (Li, Robinson, et al., 2003; Matthews, Hall, Wilkinson, & Robbins, 1996) in response to drug administration in MS rats. The obvious discrepancies in drug administration findings suggest fundamental methodical disparities. For example, one of the possible factors may be the dose of the drug administered. Matthew, Rossins, Everitt, and Caine (1999) reported retarded drug acquisition in MS rats for lower cocaine doses compared to higher doses. Age could be another possible factor as the effects of MS were more obvious on adolescent compared to adult rats (Marin & Planeta, 2004). The pronounced effect of MS during early age was also corroborated by EPM and play behavior findings in our study. Thermoregulation during MS has also been shown to play a significant role as Zimmerberg and Shartrand (1992) reported that rats maternally separated at 20 ^{o}C exhibited enhanced AMPH-induced behavioral sensitization compared to rats separated at 34 ^{o}C. Moreover, there was no significant difference in the degree of sensitization between rats separated at 34 ^{o}C and controls (mother reared) (Zimmerberg & Shartrand, 1992). In addition to the procedural differences mentioned above, the choice of control group (e.g. handled, non-handled or animal facility reared) also makes a difference in the interpretation of results as well as comparison among studies reported (reviewed in Moffett et al., 2007; Pryce & Feldon, 2003).

5.5.3. Anatomy

The structural alteration (i.e. reorganization of spine density) in the NAc and subregions of the PFC in response to MS experience, AMPH administration, and

interaction between the two was investigated. Previously, we have reported that both complex housing and psychomotor stimulants increase the spine density in the NAc (Kolb, Gorny, et al., 2003; Robinson & Kolb, 2004). However, when psychostimulant (i.e. cocaine, amphetamine, or nicotine) administration is followed by experience in an enriched environment, the prior drug exposure blocks the increase in spine density associated with enriched environment (Hamilton & Kolb, 2005; Kolb, Gorny, et al., 2003). In the present study, we reversed the order of experiential factors such that drug exposure followed the MS experience. MS increased spine density in the NAc and subregions of the PFC. In contrast, a decrease was observed in the brain weights in MS rats. The MS-associated increase in spine density could be a compensatory mechanism for the lighter brain weights. Repeated AMPH administration modulated spine density in the cortical and subcortical regions; however, MS exposure blocked the drug-induced alteration observed in control rats.

5.5.3.1. Nucleus accumbens

The spine density in the shell region of NAc was increased as a result of MS experience. The experience-dependent increase in the spine density was observed in saline-treated rats regardless of sex but the MS-dependent increase was not observed in AMPH-treated males. Furthermore, repeated AMPH administration increased the spine density the NAc in control rats but MS blocked the drug-induced alteration in the NAc regardless of sex.

NAc plays an important role in the processing of reward and motivation (reviewed in Cardinal, Parkinson, Hall, & Everitt, 2002; Day & Carelli, 2007).

Consequently, any rewarding experience (e.g., exposure to psychostimulants) modulates not only the function (Roitman, Wheeler, & Carelli, 2005) but also reorganizes the synaptic organization of the region (Robinson & Kolb, 2004). Similarly, NAc is also sensitive to other experiences. For example, environmental enrichment and stress reorganized synaptic input to NAc through modulation of the spine density, and dendritic growth (Kolb, Gorny, et al., 2003; Morales-Medina, Sanchez, Flores, Dumont, & Quirion, 2009). Interestingly, the AMPH-induced increase in the spine density observed in the shell region of the NAc was blocked by MS experience. The MS-associated interference with AMPH-induced increase in the spine density is in accordance with our previous reports that showed an interaction between drug exposure and enrichment experience such that prior AMPH exposure blocked the increase in spine density associated with subsequent experience of housing in an enriched environment (Kolb, Gorny, et al., 2003).

5.5.3.2. Prefrontal cortex

MS experience increased the spine density on the apical dendrites of the Cg3 region of the mPFC without affecting the spine density on the basilar dendrites (Zehle, et al., 2007). Similarly, an experience-dependent increase in the spine density was observed on the basilar dendrites of the pyramidal neurons in the AID region of the OFC. The MS-induced increase in the mPFC was also reported, employing combined light and electron microscopy, in *Octodon degus* (Helmeke, Ovtscharoff, Poeggel, & Braun, 2001). Similarly, MS after the stress hyporesponsive period increased the spine density on the pyramidal neurons of the mPFC (Bock, et al., 2005; Gos, Bock, Poeggel, & Braun, 2008).

However, social isolation before and after weaning, where pups were separated from the dam as well as littermates, decreased the spine density in the mPFC (Monroy, Hernandez-Torres, & Flores, 2010; Silva-Gomez, Rojas, Juarez, & Flores, 2003). Social isolation, compared to MS, is an intense stress experience for newborn pups as they are separated from the dam as well as the littermates. Interestingly, Weiss, et al. (2001) reported that social isolation enhanced AMPH-induced behavioral sensitization whereas, maternal separation, similar to our findings, did not affect the degree of drug-induced behavioral sensitization.

Repeated AMPH administration resulted in increased spine density in the mPFC and decreased in the OFC (Robinson & Kolb, 2004). However, MS experience blocked the drug-induced alteration in the PFC. Similar to MS, prenatal stress experience also resulted in the blockade of drug-induced alteration in the spine density observed in control rats (Muhammad & Kolb, 2011b). The experience-dependent interference with the drug-induced structural plasticity could be related, for instance, to basic fibroblast growth factor (FGF-2), a protein involved in synaptic plasticity. Fumagalli and colleagues showed that cocaine, a psychostimulant, increased the FGF-2 levels in the PFC. However, stress followed by drug exposure interfered with the drug-induced FGF-2 expression (Fumagalli, et al., 2008).

In conclusion, MS for three hours from postnatal day 3 to 21 modulated anxiety-like and social behaviors in male rats. Chronic AMPH administration resulted in a steady increase in locomotor activity (i.e. behavioral sensitization), which persisted at least for two weeks. However, MS did not influence the degree of AMPH-induced behavioral sensitization. Moreover, MS increased the spine density in the NAc, the medial PFC, and

the OFC regardless of the drug exposure. Repeated AMPH administration increased the spine density in the NAc and the medial PFC with a decrease in the OFC. Interestingly, though MS did not influence drug-induced behavioral sensitization yet blocked the AMPH-induced alteration in brain regions implicated in drug addiction.

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CHAPTER 6

Stress during development neuroanatomically interacts with subsequent drug exposure at cortical and subcortical levels*

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Abstract

The interference of psychostimulant exposure with subsequent rearing environment led us to investigate how early life stress followed by a psychostimulant administration would interact at neuroanatomical level. Rats, exposed to stress during embryonic development (prenatal stress; PS) or soon after birth (maternal separation; MS), were repeatedly administered with amphetamine (AMPH) as adults. At the end of behavioral testing, the rats were sacrificed and the brains were studied for structural alteration at neuronal level in the medial prefrontal cortex (mPFC), the orbital frontal cortex (OFC), and the nucleus accumbens (NAc). The findings show that the PS experience increased the dendritic branching and length in the mPFC apical and basilar dendrites. In contrast, a PS-associated decrease in dendritic branching and length was observed in the basilar branches of the OFC. However, the dendritic alterations in the subregions of the PFC were more robust in the saline-treated rats. Similarly, PS increased the dendritic growth in the NAc that was limited to saline-treated rats. Saline-treated controls and AMPH-treated PS showed a sex-dissociation where males have complex dendritic morphology in the NAc. MS resulted in overall increase in the dendritic growth in the subregions of the PFC and the NAc. Repeated AMPH administration increased the dendritic branching and length in the mPFC and the NAc but an overall decrease was observed in the OFC region. Interestingly, the drug-induced increase in the subregions of the PFC and the NAc was blocked by stress during development.

Key words: plasticity, metaplasticity, maternal separation stress, addiction, amphetamine

6.2. Introduction

Although genetic predetermination sets the stage for brain development, various experiential factors play a contributory role in offsetting some of the developmental processes (e.g., Hubel & Wiesel, 1970). Manipulation in the rearing environment, especially during development, has enduring impact via programming the brain circuits. Studies in rodents, for instance, have demonstrated that early adverse experience (e.g., stress) produce persistent structural alteration in various brain regions (e.g., hippocampus) that could be associated with some of the stress-associated impaired behaviors (e.g., learning and memory) (reviewed by Weinstock, 2008).

Consequently, the experience-induced reorganization of the brain circuits modulates the response to a subsequent experience, the process termed metaplasticity. Metaplasticity, manifested in the brain at various levels, can be studied employing different techniques (e.g., neuroanatomical) (Abraham, 2008; Kolb, et al.). Previous reports indicated that prior experience (e.g., enriched environment) alter the response to subsequent experience (e.g., drug exposure) at the structural level in individual neurons (Hamilton & Kolb, 2005; Kolb, Gorny, et al., 2003; Zehle, et al., 2007). For instance, Kolb and associates reported that psychostimulant drugs (e.g., amphetamine), similar to rearing in an enriched environment, increased the spine density in the nucleus accumbens (NAc) (Kolb, Gorny, et al., 2003; Norrholm, et al., 2003; Robinson & Kolb, 1997). However, prior drug exposure followed by environmental enrichment prevented the increase in spine density associated with the enrichment experience (Hamilton & Kolb, 2005; Kolb, Gorny, et al., 2003). In these experiments, the drug exposure, limited to adult rats, was followed by rearing experience in the enriched environment. Here we asked

what would be the neuroanatomical outcome if the order of experiential factors (i.e. drug and rearing environment) is switched such that a rearing environment manipulation is followed by exposure to a psychostimulant? With the marked influence of early adverse experience on developing brain in mind, we manipulated the rearing environment of the rats through pre- and postnatal stress exposure followed by repeated amphetamine (AMPH) administration in adulthood.

In two separate experiments, rats were either prenatally stressed (PS) during embryonic development by exposing a pregnant dam to stress or newborn pups were deprived of maternal care (i.e. maternal separation; MS) for 3 hours per day. The rats exposed to pre- or postnatal stress were exposed to repeated AMPH administration as adults. The metaplasticity as a result of stress and drug exposure was studied at behavioral and neuroanatomical levels. The AMPH-induced behavioral sensitization and the spine density findings have been reported previously (Muhammad & Kolb, 2011a; Muhammad & Kolb, 2011b). The findings show that repeated AMPH administration resulted in the development and persistence of behavioral sensitization regardless of early stress or sex. The spine density findings in the NAc and the subregions of the prefrontal cortex show some interesting trends. For example, PS increased the spine density in the NAc but in contrast a decrease was observed in the medial prefrontal cortex (mPFC). The opposite spine density trend between the NAc and the mPFC was in contrast to previous reports indicating similar directions of morphological alteration in both regions. For example, repeated psychostimulant administration increased whereas opiate exposure decreased the dendritic morphology in both regions (Robinson & Kolb, 2004). The differential spine density trend in the NAc and subregions of the PFC laid the foundation

for the present experiment to investigate if alteration in the spine density as result of early stress and subsequent drug exposure is accompanied by other measures of dendritic morphology, such as dendritic branching and length in the NAc and subregions of the PFC (i.e. mPFC and OFC).

6.3. Materials and methods

6.3.1. Animals

Long-Evans pups, stressed during gestation or separated from the dam, were randomly allotted with not more than two pups of each sex from the same litter. The control rats were not exposed to gestational or maternal separation stress. The pups along with their respective dams were housed in the breeding colony at the Canadian Centre for Behavioural Neuroscience, University of Lethbridge, Alberta, Canada. After weaning, the rats were housed in standard shoe-box cages with the same sex in a group of two in temperature- and humidity-controlled room. Standard rat food and water were provided *ad lib*. All the rats were left undisturbed till the commencement of behavioral testing and drug administration.

6.3.2. Prenatal stress

Pregnant rats were subjected to stress during the second week of gestation from day 12 to 16 twice a day for 10 minutes. The stress procedure was adopted from Wong et al. (2007). Briefly, pregnant rats, transported to a separate room for stress procedure, were placed on an elevated Plexiglas platform (1 m tall, 21 X 21 cm). The rats that

occasionally jumped from the platform were placed back immediately. At the end of stress procedure, rats were transported back to their home cages.

6.3.3. Maternal separation

The MS procedure, carried out between postnatal (P) 3-21, was conducted following the protocol described earlier (Plotsky & Meaney, 1993). Briefly, rat pups were transported in a box with new bedding to a separate room and placed on a warming pad with a temperature of ~ 34˚C. The rat pups were separated daily for 3 hours, approximately the same time of the day. The experimenter remained with the pups during the separation period to ensure the pups were comfortable and the temperature was properly maintained.

6.3.4. Amphetamine administration

Adult rats (P80) were administered with D-amphetamine sulfate (Sigma Aldrich, St. Louis, MO, USA) dissolved in sterile 0.9% saline. The rats were administered with AMPH (1 mg/kg body weight, i.p.) or 0.9% saline administration, both at a volume of 1 ml/kg. The drug was administered once a day for 14 consecutive days followed by an AMPH challenge after a withdrawal period of 2 weeks.

6.3.5. Anatomy

6.3.5.1. Perfusion and staining

Approximately 24 hours post AMPH challenge, the rats were given an overdose of sodium pentobarbital solution i.p. and perfused with 0.9% saline solution

intracardially. The brains removed from the skull were trimmed by cutting the olfactory bulb, optic nerves and spinal cord. The brains were then weighed and preserved in Golgi-Cox solution for 14 days followed by transfer to 30% sucrose solution at least for 3 days. The brains were sliced at a thickness of 200 μm on a Vibratome and fixed on gelatinized slides. The slides mounted with brain sections were processed for Golgi-Cox staining, following the protocol described by Gibb and Kolb (1998).

6.3.5.2. Dendritic analyses

Individual neurons were traced from Golgi-Cox stained brain sections using a camera lucida mounted on a microscope. Brain regions selected for neuron tracing were the shell region of the nucleus accumbens (NAc), Cg3 (layer III) region of the anterior cingulate, and dorsal agranular insular cortex (AID, layer III) described by Zilles (1985). A total of ten cells, five from each hemisphere, were traced from each brain region. The cells traced met the criteria of being thoroughly stained with solid dendritic tree and without overlapping another cell or blood vessel. The neurons traced were counted for the number of branch bifurcation (dendritic branching) and concentric rings for Sholl analysis (dendritic length). For details of the dendritic analyses methods please refer to (Kolb, Forgie, Gibb, Gorny, & Rowntree, 1998) (Figure 6. 1).

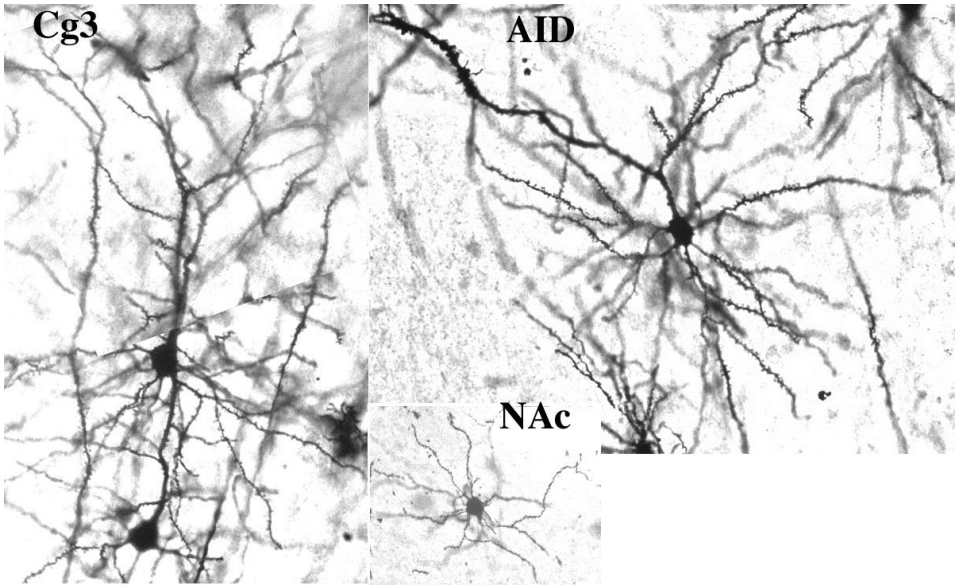

Figure 6.1. Photomicrographic (200X) examples of Golgi stained dendritic segments of neurons in Cg3, AID, and NAc.

6.4. Results

The anatomical data were analyzed using experience (PS/MS or control), sex, and drug (AMPH or saline) and hemisphere as independent factors. However, sex and hemisphere as factors were collapsed in the absence of main effects or an interaction with the other factors for the prefrontal measures. Sex did interact with other factors in the nucleus accumbens so this factor was maintained in the analysis of nucleus accumbens. Furthermore, all ANOVAs were followed by Bonferroni's post hoc test for multiple comparisons.

6.4.1. Prenatal stress

6.4.1.1. Medial prefrontal cortex

The dendritic branching and length of the pyramidal neurons in the Cg3 region of the mPFC was increased as a result of PS experience. The increase was observed in both

apical and basilar dendrites. However, the PS-associated dendritic growth was more robust in saline-treated rats. Similarly, repeated AMPH administration increased the dendritic branching and length in controls. However, PS prevented the drug-induced increase in the dendritic growth observed in controls (Table 6.1A & C).

When subjected to a two-way ANOVA (Experience X Drug), the total number of the Cg3 apical dendritic branches revealed a main effect of experience [F (1, 92) = 9.68, p = 0.002] and drug [F (1, 92) = 4.99, p = 0.028] with no interaction between the two [F (1, 92) = 1.04, p = 0.310]. Similarly, the Cg3 basilar branches revealed a main effect of experience [F (1, 92) = 15.67, $p < 0.001$], drug [F (1, 92) = 5.05, p = 0.027], and an interaction between the two [F (1, 92) = 6.05, p = 0.016]. Pair-wise comparison showed that PS increased the apical (p = 0.004) and basilar branching ($p < 0.001$) only in saline-treated rats. Similarly, repeated AMPH compared to saline administration in controls increased the apical (p = 0.024) and basilar dendritic branches ($p < 0.001$). However, PS prevented the drug-induced increase as no significant difference was observed between the saline- and AMPH-treated rats (Figure 6. 2).

A two-way ANOVA (Experience X Drug) of the Cg3 apical dendritic length revealed a main effect of experience [F (1, 92) = 24.30, $p < 0.001$] with no main effect of drug [F (1, 92) = 0.95, p = 0.331] nor an interaction between the two [F (1, 92) = 0.01, p = 0.939]. The Cg3 basilar length revealed a main effect of experience [F (1, 92) = 13.12, $p < 0.001$], a marginal effect of drug [F (1, 92) = 3.19, p = 0.07], and an interaction between the two [F (1, 92) = 3.84, p = 0.05]. PS increased the apical dendritic length in both saline- and AMPH-treated rats. However, the PS-associated increase in the basilar dendrites was limited to only saline-treated rats. Repeated AMPH compared to saline

administration in controls increased the basilar (but not apical) dendritic length, which was prevented by PS experience (Figure 6. 2).

6.4.1.2. Orbital frontal cortex

Unlike mPFC, the dendritic branching and length in the AID region of the OFC was *decreased* as a result of PS experience. However, similar to the mPFC, the effect was limited to the saline-treated rats. Repeated AMPH administration decreased the dendritic length (but not branching) in the OFC, however PS experience blocked this effect (Table 6.1A & C).

A two-way ANOVA (Experience X Drug) of the total number of the AID apical dendritic branches revealed a main effect of experience [F (1, 92) = 9.78, p = 0.002] with no main effect of drug [F (1, 92) = 2.22, p = 0.140] nor an interaction between the two [F (1, 92) = 2.10, p = 0.150]. The AID basilar dendritic length revealed a main effect of experience [F (1, 92) = 9.38, p = 0.003] with no main effect of drug [F (1, 92) = 2.11, p = 0.150] but there was an interaction between the two [F (1, 92) = 5.97, p = 0.016]. PS increased the dendritic branching (p = 0.002) and length (p < 0.001) only in saline-treated rats. Similarly, repeated AMPH compared to saline administration in controls increased the branching (p = 0.040) and length (p < 0.007). However, PS prevented the drug-induced increase in AID dendritic branching and length (Figure 6. 2).

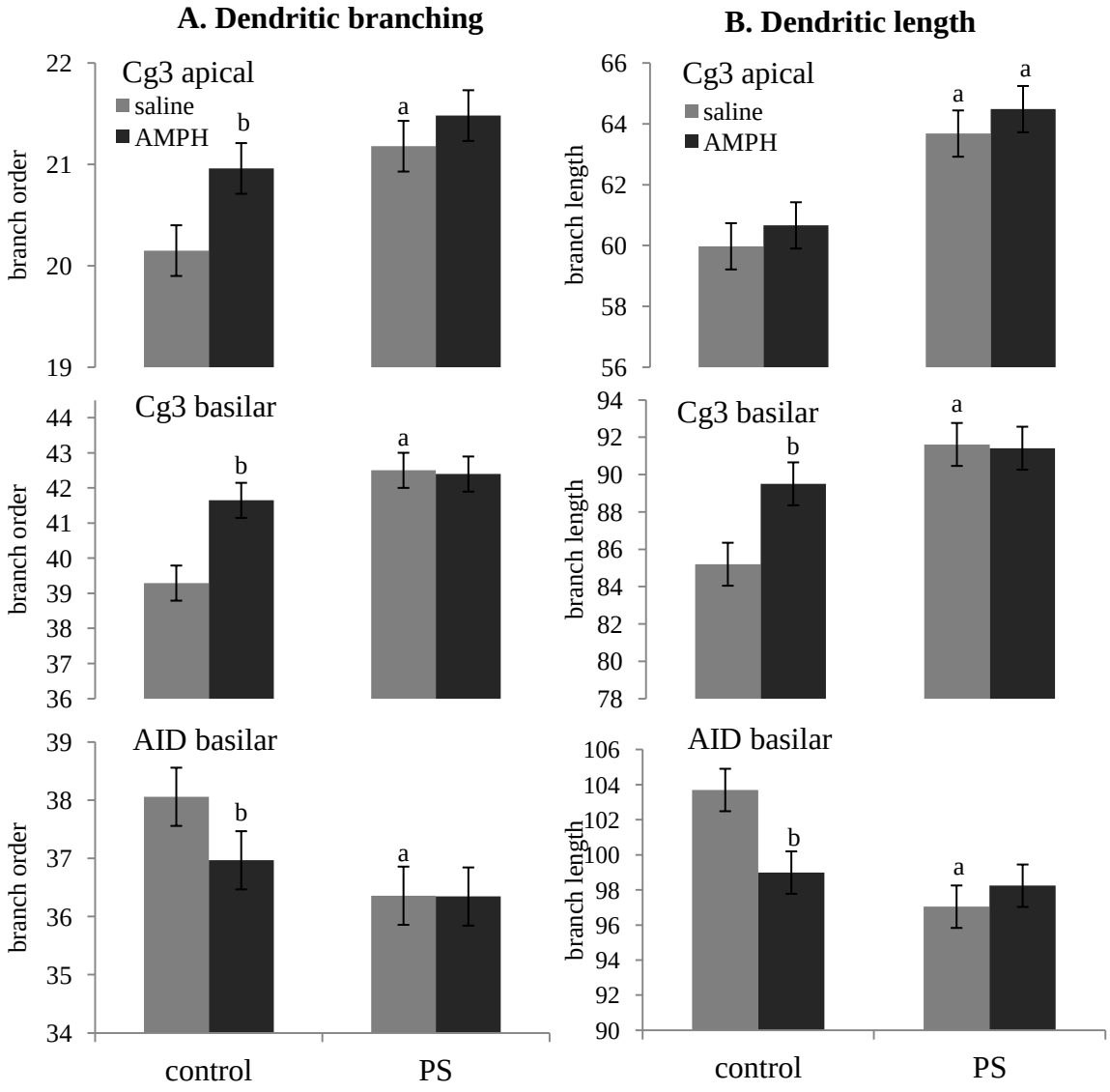

Figure 6.2. Mean (± SEM) total number of branch bifurcations (A. dendritic branching) and (B) dendritic length in the Cg3 region of the medial prefrontal cortex and the AID region of the orbital frontal cortex. PS increased the dendritic branching and length in the apical and basilar Cg3 with a decrease in the basilar AID region ([a] all *p*s =< 0.05). The increase in dendritic growth was more robust in saline-treated rats. In addition, repeated AMPH administration increased the number of dendritic branching and length in the apical and basilar Cg3 with a decrease in the basilar AID region of the control rats ([b] all *p*s =< 0.05) which was prevented by the PS experience.

6.4.1.3. Nucleus accumbens

There was an effect of sex related to NAc dendritic branching and length. Males showed increased branching in saline-treated controls and AMPH-treated PS rats. PS increased the dendritic branching in the NAc of saline-treated rats regardless of sex. Repeated AMPH administration increased the NAc dendritic growth in controls. But PS experience blocked the increase in dendritic growth associated with drug except for a decrease in dendritic branching in AMPH-treated PS females (Table 6. 2A, B, & C).

When subjected to a three-way ANOVA (Sex X Experience X Drug) , the NAc dendritic branching revealed a main effect of sex [F (1, 88) = 11.28, p = 0.001] and experience [F (1, 88) = 35.67, $p < 0.001$] with no main effect of drug [F (1, 88) = 2.73, p = 0.102]. There was an interaction between experience and drug [F (1, 88) = 8.62, p = 0.004] and among sex, experience, and drug [F (1, 88) = 3.93, p = 0.050]. Males compared to experience- and drug-matched females revealed complex NAc dendritic branching in control saline-treated (p = 0.006) and PS AMPH-treated groups (p = 0.013). AMPH-treated control males compared females showed a marginal increase in branching (p = 0.068). PS compared to sex- and drug-matched controls increased the NAc dendritic branching in saline-treated males (p = 0.006) and females ($p < 0.001$). The effect of PS was marginal on AMPH-treated males (p = 0.067), however AMPH-treated females were not affected. Repeated AMPH administration in control females increased the NAc dendritic branching (p = 0.007). In contrast, a decrease was observed in AMPH-treated PS females (p = 0.035). Control males showed a marginal increase in branching associated with AMPH administration (p = 0.074) (Figure 6. 4).

When subjected to a three-way ANOVA (Sex X Experience X Drug), the NAc dendritic length revealed a main effect of sex [F (1, 88) = 14.37, $p < 0.001$], experience [F (1, 88) = 37.47, $p < 0.001$] and drug [F (1, 88) = 5.13, $p = 0.026$]. There was an interaction between sex and drug [F (1, 88) = 15.10, $p < 0.001$]. Pair-wise comparison revealed that compared to experience- and drug-matched females, males showed increased NAc dendritic length in control saline-treated ($p = 0.006$) and PS AMPH-treated groups ($p = 0.013$). PS compared to sex- and drug-matched controls increased the NAc dendritic length in saline-treated males ($p < 0.001$) and females ($p < 0.001$). The effect of PS was marginal on AMPH-treated males ($p = 0.066$), however AMPH-treated females were not affected. Repeated AMPH administration in controls increased the dendritic length in males ($p = 0.031$) and females ($p < 0.001$). However, PS experience prevented the AMPH-associated increased in NAc dendritic length (Figure 6. 4).

6.4.2. Maternal separation

6.4.2.1. Medial prefrontal cortex

The dendritic branching and length of the pyramidal neurons in the Cg3 region of the mPFC was increased as a result of MS experience. The increase was observed in basilar dendritic branching and length. However, the MS-associated dendritic growth was more robust in saline-treated rats. Similarly, repeated AMPH administration increased the basilar dendritic branching and length in controls. MS prevented the drug-induced increase in the dendritic growth observed in controls (Table 6.1 B & C).

When subjected to a two-way ANOVA (Experience X Drug), the total number of the Cg3 apical dendritic branches revealed no main effect of experience [F (1, 92) = 0.33,

$p = 0.568$] and a main effect of drug [F (1, 92) = 7.97, $p = 0.006$] with no interaction between the two [F (1, 92) = 0.15, $p = 0.702$]. In contrast, the Cg3 basilar branches revealed a main effect of experience [F (1, 92) = 13.68, $p < 0.001$], no main effect of drug [F (1, 92) = 1.65, $p = 0.202$], and an interaction between the two [F (1, 92) = 14.46, $p < 0.001$]. Pair-wise comparison showed that MS increased the basilar branching ($p < 0.001$) only in saline-treated rats. Similarly, repeated AMPH compared to saline administration in controls increased the apical ($p = 0.026$) and basilar dendritic branches ($p = 0.001$). However, MS prevented the drug-induced increase as no significant difference was observed between the saline- and AMPH-treated rats (Figure 6. 3).

A two-way ANOVA (Experience X Drug) of the Cg3 apical dendritic length revealed a main effect of experience [F (1, 92) = 4.42, $p = 0.038$] with no main effect of drug [F (1, 92) = 2.48, $p = 0.119$] nor an interaction between the two [F (1, 92) = 0.46, $p = 0.500$]. In contrast, the Cg3 basilar length revealed no main effect of experience [F (1, 92) = 3.10, $p = 0.082$] with a main effect of drug [F (1, 92) = 3.99, $p = 0.049$] and an interaction between the two [F (1, 92) = 6.07, $p = 0.016$]. MS increased the apical dendritic length only in AMPH-treated rats ($p = 0.05$) and basilar dendritic length only in saline-treated rats ($p = 0.004$). Repeated AMPH compared to saline administration in controls increased the basilar (but not apical) dendritic length ($p = 0.002$), which was prevented by MS experience (Figure 6. 3).

6.4.2.2. Orbital frontal cortex

An MS-associated increase was observed in the dendritic branching and length in the AID region of the OFC. However, similar to the mPFC, the effect was limited to the

saline-treated rats. Repeated AMPH administration decreased the dendritic length (but not branching) in the OFC but MS experience blocked this effect (Table 6.1 B & C).

A two-way ANOVA (Experience X Drug) of the total number of the AID apical dendritic branching revealed a main effect of experience [F (1, 92) = 7.31, p = 0.008] and drug [F (1, 92) = 4.19, p = 0.043] with no interaction between the two [F (1, 92) = 0.31, p = 0.579]. The AID basilar dendritic length revealed a main effect of experience [F (1, 92) = 20.96, $p < 0.001$] and drug [F (1, 92) = 4.39, p = 0.039] with no interaction between the two [F (1, 92) = 1.58, p = 0.211]. MS increased the dendritic length in both saline- and AMPH-treated rats and branching only in saline-treated rats (p = 0.023). Similarly, repeated AMPH compared to saline administration in controls increased the dendritic length ($p < 0.020$) with a marginal increase in branching (p = 0.06). However, MS prevented the drug-induced increase in AID dendritic branching and length (Figure 6. 3).

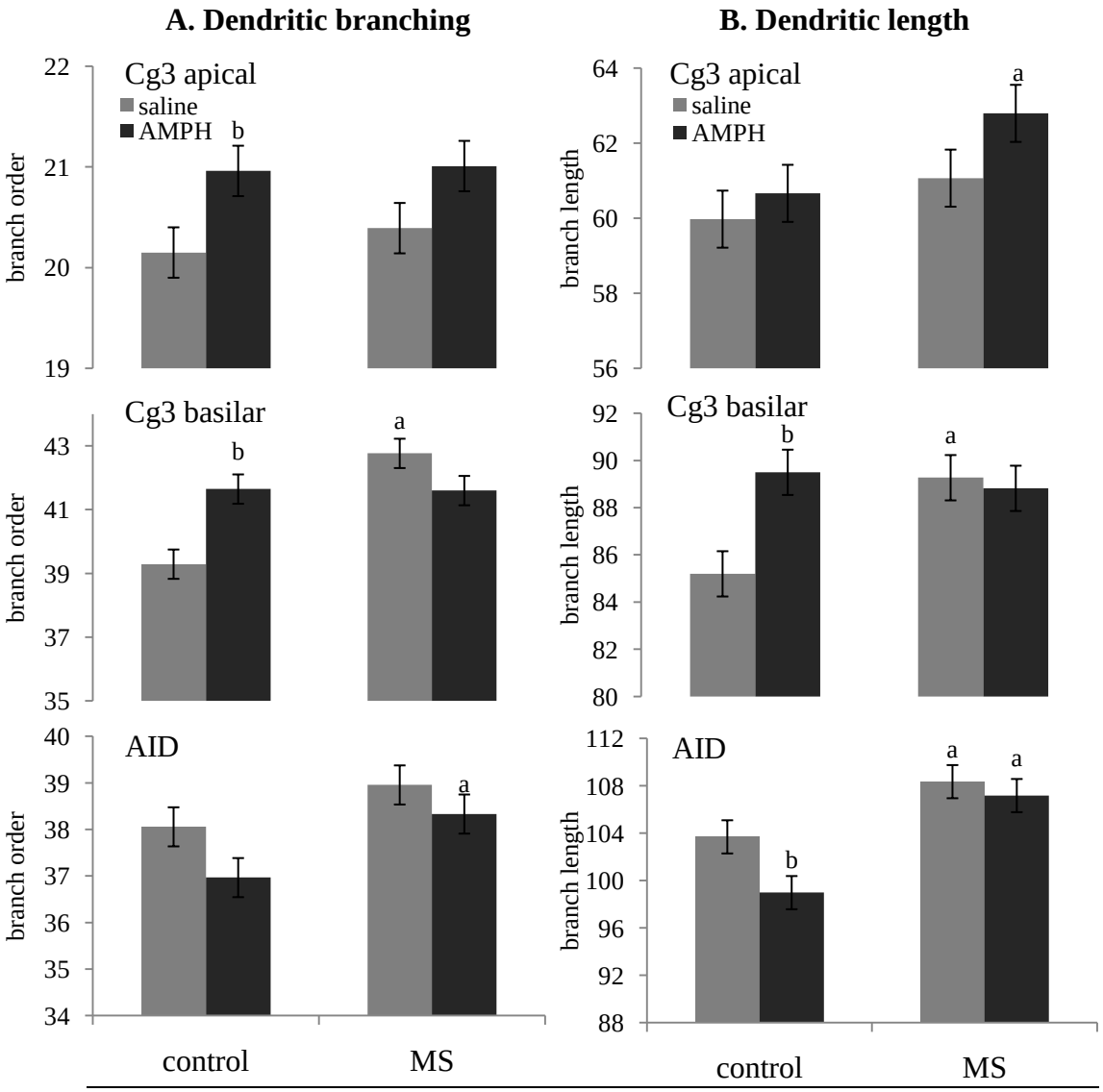

Figure 6.3. Mean (± SEM) total number of branch bifurcation (A. dendritic branching) and (B) dendritic length in the Cg3 region of the medial prefrontal cortex, and the AID region of the orbital frontal cortex. MS increased the dendritic growth in the Cg3 and the AID region ([a] all *p*s =< 0.05). Repeated AMPH administration increased the number of dendritic branches and length in the apical and basilar Cg3 with a decrease in the basilar AID region of the control rats ([b] all *p*s =< 0.05) which was prevented by the MS experience.

6.4.2.3. Nucleus accumbens

There was an effect of sex related to NAc dendritic branching and length. Males showed increased branching and length in saline-treated controls. MS increased the dendritic branching in AMPH-treated males whereas saline-treated females showed an increase in both branching and length. Repeated AMPH administration increased the NAc dendritic branching and length in controls, however MS prevented the drug-induced increase in dendritic growth (Table 6. 2 A, B, & C).

When subjected to a three-way ANOVA (Experience X Sex X Drug), the NAc dendritic branching revealed no main effect of experience [F (1, 88) = 0.10, p = 0.919] nor sex [F (1, 88) = 0.61, p = 0.438] with a main effect of drug [F (1, 88) = 5.07, p = 0.027]. There was an interaction between experience and sex [F (1, 88) = 14.56, p < 0.001] and experience and drug [F (1, 88) = 5.13, p = 0.026]. Males compared to experience- and drug-matched females revealed complex NAc dendritic branching in saline-treated controls (p = 0.007) with a marginal increase in saline-treated MS group (p = 0.06). MS compared to sex- and drug-matched controls decreased the NAc dendritic branching in AMPH-treated males (p = 0.009) whereas an increase was observed in saline-treated females (p = 0.001). Repeated AMPH administration in control females increased the NAc dendritic branching (p = 0.008) with a marginal increase observed in males (p = 0.07). However, MS prevented the drug-associated increase in dendritic branching observed in controls (Figure 6. 4).

When subjected to a three-way ANOVA (Experience X Sex X Drug), the NAc dendritic length revealed no main effect of experience [F (1, 88) = 1.36, p = 0.246] nor sex [F (1, 88) = 0.521, p = 0.472] with a main effect of drug [F (1, 88) = 11.21, p =

0.001]. There was an interaction between Experience and Sex [F (1, 88) = 8.22, p = 0.005] and Experience and Drug [F (1, 88) = 5.50, p = 0.021]. Pair-wise comparison revealed that males compared to experience- and drug-matched females showed increased NAc dendritic length in control saline-treated (p = 0.010). MS compared to sex- and drug-matched controls increased the NAc dendritic length only in saline-treated females ($p < 0.001$). Repeated AMPH administration in controls increased the dendritic length; however, MS experience prevented the drug-associated increase (Figure 6. 4).

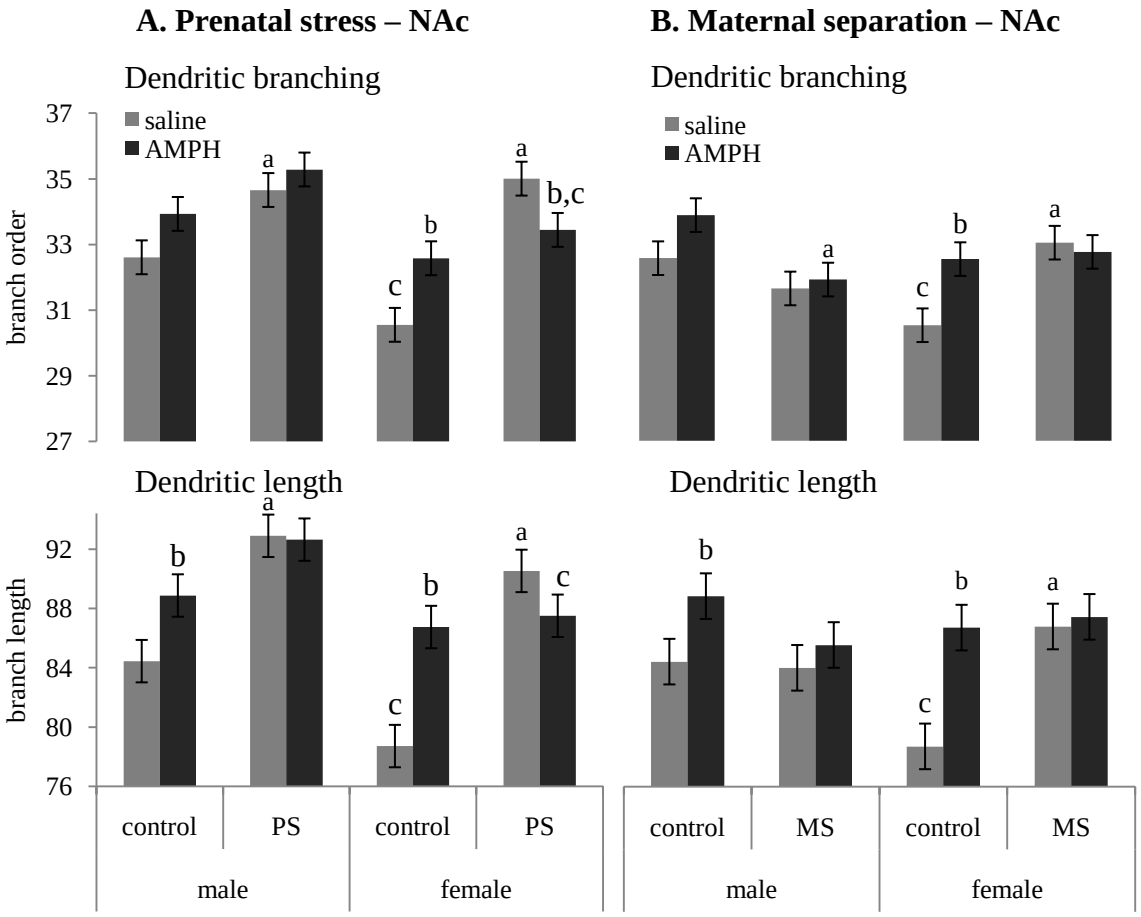

Figure 6.4. Mean (± SEM) total number of branch bifurcation (dendritic branching) and dendritic length in the nucleus accumbens of rats exposed to (A.) prenatal stress (PS) or (B.) maternal separation (MS). Males in saline-treated controls and AMPH-treated PS groups showed increased dendritic branching and length compared to experience- and drug-matched females ([c] all *p*s ≤ 0.05). PS increased the dendritic branching and length in saline-treated males and females ([a] p = 0.006 and < 0.001, respectively). Males compared to females in the saline-treated controls showed increased dendritic growth ([c] all *p*s ≤ 0.05). MS increased dendritic branching and length in saline-treated control females, however a decrease in branching was observed AMPH-treated males ([a] all *p*s =< 0.05). Repeated AMPH administration increased the dendritic growth in the NAc of control rats ([b] all *p*s =< 0.05) which was prevented by early stress experience.

6.5. Discussion

We investigated the effect of early stress (pre- versus postnatal) exposure followed by adult AMPH administration on the dendritic morphology (i.e. dendritic branching and length), two measures of structural plasticity, in cortical and subcortical regions (i.e. PFC and NAc). Previous reports indicated that exposure to either psychostimulants (i.e. cocaine, amphetamine, and nicotine) or environmental enrichment increase the dendritic growth (i.e. dendritic branching and length) and spine density in the NAc in adult rats. However, when drug administration was followed by environmental enrichment, prior drug exposure blocked the increase in dendritic measures associated with enriched environment (Hamilton & Kolb, 2005; Kolb, Gorny, et al., 2003). The present experiments studied the effect of early stress exposure followed by adult AMPH administration to investigate the neuroanatomical interaction of prior experience with subsequent drug exposure. Our findings suggest that the dendritic morphology was modulated by both early stress and AMPH administration in the NAc and the PFC. However, stress and AMPH administration prevented the drug-associated reorganization of the dendritic morphology, observed in the control rats.

The PS experience increased the dendritic branching and length on pyramidal neurons in the Cg3 region of the mPFC. The increase was observed in both apical and basilar branches. However, repeated AMPH administration blocked the increase in dendritic growth associated with PS experience (except for Cg3 apical dendritic length) as an increase was only observed in saline-treated rats. Contrary to the Cg3 regions, a PS-associated decrease in the dendritic branching and length was observed in the AID region. But similar to the Cg3, the decrease in dendritic growth was limited to saline-

treated rats (Table 6.1A). The dendritic morphology in the NAc was affected by sex where males showed increased branching in saline-treated control and AMPH-treated PS groups. However, PS increased the dendritic branching in the NAc of saline-treated rats regardless of sex (Table 6. 2A). The enhanced dendritic growth was also accompanied by an increase in the spine density, based on the previous report (Muhammad & Kolb, 2011b).

The increase in dendritic growth in the Cg3 region was associated with a decrease in the spine density, reported previously (Muhammad & Kolb, 2011b). It appears that the decrease in Cg3 spine density may have been compensated by an increase in the dendritic growth. This could be a possible explanation for the previous differential spine density findings in the NAc and the mPFC regions, where the spine density increased in the NAc and decreased in the Cg3 region (Muhammad & Kolb, 2011b). Previous reports indicated that the structural alterations always followed similar direction in both Cg3 and NAc. For instance, a change in the dendritic growth and or spine density, regardless of an increase or decrease, in one region was always correlated with a similar tendency in the other region (Robinson & Kolb, 2004). However, the present findings show that the two regions can show differential patterns of change. Dendritic growth did not complement the decrease in spine density in the mPFC regions. Instead the spine density decrease in the mPFC, in contrast to an increase in the NAc, was associated with enhanced dendritic growth.

Table 6.1. Summary of the dendritic branching and length findings in the apical and basilar Cg3 region of the medial prefrontal cortex and the AID region of the orbital frontal cortex

	Cg3A		Cg3B		AID	
	Branching	Length	Branching	Length	Branching	Length
A. PS						
Saline	↑	↑	↑	↑	↓	↓
AMPH	–	↑	–	–	–	–
B. MS						
Saline	–	–	↑	↑	–	↑
AMPH	–	↑	–	–	↑	↑
C. AMPH						
Control	↑	–	↑	↑	–	↓
PS	–	–	–	–	–	–
MS	–	–	–	–	–	–

The arrows show the direction of significant effect (all *p*s <= 0.05) whereas the hyphen '–' shows a non-significant effect for the dendritic branching and length in the (A) PS and (B) MS rats. The section C shows the effect of repeated amphetamine (AMPH) compared to saline administration in controls, PS, and MS rats.

Unlike PS where the dendritic growth followed opposite directions in the subregions of the PFC, MS experience resulted in a similar direction of increased

dendritic growth. It shows that the changes associated with pre- *vs*. postnatal stress were opposite in direction in the AID region. These studies indicate an experience-dependent differential neuroanatomical response in the subregions of the PFC depending on the stage of brain development. Comparable examples can also be found in previous reports where a similar experience before or after birth produced differential neuroanatomical changes. For instance, complex housing experience before birth decreased the dendritic length and increased the spine density (R. Gibb & B. Kolb, unpublished observations), however the reverse was true for the same experience at weaning (Kolb, Gibb, & Gorny, 2003).

Repeated AMPH administration increased the dendritic branching and length in the medium spiny neurons in the NAc shell region (Table 6.2B). Similarly, the dendritic growth was increased in the mPFC but an overall decrease was observed in the OFC region (Table 6. 1C). The dendritic morphology findings in the cortical and subcortical regions were consistent with the previous reports (reviewed in Robinson & Kolb, 2004). Interestingly, the drug-induced dendritic alteration in the NAc and subregions of the PFC was blocked by both PS and MS experiences. Previous reports also indicate that, for instance, maternal separation followed by chronic restraint stress blunted the subsequent stress-induced reduction in the CA3 apical dendritic length (Eiland & McEwen, 2010). Similarly, a study related to stress and drug interaction suggested that prior stress interfered with the cocaine-induced modulation of FGF-2 expression, a protein associated with neuronal plasticity, in the PFC (Fumagalli, et al., 2008).

Table 6.2. Summary of the dendritic branching and length findings in the shell region of the nucleus accumbens (NAc)

NAc	Branching		Length	
	male	female	male	female
A. PS				
Saline	↑	↑	↑	↑
AMPH	—	—	—	—
MS				
Saline	—	↑	—	↑
AMPH	↓	—	—	—
B. AMPH				
Control	—	↑	↑	↑
PS	—	↓	—	—
MS	—	—	—	—
C. Male	saline	AMPH	saline	AMPH
Control	↑	—	↑	—
PS	—	↑	—	↑
MS	—	—	—	—

The arrows show the direction of significant effect (all *p*s <= 0.05) whereas the hyphen '—' shows a non-significant effect for dendritic branching and length in the (A) PS and MS males and females compared to their respective drug- and sex-matched controls. (B) AMPH show the effect of repeated amphetamine compared to saline administration whereas (C) Males compared to females in controls, PS, and MS rats.

Our findings suggest that stress during development alters the cytoarchitecture (i.e. dendritic branching and length) of the cortical and subcortical regions. However, the nature of the differences is quite diverse depending on the sex, brain region, and stage of brain development. Similarly, repeated psychomotor stimulant administration reorganizes the brain circuitry through an alteration in the dendritic growth. However, early stress experience interacts with later psychomotor stimulant exposure in preventing the drug-associated cortical and subcortical modulation. One curious finding is that although prenatal stress lasted only five days it had larger effects on dendritic organization than maternal separation that lasted for 21 days. The two types of early stress had opposite interactions with later amphetamine exposure in the NAc and the OFC. In addition, whereas PS increased the effect of amphetamine on mPFC neurons, MS had very little effect on these neurons' structure. It is clear that the relationship between early stress experiences and later drug effects is complex indeed.

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CHAPTER 7

General Discussion

The effect of experience (i.e., TS or stress) during pre- or postnatal development was studied on juvenile behavior, adult AMPH-induced behavioral sensitization, and structural plasticity in the cortical and subcortical brain regions in rats. The findings show that early experience: 1) feminized social behavior in males; and 2) enhanced anxiety-like behavior was observed in males exposed to stress only. Repeated AMPH administration resulted in the development and persistence of behavioral sensitization regardless of early experience or sex. However, pre- and postnatal TS attenuated AMPH-induced behavioral sensitization in rats. Stress during development, on the other hand, failed to influence AMPH-induced behavioral sensitization (Table 7.1). Neuroanatomical results revealed an experience- and sex-dependent drug-induced structural alteration in the brain, where prefrontal cortical thickness was altered only in females and striatum size only in males (Table 7.2). Similarly, spine density and dendritic morphology in the nucleus accumbens and subregions of the prefrontal cortex (Table 7.3, 7.4) were altered depending on the pre- or postnatal stress experience, drug, or an interaction between the two. Repeated drug administration structurally altered cortical and subcortical regions whereas early experience, both TS and stress, prevented the drug-associated changes (Table 7.2, 7.3, & 7.4). I shall consider behavioral changes, drug-induced sensitization, and anatomical changes separately below.

Table 7.1. Summary of the findings for the juvenile behavior (A-D), amphetamine (E) and brain weight (F) for pre- and postnatal TS, PS, and MS

	Prenatal TS		Postnatal TS		Prenatal stress		Maternal separation	
	Male	Female	Male	Female	Male	Female	Male	Female
A. Play								
Attacks	↓	—	↓	—	—	—	↓	—
Complete rotation	—	—	↓	↓	—	—	—	↓
Evasion	—	—	↑	—	—	↑	—	—
Partial rotation	—	—	—	—	↑	—	—	↑
B. EPM								
Time in closed arms	—	—	—	—	↑	—	↑	—
C. NOR	—	—	—	—	—	—	—	—
D. Locomotor activity	—	↑	—	—	—	—	—	—
E. Amphetamine								
Acute administration	—	↓	↓	↓	—	—	—	—
Behavioral sensitization	↓	↓	↓	↓	—	—	—	—
F. Brain weight	↓	—	↓	—	↓	—	↓	—

The arrows show the direction of significant effect (all *p*s <= 0.05) whereas the hyphen '—' shows a non-significant effect.

7.1 Behavior

7.1.1. Play behavior

Rats engage in play behavior with increased frequency and duration during the juvenile period, between postnatal days 30 to 40, compared to adults (Thor & Holloway,

1984). The play behavior is comprised of a series of playful attacks, generally targeting the nape of the neck. The attack is faced by the pair mate, either as complete or partial rotation defense. However, sometimes the rat, instead of facing, evades the attack (Pellis & Pellis, 1990). In addition, the features of play behavior are different in juvenile and adult rats. For example, attack at nape seen in juveniles signifies play behavior as opposed to aggression (e.g., attack at the back) exhibited by adults. Similarly, the patterns of defense in response to an attack are also tailored to the developmental stage where juveniles exhibit complete rotation defense more frequently (Pellis & Pellis, 1997). The complete rotation defense keeps up the momentum of the play by encouraging the partner to engage in a similar reciprocal social interaction. In addition, juvenile play fighting is sexually dimorphic where males initiate playful attacks more frequently than females (Pellis & Pellis, 1990).

The play behavior in our study, scored as the frequency of attacks and the probability of either complete rotation defense (i.e., supine posture) or evasion, was modulated by early experience in a sex-dependent manner. We observed a sex difference in the frequency of playful attacks in control rats with males engaged more frequently in the play (Pellis & Pellis, 1990). However, the frequency of playful attacks was significantly reduced by early experience in males with a resultant feminization of play behavior. In contrast, the frequency of playful attacks was not influenced by early experience in females. Previous reports also point to experience-dependent modulation of play mostly in males with less robust findings in females. Previous studies in rats, exposed to either stress or stimulation (i.e., handling), indicate that regardless of an adverse or favorable experience, males exhibited female-like play behavior with no effect

of the experience on females (Arnold & Siviy, 2002; Meaney, Stewart, & Beatty, 1982; Morley-Fletcher, et al., 2003; Parent & Meaney, 2008).

Rats respond to a play attack either by facing an attack (e.g., complete rotation defense) or evasion by moving away from the attacker. The probability of either complete rotation or evasion reflects the level of interest in social interaction. Whereas juvenile rats generally respond to an attack by adapting a supine position (i.e., complete rotation defense) to keep the play bouts going, the evasion usually reveals a lack of interest in play behavior. Our findings suggest that the probability of complete rotation defense was significantly modulated by postnatal TS and stress (i.e., MS) experience. Postnatal TS experience reduced the probability of complete rotation defense in both sexes whereas only MS females exhibited a decrease. In contrast to our findings, Veenema and Neumann (2009) reported augmented aggression in maternally separated juvenile male rats. But they employed a different play paradigm where rats were exposed to an unknown play partner in their home cage. The context (e.g., home cage *vs.* play box) could potentially alter the social behavior.

The modulation of play behavior (e.g., through altered frequency of playful attacks or defense strategy), by early experiences points to the reorganization of brain structurally and functionally. For example, the decreased frequency of play attacks and complete rotation defense coupled with enhanced evasions in the postnatal TS group shows that the play is less rewarding for the rats. The lack of interest in play could possibly be the result of the reorganization of the reward system (e.g., the PFC and/or NAc). Our findings of the attenuated AMPH sensitization and lack of interest in exposure to novelty by postnatal TS rats supports this idea. In addition, previous studies reported

that modifications of social behavior play an important role in the reorganization of brain architecture. For example, Bell, Pellis, & Kolb (2010) showed that the structure of pyramidal neurons in the medial and orbital prefrontal regions, implicated in reward processing, is significantly modified by manipulating the play behavior. Thus, the modifications in play behavior in the current studies could have contributed to the structural differences observed in adulthood (see below).

7.1.2. Elevated plus maze

The anxiety-like behavior, tested in elevated plus maze, was substantially influenced by stress during development in males with no effect on females. In contrast, tactile stimulation did not modulate anxiety-like behavior. Stress during development (i.e., PS and MS) produced enhanced anxiety-like behavior in males indicated by more time spent in the closed arms of the maze. Wigger and Neumann (1999) also reported similar sex-dependent differences in emotional behavior, where male compared to female rats were more affected by maternal separation. The findings for the females are in accordance with Zargon and Weinstock (2006), although they failed to find an effect of prenatal restraint stress on anxiety-like behavior in males.

Furthermore, TS, either pre- or postnatal, did not influence emotionality in rats (Cirulli, et al., 2010). The findings for TS females were not in accordance with Maruoka et al., (2009) who reported reduced anxiety-like behavior in mice raised in an enriched environment. The possible reason for such inconsistency could be the differences between stimulation paradigm (i.e., TS vs. enriched environment) in addition to other procedural differences including experimental animal species and age at the time of both

the experience and the testing. Age has been shown as one of the modulating factor, as the beneficial effects of stimulation were more observable at later age (Meaney, et al., 1988), which is also supported by attenuated AMPH-induced behavioral sensitization observed in our TS rats (Table 7.1).

7.1.3. Novel object recognition

Object temporal order memory was tested in the recency version of NOR. During the test trial rats were allowed to explore previously explored objects one each from trial 1 (i.e., old familiar) and trial 2 (i.e., recent familiar). Normally, rats remember the temporal order of objects explored and tend to spend more time exploring the old familiar object. Our findings suggest that the cognitive performance was not influenced by early experience. Previous studies related to the effect of early experience on the cognitive performance indicated mixed findings (reviewed by Weinstock, 2008). For example, prenatal stress during the last week of gestation (Lemaire, et al., 2000) or maternal separation (Aisa, et al., 2007) in rats resulted in impaired spatial memory. In contrast, others reported no impairment (Vallee, et al., 1997) or even improved learning associated with mild prenatal stress (Fujioka et al., 2001). The possible reasons for such discrepancy might be related to procedural differences such as intensity and duration of the stress procedure or the cognition testing paradigm. For instance, intense restraint stress for 45-minutes during the last week of gestation impaired (Lemaire, et al., 2000), whereas mild prenatal stress for one day improved, learning and memory (Fujioka, et al., 2001).

7.1.4. Open field locomotion

As reported by many others, exploratory behavior, tested as open field locomotion, was not significantly modulated by early TS or stress experience in either males or females with the exception of prenatal TS females (Brake, et al., 2004; Caldji, et al., 2000; Lee, et al., 2007; Li, Robinson, et al., 2003; Marmendal, et al., 2004; Zimmerberg & Shartrand, 1992). The exploratory behavior exhibited by prenatal TS female group was in accordance with Maruoka et al., (2009) where prenatal environmental enrichment increased exploratory behavior only in females without affecting males. The exploration, partly mediated by dopamine, might not be substantially affected by early stimulation or stress as previous work has shown that, for example, neonatal handling or MS experience in rats did not alter dopamine activity in the midbrain (Madruga, Xavier, Achaval, Sanvitto, & Lucion, 2006) or dopamine transporter in the nucleus accumbens region (Brake, et al., 2004). Furthermore, the possibility of insignificant modulation of exploratory behavior could be the nature of the tasks itself, for example handled rats perform well in tasks involving conflicting situations as compared to 'simpler' tasks (e.g., exploration) (Nunez, et al., 1996).

7.2. Amphetamine sensitization

The long-term influence of early experience on drug-induced behavioral sensitization was investigated by repeated AMPH administration. Repeated administration of psychostimulant drugs (e.g., cocaine, nicotine, and AMPH) results in the development of behavioral sensitization (Kalivas & Stewart, 1991; Robinson & Becker, 1982) that persists beyond drug exposure for several months in rodents (Kolb,

Gorny, et al., 2003). The dose, duration, and route of AMPH in our study were selected based on previous reports that indicated the development and persistence of behavioral sensitization with the similar dose and route (Mattson, et al., 2007; Singer, et al., 2009).

Chronic AMPH administration for two weeks in our studies resulted in the development of behavioral sensitization, measured as locomotor activity, regardless of sex or early experience. Behavioral sensitization also persisted, assessed by AMPH challenge injection, in AMPH-treated rats at least for two weeks. TS experience during development however, resulted in attenuated AMPH sensitization in both male and female groups compared to sex-matched controls. Similar to the development of sensitization, TS rats exhibited diminished locomotor response to APMH challenge two weeks after the last drug injection. Similarly, Lovic, Fleming, and Fletcher (2006) reported the attenuation of AMPH-induced sensitization by tactile stimulation provided to artificially reared rats that were both mother and social deprived. The increased locomotor activity in response to AMPH administration exhibited by artificially reared rats was reversed by TS. Sensory stimulation experience during development has a favorable outcome related to stimulant-induced sensitization. For instance, previous studies have shown that handling (Campbell & Spear, 1999), maternal licking and grooming (Francis & Kuhar, 2008), and environmental enrichment (Bardo, et al., 1995; Bardo, et al., 2001) resulted in attenuated sensitization response to AMPH. The attenuated drug-induced sensitization, reported in the literature, has been observed across all drug abuse models including self-administration, conditioned place preference, and behavioral sensitization. Similar to rodents, enrichment in human (e.g., exercise) and

non-human primates showed a similar tendency of attenuated stimulant sensitization (Morgan, et al., 2002; Ussher, et al., 2000).

Similar to early stimulation, PS and MS in rats resulted in the development and persistence of AMPH-induced behavioral sensitization. In contrast, we failed to find an influence of developmental stress on acute locomotor response (Henry, et al., 1995) or on the development of drug-induced behavioral sensitization (Weiss, et al., 2001). Similarly, Van Waes et al. (2010) also reported that PS did not affect spontaneous preference and motivation for alcohol consumption. Previous studies related to the influence of developmental stress on drug-induced behavioral sensitization in rodents indicated mixed findings. For example, there are reports of attenuated sensitization in response to cocaine administration in MS rats (Li, Robinson, et al., 2003; Matthews, et al., 1996). In contrast, early stress enhanced AMPH-induced behavioral sensitization (Deminiere, et al., 1992; Henry, et al., 1995; Rots, et al., 1996), as well as cocaine and AMPH self-administration (Deminiere, et al., 1992; Kikusui, et al., 2005; Kippin, et al., 2008). The augmented drug response reported might be, in addition to the severity and frequency, related to possible factors such as dose of the drug administered. For instance, Matthew, Rossins, Everitt, and Caine (1999) reported retarded drug acquisition in MS rats for lower cocaine doses compared to higher doses. Age could be another possible factor as the effect of MS is more obvious on adolescent compared to adult rats (Marin & Planeta, 2004). The pronounced effect of MS during early age is also supported by the behavioral findings in our study. Thermoregulation during MS has also been shown to play a significant role as Zimmerberg and Shartrand (1992) reported that rats maternally separated at 20^{o}C exhibited enhanced behavioral sensitization compared to rats separated at 34^{o}C.

Furthermore, there was no difference between rats separated at 34^{o}C and controls (mother reared) in terms of AMPH-induced behavioral sensitization (Zimmerberg & Shartrand, 1992).

Several factors play a contributory role in the vulnerability to drug self-administration, and drug-induced behavioral sensitization. The possible reasons for attenuated AMPH sensitization exhibited by TS rats could be the result of alterations in several interlinked molecular processes in the brain (i.e., regulation of dopamine and growth factors, and modulation of HPA axis). For example, glucocorticoid levels have been positively correlated with enhanced stimulant-induced sensitization and self-administration (Marinelli et al., 1994; Piazza et al., 1996). Stimulation during development, for example maternal licking and grooming, has been shown to upregulate glucocorticoid receptors in the hippocampus and prefrontal cortex. These regions are involved in the negative feedback mechanism of HPA-axis to help shut down the stress reaction (Meaney, et al., 1985). The higher receptor density might be one of the factors directly or indirectly contributing to attenuated AMPH-induced behavioral sensitization of TS rats. In contrast, studies related to the effect of MS on the levels of glucocorticoids reported no significant MS-induced alteration (Marmendal, et al., 2004).

Similarly, the mesocorticolimbic dopamine system (including receptor type and transporter) plays a modulatory role in drug-induced sensitization as well as self-administration (Hooks & Kalivas, 1995). For example, dopamine release in the mesolimbic circuit plays a major role in drug abuse (Hooks & Kalivas, 1995), in addition to modulating locomotor activity, an index of behavioral sensitization (Koob, et al., 1981). Nevertheless to mediate its effect, dopamine relies on other related molecules such

as the dopamine transporter and the D1- and D2-like receptors. Stimulation (e.g., environmental enrichment) during development, has been reported to attenuate locomotor activity, an effect mediated by down regulation of dopamine transporters (Bezard, et al., 2003). However, MS in rats has no substantial effect on the levels of dopamine molecules in the ventral tegmental area (Madruga, et al., 2006).

Brain derived neurotrophic factor (BDNF) plays a role in the growth and survival of neuronal populations and drug-associated structural plasticity in the brain reward system (reviewed by Russo, Mazei-Robison, Ables, & Nestler, 2009). In addition, higher levels of BDNF in the hippocampus act as an antidepressant (Shirayama, Chen, Nakagawa, Russell, & Duman, 2002). Interestingly, MS upregulated, whereas handling did not alter, the expression of the BDNF molecule in the hippocampus (Cirulli, et al., 2010; Greisen, Altar, Bolwig, Whitehead, & Wortwein, 2005). Whereas depression and drug addiction frequently co-occur (Gilman & Abraham, 2001), the possible antidepressant effect of MS via up regulation of the BDNF might account for some of the compensatory mechanisms against enhanced drug-induced behavioral sensitization in rats.

7.3. Anatomy

Experience (e.g., environmental enrichment) reorganizes brain regions structurally, similar to psychostimulants, by increasing dendritic growth and spine density, in various brain regions. However, prior psychostimulant administration followed by rearing in an enriched environment results in preventing the structural plasticity associated with enrichment (Hamilton & Kolb, 2005; Kolb, Gorny, et al., 2003). Based on the interaction

of drug with enrichment experience, we wondered how an experience (i.e., TS or stress) during development followed by drug (i.e., AMPH) administration would modulate the neuroanatomy of the cortical and subcortical regions. We measured the prefrontal cortical thickness and striatum size in TS rats. The spine density and dendritic morphology in the nucleus accumbens and subregions of the PFC was examined in rats exposed to stress during development (i.e., PS and MS).

Table 7.2. Summary of the prefrontal cortical thickness findings in the medial (Cg3) and orbital (AID) regions and striatum size in the anterior and posterior regions

	Prefrontal cortex				**Striatum**			
	Medial (Cg3)		Orbital (AID)		Anterior		Posterior	
	Male	Female	Male	Female	Male	Female	Male	Female
A. Prenatal TS								
Saline	–	–	–	↓	↑	↑	↑	–
AMPH	–	↑	–	↑	↑	↑	–	↑
B. Postnatal TS								
Saline	–	–	–	–	↑	–	–	–
AMPH	–	↑	–	↑	↑	–	–	–
C. AMPH								
Control	–	↓	–	↓	–	–	↑	↑
Prenatal TS	–	–	–	–	–	–	–	–
Postnatal TS	–	–	–	↑	–	–	–	–

The arrows show the direction of a significant effect (all *p*s <= 0.05) whereas the hyphen '–' shows a non-significant effect for the (A) prenatal TS and (B) postnatal TS males and females compared to the respective drug- and sex-matched controls. Section (C) AMPH shows the effect of repeated amphetamine compared to saline administration in controls, pre- and postnatal TS rats.

7.3.1. Prefrontal cortical thickness

The prefrontal cortical thickness measures suggest that early TS experience, AMPH, and an interaction between the two altered the thickness of the PFC subregions in a sex-dependent manner. Whereas AMPH reduced the prefrontal cortical thickness in control females, postnatal TS mitigated the drug-induced reduction in thickness. However, we did not observe any substantial alteration in prefrontal cortical thickness in males, which is consistent with a study of frontal lobe volume in human male addicts and controls (Bartzokis, et al., 2000).

Cg 1 and Cg 3, regions of anterior cingulate cortex, form part of the medial PFC whereas AID, a region of the insular cortex, forms part of the OFC in rats. The role of the PFC subregions (i.e., medial and OFC) is well established in drug addiction in experimental animals (reviewed by Porrino & Lyons, 2000) and humans (reviewed by Volkow, et al., 2003). In addition to drug-induced modulation in the functions of the PFC region, structural alteration (e.g., spine density) has also been reported in rodents (Robinson & Kolb, 2004) and monkeys (Selemon, et al., 2007). We are unaware of any published study that investigated drug-induced alteration in the prefrontal cortical thickness in rodents or in monkeys. However, human imaging studies reported structural alteration in the frontal lobe associated with drug abuse (Kim, et al., 2006).

Our study suggests that the prefrontal cortical thickness in both medial and orbital subregions was substantially altered by TS, AMPH exposure, and/or the interaction between the two in females. Daily AMPH administration for two weeks resulted in a reduction in cortical thickness in both mPFC and OFC subregions in the control group. Our findings support the studies related to drug-induced structural alteration in humans.

Imaging studies in patients with a history of AMPH or methamphetamine abuse reported a decrease in cortical gray matter density or volume in the anterior prefrontal cortex (Kim, et al., 2006; Schwartz, et al., 2010) and medial orbital cortex (Tanabe, et al., 2009). Furthermore, structural abnormalities in the frontal lobe after AMPH or methamphetamine abuse were correlated with impaired cognitive performance (e.g., Wisconsin Card Sorting Test) (Kim, et al., 2006; Rogers, et al., 1999; Schwartz, et al., 2010). Similar to drug addiction, other PFC-related neurological disorders such as schizophrenia also resulted in reduced cortical thickness of the frontal region (Kuperberg, et al., 2003; Rimol, et al., 2010).

While AMPH exposure decreased the prefrontal cortical thickness in the control female group, both pre- and postnatal TS experience prevented the thinner cortex effect as there was no difference between the saline- and AMPH-treated TS group. Furthermore, there was an interaction between early experience and later drug exposure. Whereas TS in the saline group did not alter the thickness, AMPH administration increased the prefrontal cortical thickness. Similar to our findings, environmental enrichment ameliorated the decrease in cortical thickness resulting from neonatal frontal cortical injury (Comeau, et al., 2008) or early social isolation stress (Hellemans, et al., 2004). The drug-induced reduction in the prefrontal cortical thickness and the alleviation by TS needs further investigation, for instance at immunohistological levels, as the findings may not necessarily reflect an increase in neuropil but could be due to an increase in the glial population. Epigenetic investigation could also be helpful to examine the experience-dependent gene expression profile.

7.3.2. Striatum size

The striatum is divided into dorsal (i.e., caudate nucleus and putamen) and ventral (i.e., nucleus accumbens) regions and both the dorsal and ventral striatum have been implicated in drug addiction in experimental animals (Gerdeman, et al., 2003; Ito, et al., 2002; Li, Kolb, et al., 2003). Similarly, human imaging studies also confirmed the involvement of the striatum in addiction (Volkow, et al., 2003).

Our findings suggest the influence of early experience or drug was robust only in males. TS but not AMPH administration structurally altered the anterior striatum. In contrast, the posterior striatum was modified in the control group by AMPH administration but not by early experience. Postnatal TS in males resulted in an enlarged striatum in both saline- and AMPH-treated groups. Conversely, the posterior striatum became larger as result of AMPH administration in male controls, and postnatal TS experience prevented the enlarged striatal effect. The findings for male rats are consistent with the imaging reports of human amphetamine abusers (Chang, et al., 2005; Jernigan, et al., 2005). Interestingly, Jernigan et al., (2005), similar to our findings in males, failed to find an effect of methamphetamine on cerebral cortical thickness. Similarly an increase in striatal volume has also been reported in cocaine abusers (Jacobsen, et al., 2001). The enlarged striatum could be the result of enhanced dopamine release in the striatum observed with repeated AMPH administration (Robinson & Becker, 1982).

There is a sex-specific dissociation between prefrontal cortical thickness and striatum size in our study. We found an AMPH-induced decrease in cortical thickness in control female rats that was prevented by postnatal TS whereas there was no effect of experience or drug on the striatum. In contrast to females, AMPH administration in males

enlarged the posterior striatum in controls and this was prevented by TS. Furthermore, TS in males enlarged the anterior striatum in both saline- and AMPH-treated groups. However, there was no influence of early experience or drug on the prefrontal cortical thickness in males. The sex-dependent cortical and subcortical structural alteration could be related, for instance, to differential epigenetic modulation (e.g., of ERα promoter region) in male and female rats associated with somatosensory stimulation during early brain development (reviewed by McCarthy, et al., 2009). In addition, TS during development might modulate key molecules (e.g., MeCP2) in the brain that are expressed differentially in both sexes and are linked with neuronal plasticity. MeCP2 was not only associated with structural alterations in the brain (Zhou, et al., 2006) but also modulated AMPH-induced conditioned place preference (Deng, et al., 2010). Interestingly, MeCP2 alteration in the brain reorganized juvenile play behavior in a sex-dependent manner such that males showed female-like play, similar to our finding (Kurian, et al., 2008).

The areal difference could be related to different neuronal population in the PFC and the striatum. Pyramidal cells represent the majority of the neuronal population in the PFC, whereas the striatum has medium spiny neurons in abundance. However, the opposite results for both sexes add to the complexity of the experience and drug interaction. Previous reports indicated sex-specific structural differences with repeated drug abuse in humans. For example, Chang et al. (2005) reported enlargement of the posterior corpus callosum in female methamphetamine abusers but not in males. Likewise, a study related to methamphetamine-induced dopamine depletion in the striatum reported that female compared to male mice demonstrated less depletion (Dluzen, et al., 2003). The sex-specific structural alteration could be related to gonadal

hormones. Similar to psychostimulants (Robinson & Kolb, 2004), stress exposure resulted in impaired functions and altered dendritic organization in the PFC (Liston, et al., 2006), although in this case there was a reversed effect, namely increased dendritic length in OFC and decreased length in mPFC. Previous reports indicated that females exhibited enhanced sensitivity to stress-induced PFC dysfunction that was dependent on high levels of estrogens (Shansky, et al., 2003). Likewise, stress (Riva, et al., 1995) and psychostimulants (e.g., cocaine) (Fumagalli, et al., 2006) modulated FGF-2 expression in various brain regions. Stress exposure, however, interacted with cocaine administration in region-dependent modulation of FGF-2 in the PFC and the striatum (Fumagalli, et al., 2008).

7.3.3. Spine density and dendritic morphology

Spines, small projections on the dendrites, are the excitatory contact points between pre- and postsynaptic neurons. Similarly, the dendritic morphology (i.e., branching and length) reflects the organization of brain connectivity. The longer and more branchy the dendrites are, the more space there is for synaptic contacts. Any experience-associated change in the spine density and dendritic morphology reflects alteration in the synaptic inputs, which could potentially alter the computational capacity, thus modifying the functional output of the region. In addition to experience-dependent modulation, the structural alteration has been reported with normal physiological phenomena (e.g., aging and hormonal variation) as well as in pathological conditions (e.g., schizophrenia) (Garey et al., 1998; Kolb & Stewart, 1991; Markham & Juraska, 2002; Prange-Kiel, Fester, Zhou, Jarry, & Rune, 2009). The spine density and dendritic morphology thus make ideal

measures to study the long term structural plasticity associated with experience or an interaction of experiential factors (Alvarez & Sabatini, 2007; Bhatt, Zhang, & Gan, 2009; Kolb, Gorny, et al., 2003). We assessed the spine density and dendritic morphology in the shell regions of the NAc and the Cg3 (layer III) region of mPFC and the AID (layer III) of the OFC, regions known to be reorganized structurally in response to both experience and drug.

Table 7.3. Summary of the spine density, dendritic branching and length findings in the apical and basilar Cg3 region of the medial prefrontal cortex and the AID region of the orbital frontal cortex

	NAc	**Cg3A**			**Cg3B**			**AID**		
	Spines	Spines	Branch	Length	Spines	Branch	Length	Spines	Branch	Length
A. PS										
Saline	↑	↓	↑	↑	↓	↑	↑	–	↓	↓
AMPH	↑	↓	–	↑	↓	–	–	–	–	–
B. MS										
Saline	↑	↑	–	–	–	↑	↑	↑	–	↑
AMPH	↑	↑	–	↑	–	–	–	↑	↑	↑
C. AMPH										
Control	↑	↑	↑	–	↑	↑	↑	↓	–	↓
PS	–	–	–	–	–	–	–	–	–	–
MS	–	–	–	–	–	–	–	–	–	–

The arrows show the direction of significant effect (all *p*s <= 0.05) whereas the hyphen '–' shows a non-significant effect for the dendritic branching and length in the (A) PS and (B) MS rats. The (C) AMPH shows the effect of repeated amphetamine compared to saline administration in controls, PS, and MS rats.

Table 7.4. Summary of the dendritic branching and length findings in the shell region of the nucleus accumbens (NAc)

NAc	Branching		Length	
	male	female	male	female
A. PS				
Saline	↑	↑	↑	↑
AMPH	–	–	–	–
MS				
Saline	–	↑	–	↑
AMPH	↓	–	–	–
B. AMPH				
Control	–	↑	↑	↑
PS	–	↓	–	–
MS	–	–	–	–
C. Male	saline	AMPH	saline	AMPH
Control	↑	–	↑	–
PS	–	↑	–	↑
MS	–	–	–	–

The arrows show the direction of significant effect (all *p*s <= 0.05) whereas the hyphen '–' shows a non-significant effect for dendritic branching and length in the (A) PS and MS males and females compared to their respective drug- and sex-matched controls. (B) AMPH shows the effect of repeated amphetamine compared to saline administration whereas (C) Males compared to females in controls, PS, and MS rats.

Our findings suggest that stress during development (i.e., PS and MS) increased the spine density in the NAc regardless of sex and drug administered (Table 7.3). The dendritic morphology in the NAc was affected by the sex where males showed increased branching in saline-treated control and AMPH-treated PS (but not MS) groups. However, PS increased the dendritic branching in the NAc of saline-treated rats regardless of sex (Table 7.4). There is very limited published research related to early experience-dependent structural alteration in the nucleus accumbens. The only study available, to our knowledge, regarding the influence of PS on the spine density in the NAc, in contrast to our findings, reported a decrease in the spine density in Sprague-Dawley male rats (Martínez-Téllez, et al., 2009). In addition to other procedural differences, the severity of stress in terms of the duration, frequency, and paradigm, could be a possible factor in the reported discrepancy.

The stress experience during development increased the dendritic branching and length on pyramidal neurons in the Cg3 region of the mPFC. The increase was observed in both apical and basilar branches in PS and mostly in the basilar branches in MS rats. However, repeated AMPH administration blocked the increase in dendritic growth associated with the stress (except for Cg3 apical dendritic length) as an increase was only observed in saline-treated rats. Contrary to the Cg3 regions, a PS-associated decrease in the dendritic branching and length was observed in the AID region. But similar to the Cg3, the decrease in dendritic growth was limited to saline-treated rats. In contrast to PS though, an overall increase in the AID dendritic morphology was observed in rats exposed to MS (Table 7.3).

The PS-associated differential response in the subregions of the PFC (i.e., mPFC and OFC) is interesting. Previous studies also reported structural alteration in the opposite direction in response to psychostimulant or opiate drug exposure (Robinson & Kolb, 2004) and chronic adult stress (Liston, et al., 2006). Interestingly, in contrast to ours, Liston et al., (2006) reported a stress-dependent decrease in the mPFC dendritic growth and an increase in the OFC region. However, they exposed *adult* rats to a severe restraint stress in their experiment. Similar to our prenatal experience manipulation, exposing rats to ethanol during prenatal brain development increased the dendritic length in the mPFC with a decrease in the OFC in rats as adults (Hamilton et al., 2010).

In response to MS experience and subsequent AMPH administration, an enhanced dendritic growth was observed in the Cg3 as well as the AID region (Table 7. 3). Previous studies reported dendritic remodeling depending on factors such as the duration and separation procedure (e.g., separation *vs.* isolation). For instance, Bock et al. (2005) reported an increase in basilar dendritic length in the mPFC in rats maternally separated during P5-7 or 14-16 whereas a decrease in apical length was observed in rats separated during P1-3. Similarly, a decrease in dendritic length in the Cg1 region of the mPFC was observed in MS rats individually isolated from the littermates (Monroy, et al., 2010).

Unlike PS where the dendritic growth was followed by an opposite changes in the subregions of the PFC, MS experience resulted in a similar direction of increased dendritic growth in both regions. These data thus show that the changes associated with pre- *vs*. postnatal stress were opposite in direction in the AID region. These studies indicate an experience-dependent differential neuroanatomical response in the subregions of the PFC depending on the stage of brain development. Comparable examples can also

be found in previous reports where a similar experience before or after birth produced differential neuroanatomical changes. For instance, complex housing experience before birth decreased the dendritic length and increased the spine density (R. Gibb & B. Kolb, unpublished observations) whereas the reverse was true for the same experience at weaning (Kolb, Gibb, et al., 2003).

Repeated AMPH administration increased the dendritic branching and length in the medium spiny neurons in the NAc shell region (Table 7. 4). Similarly, the dendritic growth was increased in the mPFC whereas an overall decrease was observed in the OFC region (Table 7. 3). The dendritic morphology findings in the cortical and subcortical regions were consistent with the previous reports (reviewed in Robinson & Kolb, 2004). Interestingly, the drug-induced dendritic alteration in the NAc and subregions of the PFC was blocked by both PS and MS experiences. This is in accordance with the previous Kolb lab findings where prior drug exposure prevented the subsequent experience-associated neuroanatomical changes. Previous reports also indicate that, for instance, maternal separation followed by chronic restraint stress blunted the subsequent stress-induced reduction in the CA3 apical dendritic length (Eiland & McEwen, 2010). Similarly, a study related to stress and drug interaction suggested that prior stress interfered with the cocaine-induced modulation of FGF-2 expression, a protein associated with neuronal plasticity, in the PFC (Fumagalli, et al., 2008).

Our findings suggest that stress during development alters the cytoarchitecture (i.e., dendritic branching and length) of the cortical and subcortical regions. However, the nature of differences is quite diverse depending on the sex, brain region, and stage of brain development. Similarly, repeated psychostimulant administration reorganizes the

brain morphology through alteration in the dendritic growth. However, early stress experience interacts with later psychostimulant exposure in preventing the drug-associated cortical and subcortical modulation. One curious finding is that prenatal stress that lasted only five days had larger effects on dendritic organization than maternal separation that lasted for 21 days. The two types of early stress had opposite interactions with later amphetamine exposure in the NAc and the OFC. In addition, whereas PS increased the effect of amphetamine on mPFC neurons, MS had very little effect on these neurons' structure. It is clear that the relationship between early stress experiences and later drug effects is complex indeed.

7.4. Conclusion

Based on the findings it might be concluded that experience during development modulated juvenile behavior involving conflicting situations (e.g., social behavior) as compared to 'simpler' tasks (e.g., exploration). The behavioral modulation was sex-dependent with more robust findings in males. In general, experience during development resulted in the feminization of play behavior while stress enhanced anxiety-like behavior in males. Repeated AMPH administration resulted in the development of behavioral sensitization and a challenge after a withdrawal period showed the persistence of sensitization in rats regardless of sex and early experience. Remarkably, TS during development resulted in a favorable outcome of attenuated drug-induced behavioral sensitization. However, stress during development failed to influence AMPH-induced behavioral sensitization.

Synaptic plasticity was studied at the structural level in the cortical and subcortical regions implicated in drug addiction. Early experience followed by drug exposure produced structural alteration in NAc (and striatum) and subregions of the prefrontal cortex. However, early experience prevented the changes associated with later drug exposure in both cortical and subcortical regions. It seems that there is a limit on the magnitude of structural change in the brain. Consequently the subsequent experience of drug administration failed to alter the structural changes associated with prior experience of TS or stress.

The present dissertations highlighted the role of a favorable or an adverse experience, before and soon after birth, in modulating the subsequent response to drug-induced behavioral sensitization and associated anatomical changes in the brain. The attenuated behavioral sensitization as a result of early favorable experience (i.e., TS) might be related to the interference with drug-induced structural reorganization of the PFC and the striatum and therefore, may play a protective role against stimulant-induced behavioral sensitization.

The present findings have practical implication for child development practices. Demonstrating the favorable role of TS, suggests that it may underpin the importance of parenting in a child upbringing. The findings show that parenting styles needs to adapt to provide more interaction with the child where touch and sensory stimulation should carry more weight, in addition to regular childcare. Based on the outcome of the dissertation, public policies need to accommodate early childhood intervention to eradicate, or at least reduce, later drug addiction, which is hard to treat owing to the relatively less plasticity in

adult brain. The favorable outcomes of the tactile stimulation could also be extended to other neurological disorders (e.g., autism) that have roots in brain development.

7.5. Future directions

The present thesis answered some of the questions regarding the association between early experience and later drug sensitization. But on the other hand, it opened the door to other related questions that need to be investigated. Following are some of highlights for the possible future directions.

- We investigated the effect of early TS or stress before or after birth but in reality pre- and postnatal experiences could be additive or even interactive. Therefore, the experience of TS or stress needs to be investigated simultaneously during both pre- and postnatal brain development.
- Research related to the effect of early experience on later drug-associated brain plasticity should also be extended to experiential factors, either a favorable or an adverse, other than drugs to see if drugs are somehow special or is a general effect on later plasticity. Furthermore, the effect of early drug exposure needs to be investigated on later plasticity associated with a drug(s) as well as other experiential factors.
- Juvenile behaviors need more exhaustive testing (e.g., running rats at multiple ages) to examine a strong link, if there is any, between early experience and later drug abuse and to determine if there are behavioural markers in juveniles that predict later drug abuse.
- Although behavioral sensitization bears similarities with drug self-administration it still lacks the face validity of drug self-administration. Therefore, the effect of

early experience should be extended to other models of drug addiction (i.e. self-administration and conditioned place preference).

- We studied the effect of early experience on only one drug (i.e. amphetamine) but humans have the problems of concurrent multi drug abuse as well as the tendency to begin abusing one drug (e.g., nicotine) and then switch to another (e.g, cocaine). Therefore, early experience should be investigated in relation to multiple psychoactive drug administration.
- Previous findings show that prior drug exposure blocked the neuroanatomical alteration associated with learning with impaired learning and memory. Although we found similar trends, by switching the order of experiential factors, where prior experience blocked the drug associated changes in the brain. But the question as to how these brain changes are reflected in behavior needs further behavioral investigation (e.g., on cognitive performance) following drug exposure.
- Neuroplasticity associated with various experiential factors is traditionally studied employing morphological techniques. However, Epigenetics is increasingly becoming popular as a latest and emerging technique to study the effects of different experiential factors on human physical and mental health. The investigation of genes responsible for the changes in the brain and behavior associated with early experience, drug exposure, and an interaction between the two will help to target those genes for possible drug addiction treatment in humans.

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