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Early life stress, HPA axis function and sensitivity to psychomotor stimulants in nonhuman primates

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Early life stress, HPA axis function and sensitivity to psychomotor stimulants in nonhuman primates

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B.A., University of New Hampshire, 2000

Advisor: Leonard L. Howell, Ph.D.

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Abstract

Early life stress, HPA axis function and sensitivity to psychomotor stimulants in nonhuman primates

By Sarah Ewing Corcoran

Substance abuse and dependence are major public health concerns and exposure to stressors, particularly early in life, appears to be a strong determinant of increased vulnerability to drug use in humans. These studies utilized a nonhuman primate model of early life stress, in the form of maternal separation, to determine if changes in sensitivity to and increases in the propensity to self-administer psychostimulant drugs were a result of exposure to these stressors. The present studies also examined the long-term effects of maternal separation on behavioral and HPA axis reactivity in rhesus monkeys. As compared with controls, maternally separated monkeys exhibited reduced behavioral output and reduced rates of responding during acquisition and maintenance of cocaine self-administration and across a range of doses of both cocaine and amphetamine. Maternally separated monkeys also failed to exhibit the stimulant-induced increases in motor activity that were observed in control monkeys. However, these differences could not be explained by differences in dopamine D_2 receptor binding potential or in drug-induced increases in extracellular dopamine. Control and maternally separated monkeys also did not differ in behavioral reactivity as measured with acoustic startle under baseline conditions or following stimulant challenges. No differences between groups were seen in HPA axis response to an acute injection of cocaine. These results do not provide support for early life stress leading to enhanced sensitivity to the reinforcing and psychomotor effects of stimulants in the nonhuman primate model employed.

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Abbreviations

2DG	2-deoxyglucose
5-HIAA	5-hydroxyindoleacetic acid
5-HT	serotonin
ACTH	adrenocorticotropin hormone
ADHD	attention deficit hyperactivity disorder
AMPH	amphetamine
ANOVA	analysis of variance
B_{max}	receptor density
cAMP	cyclic AMP
CNS	central nervous system
COMT	catechol-O-methyl-transferase
CRF	corticotropin releasing factor
CSF	cerebrospinal fluid
DA	dopamine
DAT	dopamine transporter
DSM IV	Diagnostic and statistical manual of mental disorders, volume IV
EH	early handling
FDG	fluorodeoxyglucose
FI	fixed interval
FR	fixed ratio
GCs	glucocorticoids
GR	glucocorticoid receptor
H	handled
HPA	hypothalamic-pituitary-adrenal

HPLC	high performance liquid chromatography
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
ISI	inter-stimulus interval
K_D	dissociation equilibrium constant (affinity)
L-DOPA	3, 4-dihydroxy-L-phenylalanine
MAO	monoamine oxidase
MR	mineralocorticoid
MRI	magnetic resonance imaging
mRNA	messenger RNA (ribonucleic acid)
MS	maternally separated
NET	norepinephrine transporter
NH	non-handled
NIDA	National Institute of Drug Abuse
NSDUH	National Survey on Drug Use and Health
PET	positron emission tomography
PKA	protein kinase A
PND	post natal day
PR	peer reared
PVN	paraventricular nucleus of the hypothalamus
ROI	region of interest
SAMHSA	Substance Abuse and Mental Health Service Administration
SCN	superchiasmatic nucleus
SEM	standard error of the mean
SERT	serotonin transporter
TAC	time activity curve

TH	tyrosine hydroxylase
TTX	tetrodotoxin
VTA	ventral tegmental area

Specific Aims

1. **Characterize the effects of early rearing history on monoamine function**

2. **Determine the effectiveness of cocaine and amphetamine to function as reinforcers**

3. **Determine the effectiveness of cocaine and amphetamine to enhance locomotor activity, acoustic startle and plasma cortisol**

4. **Document differences in basal brain metabolism and D_2 receptor binding**

Chapter 1: Introduction and Background

Substance Abuse and Dependence

Substance abuse is defined as a pattern of substance use that leads to significant impairment of functioning, with recurrent use resulting in failure to fulfill major obligations at home, work or school, legal problems and use continues despite significant social or interpersonal problems (DSM IV). Substance dependence is defined as substance abuse with an increase in tolerance, where more of the drug is necessary to achieve the same effect, and withdrawal symptoms occur when substance use is stopped (DSM IV). Both substance abuse and dependence are major public health concerns in the United States. In 2007, there were 22.3 million Americans with substance dependence or abuse. Of these, 1.6 million were classified as being dependent on or abusing cocaine (NSDUH, SAMHSA, 2007).

Each day, approximately 2,500 Americans will try cocaine for the first time (NSDUH, SAMHSA, 2007). Of those who initially try cocaine (intranasally), it is estimated that only about 10-15% become abusers (Gawin 1991; Anthony et al. 1994; Wagner and Anthony 2002; O'Brien and Anthony 2005). Gawin (1991) suggested that individual differences in the progression to addiction could provide crucial insights for prevention and treatment. However, reliable predictors of later heavy substance abuse have not been found in light cocaine users who have not yet developed dependence (Gawin 1991).

Treatment and prevention

Despite a number of medications that have shown promise in animal models, there are currently no successful treatments available for stimulant abuse and dependence in humans (Carroll et al. 1999). As a result, more focus has been directed toward identifying vulnerability or risk factors and developing prevention strategies. Targeting individuals with the greatest risk of developing substance abuse and dependence would be the most effective drug abuse prevention program. However, to do this, the individual differences or environmental influences that put some people at greater risk to become drug abusers must be elucidated.

Risk Factors

There are clearly differences in vulnerability to drug use and dependence and some of the predisposing factors appear to be present very early in life (Glantz and Leshner 2000) and the interaction of the individual with his or her environment seems to be a strong determinant of vulnerability to drug use (Glantz 1992). Twin studies exploring heritability estimates for drug abuse and dependence have found that environmental factors also play an important role (Kendler et al. 2000; Vanyukov and Tarter 2000).

Environmental factors are strongly represented in the risk and protective factors for drug abuse proposed by The National Institute on Drug Abuse (NIDA: NIDA notes 2002). Among the proposed risk factors were a chaotic home environment, ineffective parenting and lack of parent-child attachment, all of which could be considered forms of early life stress. Protective factors included strong, positive family bonds and parents that were involved in the child's life and monitored the child's activities. Ellickson and

Morton (1999) also found that coming from a disrupted family adds to the risk of hard drug use. A very strong association exists between childhood adversity and depressive symptoms, antisocial behavior and drug use during the early transition to adulthood and emphasized the public health impact of childhood adversity (Schilling et al. 2007). Turner and Lloyd (1995, 2003) also found that increases in exposure to childhood adversity are significantly associated with an increased risk for drug dependence.

The early life stress linked to these risk factors may directly lead to the initiation of drug use as a coping mechanism. Widom et al. (1999) have described a number of psychological variables that could lead to substance use as a coping strategy. These include using the substance as an emotional or psychological escape from the negative environment, as a form of self-medication, for self-enhancement, to reduce feelings of isolation and loneliness or as a self-destructive behavior arising from a poor self-concept (Widom et al. 1999).

However, the exposure to early life stressors may also elicit changes in the brain that alter the inherent sensitivity to the drug, thus impacting the initial subjective response to the drug which can, in turn, impact subsequent use. Individual differences exist in human drug intake and in the reinforcing effects of abused drugs. In a study by De Wit et al. (1986) the subjective effects of amphetamine were compared in those subjects that consistently chose amphetamine over placebo and in those who preferred placebo over amphetamine. They found that that the two subject groups showed markedly different subjective responses to the stimulant drug; the choosers reported increased positive mood and euphoria but the non-choosers reported only increased anxiety and depression (De Wit et al. 1986). These differences, particularly in the initial subjective responses to

cocaine can predict cocaine abuse in humans (Lambert et al. 2006). This group found that subjects with greater feelings of "liking" and "wanting" following initial cocaine use were more likely to develop higher lifetime cocaine use and to meet criteria for cocaine dependence than those who did not report these feelings upon initial use (Lambert et al. 2006). Understanding these individual differences in the subjective and reinforcing effects of drugs of abuse may shed light on the factors involved in high risk for drug abuse (De Wit et al. 1986) and thus aid in prevention strategies.

Psychostimulants

Cocaine is an alkaloid derived from the Erythroxylon coca shrub found in South America (Johanson and Fischman 1989). It acts as a local anesthetic and is considered a sympathomimetic agent because it mimics the actions of adrenaline, producing vasoconstriction, tachycardia and stimulation of the central nervous system leading to loss of appetite, restlessness and increased motor activity. It is also a powerful psychostimulant with strong reinforcing properties. It penetrates the brain rapidly (Johanson and Fischman 1989) and has a biological half-life of 40-90 minutes (Barnett et al. 1981; Javaid et al. 1983; Ambre 1985; Chow et al. 1985; Jeffcoat et al. 1989). Cocaine is rapidly and almost completely metabolized by enzymes (cholinesterases) in both the plasma and in the liver into two major metabolites, benzoylecgonine and ecgonine methyl ester which are excreted in urine (Vitti and Boni 1995). Cocaine binds to monoamine (dopamine, serotonin and norepinephrine) transporters with roughly equipotent affinity (Ritz and Kuhar 1989; Han and Gu 2006) and blocks the reuptake of released neurotransmitter molecules back into pre-synaptic neurons (Heikkila et al.

1975). This ultimately potentiates the synaptic actions of monoamines by increasing their concentration in the synapse and extending the stimulation of pre- and post-synaptic receptors. Monoamines in the synapse are the result of impulse dependent release from vesicles.

Despite actions on serotonin and norepinephrine, the affinity of cocaine at the dopamine transporter appears to be crucial to their reinforcing and psychostimulant properties (Ritz et al. 1987; Ritz and Kuhar 1989). It was proposed that the inhibition of dopamine uptake via dopamine transporter blockade and subsequent increase of synaptic dopamine was the mechanism underlying the reinforcing effects of psychostimulant drugs (Wise 1984). This was confirmed by Ritz et al. (1987) who performed multiple regression analysis relating the log of relative concentration needed to inhibit binding and the log of behaviorally active doses and found that DAT inhibition was significantly and positively associated with the reinforcing effects of cocaine. In contrast, they found that there was little relation between serotonin transporter (SERT) and norepinephrine transporter (NET) inhibition and cocaine-reinforced behavior (Ritz et al. 1987).

Clinical findings have further implicated the importance of dopamine and blockade of the dopamine transporter in the reinforcing effects of cocaine. Volkow et al. (1997) found a positive correlation between levels of dopamine transporter occupancy using PET imaging and the subjective effects or the "high" produced by cocaine. They found that at least 47% of dopamine transporters had to be blocked in order for the subject to perceive the euphoric effects of cocaine, and that the doses typically abused by humans block between 60 and 77% of dopamine transporters (Volkow et al. 1997).

Amphetamines are synthetic compounds that are also sympathomimetic agents and potent psychomotor stimulants with strong reinforcing properties. Amphetamine was first synthesized in 1887 and has been used to treat narcolepsy, obesity and ADHD (Seiden et al. 1993). Like cocaine, amphetamine can bind to and block the dopamine transporter (DAT) (Volkow et al. 2004; Han and Gu 2006). However, in contrast to cocaine, it is taken up through the transporter and causes a conformational change by which the direction of transport reverses in the plasma membrane and sends dopamine from the cytosol to the extracellular space essentially acting as a reverse transporter (Fischer and Cho 1979; Rudnick and Clark 1993; Khoshbouei et al. 2003). Amphetamine is also thought to redistribute dopamine from the vesicles to the cytosol by disrupting the storage of dopamine in vesicles by collapsing the vesicular proton gradient (Sulzer et al. 1990, 1995, 2005; Fleckenstein 2003). Amphetamine causes release of cytosolic dopamine rather than vesicular dopamine and thus dopamine release is not impulse dependent (Hurd and Ungerstedt 1989). This lack of impulse dependence has been demonstrated in studies using in vivo microdialysis; amphetamine-induced dopamine release in the presence of tetrodotoxin (TTX), which blocks action potentials, was 94% of that without TTX (Nomikos et al. 1990). Also, pretreatment with gamma-butyrolacetone (which inhibits dopamine cell firing) failed to alter amphetamine-induced dopamine release in vivo (Carboni et al. 1989).

Further confirmation of dopamine's involvement in the reinforcing effects of psychostimulants came from microdialysis experiments that found that drugs abused by humans increased extracellular dopamine in the nucleus accumbens and dorsal striatum of rats (Di Chiarra and Imperato 1988). Additionally, the reinforcing effects of cocaine

and amphetamine could be blocked by selective dopamine receptor blockers but not by selective norepinephrine receptor blockers (Davis and Smith 1975; Risner and Jones 1976, 1980; Yokel and Wise 1975, 1976). Selective dopaminergic lesions also attenuate the reinforcing effects of cocaine and amphetamine, but lesions of the noradrenergic or serotonergic systems do not (Wise and Rompre 1989). Treatment with α-methyl-p-tyrosine treatment, which blocks dopamine synthesis, leads to an attenuation of subjective effects of euphoria associated with psychomotor stimulants in humans (Jonsson et al. 1971) and also blocks the reinforcing effects of methamphetamine in animals (Pickens et al. 1968).

Dopamine neurochemistry

Dopamine is a monoamine that is involved in a large number of processes including movement, attention, learning, and the reinforcing effects of drugs of abuse. Dopamine is synthesized from the essential amino acid tyrosine which is converted to L-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (TH). L-DOPA is then converted to dopamine by the enzyme dopa-decarboxylase (Elsworth and Roth 1997). Additional steps in this pathway can then convert dopamine to norepinephrine and ultimately to epinephrine.

Dopamine acts as a neuromodulator, it is unable to trigger or inhibit the generation of action potentials by directly operating ion channels and thus depolarizing or hyper-polarizing the neuronal membrane (Di Chiara et al. 1994). Instead, DA receptors, when activated, alter the responsiveness of the neuronal membrane to fast neuro-

transmission by modifying the voltage dependence of voltage-operated ion channels (Kitai and Surmeir 1993; Di Chiara et al. 1994).

There are 3 major dopaminergic pathways in the brain. The nigrostriatal dopamine system has cell bodies located in the substantia nigra that project to the striatum (caudate nucleus and putamen). This pathway is particularly important in the control of movement. The mesolimbic/mesocortical dopamine system's cell bodies are located in the ventral tegmental area (VTA) and project to the limbic system (nucleus accumbens, amygdala and hippocampus) where they are important for the reinforcing effects of drugs of abuse, or the prefrontal cortex, where they are involved in memory, planning and problem solving. Finally, the tuberoinfundibular (tuberohypophyseal) dopamine system consists of short neurons that project from the ventral hypothalamus to the median emminance and pituitary gland, where they regulate hormone secretion (Stahl 1996).

Dopamine receptors can be classified as either D_1-like or D_2-like, both of which are G-coupled transmembrane receptors. D_1-like receptors (D_1 and D_5) act via G_s proteins to increase adenylate cyclase which produces the intracellular 2nd messenger cyclic AMP (cAMP). cAMP activates protein kinase A (PKA; a cAMP-dependent protein kinase) which phosphorylates a number of intracellular targets such as Ca^+ channels and transcription factors. The D_2-like receptors (D_2, D_3 and D_4) act via G_i/G_o proteins to decrease adenylate cyclase activity.

D_1 receptors are found in most areas receiving dopaminergic innervation such as the striatum, limbic system, thalamus and hypothalamus. D_5 receptors are more weakly expressed, but can be found in the limbic system and striatum. D_2 receptors, like D_1, are

found in most areas receiving dopaminergic innervations, the striatum, nucleus accumbens, substantia nigra, VTA, hypothalamus and the pituitary gland. D_3 receptors are located in limbic areas, nucleus accumbens, substantia nigra, VTA and thalamus. D_4 receptors are more weakly expressed but are found in limbic and cortical areas.

D_2 receptors are found both on post-synaptic neurons and on pre-synaptic neurons where they serve as autoreceptors (Sesack et al. 1994). Activation of auto-receptors can slow the firing rate of dopamine neurons or slow the synthesis and release of dopamine depending on their location on the pre-synaptic neuron.

Excess dopamine is normally removed from the synapse through enzymatic degradation (monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT)) or by re-uptake back into the pre-synaptic neuron via the dopamine transporter. The dopamine transporter (DAT) consists of 12 membrane spanning domains and is a member of the family of Na^+/Cl^- dependent plasma membrane transporters (Amara and Arriza 1993; Rudnick and Clark 1993; Reith et al. 1997). Uptake of dopamine requires extracellular sodium (Na^+) and chloride (Cl^-), as transporters use the cellular Na^+ gradient that is maintained by the Na^+/K^+ ATPase. Two Na^+ ions and one Cl^- ion are co-transported with each positively charged molecule of dopamine. This process begins with the binding of Na^+ to the DAT, followed by the binding of extracellular dopamine and ultimately the translocation of the dopamine into the pre-synaptic terminal (Krueger 1990; Sonders et al. 1997; Rudnick 2002).

As previously described, both cocaine and amphetamine act by binding to the dopamine transporter and blocking the re-uptake of dopamine into the pre-synaptic neuron, resulting in an excess of dopamine in the synapse (Ritz et al. 1987). In addition

to blocking dopamine re-uptake, amphetamine can also induce dopamine release into the synapse (Hurd and Ungerstedt 1989). This enhanced synaptic dopamine, particularly in the mesolimbic dopamine system, is thought to be related to the reinforcing effects of psychostimulant drugs (Koob and Bloom 1988). The mesolimbic dopamine system, which projects from the VTA to limbic areas including the nucleus accumbens, appears to be crucial for responses to rewarding stimuli (Wise 1980; Ritz 1987; Wise and Bozarth 1987; Koob and Bloom 1988; Robinson and Berridge 1993; Mas et al. 1995; Berridge 1996).

Lesions of the DA projection to the nucleus accumbens decrease or extinguish i.v. self-administration behavior depending on the self administered dose of psychostimulant (Roberts et al. 1977, 1980, 1982). Dopamine depleting lesions in the nucleus accumbens markedly attenuate self-administration of both cocaine and d-amphetamine (Lyness et al. 1979; Roberts et al. 1980; Pettit et al. 1984; Caine and Koob 1994; Gerrits and Van Ree 1996). Neurotoxic lesions of DA terminals in nucleus accumbens (which may also damage DA fibers continuing to the frontal cortex) impair ongoing stimulant self-administration (Roberts et al. 1977; Lyness et al. 1979; Roberts et al. 1980) and impair the learning of self-administration in naïve animals (Lyness et al. 1979). Neurotoxic lesions in the VTA, the site of origin for nucleus accumbens dopamine innervations, also cause impairments of ongoing stimulant self-administration (Roberts and Koob 1982). Kainic acid lesions in the nucleus accumbens, which specifically destroy dopamine cell bodies but do not affect the cortical dopamine projections that pass near the nucleus accumbens, also attenuate stimulant self-administration (Zito et al. 1985).

HPA axis interaction with dopamine system

The hypothalamic-pituitary-adrenal (HPA) axis plays a prominent role in mediating physiological responses to stressors. Glucocorticoids (cortisol or corticosterone) are steroid hormones that are released from the adrenal glands as the final step in the HPA axis response. They act throughout the body and brain where they bind to specialized glucocorticoid receptors. These glucocorticoid receptors (GRs) have been found on dopamine neurons in the hypothalamus, VTA and nucleus accumbens (Harfstrand et al. 1986). This group reports that 40-75% of dopaminergic cells in the midbrain express the GR, with the most GR immunoreactive cells found in the A10 area as opposed to A8 and A9 (Harfstrand et al. 1986).

Increases in glucocorticoids interact with dopamine neurons to facilitate dopamine release (Barrot et al. 2000) and alter the synthesis and metabolism of dopamine by acting on tyrosine hydroxylase and monoamine oxidase and decreasing dopamine re-uptake (Piazza et al. 1996b). Increases in glucocorticoid levels, within the levels induced by exposure to stressors, enhance extracellular concentration of dopamine in the nucleus accumbens and induces dopamine dependent locomotor activity (Piazza et al. 1996a). The sensitivity of postsynaptic dopamine D_1 and D_2 receptors can also be affected by glucocorticoids, with suppression of glucocorticoids leading to decreases in receptor binding of antagonist ligands as measured by autoradiography and increases in glucocorticoids resulting in increased receptor binding (Biron et al. 1992). Glucocorticoids can also modulate the density of nigrostriatal and nucleus accumbens dopamine receptors (Biron et al. 1992). Faunt and Crocker (1988) also found that glucocorticoids alter responses to dopamine receptor agonists in rats. They found that

animals given chronic corticosterone exhibited decreased responses to dopamine agonists (mixed D_1/D_2 agonist apomorphine and selective D_2 agonist LY171555), which returned to normal after withdrawal from the hormone treatment. This finding suggests that glucocorticoid effects on central dopamine systems are mediated, at least in some part by dopamine D_2 receptors. This group also found that adrenalectomized rats showed an increased responsiveness to apomorphine and LY171555 which improved (decreased) following 7 days of corticosterone treatment. These findings support the hypothesis that glucocorticoids decrease dopamine receptor responsiveness and increased responsiveness is seen only when the concentration of those hormones is chronically reduced. This study (Faunt and Croker 1988) also found no significant difference in the concentration of either D_1 or D_2 binding sites or in their respective K_D's following adrenalectomy and/or chronic corticosterone treatment as compared with their respective controls. They suggest that corticosterone may act at a site distal to dopamine receptors to bring about the observed changes in dopamine mediated behavioral responsiveness (Faunt and Crocker 1988). Glucocorticoids may also act downstream by increasing the activity of adenylate cyclase (Mobley and Sulser 1980; Saito et al. 1989) a main component of the second messenger system coupled to DA receptors (Schwartz et al. 1992).

Decreases in glucocorticoids can lead to subsequent decreases in mesoaccumbens dopamine transmission (Piazza et al. 1996b). Specifically, decreased glucocorticoids was accompanied by decreased extracellular concentrations of basal dopamine in the nucleus accumbens, decreased release of dopamine in response to a depolarizing stimuli and decreased locomotor response to morphine and apomorphine (a direct dopamine agonist) (Piazza et al. 1996b). These findings taken together suggest that glucocorticoids modify

both the pre-synaptic and post-synaptic side of dopamine transmission (Piazza et al. 1996b).

The mesolimbic dopamine system appears to be highly susceptible to stress. Following a stressor there is a biphasic alteration of dopamine in the nucleus accumbens with an initial increase followed by a decrease to below control levels (Puglisi-Allegra et al. 1990, 1991). A 15 minute exposure to tail shock elicited a 40% increase in dopamine in the nucleus accumbens septi (Abercrombie et al. 1989) and restraint stress increased dopamine outflow in the nucleus accumbens by 45% as measured with intracerebral dialysis (Imperato et al. 1991). Immobilization stress affects dopamine turnover in mice, resulting in alterations of the ratio of metabolite to transmitter in the striatum and nucleus accumbens (Cabib et al. 1988). Sorg and Kalivas (1991) found that repeated exposure to the same stressor enhanced the mesolimbic dopamine response to a psychostimulant challenge. The interaction of the HPA axis and glucocorticoids with mesolimbic dopamine systems has led many researchers to explore drug abuse in the context of stress.

Stress and HPA axis function

"Stress" is a term that is commonly used but often difficult to define. A stressor has the ability to elicit a behavioral response of emotional arousal or hyper-alerting which prepares the organism for flight, struggle or strenuous exertion in a threatening situation (Mason 1971). Stress has been defined as an organism's response involving generalized activation, promoted by any stimulus that has properties of novelty, threat, conflict or homeostatic imbalance (Ursin 1978). More recently this was expanded upon such that

stress is defined as a real or interpreted threat to the physiological or psychological integrity of an individual that results in physiological and or behavioral responses (McEwen 2000). In biomedicine, stress often refers to situations in which adrenal glucocorticoids (GCs) and catecholamines are elevated because of an experience (McEwen 2000).

The hypothalamic-pituitary-adrenal (HPA) axis plays a prominent role in mediating physiological responses to stressors. The hypothalamus releases corticotropin releasing factor (CRF) which acts on the pituitary gland leading to the release of adrenocortiocotropic hormone (ACTH). ACTH acts on receptors in the adrenal cortex which ultimately results in the release of glucocorticoids, cortisol or corticosterone (in rodents), which are steroid hormones. When glucocorticoids bind to glucocorticoid and mineralocorticoid receptors (GR and MR) the ligand-receptor complex translocates into the nucleus where it binds to specific sites on promoters of responsive genes and acts as a transcription factor. Mineralocorticoid receptors (type I) have high affinity for glucocorticoids, and are important in the proactive maintenance of HPA axis basal activity and they maintain the circadian rhythmicity of glucocorticoid secretion, particularly in diurnal trough (Sanchez 2006). Glucocorticoid receptors (type II) have a low affinity for glucocorticoids and are primarily responsible for reactive negative feedback during the circadian peak and following acute stressors (Sanchez 2006).

Receptor distribution

The distribution of mineralocorticoid and glucocorticoid receptors in the CNS varies in a brain region and species specific manner (Sanchez 2001). Mineralocorticoids

are principally located in the septo-hippocampal system in rodents but are more widely distributed in nonhuman primates (Lopez et al. 1999). Glucocorticoid receptors are widely distributed throughout the central nervous system of rodents (Marinelli and Piazza 2002; Reul and de Kloet 1985) and are found in high density in hypothalamic and cortical areas of nonhuman primates (Sanchez et al. 2000). Nonhuman primates have been shown to have lower than expected levels of GRs in the hippocampus but a high density in the paraventricular nucleus (PVN), prefrontal, entorhinal and cerebellar cortex (Sanchez et al. 2000). It is thought that the high level of both MRs and GRs in these cortical and hypothalamic areas may be important targets of glucocorticoids and suggest a potentially important role in their modulation of cognition, mood and social behavior (Sanchez et al. 2000, 2001). Outside of the CNS, GRs are expressed in most mammalian tissues and MRs are expressed in many peripheral tissues including the kidney, colon and heart.

Actions of glucocorticoids and mineralocorticoids

The final effect of the steroid hormones is to mobilize energy substrates during stress and regulate activity of HPA axis via negative feedback systems. Glucocorticoids in the periphery help to break down protein and convert it to glucose, help make fats available for energy, increase blood flow thereby increasing availability of energy substrates and stimulating behavioral responsiveness, they also decrease secretion of sex steroid hormones all to help the organism deal with the stressful conditions (Marinelli and Piazza 2002) and protect itself from the primary response to the stressor (Munck et al. 1984; Dallman et al. 1989; Reul et al. 1990). The mineralocorticoid hormone,

aldosterone, is primarily involved in sodium and water balance through its actions on the kidney and hypothalamus.

Additionally, glucocorticoids, when bound to their receptors, translocate in to the nucleus where they bind to glucocorticoid response elements in the promoter region of specific genes and act as transcription factors. Given the extensive distributuion of glucocorticoid and mineralocorticoid receptors, circulating glucocorticoids can yield a wide range of effects, such as those on the dopamine system described previously.

Negative feedback

A negative feedback loop keeps the HPA axis in balance. When stressed, the system is highly activated but is then downregulated quickly by negative feedback from the released glucocorticoids acting on the brain (particularly the hypothalamus) and pituitary gland, which returns the output of the HPA axis to basal levels. Glucocorticoid receptor (GR) mediated negative feedback acts upon the PVN to inhibit biosynthesis and release of CRF and inhibits release of ACTH from the anterior pituitary either directly or indirectly by acting through brain stem catecholaminergic nuclei such as the locus coerelus, ultimately resulting in the termination of stress-induced HPA axis activation (Makino et al. 2002). Some feedback effects, which may be GR independent, occur within seconds to minutes while other effects take hours. These delayed effects most likely involve gene transcription via GRs. ACTH secretion is regulated by two feedback mechanisms, a rate sensitive fast feedback which occurs 5 to 30 min after steroid administration and a proportional delayed feedback which appears after one or more hours (Dallman and Yates 1969; Jones 1977). In chronic stress situations, down-

regulation of glucocorticoid receptors in the PVN and in other brain structures results in a failure to restrain hyperactivity of the HPA axis (Makino et al. 2002).

Circadian rhythmicity of HPA axis

The HPA axis follows a circadian rhythm that appears to be entrained to the rest-activity cycle of the organism, with the secretory peak occurring just before the active phase even when the relation of activity to the lighting cycle is reverse, as in humans working on night shifts or in rats fed only during the day (Morimoto et al. 1977). The circadian rhythm derives from connections between the PVN and the suprachiasmatic nucleus (SCN) which acts as the endogenous pacemaker (Moore and Eichler 1972). In humans, the nadir for cortisol typically occurs around midnight. Cortisol levels then begin to rise about 2-3 hours after sleep onset and continue to rise into the early morning hours. The peak of cortisol typically occurs at about 0900 or shortly after waking and as the day continues, cortisol levels gradually decline (Van Cauter et al. 1995, 1999; Buckley and Schatzberg 2005).

MR function predominates in the early nocturnal period and is most prominent at the time of nocturnal nadir, whereas the effects GRs dominate in the morning when the cortisol levels are at their highest (Spencer et al. 1998).

Exposure to chronic stress

From early on, the dual nature of stress was recognized. In the short-term it leads to adaptive changes that help the organism respond to stressors, but in the long-term it can induce maladaptive changes (Selye 1950). Most of the harmful effects of stress are thought to be produced by the prolonged secretion of glucocorticoids (Selye 1976). The

short-term effects of glucocorticoids are essential but the long-term effects are damaging, leading to increased blood pressure, damage to muscle tissue, steroid diabetes, infertility, inhibition of growth, inhibition of inflammatory responses and suppression of the immune system (Selye 1976).

Exposure to chronic stress leads to increased activity of the HPA axis, but this increased activity can lead to a compensatory downregulation at different levels of the axis, thus resulting in a hypo-functional HPA axis at a later point. Long-term increases in glucocorticoids can lead to a downregulation of GRs. Repeated stress induces a selective decrease in hippocampal GR number (Sapolsky et al. 1984b). Decreases in GRs and MRs may desensitize the HPA axis to feedback regulation and give rise to a more sustained release of glucocorticoids under basal and or stressful conditions (Maccari et al. 1991).

Negative feedback modifications induced by stressful life events involve the downregulation of glucocorticoid receptors. It has been reported that hippocampal corticosteroid receptors are selectively modified by environmental experiences (Sapolsky et al. 1984a, b). Rats that underwent maternal separation for 3h/day during post natal days (PND) 2-14 exhibited increased hippocampal mineralocorticoid receptor mRNA density and decreased cortical and hippocampal glucocorticoid receptor mRNA as compared with a group of rats that were exposed to only a brief handling but no maternal separation (Ladd et al. 2004). This decrease in glucocorticoid receptors ultimately results in impaired reactive inhibition of the the HPA axis leading to an exaggerated endocrine response to stress.

Maternal Separation as an early life stressor

The mother-infant bond is the strongest social attachment formed in most mammals (Bowlby 1982). Rhesus monkey infants spend the first months of life in close physical contact with their mother (Pryce 1995). The mother-infant pair remains in physical contact for nearly a year until a sibling is born. Even after the first year the juvenile maintains a close relationship with its mother. The primary early social relationship is with its mother and the integrity of this relationship is crucial for proper emotional, cognitive and neurobiological development. Maintaining contact or proximity with their mother also enables the infant to control and modulate its physiological and behavioral responses to stress and its arousal levels (Sanchez 2006).

Disruption of this mother-infant bond produces profound behavioral and physiological responses, making it an effective stressor. Various forms of maternal separation have been used in both rodents and nonhuman primates as models of early life stress. Outcomes of maternal separation in the rat depend upon the specific type, timing and duration of the separation as well as the timing of the testing.

Single 24 hour maternal separation: Neonatal rats were subjected to a single 24 hour maternal separation on either post natal day (PND) 3 or PND 11 and were tested on PND 20. When the separation occurred on PND 3, animals were hyper-responsive to stress but the animals that were separated on PND 11 were hypo-responsive to stress when ACTH and corticosterone levels were tested following a mild stressor on PND 20 (van Oers et al. 1998, 1999). Alternatively, when an additional group that had the separation on PND 7 was tested, the animals were relatively unaffected (van Oers et al. 1998, 1999). Lehmann et al. (2002) found that a single 24 hour maternal separation on

PND 4, 9 or 18 all resulted in increased corticosterone in response to restraint stress in adult rats.

Repeated or periodic maternal separations: Periodic maternal separations (4.5h/day) during the first 3 weeks of life resulted in lower corticosterone levels during restraint stress later in life and a potentiation of negative feedback function (Ogawa et al. 1994). This enhanced negative feedback sensitivity to glucocorticoids persisted in adult rats (Muneoka et al. 1994). Other studies using repeated maternal separations have yielded conflicting results. Maternal separations (5h/day) from PND 2-6 resulted in increased corticosterone levels under resting conditions and following a 30 min exposure to novelty (Biagini et al. 1998). In a study by Plotsky and Meaney (1993), rats that were exposed to maternal separations (3h/day) from PND 2-14 were hyper-responsive to stressors, exhibited increased ACTH and corticosterone following exposure to a mild stressor and resistance to glucocorticoid negative feedback.

Handled vs. non-handled: Early handling (EH) serves as another variation of maternal separation where mothers and infants are separated and exposed to human contact and a different physical environment for 15 minutes daily. Animals that are considered non-handled (NH) are left with their mothers and are not exposed to human physical contact and are exposed to only minimal distal human disturbance. The EH-NH model (Denenberg 1964; Levine 1957) has demonstrated that relative to NH, EH leads to adult offspring that are less anxious and fearful when exposed to environmental challenge (Caldji et al. 2000; Pryce et al. 2001, 2003). EH adults exhibit an attenuated pituitary-adrenal endocrine stress response to environmental challenge, with lower peak ACTH and corticosterone stress responses and more rapid post-stress return to basal levels

(Meaney et al. 1996; Liu et al. 2000; Plotsky and Meaney 1993). Outcomes of maternal separation models in nonhuman primates also depend on the specific type, timing and duration of the separations.

Maternal privation: Maternal privation consists of complete and continuous absence of the mother beginning shortly after birth. After 3-6 months, infant rhesus monkeys are placed with other maternal privation infants for peer rearing. When tested as juveniles or adolescents, animals exposed to maternal privation can exhibit lower basal and stress related HPA axis activity (Clarke 1993) or higher basal HPA axis activity (Higley et al. 1992) and phase shifts in the HPA axis circadian rhythm (Boyce et al. 1995). They have also been shown to exhibit chronically reduced basal CSF norepinephrine levels as compared with mother reared animals (Kraemer 1992).

Other studies, in which rhesus monkeys were singly reared, demonstrated higher basal levels of plasma cortisol at 19 months of age (Champoux et al. 1989; Sackett et al. 1973). However, when these animals were tested at 4 years of age, no differences in HPA axis function were found (Meyer and Bowman 1972).

Peer or nursery rearing: Monkeys reared on cloth surrogate mothers exhibited low early morning cortisol levels and alterations in normal circadian rhythm of cortisol when tested at approximately 5 months old, suggesting that these animals may be phase delayed in their HPA axis rhythm (Boyce et al. 1995). Monkeys separated from their mothers but reared with other infants in peer groups or in a nursery setting have been found to exhibit reduced (Clarke 1993) or increased (Higley et al. 1991, 1992) basal HPA axis function. Increased HPA axis function in response to a separation stressor has also been found in these animals (Fahlke et al. 2000).

Repeated maternal separation: Infant marmosets subjected to repeated maternal separations exhibited elevated cortisol levels and signs of sympathetic activation initially followed by lower than normal cortisol levels later in life (Dettling et al. 2002; Pryce et al. 2005). Similarly, squirrel monkeys exposed to repeated maternal separations exhibited attenuated increases in cortisol and fewer vocalizations in response to later maternal separations or brief social isolations (at 2-3 years), despite having shown robust increases in cortisol immediately after the separation challenges (Levine et al. 1997; Lyons et al. 1999; Lyons et al. 2000). In rhesus monkeys, repeated maternal separations produced increased cortisol release in response to acute separation challenges; the same monkeys exhibited flattened diurnal cortisol rhythms and increased acoustic startle reactivity when tested at a later age (Sanchez et al. 2005).

Variable foraging demand: The variable foraging demand model is not specifically a model of maternal separation but does serve as a model of early life stress. In this paradigm mothers are exposed to a predictable, abundant food supply, a predictable scarce food supply or a combination of the two on an unpredictable schedule. The offspring of the mothers exposed to the unpredictable foraging condition demonstrate insecure patterns of attachment, are less socially competent, are hyper-responsive to stressful stimuli and exhibit persistent alterations in CNS levels of CRF and biogenic amines (Coplan et al. 1996, 1998).

In summary, maternally separated animals show altered HPA axis responsivity to stress (Plotsky and Meaney 1993; van Oers, de Kloet et al. 1998; van Oers, de Kloet et al. 1999), altered HPA axis negative feedback (Plotsky and Meaney 1993; Muneoka et al.

1994; Ogawa et al. 1994; Lyons et al. 2000) and flattened diurnal cortisol rhythm (Gunnar and Vazquez 2001; Sanchez et al. 2005).

In addition to effects on the HPA axis, maternally separated rats have fewer dopamine transporters particularly in the ventral striatum (Meaney et al. 2002). In a study using in vitro receptor autoradiography with [^{3}H]BTCP they found that handled (H) and non-handled (NH) rats had DAT levels that were 250% higher than in maternally separated (MS) rats in the nucleus accumbens and the DAT levels in the caudate putamen of H and NH rats were almost twice that of the MS rats (Meaney et al. 2002). Fewer dopamine transporters could result in greater and more persistent increases in synaptic dopamine in response to many stimuli, including stress and increased behavioral responses to either stress or cocaine (Meaney et al. 2002).

Stress and drug abuse

Stress appears to modify the reinforcing properties of drugs of abuse (Piazza and LeMoal 1998) most likely by acting on mesencephalic dopamine neurons. Exposure to repeated stress induces a long-lasting increase in the release of dopamine (Kalivas and Stewart 1991). Increases in glucocorticoids, which are released in response to stress, also lead to increased dopamine release in the nucleus accumbens (Piazza et al. 1996a).

Alterations in HPA axis function have been linked to the propensity to self-administer drugs of abuse, probably through the interactions of glucocorticoids and dopamine. Maccari and colleagues (1991) found that animals that readily acquire drug self-administration have longer lasting release of corticosterone after environmental stress. They also determined that intravenous corticosterone injections induce AMPH

self-administration in animals that do not acquire this behavior spontaneously (Maccari et al. 1991). A decrease in corticosterone feedback in the rat has also been found to predispose individual animals to develop intravenous amphetamine self-administration (Piazza et al. 1991).

Life events involving repeated stress, which induce dysregulation of feedback mechanisms, may also increase vulnerability to acquire drug self-administration by altering the number of glucocorticoid receptors. Maccari and colleagues (1991) demonstrated that the number of corticosteroid receptors is profoundly reduced (by more than 50%) in subjects with a stress-induced increase in vulnerability to the drug (Maccari et al. 1991; Maccari et al. 1995). This group also found that when they compared rats living in different housing conditions, those in the more stressful condition had a reduced number of mineralocorticoid receptors in the hippocampus, longer durations of corticosterone increases following exposure to novel environments and a greater vulnerability to acquire amphetamine self-administration (Maccari et al. 1991).

Behavioral sensitization to stress and drugs is associated with augmented nucleus accumbens dopamine release (Kalivas and Stewart 1991; Doherty and Gratton 1992). Repeated exposure to the same stressor has been shown to promote enhanced mesoaccumbens DA response to psychostimulant challenges and this effect has been related to enhanced motor response to the drug (Sorg and Kalivas 1991). Cross sensitization occurs between stress and drugs of abuse. Repeated exposure to stress enhances behavioral responses to subsequent stress but also to drugs of abuse (Sorg and Kalivas 1991; Robinson & Becker 1986; Stewart & Badiani 1993). Repeated exposure to tail pinch stress in rats was sufficient to produce a virtually identical sensitization of

amphetamine-induced stereotypies as seen during long-term amphetamine administration (Antelman et al. 1980). Conversely, a single injection of amphetamine also resulted in a persistent sensitization of tail pinch-induced behavior (Antelman et al. 1980). Based on these findings, the authors suggest that given the interchangeability of amphetamine and stress to induce sensitization, it might be predicted that that individuals with vulnerability to stress would show an enhanced response to amphetamine (Antelman et al. 1980). Maternally separated animals that received repeated saline treatments showed significantly elevated locomotor activity following an amphetamine challenge. This response was comparable to that of animals that had received repeated amphetamine treatments, so it appears that the maternally separated animals became sensitized to amphetamine following repeated saline administration with the stress of the injections enough to cross-sensitize to the drug (Meaney et al. 2002).

Alternatively, it is possible that exposure to stress could decrease an organism's sensitivity to rewarding or reinforcing stimuli. Stressful experiences reduce the rewarding value of intracranial self-stimulation in the mesocorticolimbic system (Zacharko and Anisman 1991). Papp et al. (1991) found that exposure to a repeated variable stress paradigm induced hyposensitivity to the behavioral activating effects of amphetamine.

Suppression or reduction of circulating glucocorticoids may decrease the reinforcing effects of psychostimulants. Adrenalectomy (removal of the adrenal gland where glucocorticoids are released) abolished acquisition of cocaine self administration over a range of cocaine doses (Goeders and Guerin 1996). This was partially reversed when corticosterone was added to the drinking water, suggesting that corticosterone is

necessary for the acquisition of cocaine self-administration. Metyrapone pretreatment (inhibits synthesis of corticosterone) decreased ongoing cocaine self-administration suggesting that corticosterone may also be necessary for the maintenance of self-administration as well (Goeders and Guerin 1996). Adrenalectomy and metyrapone also decreased locomotor response to cocaine (Piazza et al. 1994).

The GR may also play a role in altered sensitivity to psychostimulants. Glucocorticoid receptor knockout mice (CNS only) generated with a Cre/LoxP system, can learn drug self-administration but their dose-response functions were flattened, leading the authors to suggest a decreased motivation to self-administer cocaine as compared with wild-type animals (Deroche-Gamonet et al. 2003). Administration of a GR antagonist (mifepristone) dose-dependently reduced the motivation to self-administer cocaine while responding on a progressive-ratio (PR) schedule (Deroche-Gamonet et al. 2003). Using a different GR transgenic mouse model that produced approximately 50% reduction of GR mRNA levels in the brain also indicated that a reduction in GR levels leads to a reduction in the sensitivity to cocaine (St-Hilaire et al. 2003). Taken together both studies show that a reduction in GR levels in the brain reduces the behavioral and molecular sensitivity to cocaine.

Summary and rationale

In summary, early life stress, specifically in the form of maternal separation, has been shown to have profound, long-lasting effects on both behavior and HPA axis function. It has been proposed that alterations in HPA axis function may, in turn, alter the reinforcing effects of psychostimulant drugs, thus increasing the propensity to use and

potentially abuse drugs. While the relationship between stress and drugs of abuse has been fairly well characterized in rodent models, little work has been done in nonhuman primates. The present study utilizes a group of rhesus monkeys that, as infants, experienced repeated maternal separations and exhibit a resulting phenotype of altered stress reactivity and HPA axis function. It was hypothesized that monkeys exhibiting this phenotype will also be more sensitive to the reinforcing effects of psychostimulants. Therefore, the goal of this project was to characterize the neurochemical and reinforcing effects of cocaine and amphetamine and the long term effects of early life stress on behavior and HPA axis reactivity in this group of monkeys.

In order to accomplish this, sensitivity to psychostimulant drugs was assessed thru drug self-administration and stimulant-induced locomotor activity. The dopaminergic system, which is crucial in the reinforcing and motor effects of psychostimulant drugs, was characterized with PET imaging to determine dopamine D_2 receptor binding potential and in vivo microdialysis to assess stimulant-induced increases in extracellular dopamine. To further characterize the long-term effects of maternal separation in this group of monkeys, PET imaging with [^{18}F]fluorodeoxyglucose was used to examine cerebral glucose metabolism, acoustic startle was used to assess behavioral reactivity and measures of plasma cortisol and ACTH were utilized to characterize the function and reactivity of the HPA axis.

Results from these experiments provided information on the long-term impact of early lifes stress, in the form of maternal separation, and the resulting effects on sensitivity to psychostimulants in a nonhuman primate model. Understanding the factors

and underlying mechanisms that modulate or predict one's vulnerability to use and abuse may prove useful in drug abuse prevention efforts.

Chapter 2: Subject information and maternal separation procedure

General: A group of nine female rhesus monkeys (*Macaca mulatta*), weighing 6.5-9.5 kg served as subjects for these studies. The group consisted of 5 controls (RJj-7, RUj-7, RRq-7, RFj-7 and RRk-8) and 4 maternally separated monkeys (RMq-8, RWp-8, RZo-7 and RJu-7). Monkeys were housed in individual home cages with access to food (monkey chow, Ralston Purina, St. Louis, MO) and water between experimental sessions. Animal care procedures strictly followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Emory University.

Only 3 control monkeys were involved in drug self-administration experiments due to issues with maintaining venous catheters. For dopamine D_2 receptor binding potential studies only 4 control monkeys took part due to subject RFj-7 (control) having medical complications under isoflurane anesthesia. Two subjects per group were involved in microdialysis experiments since 5 monkeys lost their dialysis head caps before any or all of the data could be collected. Subject RJj-7 (control) was administered amitryptiline to reduce the frequency of self-injurious behavior which had been observed in her previously and was subsequently excluded from analysis in acoustic startle experiments. Amitryptiline, a tricyclic antidepressant, has been shown to reduce startle amplitude in humans (Phillips et al. 2000). For a complete summary of subject participation see Tables 1 and 2.

Prior to the acoustic startle and stimulant-induced cortisol and ACTH experiments 7 of the 9 monkeys (excluding 2 control subjects, RFj-7 and RRk-8) had a history of drug self-administration with both cocaine and amphetamine. Self-administration experiments commenced when the monkeys were 5-6 years old and lasted approximately 1-1.5 years; this period included acquisition and a maintenance period of cocaine self-administration and dose-response functions for both cocaine and amphetamine. During this period of drug self-administration the average intake of cocaine ranged from 100-150 mg/kg and 30-50 mg/kg of amphetamine. Self-administration sessions occurred once per day, 5-7 days per week. For a detailed experimental timeline, see Figure 1.

Maternal separation paradigm: The group of maternally separated monkeys experienced repeated, periodic maternal separations as infants, as described in detail previously (Sanchez et al. 2005). The infants involved in this study were born to adult female monkeys living in stable social groups consisting of 4-7 adult females and 1 adult male sire per group. These social groups were maintained in indoor/outdoor run-type facilities at the Yerkes National Primate Research Center Field Station. Maternal separations took place between 3 and 6 months postpartum and consisted of repeated separations of systematically varied durations (0.5, 3.0 and 6.0 hours) following a counter-balanced design. Separations were scheduled once per day between 8:00am and 11:00am (onset of separation was variable/unpredictable within this time window), 2-3 times per week to yield a total of 36 separations per infant in 90 days. Separations were achieved by herding the entire social group into a small pre-capture area and then releasing individuals back into their home run through a capture tunnel. Every member

of the social group was disturbed during separation, but only one animal (mother) was removed from the group at a time. Initially mother and infant were captured as a pair and transferred to a restraint cage where the infant was removed from his/her mother. Infants were then immediately returned to their social group. Mothers were transported to a separate facility in an adjacent building where they remained for the duration of the separation and were provided food and water. However, within several separations all experimental mothers routinely separated from infants in the pre-capture area and entered the transport cages alone with minor prompting. In this case the infants were not captured and remained with their social group. At the end of each separation the mothers were returned to their social group and were reunited with their infants and social groups. At the completion of the separation protocol, social groups remained undisturbed by research staff for a minimum of 6 months. Monkeys were then transferred to pair housing (one maternally separated and one control monkey) at the Yerkes Main Station where additional studies were conducted.

Figure 1: Timeline of all experiments completed in this group of subjects, including approximate age of the subjects at each experimental timepoint.

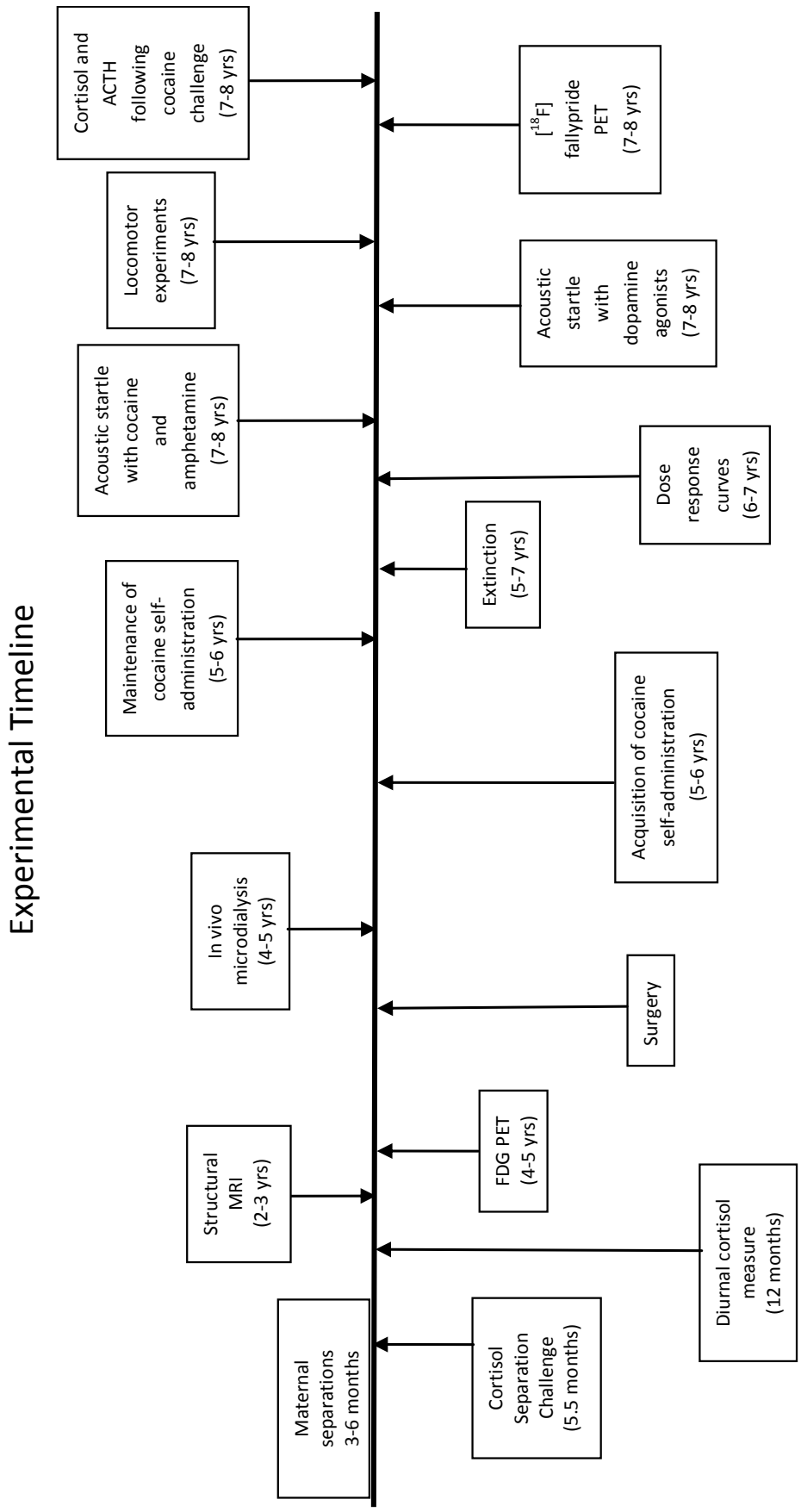

Experimental Timeline
Maternal separations 3-6 months
Structural MRI (2-3 yrs)
In vivo microdialysis (4-5 yrs)
Maintenance of cocaine self-administration (5-6 yrs)
Acoustic startle with cocaine and amphetamine (7-8 yrs)
Locomotor experiments (7-8 yrs)
Cortisol and ACTH following cocaine challenge (7-8 yrs)
Cortisol Separation Challenge (5.5 months)
FDG PET (4-5 yrs)
Surgery
Acquisition of cocaine self-administration (5-6 yrs)
Extinction (5-7 yrs)
Dose response curves (6-7 yrs)
Acoustic startle with dopamine agonists (7-8 yrs)
[18F] fallypride PET (7-8 yrs)
Diurnal cortisol measure (12 months)

Table 1: Summary of the order of experiments described in Chapter 3 and the particular subjects involved in each experiment. The X indicates that the experiment was completed in that subject; while a darkened box indicates that the subject did not complete the experiment.

Animal	Group	In vivo microdialysis	Acquisition of cocaine self-administration	Maintenance	Extinction	Dose response	Locomotor	PET with [^{18}F]fallypride
RRq-7	**CON**		X	X	X	X	X	X
RJj-7	**CON**	X	X	X	X	X	X	X
RJu-7	**MS**	X	X	X	X	X	X	X
RZo-7	**MS**	X	X	X	X	X	X	X
RUj-7	**CON**	X	X	X	X	X	X	X
RFj-7	**CON**						X	
RRk-8	**CON**						X	X
RMq-8	**MS**		X	X	X	X	X	X
RWp-8	**MS**		X	X	X	X	X	X

Table 2: Summary of the order of experiments described in Chapter 4 and the particular subjects involved in each experiment. The X indicates that the experiment was completed in the subject; the darkened boxes indicate that data from that subject was excluded from analysis.

Animal	Group	FDG PET	Acoustic startle with cocaine and amphetamine	Acoustic startle with dopamine agonists	Cortisol and ACTH following cocaine challenge
RRq-7	**CON**	X	X	X	X
RJj-7	**CON**	X			X
RJu-7	**MS**	X	X	X	X
RZo-7	**MS**	X	X	X	X
RUj-7	**CON**	X	X	X	X
RFj-7	**CON**	X	X	X	X
RRk-8	**CON**	X	X	X	X
RMq-8	**MS**	X	X	X	X
RWp-8	**MS**	X	X	X	X

Chapter 3: Effects of early life stress on the reinforcing effects of psychostimulant drugs

Abstract

Early life stress can have effects on behavior and stress reactivity which are linked to enhanced sensitivity to stimulants in rodents. The present study investigated whether rhesus monkeys that experienced early life stress (n=4) would show altered sensitivity to the reinforcing effects of stimulants as compared to controls (n=5). Maternally separated monkeys experienced periodic maternal separations between 3-6 months of age and exhibited a phenotype of altered hypothalamic-pituitary-adrenal (HPA) axis function and hyper-responsivity to stress predictive of an enhanced propensity to stimulant use. Monkeys were trained to self-administer cocaine (0.1mg/kg/injection) under a second order schedule of i.v. drug delivery. The rate of acquisition and subsequent dose-effect determinations for cocaine (0.01-1.0 mg/kg/injection) and amphetamine (0.003-0.3 mg/kg/injection) provided complementary measures of reinforcing effectiveness. In addition, stimulant-induced increases in home cage activity were quantified with actiwatch devices, dopamine D_2 receptor binding potential was quantified with PET neuroimaging, and stimulant-induced increases in extracellular dopamine in the caudate nucleus were quantified with in vivo microdialysis. As compared to controls, maternally separated monkeys showed reduced behavioral output during the acquisition of self-administration and in the dose-response curves for both stimulants and significantly lower response rates during maintenance of cocaine self-administration. Maternally separated monkeys also failed to exhibit stimulant-induced increases in motor activity

seen in controls. Groups did not differ in dopamine D_2 receptor binding potential or drug-induced increases in dopamine which could have explained the differences in their sensitivity to stimulants. Taken together the results do not provide support for early life stress leading to enhanced vulnerability to stimulant use in the nonhuman primate model employed.

Introduction

Early life stress has been shown to have profound, long-lasting effects on behavior and hypothalamic-pituitary-adrenal (HPA) axis function in animal models. Periodic maternal separation has been used in both rodents and nonhuman primates as an animal model of early life stress. Studies in rodents undergoing maternal separation have shown conflicting results depending on the timing and duration of the separations (van Oers et al. 1998, 1999; Plotsky and Meaney 1993). The effects of maternal separation on HPA axis function have been fairly well documented in nonhuman primates, particularly in New World monkeys. Studies in squirrel monkeys that have experienced periodic maternal separations in infancy show a decreased stress response following a social isolation challenge at 2-3 years of age, despite having shown robust increases in cortisol following the separation challenges (Levine and Mody 2003). Although research of this kind is limited in Old World monkeys, recent work in the rhesus monkey has demonstrated that repeated maternal separations produce increased cortisol release in response to acute separation challenges, but the same monkeys showed a flattened diurnal cortisol rhythm when tested at a later age (Sanchez et al. 2005). This flattened diurnal

cortisol rhythm later in life has also been seen in humans exposed to early life stressors (Gunnar and Vazquez 2001).

It has been proposed that alterations in HPA axis function, such as those resulting from exposure to early life stress, may in turn alter the reinforcing effects of psychostimulant drugs. Dopamine, particularly in the mesolimbic dopamine system, has long been associated with the reinforcing effects of psychostimulants and other drugs of abuse (Wise 1996). The reinforcing properties of psychostimulant drugs are thought to be related to elevated synaptic dopamine levels induced by these drugs (Koob and Bloom 1988). Glucocorticoids have been shown to interact with dopamine neurons to facilitate dopamine release (Barrot et al. 2000) and alter the synthesis and metabolism of dopamine (Piazza et al. 1996a, b). Altered responsiveness to both D_1 and D_2 dopamine receptor agonists is also associated with glucocorticoid treatment (Faunt and Crocker 1988). Accordingly, alterations in dopamine mediated by interactions with glucocorticoids, particularly in the mesolimbic pathway, may therefore change the reinforcing effects of psychostimulants.

Drug self-administration

Drug self-administration serves as a model for many aspects of human drug use. In general, an animal or human subject will perform a task such as a lever press to receive a reinforcer, such as an injection of drug; thus drug self-administration is viewed as an operant response reinforced by the delivery of the drug (Panlilio and Goldberg 2007). The self-administration model has proved to be a valid and reliable tool for determining the abuse liability of drugs in humans (Howell and Wilcox 2001; Panlilio and Goldberg

2007). Animals will self-administer most drugs abused by humans (Mello 1978; Griffiths et al. 1980) and drug self-administration is highly sensitive to pharmacological and environmental variables (LeSage et al. 1999). Drug self-administration is also an important tool for assessing sensitivity to the reinforcing effects of drugs. It has been proposed that the more sensitive an organism is to the reinforcing effects of a drug, the faster they will acquire self-administration behavior (Deminiere et al. 1989). Additionally, drug self-administration across a range of doses to generate a dose-response function can provide a measure of drug potency. Differences in drug potency are indicative of differences in an organism's sensitivity to that drug.

Studies in rodents have consistently shown that acute exposure to stressors can enhance the acquisition of drug self-administration and can induce reinstatement of previously extinguished drug seeking behaviors (Goeders and Guerin 1994, 1996; Goeders 2002). Research specifically examining maternal separation as the stressor has also shown enhanced drug effects in rodents. Maternally separated rats exhibited increased dopamine release in the nucleus accumbens in response to psychostimulant drug challenges (Hall et al. 1999) and these effects are long lasting and can be seen into adulthood (Kehoe et al. 1996). Maternally separated rats also exhibited greater acquisition of cocaine self-administration than control animals (Kosten et al. 2000).

In a study comparing the effects of amphetamine on groups of rats that were handled (H), non-handled (NH) or those that experienced maternal separation (MS) as infants it was found that when a range of doses of cocaine was tested in these animals, the maternally separated animals showed a dose-response curve that was shifted to the left (Meaney et al. 2002). A leftward shift in the dose-response curve is indicative of

increased drug potency or increased sensitivity to the drug such that a lower dose of the drug can maintain responding.

While the relationship between exposure to stress and drug self-administration has been fairly well characterized in rodent models, limited work has been done in nonhuman primates. However, there are comparable studies that have investigated alcohol consumption as a function of early life stress in nonhuman primates. Higley et al. (1991) found that peer-reared rhesus monkeys consumed more alcohol under baseline conditions and following a separation challenge than did their mother-reared counterparts. Similar results were found when the experiment was repeated when the monkeys were 6 years of age (Fahlke et al. 2000). While these studies lend further support to the idea that animals exposed to stressors, particularly early in life, are more likely to use drugs of abuse, they focused only on alcohol.

Stimulant-induced locomotor activity

Locomotor activity has historically been used as an index of psychostimulant effects (Gold et al. 1989) and, in rodents, has proven a reliable indicator of initial sensitivity to psychostimulant drugs (Sabeti et al. 2002, 2003). Psychomotor stimulant drugs such as cocaine and amphetamine have locomotor activating properties that are thought to be due to their ability to increase dopamine transmission in the brain. These locomotor activating properties can be reversed by drugs that block dopamine receptors in the central nervous system (Koob et al. 1975; Seiden et al. 1975; Baker et al. 1996) or through the destruction of dopamine terminals in the nucleus accumbens (Joyce and Koob 1981). Variations in both human and rodent dopamine transporter (DAT) levels

have been strongly linked with variation in both baseline and psychostimulant-induced locomotor activity (Uhl et al. 2002; Sabeti et al. 2002, 2003). Following administration with both direct and indirect dopamine agonists, stimulant-induced locomotor activity was attenuated in mice in which dopamine transporter levels had been reduced (Zhaung et al. 2001). Likewise, DAT knockouts exhibit no stimulant-induced increases in motor activity (Uhl et al. 2002). Therefore, alterations in the dopamine system can result in changes in stimulant-induced locomotor activity.

Glucocorticoids, possibly through their actions on dopamine, are thought to facilitate stimulant-induced increases in locomotor activity. Accordingly, the suppression of endogenous glucocorticoids by adrenalectomy suppresses maximal locomotor responses to cocaine and amphetamine but this can be reversed dose-dependently by corticosterone administration (Cador et al. 1993; Marinelli et al. 1994, 1997). Differences in stimulant-induced locomotor activity have also been shown in animals that have different rearing histories. Cocaine injections elicited dose-dependent increases in locomotor activity in maternally separated, handled and non-handled rats, but the magnitude of this response differed significantly between the groups. Injections of 10 and 20 mg/kg of cocaine elicited greater locomotor responses in the maternally separated and non-handled rats as compared with the handled animals (Brake et al. 2004). A similar effect has been seen in rats that were group housed after weaning. These animals exhibit significantly greater cocaine-induced locomotor activity as compared to subjects reared in isolation after weaning (Boyle et al. 1991).

Dopamine

Dopamine D_2 receptors are thought to be involved in both the reinforcing effects (Volkow et al. 1999, Morgan et al. 2002; Nader et al. 2006) and motor stimulating effects (Baker et al. 1996) of cocaine and amphetamine. There appears to be an inverse relationship between dopamine D_2 receptor availability and the effectiveness of stimulants as reinforcers. Levels of dopamine D_2 receptors were found to be associated with the reinforcing responses to methylphenidate in humans. Those with lower D_2 levels tended to 'like' the effects of methylphenidate while those with higher D_2 levels were more likely to 'dislike' methylphenidate's effects and the higher the level of D_2, the greater the intensity of the unpleasant effects (Volkow et al. 1999). In cynomolgus monkeys, animals with high levels of D_2 receptors, those that were dominant following social housing, showed very little cocaine self-administration behavior and lower cocaine intake as compared to subordinate animals with lower D_2 levels (Morgan et al. 2002).

It has been hypothesized that there is an optimal range in which dopamine D_2 stimulation can be perceived as reinforcing, too little stimulation might not be sufficient but too much may be aversive (Glick et al. 1994). Low levels of dopamine D_2 receptors could predispose an individual to use drugs as a means of compensating for decreased activation of reward circuits (Blum et al. 1996) or it could predispose individuals to psychostimulant abuse by promoting an initial pleasant response to the drug (Chausmer and Ettenberg 1997), which has been shown to predict future drug use (Lambert et al. 2006). High levels of D_2 dopamine receptors may protect an individual from drug abuse by fostering an initial unpleasant response to the drug (Chausmer and Ettenberg 1997).

The dopamine transporter, which plays an important role in the reinforcing effects of psychostimulants, also appears to be affected by exposure to early life stress. Maternally separated rats have fewer dopamine transporters particularly in the ventral striatum (Meaney et al. 2002). In studies using in vitro receptor autoradiography with both [^{3}H]BTCP and [^{3}H]WIN 35, 428, both handled (H) and non-handled (NH) rats had DAT levels that were 250% higher than maternally separated (MS) rats in the nucleus accumbens and the DAT levels in the caudate nucleus and putamen of H and NH rats were almost twice that of the MS rats (Meaney et al. 2002; Brake et al. 2004). Fewer DAT could result in greater and more persistent increases in synaptic dopamine in response to many stimuli, including stress and increased behavioral responses to either stress or cocaine (Meaney et al. 2002). Studies have also found alterations in dopamine receptor levels resulting from this rearing model. Maternally separated and non-handled rats were found to have a higher density of dopamine D_3 receptors in the shell of the nucleus accumbens when compared to handled rats (Brake et al. 2004). Maternally separated and handled rats had significantly higher levels of dopamine D_1 receptors in the caudate putamen, as measured with [^{3}H] SCH 23390 autoradiography, when compared with non-handled rats (Brake et al. 2004). No differences in groups were found based on rearing condition in [^{3}H] raclopride binding, a measure of D_2 receptor densities (Brake et al. 2004). Taken together, these studies demonstrate that exposure to early life stress can alter dopamine transporter and receptor levels which could ultimately lead to changes in the reinforcing effects of psychostimulant drugs.

In vivo microdialysis

In vivo microdialysis allows for sampling of extracellular neurochemicals in discrete brain structures and provides real time functional measures of neurochemistry in awake, behaving animals (Howell and Wilcox 2002). In vivo microdialysis has been adapted for use in nonhuman primates and allows for repeated, within subjects and even longitudinal designs. This method is not without limitations however. Analysis is limited to small molecules that actively diffuse across the dialysis membrane with a temporal resolution on the order of minutes (Howell and Wilcox 2002). Despite these limitations, in vivo microdialysis is a technique well suited for measuring changes in dopamine concentration, particularly those brought about by challenges with psychostimulant drugs.

Cocaine and amphetamine dose-dependently increase levels of extracellular dopamine in the nucleus accumbens (Pettit and Justice 1989, 1991; Carboni et al. 1989; Kalivas and Duffy 1990; Kuczenski et al. 1991; Di Chiara and Imperato 1988) and the dorsal striatum (Di Chiara and Imperato 1988). Both experimenter delivered (Carboni et al. 1989; Kalivas and Duffy 1990; Kuczenski et al. 1991) and self-administered (Pettit and Justice 1989, 1991; Ranaldi et al. 1999; Hemby et al. 1997) cocaine and amphetamine induce this dopamine release; however Hemby et al. (1997) found a greater increase in nucleus accumbens dopamine when cocaine was self-administered.

As described previously, early life stress, in the form of neonatal isolation, has been found to lead to exaggerated increases in extracellular dopamine in the ventral striatum following stimulant administration in rats (Kehoe et al. 1996, 1998; Kosten et al. 2005). In rats that were isolated from their mother and nest for 1h/day from PND 2-9 and

tested as juveniles (PND 26-29), a high dose of amphetamine induced a 4-fold increase in dopamine release in the ventral striatum (Kehoe et al. 1996). When rats were tested immediately after the separations, on PND 10, even low doses of amphetamine induced a 5-fold increase in non-handled rats and a 10-fold increase in the isolated rats (Kehoe et al. 1998). Cocaine (10 mg/kg) also increased dopamine levels in the nucleus accumbens to a greater extent in previously isolated rats as compared with non-handled control rats, however these animals didn't differ in basal dopamine levels (Kosten et al. 2005). Similar results have been found in maternally separated rats that when tested as adults showed an increased dopamine response in the nucleus accumbens following an amphetamine challenge (Hall et al. 1999).

In humans, psychological stressors have been shown to cause significant release of dopamine in the ventral striatum, as measured with PET neuroimaging (Pruessner et al. 2004). Reductions in [^{11}C]raclopride binding potential, indicating increased dopamine release, were found while subjects were engaged in a mental arithmetic task that served as a psychological stressor as compared to the resting condition (Pruessner et al. 2004). Additionally, the magnitude of the salivary cortisol response to stress was significantly correlated with the reduction in [^{11}C]raclopride binding in the ventral striatum, consistent with facilitating effect of cortisol on the firing of dopamine neurons (Pruessner et al. 2004). The greatest increases in dopamine were found in subjects with a history of low parental care as determined by self-report on psychological questionnaires (Pruessner et al. 2004).

Summary and rationale

While previous data implicate exposure to early life stress as a possible factor that increases the propensity to abuse psychostimulant drugs, this possibility has not been thoroughly explored outside of the rodent model. To date, research in the nonhuman primate has focused only on alcohol consumption. Accordingly, the present study investigated whether rhesus monkeys that experienced early life stress associated with maternal separation would show altered sensitivity to the reinforcing effects of stimulants as compared to controls. To this end, monkeys were trained to self-administer cocaine (0.1mg/kg/injection) under a second-order schedule of i.v. drug delivery. The rate of acquisition and subsequent dose-effect determinations for cocaine (0.01-1.0 mg/kg/injection) and amphetamine (0.003-0.3 mg/kg/injection) provided complementary measures of reinforcing effectiveness. In addition, stimulant-induced increases in home cage activity were quantified with actiwatch devices, dopamine D_2 receptor binding potential was quantified with PET neuroimaging, and stimulant-induced increases in extracellular dopamine in the caudate nucleus were quantified with in vivo microdialysis.

Methods and Materials

Surgery: Chronic indwelling venous catheters were implanted in monkeys under sterile surgical conditions using a previously described technique (Howell and Wilcox et al. 2001). Preoperative antibiotics [Rocephin (ceftriaxone, 25 mg/kg i.m.)] were given on the day of the surgery to prevent infection. A silicone catheter (0.65 mm i.d., 1.75 mm o.d.; Access Technologies, Skokie, IL) was implanted under a combination of Telazol

(tiletamine hydrochloride and zolazepam hydrochloride, Fort Dodge Animal Health, Fort Dodge, IA; 4.0 mg/kg i.m.) and isoflurane anesthesia using aseptic techniques. The proximal end of the catheter was implanted into the femoral or the external jugular vein and terminated at the vena cava above the right atrium and the distal end was routed under the skin and attached to a subcutaneous vascular access port (Access Technologies) located in the center of the lower back. After surgery, the monkey was returned to its home cage and received Banamine (flunixin meglumine, 1.0 mg/kg i.m.) every 6 h for 24 h postoperatively to reduce pain and discomfort associated with surgery. Catheters were flushed daily with 1.0 ml of heparinized (100 U/ml) saline to maintain patency.

Guide cannulae were also implanted bilaterally into the caudate nucleus of the monkeys under sterile conditions. Pre-operative antibiotics were given (Rocephin, 25mg/kg) and monkeys were sedated with Telazol (4.0 mg/kg). During surgery, isoflurane anesthesia (1-2%) was used. The monkey was positioned in a stereotaxic frame with coordinates derived from MRI. A small burr hole was made with a trephine drill directly above the caudate nucleus and guide cannulae were inserted to the appropriate depth. The guide cannulae were enclosed within a small plastic chamber to prevent access by the animal. Teflon screws attached to the skull and cranioplastic cement anchored the chamber and the skin around the implant site was sutured closed. All monkeys received an analgesic (Banamine 1.0 mg/kg) every 6 h for 24 h post-operatively or longer if they exhibited signs of discomfort. Stainless steel stylets were placed in the guide cannulae when not in use. Monkeys were allowed to recover from surgery for at least two weeks before initiating microdialysis experiments. At least two weeks separated microdialysis experiments on the same side to maintain tissue integrity.

Apparatus: Monkeys were seated in a commercially available primate chair (Primate Products, Miami, FL) with a panel outfitted with a response lever and stimulus lights attached to the front of the chair. While the monkey was in the chair, a Huber needle infusion set was inserted into the vascular access port. The polyvinyl chloride tubing of the infusion set was attached to a motor driven syringe pump (Harvard Apparatus PhD 2000) containing the drug solution located outside of the chamber (Med Associates, St. Albans, VT). The pump delivered a volume of 2.0 ml/infusion over 7s. Daily sessions took place in a ventilated, sound-attenuating chamber. Experimental events were controlled by a computer that also recorded data.

Drug self-administration: When the monkeys were 5-6 years old they were trained to respond on a second-order schedule of drug reinforcement. Responding was initiated using a 1-response fixed-ratio schedule (FR 1) such that each response in the presence of the red light produced an i.v. drug injection and a 15 s illumination of a white light. There was a 2 h limited hold in which the subject could complete the ratio requirement. This long limited hold ensured that the subjects would not miss any infusions in the early parts of the session. The ratio value was increased gradually as responding increased, from the initial FR 1 to FR 2, FR 5, FR 10 and ultimately FR 20. When the schedule value reached FR 20, drug injections no longer followed the completion of each FR but instead followed an increasing number of FR components an initial fixed interval (FI) duration of 30 s. A 2 s white light was presented upon completion of each FR 20 component. Once response criteria was met at this duration, the FI was increased to 60 s, and from that point on, increased in 60 s intervals until the FI duration reached 600 s.

Ultimately, the schedule was a second-order schedule of FR 20 components with drug injection following the first ratio requirement completed after 600 s had elapsed (FI 600 s [FR 20:S]). Drug administration was accompanied by a change in the stimulus light from red to white for 15 s. Monkeys had the opportunity to take 10 injections in each daily session. The unit dose remained constant at 0.1 mg/kg/infusion. However, one subject (RMq-8) was unable to maintain stable responding at the 0.1 mg/kg/infusion dose so the unit dose was decreased to 0.03 mg/kg/infusion for the acquisition experiments. All subjects went through the same acquisition paradigm with set criteria for moving on to the next level (increasing FR or FI) as shown in Table 3. The criterion for the initial phase of the training (FR 1) was set such that the monkeys were required to take 9 of 10 infusions for 5 consecutive sessions before the ratio requirement was increased. For subsequent FR and FI values the monkey had to take all 10 infusions for 3 consecutive sessions before schedule parameters were changed.

Following the acquisition of the second-order schedule, subjects were maintained on the terminal schedule (FI 600 s [FR 20:S]). However, the limited hold was reduced to 300 s and a 30 s time out, in which the stimulus lights were extinguished and responding had no consequence, was scheduled after each component until behavior was stable (<20% variance in response rates with animals taking all 10 infusions) for 10 consecutive sessions. Responding was then extinguished by substituting saline for cocaine and eliminating the presentation of the drug-paired stimulus (white light) that accompanied both the completion of a FR and the drug infusion. Extinction sessions continued until responding decreased to <20% of the response rate maintained by cocaine self-administration for 3 consecutive sessions. Following this extinction, cocaine-maintained

responding was re-initiated on the second-order schedule, but the number of components within a session was reduced from 10 to 5 in order to shorten overall session time. The unit dose of cocaine was then manipulated in random order to establish a dose-response curve that encompassed 0.01-1.0 mg/kg/infusion. Each dose was self-administered until responding was stable (<20% variance in response rates over 5 consecutive sessions). Upon completion of the dose-response function for cocaine, stable responding was re-established (<20% variance in response rates over 5 consecutive sessions) with a unit dose of 0.1 mg/kg/infusion cocaine. A dose-response curve for amphetamine was then established by manipulating the unit dose in random order to encompass 0.003-0.3mg/kg/infusion. Again, each dose was self-administered until responding was stable (<20% variance in response rates over 5 consecutive sessions). For the highest dose of cocaine (1.0mg/kg/infusion) and amphetamine (0.3mg/kg/infusion) the syringe pump was turned off after the monkey had taken the second infusion in order to limit the total drug intake during the session and prevent adverse effects at high doses. Subject RJj-7 (control) was on amitryptiline during her amphetamine dose-response curve to reduce the frequency of self-injurious behavior which had been observed in her previously.

Table 3: All subjects completed the acquisition paradigm with set criteria for moving to the next level. This table represents the sequence of schedule changes and required response criteria.

Sequence of schedule changes for acquistion of second-order drug-self administration

Schedule Parameters	Resonse criteria
FR 1	9-10 infusions for 5 consecutive sessions
FR 2	10 infusions for 3 consecutive sessions
FR 5	10 infusions for 3 consecutive sessions
FR 10	10 infusions for 3 consecutive sessions
FR 20	10 infusions for 3 consecutive sessions
FR 20 + FI 30s	10 infusions for 3 consecutive sessions
FR 20 + FI 60s	10 infusions for 3 consecutive sessions
FR 20 + FI 120s	10 infusions for 3 consecutive sessions
FR 20 + FI 180s	10 infusions for 3 consecutive sessions
FR 20 + FI 240s	10 infusions for 3 consecutive sessions
FR 20 + FI 300s	10 infusions for 3 consecutive sessions
FR 20 + FI 360s	10 infusions for 3 consecutive sessions
FR 20 + FI 420s	10 infusions for 3 consecutive sessions
FR 20 + FI 480s	10 infusions for 3 consecutive sessions
FR 20 + FI 540s	10 infusions for 3 consecutive sessions
FR 20 + FI 600s	10 infusions for 3 consecutive sessions

FR, fixed ratio; FI, fixed interval

Locomotor Activity: In order to quantify the locomotor activity, collars were outfitted with Actiwatch (Mini Mitter, Bend, OR) activity monitors for a period of one week when the monkeys were between 7-8 years old. The Actiwatch is a watch-like device weighing 16 g, encased in an aluminum housing device (weighing an additional 40 g) that was attached to the primate collar (it fits into one of the pole rings of the Primate Products nylon collar). It contains an omni-directional sensor that is sensitive to motion in all directions and this movement data is stored in the Actiwatch's 64kB of memory. Monkeys were anesthetized with Ketaset (ketamine HCl, Fort Dodge Animal Health, Fort Dodge, IA; 3-10 mg/kg i.m.) in order to attach the Actiwatch to the collar on a Thursday and were given the day to recover and acclimate to the change. Normal baseline activity was measured for the next two days (data not shown). On the third day, an i.m. injection of cocaine (1.0 mg/kg), amphetamine (0.3 mg/kg) or saline was administered in the home cage and the locomotor response to the drug challenge was recorded on the Actiwatch. All injections were given at the same time of day (9am Sunday) in order to ensure minimal disturbances. The monkeys were anesthetized with Ketaset (3-10 mg/kg i.m.) again and the Actiwatch was removed from the collar on the following Wednesday. The data were downloaded and analyzed with Actiware Sleep 3.4 (Mini-Mitter Co. Inc., Bend, OR).

Dopamine D_2 Receptor Binding Potential

Radiochemistry: [^{18}F]fallypride is a selective, high affinity D_2/D_3 PET radioligand that is readily taken up in the striatum and is rapidly cleared from the cerebellum (Christian et al. 2000). [^{18}F]fallypride was synthesized according to published methods (Mukherjee et

al. 1995). Briefly, the tosylate precursor (S)-N-[(1-Allyl-2-pyrrolidinyl) methyl]-5-(3-toluene-sulfonyloxypropyl)-2,3-dimethoxybenzamide was prepared following general procedures described previously (Mukherjee 1991). The yield of the DCC coupling reaction of 2,3-dimethoxy-5-(3-hydroxypropyl)benzoic acid and (S)-2-(aminomethyl)-N-allylpyrrolidine to provide the alcohol (S)-N-[(1-Allyl-2-pyrrolidinyl)methyl]-5-(3-hydroxy-propyl)-2,3-dimethoxybenzamine was moderate (Mukherjee et al. 1995). Compound (S)-N-[(1-Allyl-2-pyrrolidinyl) methyl]-5-(3-hydroxy-propyl)-2,3-dimethoxybenzamine was converted to the tosylate in 70% yield. Radiolabelling was carried out by nucleophillic displacement of the tosylate precursor by no-carrier-added ^{18}F to provide [^{18}F]fallypride (Mukherjee et al. 1995). Purification was carried out by reverse phase HPLC to yield radiochemically pure [^{18}F]fallypride (Mukherjee et al. 1995).

Imaging protocol: On the day of the PET study, monkeys (7-8 years old) were anesthetized in their home cage with Telazol (4.0 mg/kg) and transported to the Yerkes Imaging Center to be scanned on the MicroPET Focus scanner. Subjects were intubated and anesthesia was maintained with isoflurane (1-2%). An i.v. of lactated ringer was started with a 22 g catheter in the saphenous vein. Monkeys were positioned in the tomograph and a 10-20 minute transmission scan was obtained for attenuation detection. Once the transmission scan was complete and verified a slow bolus of approximately 6 mCi [^{18}F]fallypride was injected over 5 min at a rate of 1.0 ml/min using a motor driven syringe pump (Harvard Apparatus PhD 2000). A dynamic PET sequence was obtained over the 2 hours following injection of the radioligand. At the conclusion of the scan

monkeys were returned to their home cage to recover. Subject RFj-7 (control) was excluded from these imaging studies due to medical complications that arose in her while under isoflurane anesthesia.

In vivo Microdialysis: At the time of testing, monkeys (approximately 4-5 years old) were seated in commercially available primate chairs described above. The chair and Lexan neck plate attached to the chair limited the movement of the animal ensuring that they could not contact the probe area. Microdialysis sessions lasted approximately 4 h and were conducted in a ventilated, sound-attenuating chamber (Med Associates, St. Albans, VT). A microinjection pump (CMA/102) located outside the chamber continuously delivered artificial cerebrospinal fluid (Na_2HPO_4, 1.0 mM; NaCl, 150 mM; KCl, 3 mM; CaCl, 1.3 mM; MgCl, 1.0 mM and ascorbic acid, 0.15 mM) via FEP Teflon tubing to the probe for perfusion at a flow rate of 2.0 µl/min. During a 2-hour equilibrium period, monkeys were seated in the chair and baseline samples were collected at 10 minute intervals outside the test chamber to ensure that they were not disturbed during the experiments. Doses of cocaine (1.0 mg/kg) or amphetamine (0.3 mg/kg) were administered intravenously after the 2 hour equilibrium period to determine drug-induced increases in extracellular dopamine. A high potassium (K^+) aCSF (KCl was increased to 54 mM and NaCl was decreased to 103 mM) was substituted for the standard aCSF at the end of each session (120 min after drug administration) to induce voltage-dependent dopamine release. This dopamine response to the high K^+ solution served as an indication of site viability. Probes were tested in vitro to determine the fitness of probe efficiency and performance before and after in vivo experiments.

Small-bore high pressure liquid chromatography (HPLC) and electrochemical detection were used to quantify extracellular levels of dopamine according to well-established analytical procedures (Czoty et al. 2000, 2002). The HPLC system consisted of a small-bore (3 mm internal diameter x 100 mm) column (5 µm C18 stationary phase; Thermo Hypersil, Keystone Scientific Operations, Bellefonte, PA) with a commercially available mobile phase (ESA, Inc., Chelmsford, MA) delivered by an ESA 582 solvent delivery pump at a flow rate of 0.6 ml/min. After being loaded onto a refrigerated sample tray, samples (20 µl) were automatically mixed with 3 µl of ascorbate oxidase and 18 µl of this mixture was injected into the HPLC system by the ESA model 542 autosampler. Samples were analyzed within 12 hours of collection remaining either in the refrigerated sample tray or in a refrigerator. Electrochemical analyses were performed with an ESA dual-channel analytical cell (model 5040) and guard cell (model 5020, potential = 350 mV) and an ESA Coulchem II detector. The potential of channel 1 was set to -150 mV for oxidation and the potential of channel 2 was set to 275 mV for reduction. Chromatograms were generated using EZChrom Elite software (version 3.1, Scientific Software, Pleasanton, CA).

Drugs: Cocaine HCl (National Institute on Drug Abuse, Bethesda, MD) and d-amphetamine H_2SO_4 (Sigma Aldrich, St. Louis, MO) were dissolved in 0.9% saline. All doses are reported as salts.

Data Analysis

Drug self-administration: For acquisition studies, the mean total number of responses for each monkey was averaged from the three days (5 days for the FR1) in which they met the criteria set for the schedule parameter. Mean rates of responding for the maintenance phase of cocaine self-administration were determined for each monkey by averaging the data from the last five days of the ten day maintenance phase. Number of days required to meet extinction criteria (<20% of cocaine maintained responding for 3 consecutive sessions) was determined for each monkey. Mean rates of responding for each dose in the dose-response functions were determined for each monkey by averaging the data from the five days in which responding was stable. For all measures group data were derived and expressed as the mean ± SEM. Mean total number of responses during acquisition and mean rates of responding across a range of doses were analyzed using a two-way analysis of variance (ANOVA) with repeated measures on one factor to compare control and maternally separated animals. Only the data from schedule conditions that included FI were analyzed (without the FI there was a limit to the number of responses the animal could make). Multiple pairwise comparisons between means were performed using a Tukey's post hoc test.

Comparisons between groups for days to meet acquisition criteria at FR 1, days to meet extinction criteria, response rate in maintenance phase, rates of responding at the peak dose on the cocaine and amphetamine dose-response functions were made using the two-tailed Student's t-test for unpaired samples. For comparisons between groups on measures across dose-response functions for cocaine and amphetamine, data did not meet

the assumptions required for t-tests or ANOVA (equal variance and normally distributed data) so the Mann-Whitney U test was used.

Locomotor Activity: The Actiwatch was programmed to record activity with an epoch length of 15 s. Data from these epochs were averaged into 10 min bins. The order of the experimental sessions was randomized across monkeys. Drug-induced locomotor activity is represented as a % of the activity level during the same time period following a saline injection. Time-course of stimulant-induced activity is shown for the 60 min following the injection. For group comparisons on measures of locomotor activity following stimulant challenge, data did not meet the assumptions required for t-tests or ANOVA (equal variance and normally distributed data) so the Mann-Whitney U test was used.

PET Image analysis: PET images were co-registered to a standard adult rhesus monkey MRI image. Regions of interest (ROIs) were manually drawn over the caudate nucleus, putamen and cerebellum on the frames where these structures could be seen clearly. The cerebellum was used as a reference region (representing a concentration of free and non-specifically bound [^{18}F]fallypride with negligible specific binding). The regions of interest were then transferred to all frames to obtain time activity curves (TACs) decay-corrected to time of [^{18}F]fallypride injection. Logan analysis was used to determine binding potential (Logan et al. 1990, 1996). These binding potentials for the caudate nucleus and putamen were averaged across rearing condition (n=4 per group).

Comparisons between groups for dopamine D_2 binding potential were made using the two-tailed Student's t-test for unpaired samples.

Microdialysis: Percent of basal dopamine was calculated for each sample following drug injection. Mean percent basal transmitter was analyzed as a function of drug dose and time. Each drug dose was tested bilaterally and the data from two sides were averaged together such that each subject had one set of values for a particular experiment. Data were derived from individual monkeys and combined within treatment (rearing) condition for group statistical analyses using a two factor ANOVA with repeated measures on one factor and a Tukey's post hoc multiple comparisons test. .

For all comparisons, probability of significance was set at $p<0.05$. All statistical analyses were conducted using Sigma Stat 3.0 for Windows.

Results

Drug self-administration

Rate of acquisition was determined by the number of days needed to meet acquisition criteria. On average, control monkeys met the criteria for the FR 1 sessions (9-10 infusions for 5 consecutive sessions) in 5.67 ± 0.67 days while the maternally separated monkeys required 8.0 ± 2.0 days. This difference was not statistically significant (Table 4). After the initial FR 1 sessions, all subjects completed the criteria of the schedule condition (10 out of 10 infusions) in the minimal amount of time required (3 consecutive sessions). There was no significant group difference in rate of acquisition. However, maternally separated monkeys consistently demonstrated a reduced behavioral

output as compared to control animals once the FI schedule was introduced. Figure 2 presents the mean total number of responses per session for each schedule condition by schedule parameter for control and maternally separated monkeys during the acquisition of cocaine self-administration. Due to the long limited hold during the acquisition sessions (7200 s) and the resulting variability in session length, response rate was not the appropriate dependent measure in this experiment so total number of responses was used. No significant difference was found for responding during acquisition in the control and maternally separated monkeys ($F(10, 50) = 1.50$, $p = 0.17$). Following the acquisition of the second-order schedule, subjects were maintained on the terminal schedule (FI 600 s [FR 20: S]) with 300 s limited hold and 30 s time out. These sessions continued until response rates were stable (<20% variance) for ten consecutive sessions. Response rates from the last five days were averaged for each monkey and group means were determined (±SEM) as shown in Figure 3. The mean response rate for the maintenance phase was 0.35 ± 0.08 responses/sec for controls and 0.17 ± 0.02 responses/sec for maternally separated monkeys. The difference in response rates for control and maternally separated monkeys during the maintenance phase was statistically significant ($t(5) = 2.69$, $p = 0.04$) (Table 4).

Following acquisition and maintenance of cocaine self-administration on the second-order schedule, subjects underwent a series of extinction sessions. Extinction sessions continued until responding was less than 20% of cocaine-maintained responding (derived from the last 5 days of maintenance on the terminal schedule) for 3 consecutive sessions (Figure 4A). The dependent variable was days to extinction criteria. On average, control monkeys reached extinction criteria in 10.7± 6.2 days while the average

for maternally separated monkeys was 7.0± 1.5 days. This difference in the mean number of days to extinction for the control and maternally separated monkeys was not statistically significant ($t(5) = 0.67$, $p = 0.53$) (Table 4). During extinction sessions the drug-paired stimulus (white light) that accompanied both the completion of a FR and the drug infusion was also removed. All animals demonstrated a dramatic decrease in responding in the first component of the session (Figure 4B) prior to any saline infusion, indicating that the drug-paired stimulus was an important factor in maintaining responding. When response rate were stable, groups did not differ in their responding for saline.

When a range of cocaine doses (0.01-1.0 mg/kg/injection) was substituted for the maintenance dose of cocaine, maternally separated subjects demonstrated reduced rates of responding at all doses tested compared to controls as evidenced by the downward shift in the dose-response function (Figure 5A). The dose that maintained peak rates of responding was the same in both groups, with no rightward or leftward shifts observed in the dose-response function. On average, the rate of responding for the peak dose of cocaine in control monkeys was 0.67 ± 0.23 responses/sec and 0.26 ± 0.05 responses/sec for maternally separated monkeys; however this difference did not reach statistical significance (Table 4). Control and maternally separated groups differed significantly in responding for a range of doses of cocaine (Mann-Whitney $U= 0$, $p=0.03$). When a range of amphetamine doses (0.003-0.3 mg/kg/injection) was substituted for the maintenance dose of cocaine, maternally separated monkeys demonstrated reduced rates of responding across all doses tested, compared to controls, as evidenced by the downward shift in the dose-response function (Figure 5B). The dose that maintained peak rates of responding

was the same for both groups, with no obvious right or leftward shift in the dose-response function. On average, the response rate at the peak dose of amphetamine was 0.69 ± 0.29 responses/sec for control monkeys and 0.23 ± 0.07 responses/sec for maternally separated monkeys but this difference was not statistically significant (Table 4). Control and maternally separated groups showed a strong trend towards a difference in responding across a range of doses of amphetamine (Mann-Whitney U = 1.0, p = 0.06).

Locomotor Activity

Gross motor activity was recorded following intramuscular injection of saline, cocaine (1.0 mg/kg) and amphetamine (0.3 mg/kg). Activity following drug injection was normalized to % of activity following saline injection. Time courses of activity for the 6o min post-injection are shown in Figure 6. Control and maternally separated groups differed significantly for cocaine-induced increases in motor activity (Mann-Whitney U=1.0, p=0.004) and there was a strong trend towards a difference for amphetamine-induced increases in motor activity (Mann-Whitney U=6.0, p=0.06).

The onset of the increase in activity in control monkeys occurred approximately 30 min post-injection and returned to baseline by 120 min post-injection (data not shown).

Figure 2: Mean total number of responses per session for each schedule condition by schedule parameter for control (n=3) and maternally separated (n=4) monkeys during the acquisition of cocaine self-administration. No significant differences between groups were found for responding during acquisition of cocaine self-administration ($F(10, 50) = 1.50$, $p = 0.17$).

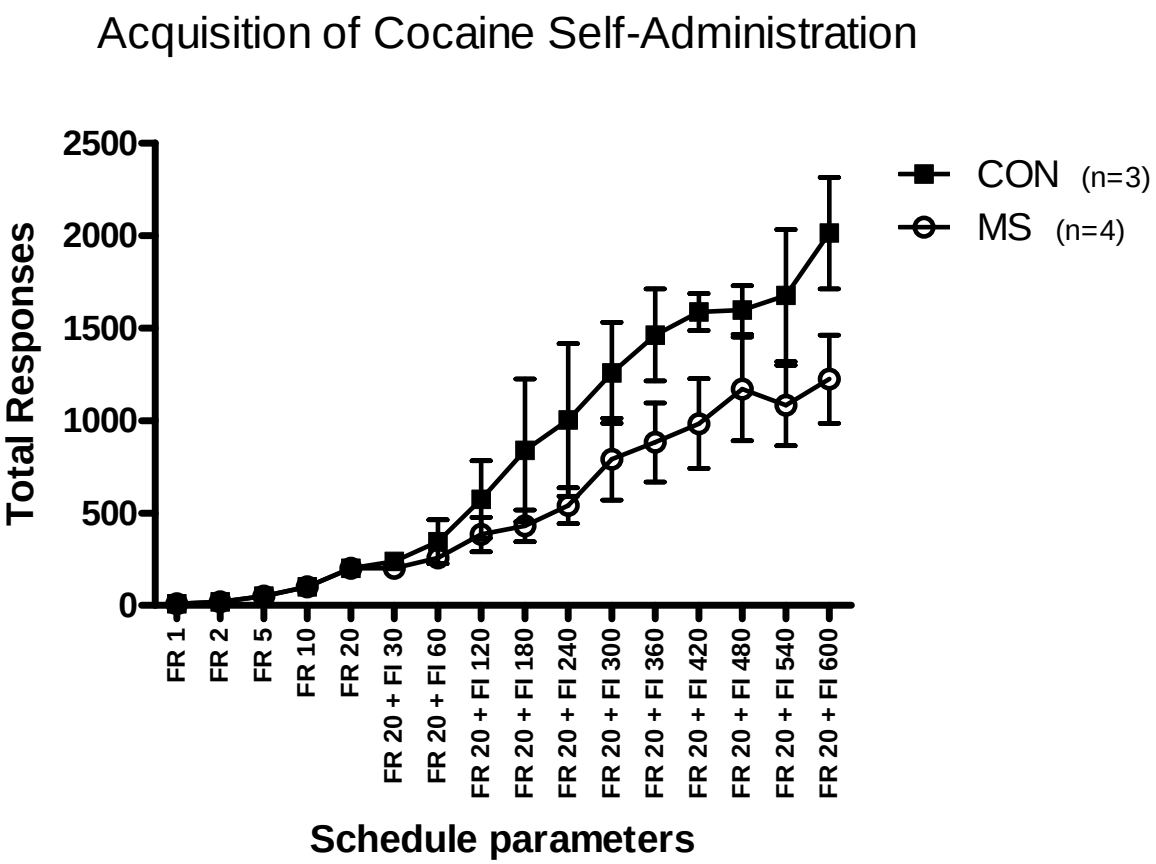

Acquisition of Cocaine Self-Administration
Total Responses
2500
2000
1500
1000
500
0
CON (n=3)
MS (n=4)
FR 1
FR 2
FR 5
FR 10
FR 20
FR 20 + FI 30
FR 20 + FI 60
FR 20 + FI 120
FR 20 + FI 180
FR 20 + FI 240
FR 20 + FI 300
FR 20 + FI 360
FR 20 + FI 420
FR 20 + FI 480
FR 20 + FI 540
FR 20 + FI 600
Schedule parameters

Table 4: Summary of group comparisons using two-tailed Students t-test for unpaired samples.

Test	Control mean (n=3)	Maternally separated mean (n=4)	t value	p value
Days to meet criteria at FR 1	5.67	8.00	0.94	0.39
Days to meet extinction criteria	10.67	7.00	0.67	0.53
Rate of responding in maintenance phase	0.35	0.17	2.69	0.04
Rate of responding at peak cocaine dose	0.67	0.26	2.02	0.10
Rate of responding at peak AMPH dose	0.69	0.23	1.81	0.13

Figure 3: Response rates averaged for the last five cocaine self-administration maintenance sessions (unit dose = 0.1mg/kg/injection). Mean response rates for maternally separated monkeys were significantly lower than those of controls ($t(5) = 2.69$, $p = 0.04$).

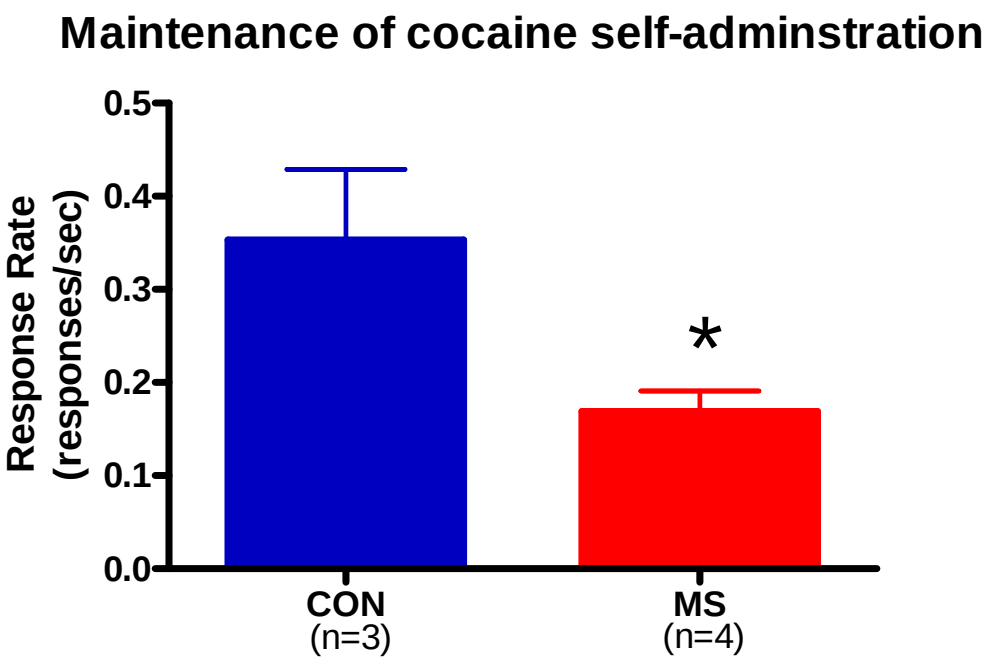
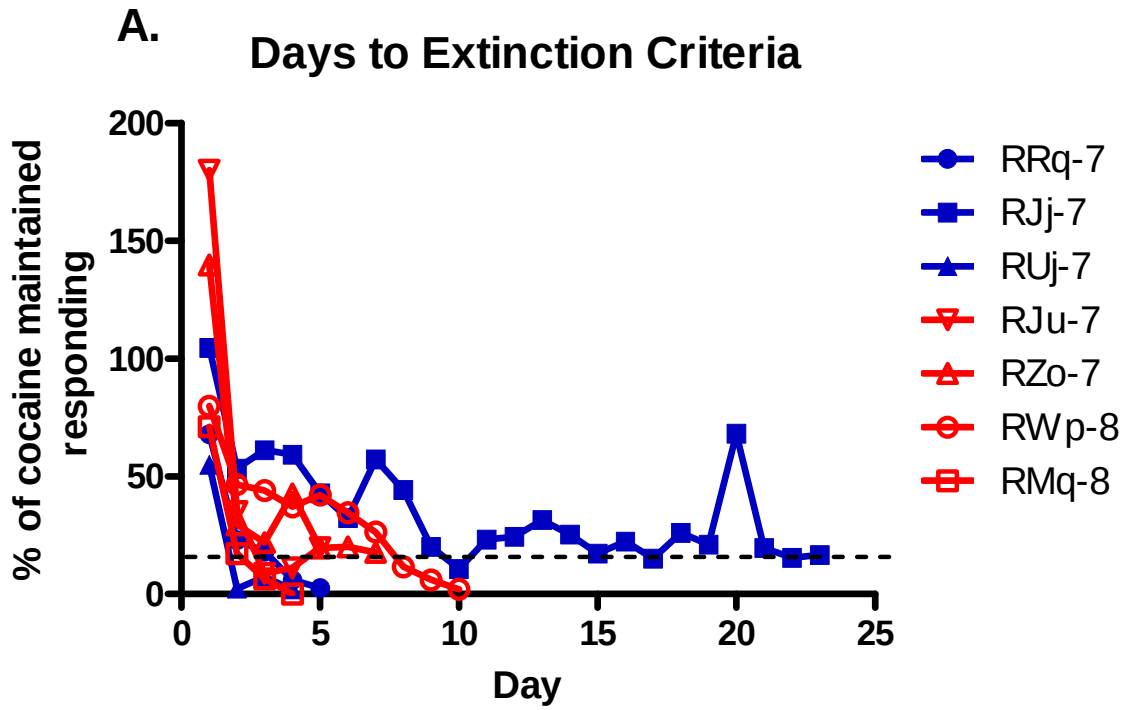

Maintenance of cocaine self-adminstration
Response Rate
(responses/sec)
0.5
0.4
0.3
0.2
0.1
0.0
*
CON
(n=3)
MS
(n=4)

Figure 4: (**A**) Number of days required to meet extinction criteria (<20% of cocaine maintained responding, as indicated by dashed line, for 3 consecutive sessions). Individual subject data are shown since one monkey was an outlier and greatly influenced the mean for the control group. Blue lines (closed symbols) represent control monkeys and red lines (open symbols) represent maternally separated monkeys. On average, control monkeys reached extinction criteria in 10.7 ± 6.17 days, while the average for maternally separated monkeys was 7.0 ± 1.47 days. (**B**) Responding in the first component of the session as a % of cocaine maintained responding. The dramatic decrease in first component responding when the drug-paired stimulus (white light) was removed demonstrates the effectiveness of the drug cues to maintain behavior.


A.
Days to Extinction Criteria
% of cocaine maintained responding
200
150
100
50
0
0
5
10
15
20
25
Day
RRq-7
RJj-7
RUj-7
RJu-7
RZo-7
RWp-8
RMq-8

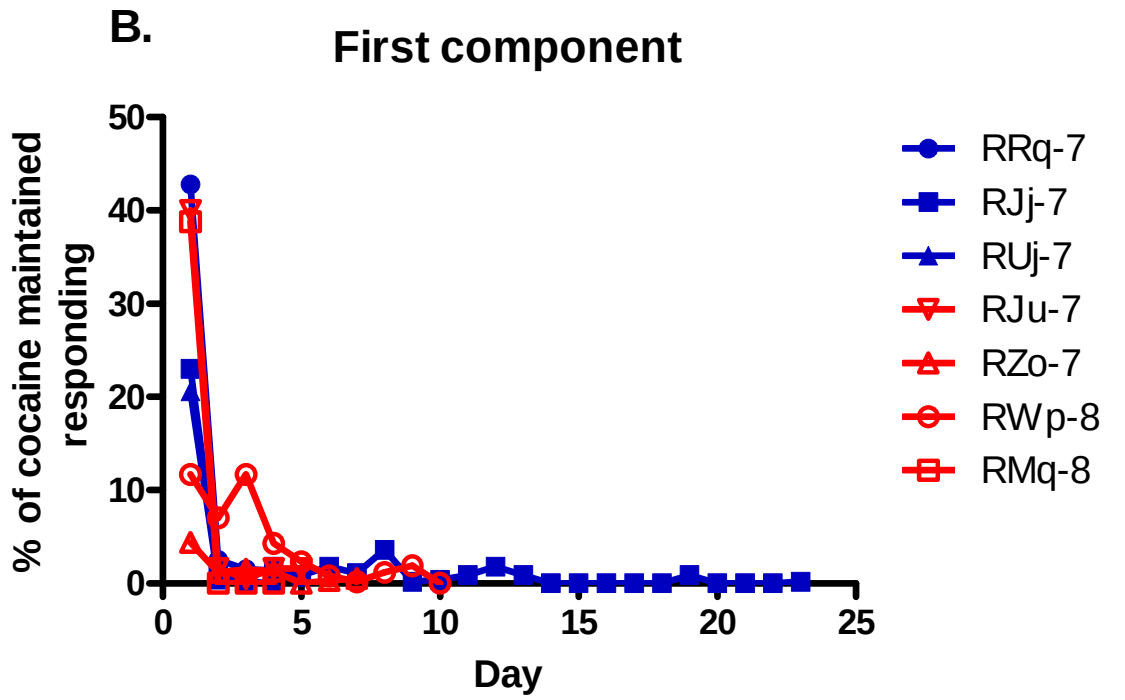
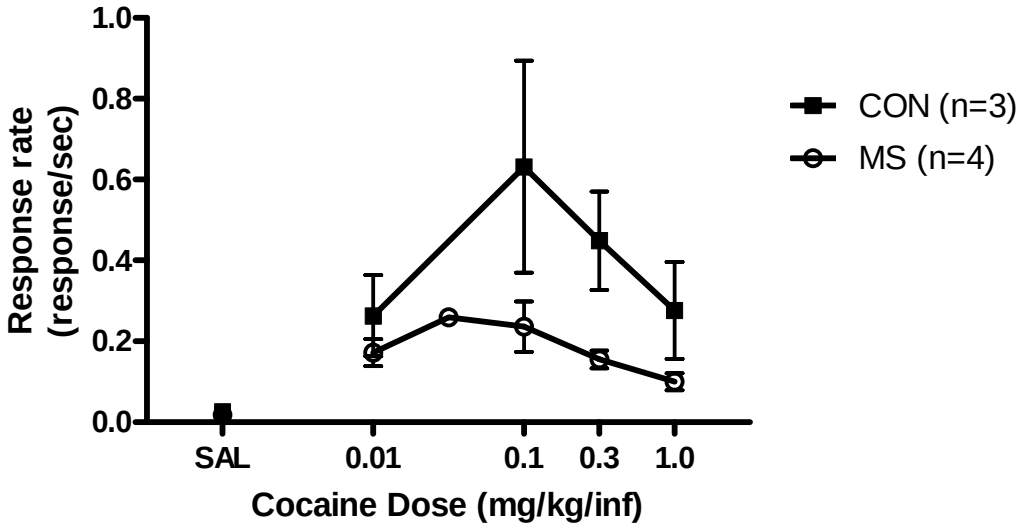

B.
First component
% of cocaine maintained responding
50
40
30
20
10
0
0
5
10
15
20
25
Day
RRq-7
RJj-7
RUj-7
RJu-7
RZo-7
RWp-8
RMq-8

Figure 5: Doses of (**A**) cocaine (0.01-1.0 mg/kg/injection) and (**B**) amphetamine (0.003-0.3 mg/kg/injection) were substituted for the maintenance dose of cocaine. Each dose was tested until response rates were stable for 5 consecutive sessions. Groups differed significantly in responding for a range of doses of cocaine (Mann-Whitney U= 0, $p = 0.03$) and there was a strong trend towards a difference in responding across a range of doses of amphetamine (Mann-Whitney U= 1.0, $p = 0.06$).

A.


Cocaine Dose-Response Curve
Response rate (response/sec)
1.0
0.8
0.6
0.4
0.2
0.0
SAL
0.01
0.1
0.3
1.0
Cocaine Dose (mg/kg/inf)
CON (n=3)
MS (n=4)

B.

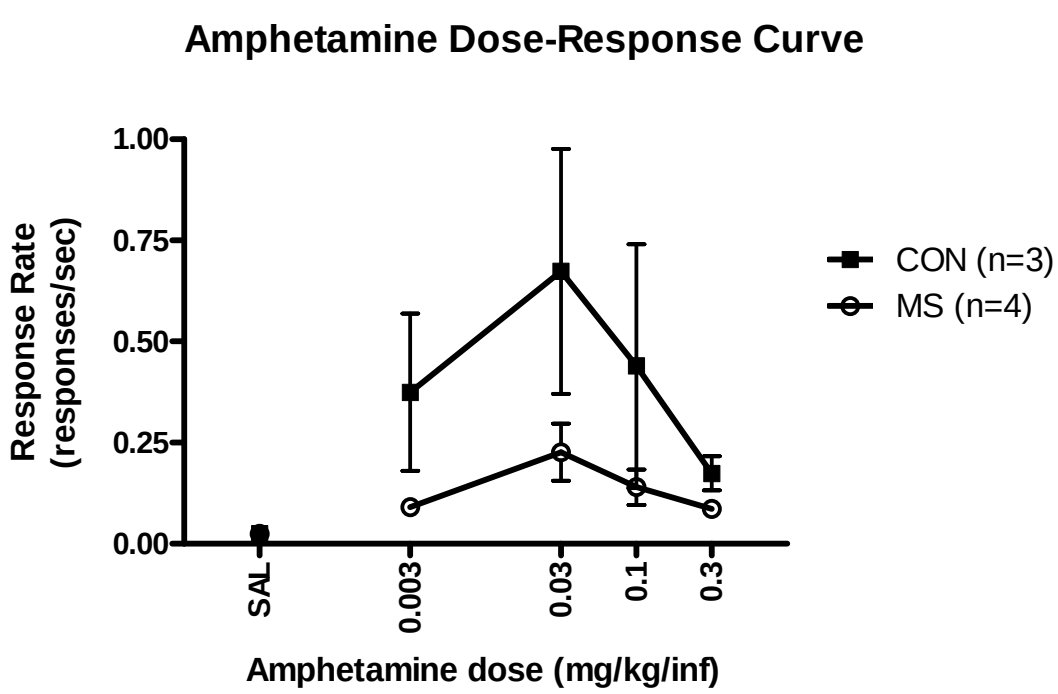
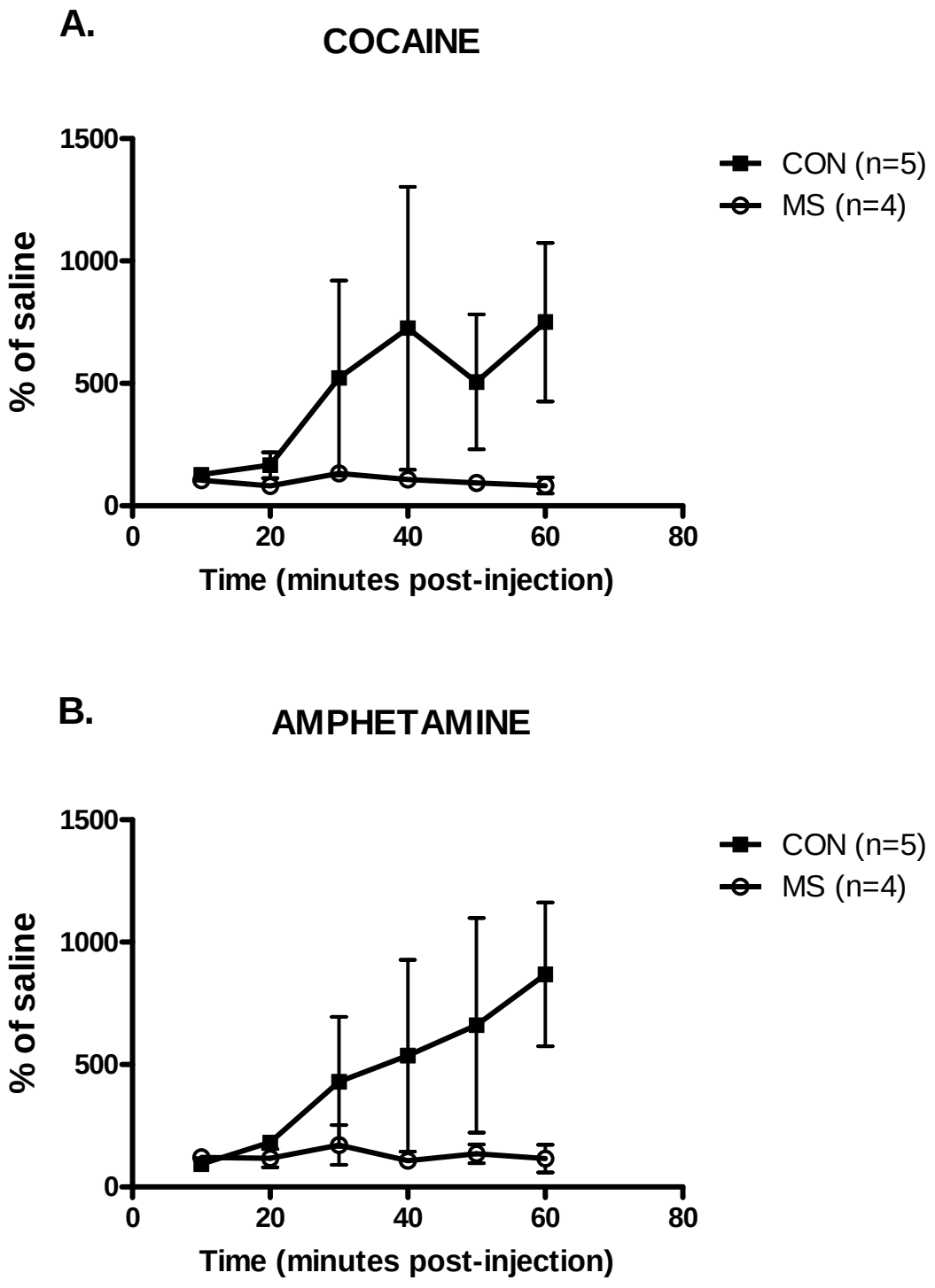

Amphetamine Dose-Response Curve
Response Rate (responses/sec)
1.00
0.75
0.50
0.25
0.00
SAL
0.003
0.03
0.1
0.3
Amphetamine dose (mg/kg/inf)
CON (n=3)
MS (n=4)

Figure 6: Gross motor activity following injection of cocaine (1.0 mg/kg) or amphetamine (0.3 mg/kg) was measured and data normalized to a % of motor activity following saline injection. Control and maternally separated groups differed significantly in (**A**) cocaine-induced increases in motor activity (Mann Whitney $U = 1.0$, $p = 0.004$) and there was a strong trend towards a difference for (**B**) amphetamine-induced increases in motor activity (Mann Whitney $U = 6.0$, $p = 0.06$).


A.
COCAINE
1500
1000
500
0
% of saline
0
20
40
60
80
Time (minutes post-injection)
CON (n=5)
MS (n=4)
B.
AMPHETAMINE
1500
1000
500
0
% of saline
0
20
40
60
80
Time (minutes post-injection)
CON (n=5)
MS (n=4)

Dopamine D_2 Receptor Binding Potential

PET imaging with the dopamine D_2 receptor radioligand [^{18}F]fallypride was used to determine dopamine D_2 receptor binding potential in the caudate nucleus and putamen of each monkey. Group means (± SEM) were derived and are presented in Figure 7. Mean binding potential in the caudate nucleus for controls was 47.99 ± 7.38 and 50.29 ± 3.48 for maternally separated monkeys. In the putamen, mean binding potential for controls was 42.92 ± 6.20 and 46.09 ± 3.04 for maternally separated monkeys. These differences were not statistically significant (caudate: $t(6) = 0.28$, $p = 0.78$; putamen: $t(6) = 0.46$, $p = 0.66$).

In vivo microdialysis

In both groups, cocaine (1.0 mg/kg) increased extracellular dopamine in the caudate nucleus to approximately 300-350% basal levels within the first two 10 min samples following drug administration. Dopamine levels returned to baseline levels approximately 60 min post-injection. Control and maternally separated monkeys did not differ statistically in their response to cocaine ($F(17, 34) = 1.18$, $p = 0.33$) (Figure 8A).

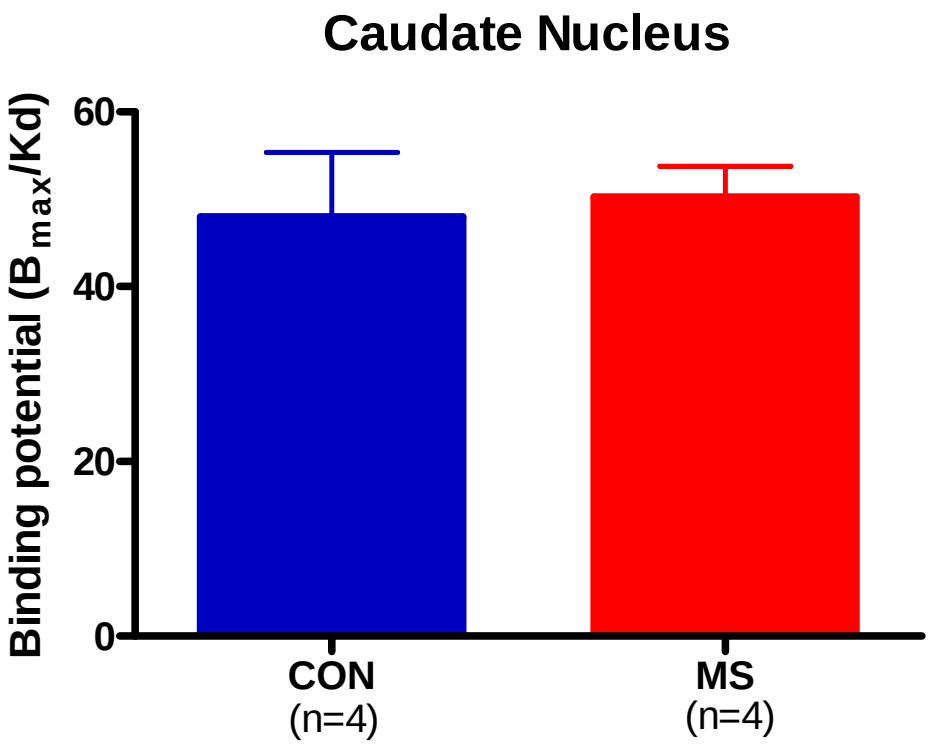
In control monkeys, amphetamine (0.3 mg/kg) increased extracellular dopamine in the caudate nucleus to approximately 275% basal levels within the first two 10 min samples following drug administration. In maternally separated monkeys, amphetamine increased extracellular dopamine in the caudate nucleus to approximately 700% basal levels within the first two 10 min samples following drug administration. In both groups, dopamine levels returned to baseline levels approximately 120 min post-injection. The

difference observed between control and maternally separated monkeys did not attain statistical significance ($F(17, 34) = 0.97$, $p = 0.51$) (Figure 8B).

Individual monkey data following cocaine administration are shown in Figure 9. There was minimal variability within subjects as well as between subjects within groups. Individual monkey data following amphetamine administration are shown in Figure 10. Due to problems maintaining dialysis implants in two of the monkeys, data from only one hemisphere were available. While there was minimal variability between control monkeys, subject RJj-7 (control) did show some variability between hemispheres. In the maternally separated monkeys there was a great amount of variability between monkeys, in addition to the large amount of variability subject RJu-7 (maternally separated) demonstrated between hemispheres.

At the completion of each microdialysis experiment a high K^+ solution, which induces voltage dependent dopamine release, was substituted for the aCSF as a way to confirm site viability. All monkeys demonstrated a robust increase in dopamine in response to the high K^+ solution. The mean high K^+ dopamine response following experiments with 1.0 mg/kg cocaine was 1171.8±371.3% for control monkeys and 490±197.8% for maternally separated monkeys. The mean high K^+ dopamine response following experiments with 0.3 mg/kg amphetamine was 759.9±16.5% for control monkeys and 658.17±521.2% for maternally separated monkeys.

Figure 7: Groups did not differ in dopamine D_2 receptor binding potential as measured with [^{18}F]fallypride binding in the caudate nucleus($t(6) = 0.28$, $p = 0.78$) or the putamen ($t(6) = 0.46$, $p = 0.66$).


Caudate Nucleus
Binding potential (B_{max}/Kd)
60
40
20
0
CON
(n=4)
MS
(n=4)

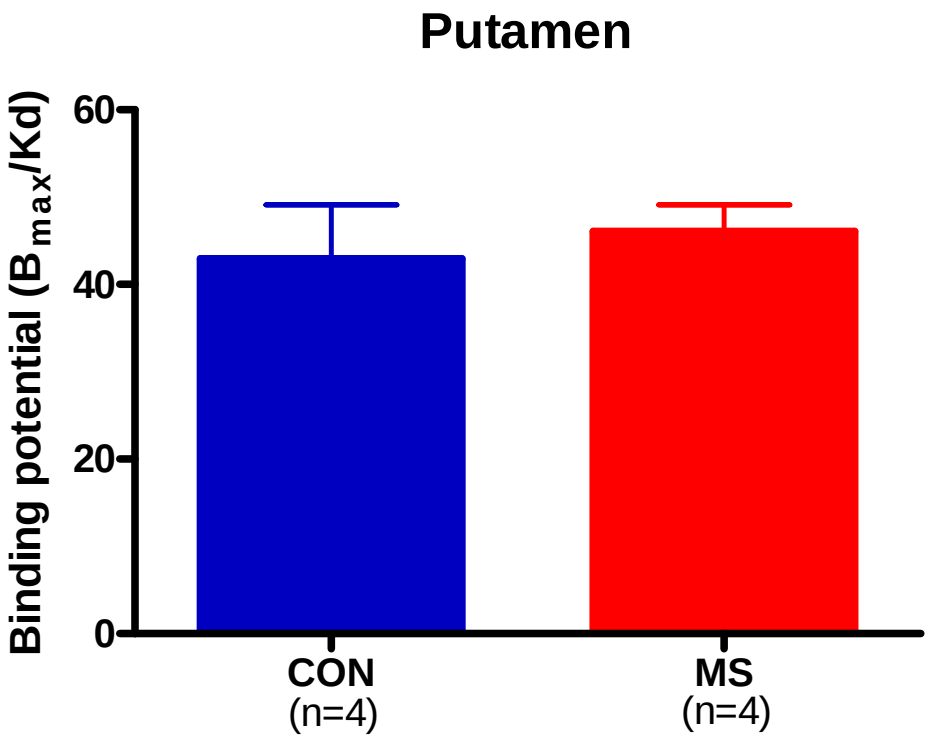
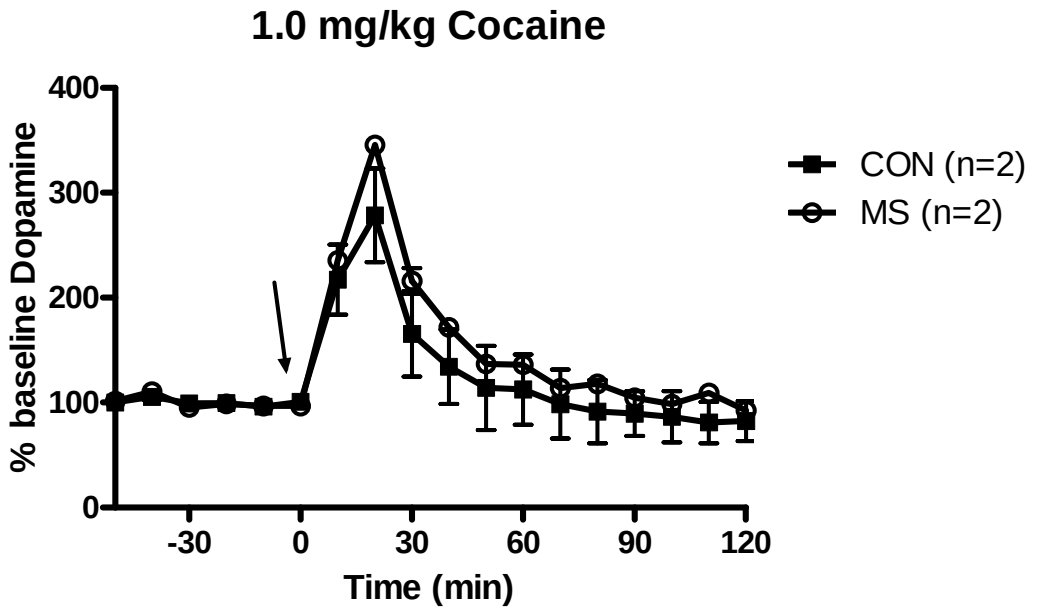

Putamen
Binding potential (B_{max}/Kd)
60
40
20
0
CON
(n=4)
MS
(n=4)

Figure 8: Effects of (**A**) cocaine (1.0 mg/kg) and (**B**) amphetamine (0.3 mg/kg) on extracellular dopamine levels in the caudate nucleus determined with in vivo microdialysis in conscious monkeys. Groups did not differ in their response to cocaine ($F(17, 34) = 1.18$ $p = 0.33$) or amphetamine ($F(17, 34) = 0.97$ $p = 0.51$).

A.


1.0 mg/kg Cocaine
% baseline Dopamine
400
300
200
100
0
-30
0
30
60
90
120
Time (min)
CON (n=2)
MS (n=2)

B.

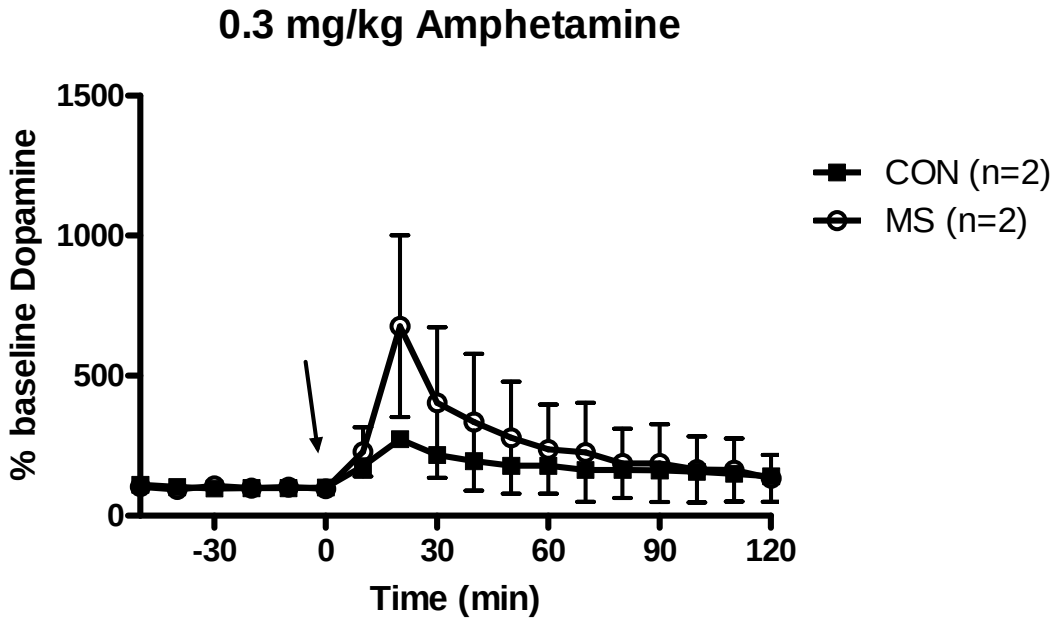

0.3 mg/kg Amphetamine
% baseline Dopamine
1500
1000
500
0
-30
0
30
60
90
120
Time (min)
CON (n=2)
MS (n=2)

Figure 9: % of baseline dopamine following a non-contingent injection of 1.0 mg/kg cocaine at time point 0, measured with in vivo microdialysis. Data for individual animals represents the mean of bilateral experiments. Group data is shown as mean ± SEM.

1.0 mg/kg Cocaine

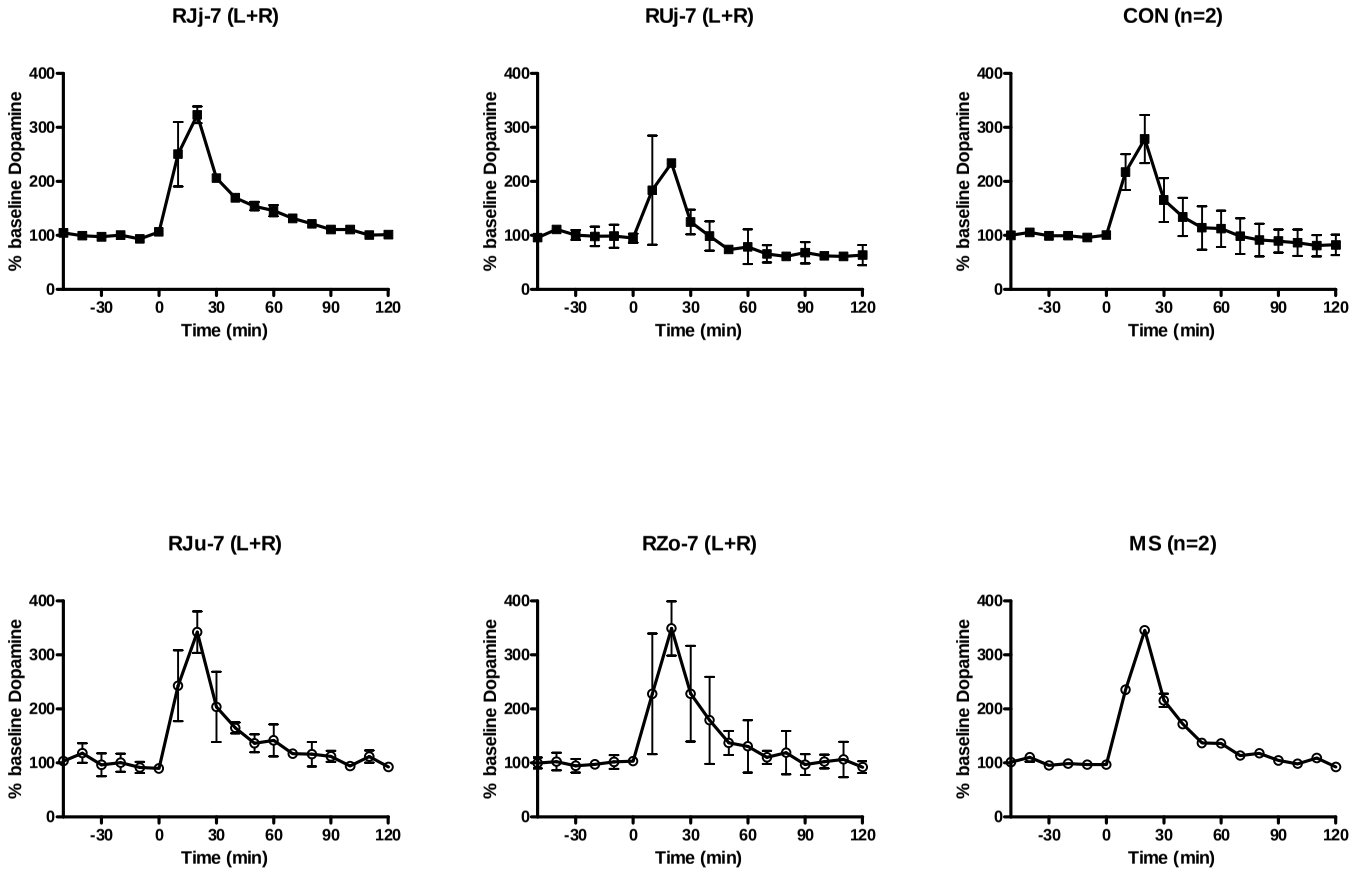
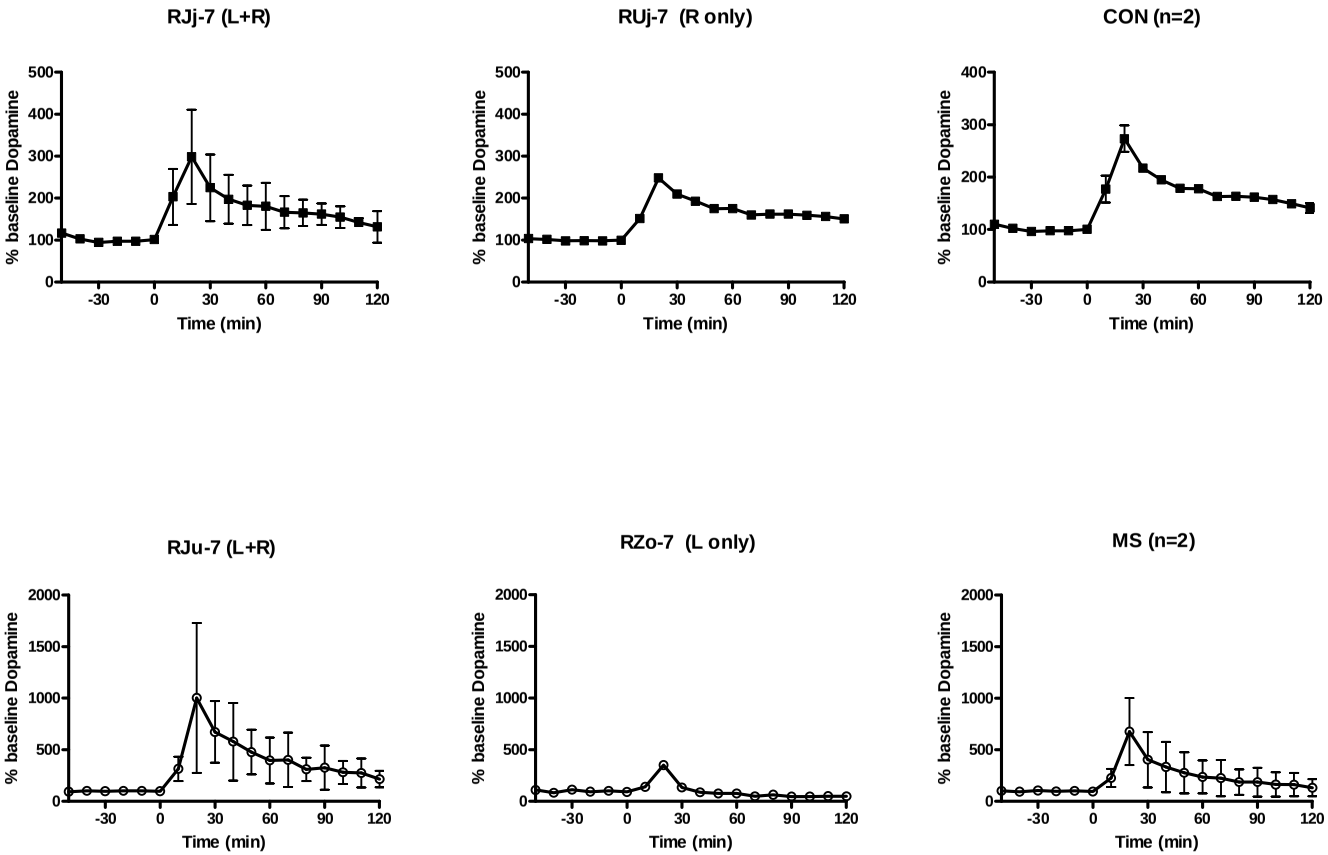


Figure 10: % of baseline dopamine following a non-contingent injection of 0.3 mg/kg amphetamine at time point 0, measured with in vivo microdialysis. Data for individual animals represents the mean of bilateral experiments for subjects RJj-7 (control) and RJu-7 (maternally separated). Data from only one hemisphere was collected from the other subjects due to problems maintaining dialysis implants. Group data is shown as mean ± SEM.

0.3 mg/kg Amphetamine



Discussion

Early life stress has been shown to have profound effects on the HPA axis and stress reactivity. A large body of literature derived from rodent studies has also shown that stressors early in life can lead to an increase in self-administration of psychostimulants and other drugs of abuse. The current set of experiments set forth to determine whether early life stress in nonhuman primates influenced the sensitivity to cocaine and amphetamine, including the propensity to self-administer each drug. Measures of cocaine- and amphetamine-induced changes in dopamine neurotransmission with in vivo microdialysis and measures of D_2 receptor binding potential with PET imaging did not find group differences. However, the behavioral effects of these psychostimulants were blunted in maternally separated monkeys compared to controls. These results do not provide support for early life stress leading to enhanced vulnerability to stimulant use in the nonhuman primate model employed.

Drug self-administration

All monkeys acquired cocaine self-administration at the same rate, as they all met the acquisition criteria in the minimum amount of time required. However, the maternally separated monkeys showed reduced behavioral output throughout the acquisition of cocaine self-administration as compared to controls. This reduced behavioral output was also seen during the maintenance phase of cocaine self-administration. Maternally separated monkeys exhibited significantly lower rates of responding as compared with controls during the last 5 sessions of a 10 day self-administration maintenance period. When saline was substituted for cocaine and the

cocaine-paired stimuli were removed in extinction sessions, 6/7 subjects showed a rapid reduction in responding and met extinction criteria within 10 days. Subject RJj-7 (control) was more resistant to extinction and required 23 days to meet extinction criteria. However, there was no significant group difference in the average number of days to meet extinction criteria. When a range of drug doses of cocaine and amphetamine was substituted for the maintenance dose of cocaine, maternally separated monkeys demonstrated reduced behavioral output and response rates across drug doses for both cocaine and amphetamine, resulting in downward shifted, flattened dose-response functions. However, the dose at which peak responding occurred for both stimulants remained the same for both groups. Although maternally separated monkeys did not differ significantly from controls on measures of acquisition and extinction, they consistently demonstrated reduced behavioral output during drug self-administration as compared to control monkeys, and they did not appear to be sensitive to changes in the unit dose of cocaine or amphetamine.

Locomotor activity

Control monkeys exhibited stimulant-induced increases in motor activity but maternally separated monkeys did not, with activity in the latter group not increasing beyond that induced by an injection of saline. Maternally separated monkeys did not appear to be sensitive to the motor-stimulating effects of cocaine or amphetamine, even though the doses administered induce robust behavioral stimulant effects in nonhuman primates. This lack of sensitivity to the motor effects of cocaine substantiates the attenuated responding found in the maternally separated monkeys during the drug self-

administration and the lack of sensitivity seen in response to a change in unit dose for both cocaine and amphetamine. Both the reinforcing and motor-stimulating effects of psychostimulants have been attributed to their actions on dopamine. Given the diminished reinforcing and motor effects found in the maternally separated monkeys it was possible that their dopaminergic system may have been hypo-functional. Additional studies specifically designed to examine aspects of the dopamine system were performed in these animals in an attempt to elucidate the source of the blunted reinforcing and motor-stimulating effects of psychostimulants.

Dopamine

As described previously, there appears to be an inverse relationship between dopamine D_2 receptor availability and stimulant reinforcement, such that drugs are more reinforcing in organisms with lower levels of D_2 receptors. In order to assess whether differences in dopamine D_2 receptors could explain the differences seen in drug self-administration behavior and stimulant-induced locomotor activity, PET imaging with the dopamine D_2 receptor radioligand [^{18}F]fallypride was used. Control and maternally separated monkeys did not differ in dopamine D_2 receptor binding potential in the dorsal striatum. Additionally, correlations between dopamine D_2 receptor levels and response rates during the maintenance phase of cocaine self-administration and for the peak doses of both cocaine and amphetamine were examined (data not shown). No relationships were found between levels of D_2 receptors and rates of responding on these measures. Therefore, dopamine D_2 receptor levels cannot explain the group differences seen in behavioral output during acquisition of cocaine self-administration, in response rates

during maintenance and across a range of doses of cocaine or amphetamine, or in drug-induced increases in locomotor activity. Similarly, control and maternally separated monkeys did not differ significantly in their response to stimulant-induced increases in extracellular dopamine in the caudate nucleus. Both groups exhibited an increase in extracellular dopamine of 300-350% in response to an injection of cocaine. Although, the maternally separated monkeys showed a greater increase in the release of dopamine (700%) as compared to the controls (275%) following an amphetamine injection, this difference did not reach statistical significance. These data are in contrast to those from studies in rodents in which maternally separated or neonatally isolated animals were found to exhibit greater increases in extracellular dopamine levels in response to stimulant challenges (Hall et al. 1999; Kehoe et al. 1996, 1998; Kosten et al. 2005). However, five of the nine monkeys lost their dialysis caps before any or all data could be collected resulting in complete data sets from only 2 monkeys, and partial data sets from an additional 2. The limited group sizes may have contributed to the lack of statistical significance in the differences seen in extracellular dopamine following amphetamine administration.

Although the majority of literature derived from rodent studies has reported enhanced sensitivity to psychostimulants following early life stress, the results of the current study do not support an increase in sensitivity to cocaine or amphetamine in rhesus monkeys that underwent maternal separation. In fact, maternally separated monkeys showed a reduced behavioral output during the maintenance phase of self-administration and in response to a wide range of doses of both cocaine and amphetamine. One interpretation of the downward displacement of the dose-response

functions and the reduced behavioral output during the maintenance phase of self-administration is that the maternally separated group was less sensitive to psychostimulants. The blunted response to the locomotor-stimulant effects of cocaine and amphetamine is consistent with the latter interpretation. However, the maternally separated monkeys showed no increase in responding at the higher end of the dose range for cocaine and amphetamine as would be expected if they had a reduced sensitivity to psychostimulants. Alternatively, these monkeys may have reduced motivation that is leading to the overall lower behavioral output on all measures. Testing the maternally separated monkeys on a progressive-ratio schedule might provide more direct evidence of the reinforcing efficacy of cocaine and amphetamine. Operant tasks with alternative reinforcers may be another way to target the reinforcing efficacy of psychostimulants in these monkeys.

Reinforcing effectiveness is thought to be mediated to some extent by the level of dopamine D_2 receptors (Volkow et al. 1999; Morgan et al. 2002; Nader et al. 2006). However, no differences were found in D_2 receptor levels between control and maternally separated monkeys so this cannot explain the differences in reinforcing effects seen in self-administration studies. The PET imaging studies were performed after the monkeys had participated in drug self-administration experiments so it is possible that inherent differences in D_2 levels did exist and were in some way normalized by stimulant exposure. A number of studies have demonstrated that chronic exposure to psychostimulants can decrease dopamine D_2 receptor availability. Cocaine abusers show significant decreases in dopamine D_2 receptor availability, which persists even 3-4 months after detoxification (Volkow et al. 1993). Cocaine self-administration decreased

dopamine D_2 receptor availability in rhesus monkeys as well; however, after a period of abstinence, 60% of the monkeys demonstrated a recovery in D_2 receptor availability (Nader et al. 2006). Similarly, long-term cocaine use changed D_2 receptor levels and cocaine reinforcement such that dominant and subordinate rhesus monkeys, who had exhibited differences in both cocaine self-administration and D_2 receptor availability after exposure to social housing, were no longer different (Czoty et al. 2004). Nader et al. (2006) also found a marked inverse relationship between D_2 receptor availability in drug-naïve rhesus monkeys and future rates of cocaine self-administration, suggesting that low D_2 receptor availability is a predisposing trait to cocaine abuse. Future experiments would benefit from the determination of dopamine D_2 levels in drug-naïve monkeys prior to drug self-administration experiments.

The phase of the menstrual cycle has been found to alter dopamine D_2 receptor availability as measured with [^{18}F]FCP. Drug naïve female cynomologus monkeys in the luteal phase had measures of D_2 receptor availability that were 12% higher in the caudate nucleus and putamen than when in the follicular phase (Czoty et al. 2008). This difference is outside the range of reported between studies variation in [^{18}F]FCP availability (approximately 2%) (Nader et al. 1999). From these PET studies it is not possible to determine whether these changes in receptor availability across the menstrual cycle were due to changes in receptor numbers, fluctuations in levels of extracellular dopamine or a combination of both. Menstrual cycle was not monitored in the monkeys that served as subjects for the present set of experiments. Therefore, differences in dopamine D_2 receptor availability may have been missed due to imaging the monkeys in different phases of their cycle. Given the findings of Czoty et al. (2008), future studies

should take into account the effect of the menstrual cycle on measures of receptor availability with PET neuroimaging.

The present study utilized a stress paradigm that is quite different from the stressors that are commonly described in the literature derived from rodent studies. Rodents are typically only a few months old when tested for acquisition of drug self-administration and thus only a few months removed from the stressors. Acute effects of the early life stress and resulting alterations in corticosterone levels are probably playing a major role in the subsequent changes seen in drug self-administration in the rodent. In the present study, the monkeys were 5-6 years removed from the maternal separations when the acquisition of cocaine self-administration sessions began. Therefore, any differences observed in sensitivity to stimulants would be related to enduring changes in neurobiology. Substantial individual differences were seen in responding during acquisition of cocaine self-administration, in dose-response functions for both cocaine and amphetamine and in motor activity following administration of both cocaine and amphetamine, particularly in the control group. It is possible that group differences may have been overshadowed by this individual variability given the small group sizes. Additionally, the manner in which the animals were reared calls into question the appropriateness of the control group to serve as true controls. The entire social group was disrupted during the maternal separations and the control and maternally separated monkeys were later pair housed for a period of time. These factors may have adversely affected the control monkeys. Another limitation in the present study was the use of only female subjects. As a result, effects of gender or the interaction of rearing and gender on drug self-administration for psychostimulants were not evaluated.

Overall, significant differences between maternally separated monkeys and controls were not observed in the rate of acquisition for cocaine self-administration or in days to reach extinction criteria. However, rates of responding during the maintenance phase of self-administration were significantly lower and dose-response functions for cocaine and amphetamine were shifted downward in maternally separated monkeys. Maternally separated monkeys consistently showed reduced behavioral output as compared to controls across the range of doses tested for cocaine and amphetamine, and failed to exhibit stimulant-induced motor activity to cocaine or amphetamine injection. However, these differences cannot be explained by dopamine D_2 binding potential or differences in stimulant-induced dopamine release. Collectively, these data do not provide support for early life stress, in the form of maternal separation, increasing sensitivity or vulnerability to the reinforcing effects of psychostimulants in the nonhuman primate model employed.

Chapter 4: Long-term impact of early life stress on behavioral and HPA axis reactivity

Abstract

Early life stress in the form of maternal separation in rhesus monkeys has been found to lead to a phenotype of a HPA axis that is basally hypo-functional but hyper-reactive when challenged in maternally separated monkeys. The present study investigated whether this maternally separation phenotype persists into adulthood. Additionally, this study explored possible differences in brain metabolism, as well as behavioral and HPA axis based on rearing history. PET imaging with [^{18}F]fluorodeoxyglucose was used as a measure of cerebral glucose metabolism under basal conditions. Baseline acoustic startle reactivity was measured to determine if the enhanced startle reactivity described in the maternally separated infants persisted into adulthood. Acoustic startle reactivity was also measured following drug challenges with cocaine (0.3 and 1.0 mg/kg), amphetamine (0.1 and 0.3 mg/kg), dopamine D_1 agonist SKF 82958 (0.3mg/kg) and D_2 agonist quinpirole (0.03 and 0.1 mg/kg) to assess behavioral reactivity and dopaminergic modulation of the startle reflex. HPA axis reactivity, as measured with plasma cortisol concentration, was also determined in response to an acute cocaine injection. No differences in cerebral glucose metabolism were found between control and maternally separated monkeys. Baseline acoustic startle reactivity was no longer different when comparing maternally separated monkeys to controls. Acoustic startle reactivity also did not differ between groups in response to any drug challenge. Plasma cortisol levels were determined following a separation challenge

and a study of diurnal cortisol rhythm in these monkeys when they were infants as part of a larger study which found elevated cortisol levels in response to separation but blunted diurnal cortisol rhythm in maternally separated monkeys as compared with controls. When the data from the nine monkeys involved in the present study were re-analyzed apart from the larger data set, no significant group differences were found but the data from the maternally separated monkeys did follow the trends of the previous study. Finally, no significant group differences were seen in HPA axis reactivity to an acute cocaine challenge. As a whole, these results suggest that the detrimental consequences of maternal separation dissipate as the animals mature to adulthood.

Introduction

Maternal separation phenotype

As previously described, maternal separation is a form of early life stress that can have a profound effect on the behavior and physiology of an organism. As infants, the monkeys involved in the present set of experiments were part of a study by Sanchez et al. (2005) examining the short-term effects of repeated, intermittent maternal separations in infancy on the HPA axis. This study found that in the rhesus monkey, maternal separations produce increased cortisol release in response to acute separation challenges at 5.5 months, but the same monkeys showed a flattened diurnal cortisol rhythm when tested at a later age (12 months old) (Sanchez et al. 2005). These monkeys also exhibited enhanced acoustic startle reactivity when tested at 20-24 months of age. The individual

differences in HPA axis reactivity to maternal separation appeared to also be predictive of startle response measured almost 18 months later (Sanchez et al. 2005).

The effects of maternal separation in these monkeys appear to be gender specific. At 5.5 months of age, infant female subjects exhibited higher basal and stress-induced cortisol levels than male subjects, independent of rearing condition (Sanchez et al. 2005). The magnitude of the response to an acute separation was highest in maternally separated female subjects compared with all other groups, suggesting a "sensitization" of the infant female HPA axis by the repeated separation experience. The maternally separated female monkeys also had the most "flattened" diurnal pattern of cortisol secretion of all the monkeys tested (Sanchez et al. 2005).

In general, the phenotype of the maternally separated monkeys, particularly the maternally separated females, is one of basal hypoactivity with regards to HPA axis function with elevated behavioral reactivity, as measured with acoustic startle reactivity. This phenotype has been shown to persist through the first 24 months of life (Sanchez et al. 2005). However, there appears to be some normalization as the monkeys age. When the diurnal pattern of cortisol secretion was re-tested at 22 months in a subset of monkeys, they found that the flattening of the diurnal pattern of cortisol secretion was still present but was smaller than when tested at 12 months and there were no longer any significant differences between control and maternally separated monkeys in % decline of cortisol secretion (Sanchez et al. 2005). However, there are no other published reports of the long-term effects of maternal separation in these rhesus monkeys and it is important to determine whether this maternal separation phenotype persists into adulthood.

The current set of experiments was designed to assess whether the maternal separation phenotype persists in the subset of monkeys (5 controls and 4 maternally separated). Additionally, this study explored possible differences in cerebral glucose metabolism based on rearing history, as well as behavioral and HPA axis reactivity to drug challenges.

PET imaging with [^{18}F]fluorodeoxyglucose

In order to assess cerebral glucose metabolism, PET imaging with [^{18}F]fluorodeoxyglucose (2-fluoro-2-deoxy-D-glucose, FDG) was used. [^{18}F]FDG is a radiolabeled glucose analog that is taken up by high glucose utilizing cells, including neurons, where it serves as a ligand for PET imaging. After it crosses the blood brain barrier and is transported into the neuron, [^{18}F]FDG is phosphorylated by the hexokinase system to deoxyglucose-6-phosphate,which is similar to glucose-6-phosphate (phosphorylated glucose), but instead of being metabolized to CO_2 and H_2O it remains intact and is trapped inside the cell with its accumulation dependent on neuronal activity (Sokoloff 1977). Radiolabeled deoxyglucose is a great tool for mapping regional brain function due to this unique metabolic behavior. Cells that are most active will take up the most glucose and will show the strongest PET signal; the density pixels represents signal intensity in areas where neurons are most active (Sokoloff 1977).

When radiotracers degrade they emit positrons which are highly unstable particles which travel a short distance and collide with electrons. This reaction releases two photons travelling in exactly opposite directions (180°apart), this is called “coincident release”. Photons are then detected by the detector ring encircling the head and are

reconstructed by the computer. The cameras are instructed to only include for final analysis those particles that are recorded simultaneously in the camera and its 180° counterpart thus enabling a more precise reconstruction of exactly where the photon originated, called "coincident detection" (Herholtz et al. 2004).

Factors affecting glucose metabolism

A number of factors have been shown to influence cerebral glucose metabolism. Of particular relevance to the current study, maternal separation has been shown to affect glucose metabolism. An acute maternal separation challenge was associated with increased activity of the right frontal cortex of juvenile rhesus monkeys as measured with FDG PET imaging (Rilling et al. 2001). In this study comparing monkeys kept with their mothers (not separated) to those separated but kept in auditory and visual contact with their mother and those separated with no contact with their mother, both separation conditions resulted in activation of the right dorsolateral prefrontal cortex (Rilling et al. 2001).

Stress in general also appears to have an effect on cerebral glucose metabolism. When corticotrophin releasing factor (CRF) was injected intracerebroventricularly into rhesus monkeys, increased glucose metabolism in the pituitary/infundibulum, amygdala and hippocampus was observed (Strome et al. 2002). These results suggest that increased central CRF tone activates limbic and stress responsive regions (Strome et al. 2002). Additionally, in humans with stress associated disorders, such as anxiety and depression, alterations are seen in glucose metabolism. Patients with anxiety disorder consistently show altered metabolism and blood flow in the hippocampus (Semple et al. 1993; Bisaga

et al. 1998). Following successful treatment with the antidepressant paroxetine, patients with major depression showed decreased hippocampal glucose metabolism, suggesting over activity in this area is associated with depression (Kennedy et al. 2001).

Alterations in glucose metabolism have also been associated with drug abuse. Chronic cocaine users exhibit decreased glucose metabolism, particularly in frontal areas, which persisted even after 3-4 months of abstinence (Volkow et al. 1992). However, the question remains as to whether the decreased metabolic activity is a result of the chronic drug use or whether inherent decreases in brain glucose metabolism could predispose individuals to abuse cocaine. Evidence for differences in glucose metabolism resulting in differences in drug use has been shown in an animal model; however, it suggests that higher levels of glucose metabolism may be a predisposing factor, at least for alcohol use. Using 2-deoxyglucose (2DG) imaging in alcohol naïve animals, Smith et al. (2001) found that rats selectively bred to be alcohol preferring showed higher cerebral glucose utilization than their alcohol non-preferring counterparts.

In the present study, differences in cerebral glucose metabolism based on rearing history and exposure to early life stressors were assessed. Monkeys were drug naïve at the time of the scan allowing for examination of pre-existing alterations in glucose metabolism that may also be predictive of differences in susceptibility to drug use.

Acoustic startle

The acoustic startle reflex is a rapid escape response elicited by a sudden and intense auditory stimulus (Davis 1984). This reflex is probably part of an organism's innate defense response system that detects changes in the surrounding environment,

especially those that may pose a potential threat. The startle pattern consists of eye-lid closure and a contraction of facial, neck and skeletal muscles as well as an arrest of ongoing behaviors and an acceleration of the heart rate (Koch 1999). The startle reflex has a very short latency; in rats the electromyographic latency in leg muscles is 6-8 msec (Davis 1980), which implies that the underlying circuit must be relatively simple. The startle reflex is thought to be mediated by a simple 3 synapse sub-cortical circuit. In the proposed circuit, the acoustic stimulus enters the ear where cochlear root neurons are activated and they in turn act upon the nucleus reticularis pontis caudalis which then acts upon spinal cord motor neurons (Davis et al. 1982; Koch et al. 1993). However, the exact pathway remains unknown and a number of other models have been proposed (reviewed in Yeomans and Frankland 1996). So far, all proposed models include synaptic relays in the cochlear nucleus, in the reticular formation and in spinal motor neurons (Yeomans and Frankland 1996). The startle reflex appears to be under the influence of the dopamine system and has been found to be a useful behavior with which to study the neural mechanisms underlying dopaminergic control of movement (Meloni and Davis 1999). In general, drugs that increase dopamine transmission increase the acoustic startle response. The dopamine agonist apomorphine increased acoustic startle amplitude in rats and this effect could be blocked by pretreatment with haloperidol or pimozide (Davis and Aghajanian 1976; Svensson 1990). Indirect dopamine agonists, cocaine and amphetamine, also increased startle amplitude and this effect was blocked with haloperidol pretreatment (Davis 1985). Dopamine antagonists and drugs that decrease dopamine synthesis, haloperidol and pimozide depress the acoustic startle response in rats (Davis et al. 1975, Sorenson et al. 1975; Kehne et al. 1978; Pohorecky et

al. 1976). However, it has been suggested that dopamine does not have a tonic excitatory effect on the acoustic startle response, but only when first stimulated by dopamine agonists does the dopamine system have an excitatory effect on acoustic startle (Davis 1980). While these findings provide support for dopamine mediating the acoustic startle response, almost nothing is known about which dopamine systems might be contributing to the increased or decreased startle response with corresponding increased or decreased dopamine transmission (Davis 1980).

Acoustic startle has been studied extensively in rodent models and a testing apparatus has recently been adapted to test whole body startle responses in nonhuman primates (Winslow et al. 2002) and eye-blink response in humans (Efferen et al. 2000). An advantage of using the startle response as a measure of behavioral reactivity is that its effects can be measured by modification of a simple reflex that has a non-zero baseline and it requires no learning (Koch 1999; Winslow et al. 2002). This is important because it allows for the response to be either enhanced or attenuated (Koch 1999). The startle reflex can be modulated by a variety of internal and external variables and the modulation is probably due to an enhancement or inhibition of the information transfer between the sensory receptors and the motor output systems (Koch 1999). No long-term habituation in startle amplitude was found between sessions within a 6 month schedule of repeated testing but it did show a modest, but significant, habituation over repeated acoustic stimulus presentations within an individual session (Winslow et al. 2002).

Factors affecting startle reactivity

The acoustic startle reflex is sensitive to stress in early development (Parr et al. 2002). Rhesus monkeys reared with same-age peers showed a greater startle response than monkeys reared with their mothers for the first year of life (Parr et al. 2002). This outcome is similar to the results of studies in rodents (Caldji et al. 2000; Krebs-Thomson et al. 2001; Varty et al. 2000) where animals that experienced early life stress showed a greater startle response than mother-reared, non-stressed animals. As previously described, maternally separated rhesus monkeys exhibited increased acoustic startle reactivity as compared with non-separated controls when tested at approximately 2 years of age (Sanchez et al. 2005). Individual differences in HPA axis reactivity to maternal separation in rhesus monkeys, as measured with plasma cortisol concentrations, were predictive of startle response measured almost 18 months later (Sanchez et al. 2005). This pronounced rearing-related difference in startle reactivity suggests that rearing experience may interact with the emergence of an anxious or fearful temperament for months, perhaps years, after the aversive experience (Sanchez et al. 2005). However, the acoustic startle response has not be described in adult monkeys that were exposed to early life stress, so it is unclear as to whether the increased startle reactivity and the previously described temperament persist.

The startle response appears to be under the influence of dopamine and in general, drugs that increase dopamine transmission also increase the acoustic startle response (Meloni and Davis 1999). Injections of cocaine (2.5-10 mg/kg) resulted in a dose-related increase in the amplitude of acoustic startle in the rat, but comparable injections of procaine actually led to a decrease in acoustic startle amplitude, indicating that the effects

of cocaine could not be attributed to its local anesthetic effects (Davis 1985). Reserpine treatment prior to cocaine injection attenuated it's excitatory effects on acoustic startle, which is consistent with studies that showed that the behavioral effects of cocaine involved stored rather than newly synthesized catecholamines (Davis 1985). High doses of d-amphetamine have also been shown to markedly increase the acoustic startle response in rodents (Davis et al. 1975). These increases can be blocked by α-methyl-p-tyrosine, indicating that amphetamine interacts with newly synthesized catecholamines (Davis et al. 1975).

The effects of acute stimulant administration on the acoustic startle response have not previously been studied in nonhuman primates or in humans. However, experiments have investigated startle reactivity in chronic cocaine users. Chronic cocaine users that had been abstinent for a period of 3 days to 6 months had noticeably diminished acoustic startle responses as compared with controls, which was consistent with a state of decreased dopamine neurotransmission (Efferen et al. 2000).

As previously described, cocaine and amphetamine which are indirect dopamine agonists, can increase the startle response. Direct dopamine agonists can also alter the startle response. Dopamine D_1 agonist SKF 82958 enhanced the acoustic startle response in rats in a dose dependent manner with 1.0 mg/kg producing the maximal amount of startle enhancement (Meloni and Davis 1999). Conversely, administration of dopamine D_2 agonists has been shown to decrease startle amplitude (Davis 1987; Peng et al. 1990). Co-activation of both D_1 and D_2 receptors can produce synergistic facilitation of some dopamine-modulated behaviors (Braun and Chase 1986; Arnt et al. 1987). When the D_1 agonist SKF 38393 and the D_2 agonist quinpirole, at doses that didn't affect startle on

their own, were co-administered they induced a significant increase in the startle response (Wan et al. 1996). Based on these findings, it has been suggested that co-activation of dopamine D_1 and D_2 receptors may be important for the stimulatory effect on startle amplitude.

As part of the maternal separation phenotype described in Sanchez et al. (2005), acoustic startle reactivity was found to be enhanced in maternally separated monkeys as compared with controls. In order to determine the persistence of this phenotype, acoustic startle reactivity was assessed in adult rhesus monkeys with a history of maternal separation. Furthermore, acoustic startle reactivity in response to pretreatments with psychostimulant drugs and dopamine receptor agonists was examined.

HPA axis function and reactivity

Acute administration of cocaine activates the HPA axis increasing the release of plasma ACTH and glucocorticoids such as corticosterone in rats (Rivier and Vale 1987; Saphier et al. 1993; Simar et al. 1996) and cortisol in rhesus monkeys (Broadbear et al. 1999a, b, c; Sarnyai et al. 1996) and humans (Vescovi et al. 1992; Heesch et al. 1995, Ward et al. 1998; see Mello and Mendelson 1997 for review).

Rats injected with cocaine i.p. showed significant increases in plasma ACTH, beta-endorphin and corticosterone concentration (Moldow and Fischman 1987; Borowsky and Kuhn 1991; Levy et al. 1991; Simar et al. 1996). Intravenous injections of cocaine also induced dose-dependent increases in ACTH (Rivier and Vale 1987). Intra-cerebroventricular and intra-hypothalamic injections of cocaine in rats elicited dose-dependent increases in plasma corticosterone (Saphier et al. 1993). Additionally, self-

administered cocaine induced significant increases in plasma corticosterone in rats (Mantsch et al. 2000).

Cocaine also dose-dependently induced increases in ACTH and cortisol release in male rhesus monkeys (Sarnyai et al. 1996). Low doses of cocaine (0.4 mg/kg) increased ACTH peak duration, peak amplitude and mean peak area in comparison to placebo control, but this dose did not increase cortisol peak amplitude. Higher doses of cocaine (0.8 mg/kg) also increased peak duration, amplitude and mean peak area of ACTH and significantly increased cortisol peak amplitude (Sarnyai et al. 1996). The effects of both non-response-contingent and response-contingent cocaine infusions have been explored in rhesus monkeys. Response-contingent cocaine delivery resulted in larger, more dose-dependent HPA axis responses than did the non-response-contingent delivery (Broadbear et al. 1999b). This group also found that when cocaine (1.0mg/kg) was administered to drug-naïve monkeys and again following drug self-administration experiments, no differences were found in cocaine's effects on ACTH or cortisol (Broadbear et al. 1999b). This finding suggests that drug history does not affect the endocrine response to an acute cocaine challenge.

In humans, acute i.v. administration of cocaine increased the secretion of cortisol and ACTH in chronic cocaine users (Mendelson et al. 1989, 1992). Cocaine injection induced a 2-fold increase in plasma cortisol that was apparent both 15 and 45 min post injection (Baumann et al. 1995). In a similar study, when cocaine (0.4 mg/kg) or saline were infused i.v. into a group of male cocaine users, ACTH increased significantly within 8-12 min following cocaine infusion and the increase was significantly correlated with increases in plasma cocaine levels. Peak levels of cortisol were measured at about 36

min following cocaine injection (Mendelson et al. 2002). A tight temporal correlation was also found between levels of plasma ACTH and plasma cocaine levels in a study by Sholar et al. (1998) which demonstrated that plasma concentrations of both ACTH and cocaine reached peak at almost the same time. It has been proposed that this temporal concordance of plasma levels of ACTH and cocaine suggests that cocaine stimulates ACTH by acting on dopaminergic systems which can help to modulate CRF release in the brain (Mendelson et al. 1992).

A growing body of evidence suggests that the HPA axis response to cocaine is mediated by a hypothalamic dopamine neuronal system which directly or indirectly innervates CRF neurons (Borowsky and Kuhn 1991). Other data suggests that the stimulatory effects of cocaine on the HPA axis may be mediated through endogenous CRF release from the hypothalamus (Sarnyai et al. 1995). When endogenous release of CRF was blocked in rats, cocaine injections elicited a significant increase in plasma corticosterone concentration suggesting that the HPA axis response to cocaine is mediated at the level of the pituitary (Moldow and Fischman 1987). This is further supported by the findings of Rivier and Vale (1987) in which i.v. injections of cocaine in adult male rats led to dose-dependent elevations in plasma ACTH which could be blocked with pretreatment with CRF antiserum. They also found that cocaine was not able to induce ACTH secretion in cultured pituitary cells, so the cocaine is not acting directly on the pituitary (Rivier and Vale 1987). Likewise, pretreatment with CRF antibodies or CRF receptor antagonists in rats dose-dependently prevented the cocaine-induced increase in corticosterone levels further supporting the hypothesis that the

activation of HPA axis by cocaine is mediated through the release of endogenous CRF (Sarnyai et al. 1992).

Although the exact mechanism of cocaine-induced activation of the HPA axis is unknown it appears to involve DA and 5-HT neurons in the brain (Borowsky and Kuhn 1991; Levy et al. 1991; Levy 1994) in addition to CRF. The findings of Borowsky and Kuhn (1991) suggest important stimulatory roles for both dopamine and serotonin in the adrenocortical stimulation by cocaine. In particular, it appears that the stimulation of HPA function by cocaine is mediated by the inhibition of DA and 5-HT uptake. Both selective D_1 antagonist SCH 23390 and selective D_2 antagonist sulpiride attenuated the ACTH response to cocaine in rats (Borowsky and Kuhn 1991), so it appears that both D_1 and D_2 receptors are involved in the dopaminergic component of this response. Furthermore, GBR 12909, a selective DA uptake inhibitor, dose-dependently stimulated adrenocortical activity and this effect was blocked with haloperidol pretreatment (Borowsky and Kuhn 1991). Treatment with the serotonin 5-HT_2 receptor antagonist ketanserin also significantly attenuated the ACTH response to cocaine, suggesting that the adrenocortical response to cocaine may be mediated in some way by serotonergic stimulation in addition to, or as a modulator of, the dopaminergic mediation (Borowsky and Kuhn 1991).

In the present study, plasma cortisol and ACTH concentrations were measured in response to acute cocaine administration as an indication of HPA axis reactivity in adult rhesus monkeys that experienced maternal separations as infants.

Summary and rationale

The present study investigated whether the maternal separation phenotype described by Sanchez et al. (2005) was evident in this subset of monkeys and if it persisted into adulthood. Maternal separation in the rodent model has been found to be long-lasting and also to increase sensitivity to psychostimulant drugs. The primary purpose of the present study was to investigate the role of rearing history on sensitivity to psychostimulant drugs. However, these drug sensitivity measures were conducted when the monkeys were adults, so understanding whether the effects of the maternal separations persist into adulthood is important for the interpretation of that data. These studies also examined behavioral and HPA axis reactivity under both baseline conditions and following psychostimulant challenges. To this end, data from the subset of monkeys used in the present study was extracted from the data set used by Sanchez et al. (2005) and analyzed to determine cortisol response to an acute maternal separation and diurnal rhythm of cortisol. Comparisons of basal glucose metabolism were designed to determine if rearing history influenced global brain function. Baseline behavioral reactivity and reactivity following pretreatment with both indirect (cocaine and amphetamine) and direct dopamine receptor agonists (SKF 82958 and quinpirole) was determined using acoustic startle. Additionally, cortisol and ACTH concentrations prior to and following acute cocaine administration were quantified.

Methods and Materials

PET imaging with [^{18}F]fluorodeoxyglucose : Monkeys, 4-5 years of age, that were drug naïve at the time of the scan, were injected with a dose of [^{18}F]fluorodeoxyglucose (FDG, 15 mCi, PETNET Solutions, Atlanta, GA) i.m. while conscious in their home cage. The monkey was left undisturbed and did not engage in any scheduled tasks for 45 minutes to allow for uptake of the FDG. Brain uptake of [^{18}F]FDG following i.m. injection in the rhesus monkey was found to be approximately linear between 0-40 min and was to at least 80% of its maximum within 40 min (Rilling et al. 2001). Therefore, 40-45 min was determined to be an appropriate time frame for a behavioral session following FDG injection due to the neural activity-dependent patterns of glucose fixation (Rilling et al. 2001). At the end of the 45 min period the animal was anesthetized with Telazol (4.0 mg/kg) and transported to the Yerkes Imaging Center to be scanned on the MicroPET Focus scanner. The monkeys were intubated and anesthetized with isoflurane (1-2%) during the scan. The imaging session lasted approximately 15 min and followed the procedures of Rilling et al. (2001). At the completion of the imaging session, monkeys were returned to their home cage to recover.

Acoustic Startle: Methods for measuring whole-body acoustic startle in the rhesus monkey were adapted from Parr et al. (2002) and Winslow et al. (2002). Monkeys began startle sessions when approximately 7-8 years old. For testing, the animals entered a transport box and were carried to the testing room. They were then relocated to a squeeze cage to facilitate transfer to a custom built Lexan restraint box (chair 25x25x56 cm) using the standard pole and collar handling procedures. The animal's collar was

fitted into a groove on the top the chair (box) and secured with a metal pin allowing the monkey to move freely within the apparatus. An internal shelf supported the monkey's back and arms while it remained in a standing position.

Animals were initially habituated first to the squeeze cage and then to the Lexan restraint chair for 3-4 sessions prior to testing with participation accompanied by a food reward. Once the animal was habituated to the chair, testing began. The Lexan restraint chair was moved to a sound attenuated custom built wooden chamber (70x48x108 cm) designed to deliver a brief acoustic startle stimulus. The chair was mounted on a platform located above an accelerometer (Endevco 2217E) located between the platform and a rubber stopper. Chair movements produced displacement of the accelerometer which produced a voltage signal that was amplified (Endevco model 104), digitized and stored on a computer (Apple Macintosh G3). Background noise (0-20 kHz, 55 dB) is produced by a white noise generator (Lafayette, Model 15800, Lafayette, ID) and delivered through speakers (Radio Shack Supertweeters; range 5-40 kHz) located in front of the platform. This, plus the noise of the ventilating fan attached to the side wall of the housing chamber produce an overall background noise level of approximately 65 dB. The startle stimulus was a 50 msec burst of white noise (5 msec rise-decay time) of varying 95, 105 and 115 dB intensities generated by a white noise generator and delivered through the same speakers as the background noise. An infrared video camera was mounted on the inside of the chamber door and the monkeys were monitored throughout the entire startle session.

Monkeys were placed in the startle chamber for a 5 min acclimation period followed by the 19 min test session. The test session consisted of 6 blocks each consisting

of noise-alone trials one at each of three intensities (95, 105 and 115 dB) presented in random order within each block. Startle amplitude was defined as the maximum accelerometer voltage that occurs during the first 200 msec after startle stimulus onset. During the inter-stimulus-interval (ISI=60 s) activity measures were taken 30 s before each startle stimulus background chair (box) movement was sampled by measuring maximal peak accelerometer output voltage over a 600 msec period in the absence of any startle stimulus. This measure was defined as basal activity to provide a measure of general activity.

Two baseline sessions with no drug pretreatment were conducted prior to any drug experiments. Following the two baseline sessions, pretreatments of saline (0.5 ml), cocaine (0.3 and 1.0 mg/kg) or amphetamine (0.1 and 0.3 mg/kg) were injected i.m. 5 min prior to the start of the startle test session. All saline and drug experiments were randomized for each monkey and were conducted twice and data were averaged together.

Additional startle experiments using the dopamine D_1 receptor agonist SKF 82958 (0.3 mg/kg) and the dopamine D_2 receptor agonist quinpirole (0.03 and 0.1 mg/kg) were conducted in the same manner described above. Prior to the drug sessions, baseline startle measures were re-established. All drug and saline experiments were randomized for each monkey, conducted twice and the replicates were averaged together. However, 0.3 mg/kg SKF 82958 was tested only once in each monkey, given the lack of drug effect on acoustic startle and the adverse responses (extreme agitation) observed in response to the high doses of this drug. In a subset of 3 monkeys, SKF 82958 dose-response curves were performed and the drug showed little or no effect on startle amplitude at the doses tested (0.1, 0.3 and 1.0 mg/kg). The initial intent was to use 1.0 mg/kg dose of SKF

82959, in addition to 0.3 mg/kg, but this experiment was discontinued after 2 animals demonstrated an adverse response (extreme agitation) to the drug.

Cortisol measures in infants: Prior to joining the current set of studies, when the monkeys were infants, they were tested on a number of measures of cortisol function (Sanchez et al. 2005). At approximately 5.5 months of age, cortisol measures were taken under baseline conditions (immediately after capture) and following a 30 min maternal separation challenge. Blood was collected from saphenous or femoral veins following ketamine anesthesia (5 mg/kg) as described in more detail in Sanchez et al. (2005). Additionally, when the monkeys were 12 months of age, diurnal rhythm of cortisol was examined. Basal blood samples were taken at 3 time points across the day; in the morning (AM, sunrise), afternoon (PM, midway between am and night points) or at night (NITE, one hour after sunset), with only one time point blood sample collected per week per subject (see Sanchez et al. 2005 for more detail). The monkeys were living under natural lighting conditions, in indoor/outdoor runs, so time points were selected from sunrise and set time charts published by the US Naval Observatory in order to use the daylight and not the clock time as a reference for diurnal samples (Sanchez et al. 2005). Data from the nine animals in the present study were examined separately to determine if they were in agreement with the group phenotypes described by Sanchez et al. (2005).

Cortisol and ACTH response to cocaine challenge: Monkeys had previously been trained to sit quietly in commercially available primate chairs. Prior to these experiments and when the monkeys were approximately 7-8 years of age, they were also habituated to

having their leg held and manipulated while seated in the primate chair. On the day of testing, i.v. catheters (Saf-T-Intima, Becton, Dickinson Co., Franklin Lakes, NJ) were inserted into the saphenous vein of the monkeys while seated in the primate chairs and were secured in place with a flexible bandage wrapped around the leg. Monkeys were left undisturbed for 15 min, to acclimate to the catheterization. After the 15 min, a baseline blood draw (2.0 ml) was drawn. An injection of cocaine (1.0 mg/kg) was administered i.v. through the catheter 10 min after the baseline blood sample (25 min after catheterization). Following drug injection and each blood draw, the catheter was flushed with 1.0 ml heparin in order to keep the catheter patent. Additional blood samples (2.0 ml) were drawn at 10, 20, 30 and 60 min post-injection. These time points were chosen based on the findings of Broadbear et al. (1999b) who demonstrated that following intravenous cocaine injection in rhesus monkeys, plasma cortisol and ACTH concentrations were elevated above baseline in blood samples taken from 20 to 60 min post-injection. Following each draw, the blood was transferred to EDTA coated Vacutainers (Becton, Dickinson Co., Franklin Lakes, NJ) and refrigerated. Blood was centrifuged at 3000 rpm for 13 min; plasma was then separated and stored in a -20°F freezer. Assays for cortisol and ACTH concentration were performed in duplicate by the Biomarkers Core Laboratory of the Yerkes National Primate Research Center of Emory University in Atlanta, GA. using commercially available RIA kits for cortisol (Diagnostic Systems Laboratories, Webster, TX) and ACTH (DiaSorin, Inc., Stillwater, MN). Normal assay range for cortisol was 0.25-60 μg/dl and 6.65-434.00 pg/ml for ACTH.

Drugs: Cocaine HCl (National Institute on Drug Abuse, Bethesda, MD) and d-amphetamine H_2SO_4 (Sigma Aldrich, St. Louis, MO) were dissolved in 0.9% saline. SKF 82958 (Sigma Aldrich, St. Louis, MO) and quinpirole, (Sigma Aldrich, St. Louis, MO) were dissolved in sterile water. All doses are reported as salts.

Data Analysis

FDG PET analysis: Prior to entry in the present set of experiments, MRI scans were performed on all monkeys. PET images were aligned to each monkey's MRI image. The registered PET images were normalized to mean whole brain activity (whole brain ROI) to control for differences in the injected dose of [^{18}F]FDG. All images within a rearing condition were combined to generate group average images. The maternally separated mean image was subtracted from the control mean average image to generate a difference image. Standard deviation images were generated for each group. A t-statistic image was formed to define statistically significant differences.

Acoustic startle: Average startle amplitudes at each of the 3 stimulus intensities were determined for each test session. Replicate sessions were then averaged such that each monkey had a mean set of startle amplitudes for each condition (± standard deviation). Data from individual monkeys were averaged across rearing condition and group data are represented as mean ± SEM. Comparisons between groups were made using a two-way ANOVA with repeated measures on one factor (stimulus intensity). Additionally, one-way within group ANOVAs were performed for acoustic startle reactivity following stimulant pretreatment and following pretreatment with dopamine agonists to determine

if the drug treatments differed from baseline or saline levels in controls and maternally separated monkeys.

Cortisol following separation challenge: Data from individual monkeys were averaged across rearing condition and group data were represented as mean ± SEM. Repeated measures analysis of variance (ANOVA) was used to compare plasma cortisol levels under basal conditions to levels following separation stress. Percent change in cortisol concentration during the separation challenge was also determined for each subject. These values were averaged across rearing condition and group means in % change in cortisol concentration were compared using the student's t-test for unpaired samples.

Diurnal rhythm of cortisol secretion: Individual monkey data were averaged across rearing condition and group data were represented as mean ± SEM. Repeated measures analysis of variance (ANOVA) was used to compare plasma cortisol concentration across the day. Percent change in cortisol concentration across the time points subject (AM-PM and AM-Nite differences) were also determined for each. These values were averaged across rearing condition and group means for % change in cortisol concentration as determined by the AM-PM and AM-Nite differences were compared using the student's t-test for unpaired samples.

Cortisol and ACTH following cocaine challenge: Average cortisol and ACTH values for each monkey were determined from replicates run in the assay. Group means for each time point were determined from individual monkey data averaged across rearing

condition and were expressed as group mean ± SEM. Comparisons between groups on baseline concentrations of cortisol were made using an unpaired student's t-test. A two-way ANOVA with repeated measures on one factor (time) was used to compare groups across all time points following cocaine administration. The same analyses were used for ACTH concentrations at baseline and across time points during the cocaine challenge.

For all analyses, probability of significance was set at $p<0.05$. All statistical analyses were conducted using Graphpad Prism 5.0 or Sigma Stat 3.0 for Windows.

Results

PET imaging with [^{18}F]fluorodeoxyglucose

PET imaging with [^{18}F]FDG was used to measure basal cerebral glucose metabolism. No significant differences in basal cerebral glucose metabolism were found when maternally separated and control monkeys were compared (Figure 11). Therefore, in the model tested, early life stress in the form of maternal separation did not appear to have an effect on basal cerebral glucose metabolism.

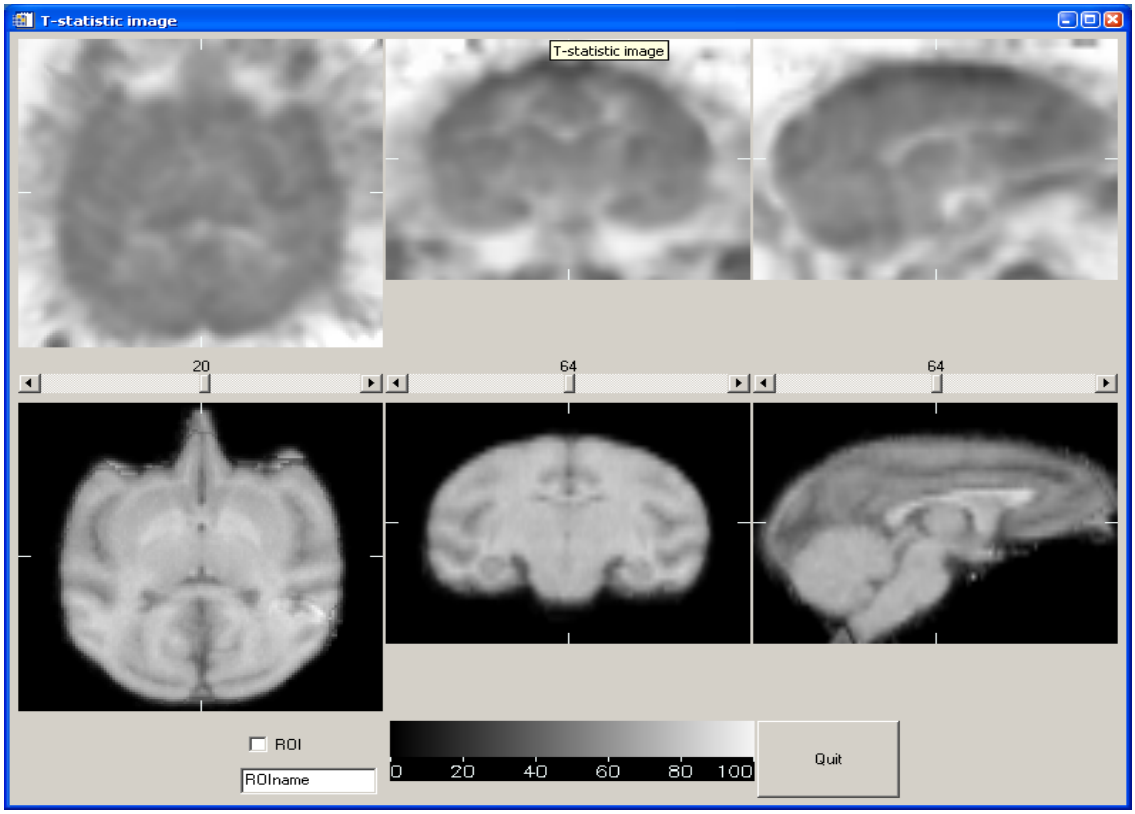
Acoustic Startle

Cocaine and amphetamine pretreatments

Both groups of monkeys demonstrated a stimulus intensity dependent increase in startle amplitude under baseline conditions and following saline pretreatments. There were no significant differences between the control and maternally separated monkeys in startle amplitude under baseline conditions ($F(1, 12) = 0.70$, $p = 0.43$) or in response to the saline pretreatment ($F(1, 12) = 0.10$, $p= 0.76$) (Figure 12 A, B).

Following pretreatments with cocaine (0.3 and 1.0 mg/kg) and amphetamine (0.1 and 0.3 mg/kg) both groups of monkeys demonstrated a stimulus intensity dependent increase in startle amplitude. There were no significant differences in startle amplitude between groups in response to a pretreatment with either a low dose (0.3 mg/kg) ($F(1, 12) = 0.87$, $p = 0.39$) or a high dose (1.0 mg/kg) ($F(1, 12) = 0.12$, $p = 0.74$) of cocaine (Figure 12 C, D). Maternally separated and control monkeys also did not differ significantly in response to pretreatments with either a low dose (0.1 mg/kg) ($F(1, 12) = 0.35$, $p = 0.57$) or a high dose (0.3 mg/kg) ($F(1, 12) = 0.25$, $p = 0.63$) of amphetamine (Figure 12 E, F). In general, neither group exhibited robust increases in startle amplitude following stimulant administration. No drug dose differed significantly from baseline or saline levels in controls ($F(5, 12) = 0.94$, $p = 0.49$) or maternally separated monkeys ($F(5, 12) = 0.84$, $p = 0.54$) (Figure 13 A, B).

Figure 11: The top panel represents the t-statistic PET image aligned to the MRI (MRI alone is shown in bottom panel). No statistically significant differences were found between control and maternally separated monkeys.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Dopamine agonist pretreatments

Prior to startle experiments using the dopamine D_1 receptor agonist SKF 82958 and the D_2 receptor agonist quinpirole, baseline startle measures were re-established. Both groups demonstrated a stimulus intensity dependent increase in startle amplitude under baseline conditions and following saline pretreatments. There were no significant differences between the control and maternally separated monkeys in startle amplitude under baseline conditions ($F(1, 12) = 0.38$, $p = 0.56$) or in response to the saline pretreatment ($F(1, 12) = 0.33$, $p = 0.59$) (Figure 14 A, B).

In response to pretreatments with quinpirole (0.03 and 0.1 mg/kg) and SKF 82958 (0.3 mg/kg) both groups demonstrated stimulus intensity dependent increases in startle amplitude. However, the magnitude of increases in startle amplitude was lower at all stimulus intensity levels. There was no significant difference in startle amplitude between groups in response to a pretreatment with either a low dose (0.03 mg/kg) ($F(1,12) = 0.12$, $p = 0.74$) or a high dose of quinpirole (0.1 mg/kg) ($F(1,12) = 0.0001$, $p = 0.99$) (Figure 14 C, D). The groups also did not differ in response to pretreatments with 0.3 mg/kg SKF 82958 ($F(1, 12) = 0.0001$, $p = 0.99$) (Figure 14 E). In general, neither group exhibited robust changes in startle amplitude following dopamine agonist administration. No drug dose induced startle reactivity that was significantly different from baseline or saline levels in controls ($F(4, 10) = 1.64$, $p = 0.24$) or maternally separated monkeys ($F(4, 10) = 2.29$, $p = 0.13$) (Figure 15 A, B), however there was a strong trend towards a quinpirole-induced decrease in startle amplitude in the latter group. It should be noted that following pretreatment with both doses of quinpirole the monkeys appeared to fall asleep.

Figure 12: Startle amplitude in response to different acoustic stimulus intensities under baseline conditions or following pretreatments with saline, cocaine (0.3 and 1.0 mg/kg) or amphetamine (0.1 or 0.3 mg/kg). Data are shown as mean ± SEM for each group. There were no significant group differences in startle amplitude under (**A**) baseline conditions ($F(1, 12) = 0.70$, $p = 0.43$), following pretreatments with (**B**) saline ($F(1,12) = 0.10$, $p = 0.76$), (**C**) 0.3 mg/kg cocaine ($F(1,12) = 0.87$, $p = 0.39$), (**D**) 1.0 mg/kg cocaine ($F(1, 12) = 0.12$, $p = 0.74$), (**E**) 0.1 mg/kg amphetamine ($F(1, 12) = 0.35$, $p = 0.57$) and (**F**) 0.3 mg/kg amphetamine ($F(1, 12) = 0.25$, $p = 0.63$).

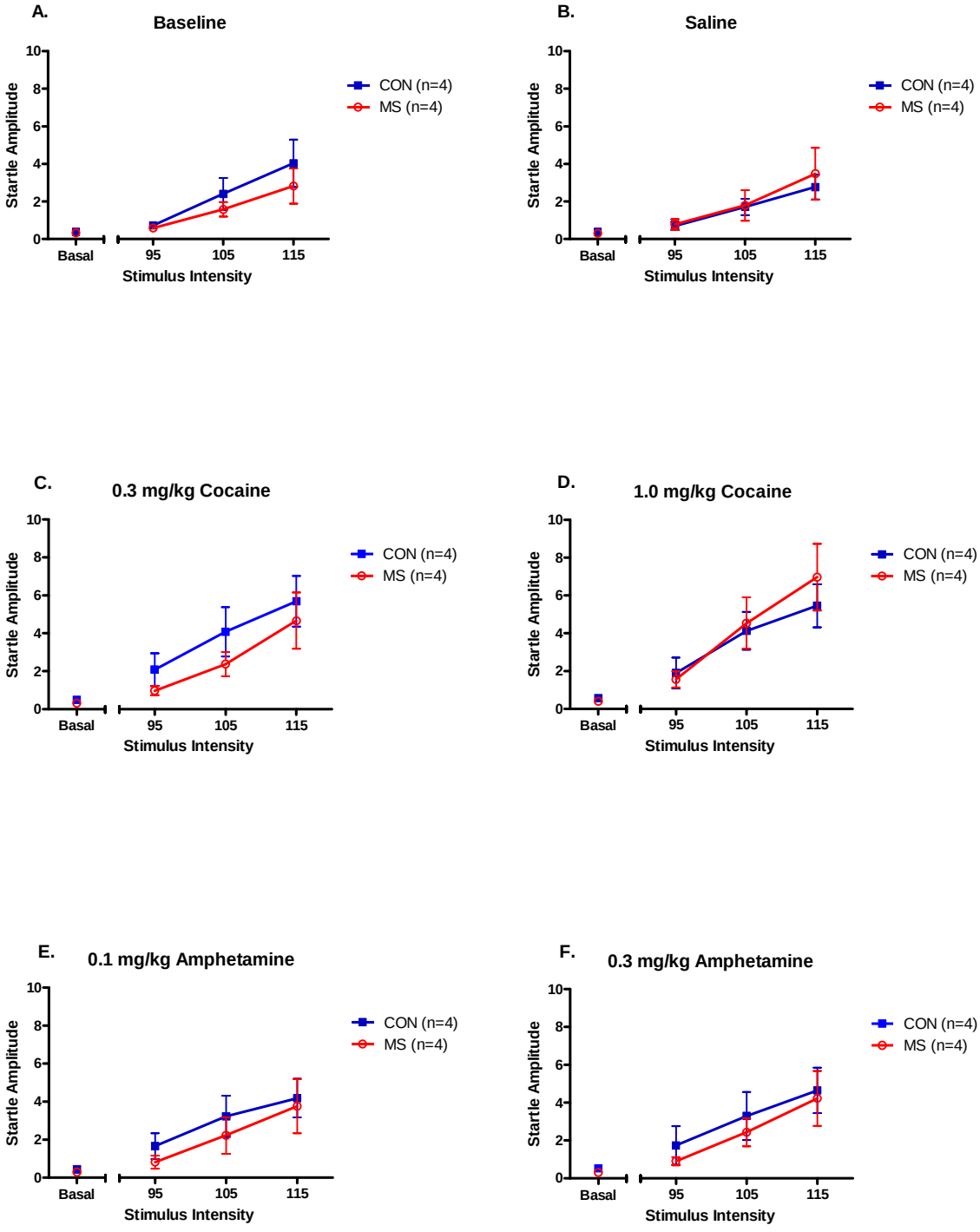

A.
Baseline
B.
Saline
C.
0.3 mg/kg Cocaine
D.
1.0 mg/kg Cocaine
E.
0.1 mg/kg Amphetamine
F.
0.3 mg/kg Amphetamine
Startle Amplitude
Stimulus Intensity
Basal
95
105
115
CON (n=4)
MS (n=4)

Figure 13: One way ANOVAs within groups were performed for the (**A**) controls and (**B**) maternally separated monkeys. No drug dose induced startle amplitudes that were significantly different from baseline or saline levels in controls ($F(5, 12) = 0.94, p = 0.49$) or maternally separated monkeys ($F(5, 12) = 0.84, p = 0.54$).

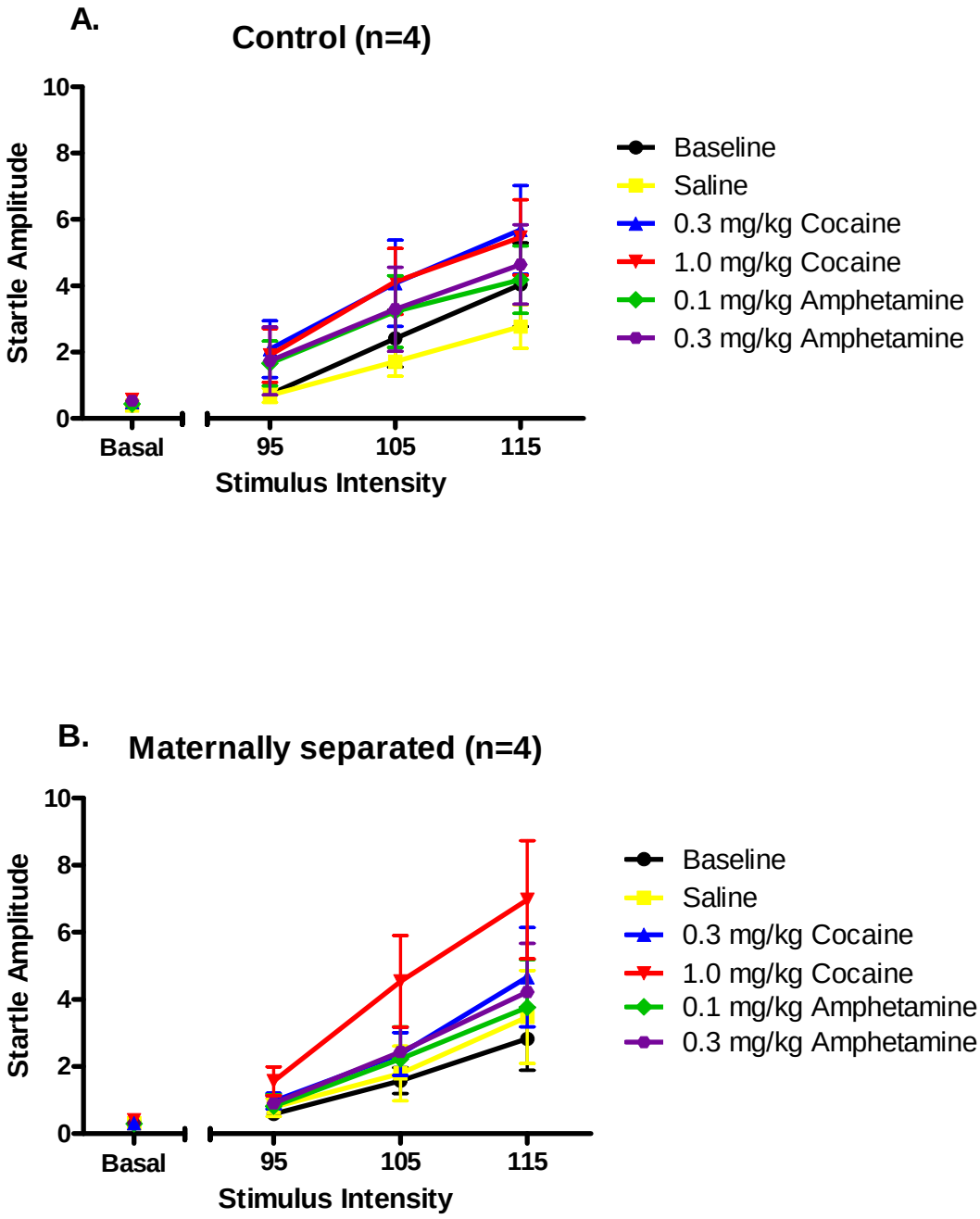
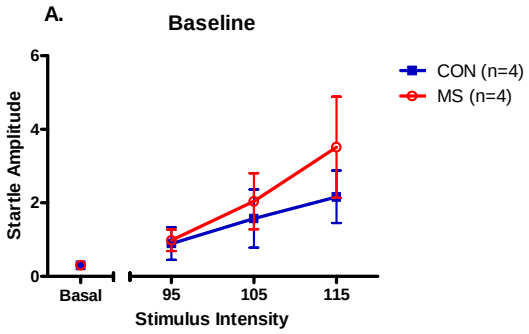
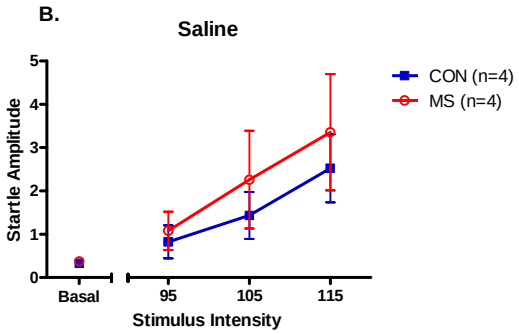
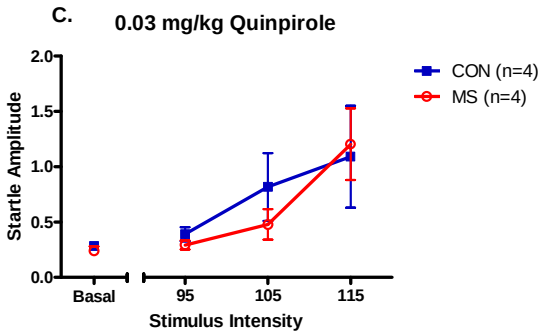
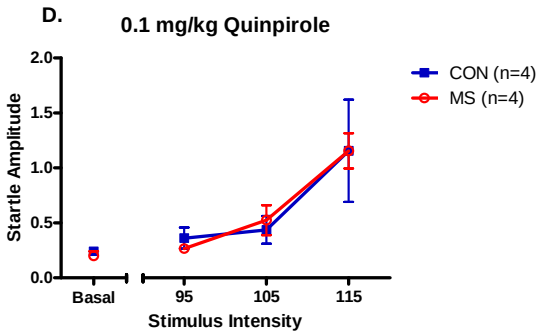

A.
Control (n=4)
Startle Amplitude
10
8
6
4
2
0
Basal
95
105
115
Stimulus Intensity
Baseline
Saline
0.3 mg/kg Cocaine
1.0 mg/kg Cocaine
0.1 mg/kg Amphetamine
0.3 mg/kg Amphetamine
B.
Maternally separated (n=4)
Startle Amplitude
10
8
6
4
2
0
Basal
95
105
115
Stimulus Intensity
Baseline
Saline
0.3 mg/kg Cocaine
1.0 mg/kg Cocaine
0.1 mg/kg Amphetamine
0.3 mg/kg Amphetamine

Figure 14: Startle amplitude in response to different acoustic stimulus intensities under baseline conditions or following pretreatments with saline, SKF 82958 (1.0mg/kg) or quinpirole (0.03 and 0.1mg/kg). Data are shown as mean ± SEM for each group. There were no significant differences between controls and maternally separated monkeys under (**A**) baseline conditions ($F(1,12) = 0.38$, $p = 0.56$), following pretreatments with (**B**) saline ($F(1,12) = 0.33$, $p = 0.59$), (**C**) 0.03 mg/kg quinpirole ($F(1,12) = 0.12$, $p = 0.74$), (**D**) 0.1 mg/kg quinpirole ($F(1,12) = 0.0001$, $p = 0.99$) and (**E**) 0.3 mg/kg SKF 82958 ($F(1,12) = 0.0001$, $p = 0.99$).


A.
Baseline
Startle Amplitude
0
2
4
6
Basal
95
105
115
Stimulus Intensity
CON (n=4)
MS (n=4)


B.
Saline
Startle Amplitude
0
1
2
3
4
5
Basal
95
105
115
Stimulus Intensity
CON (n=4)
MS (n=4)


C.
0.03 mg/kg Quinpirole
Startle Amplitude
0.0
0.5
1.0
1.5
2.0
Basal
95
105
115
Stimulus Intensity
CON (n=4)
MS (n=4)


D.
0.1 mg/kg Quinpirole
Startle Amplitude
0.0
0.5
1.0
1.5
2.0
Basal
95
105
115
Stimulus Intensity
CON (n=4)
MS (n=4)

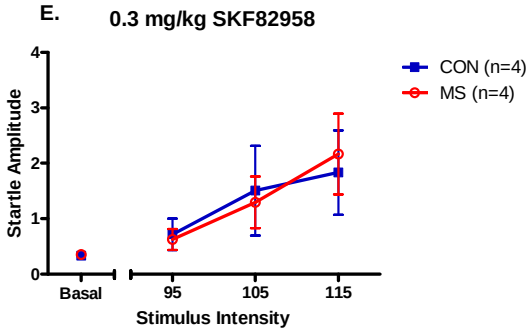
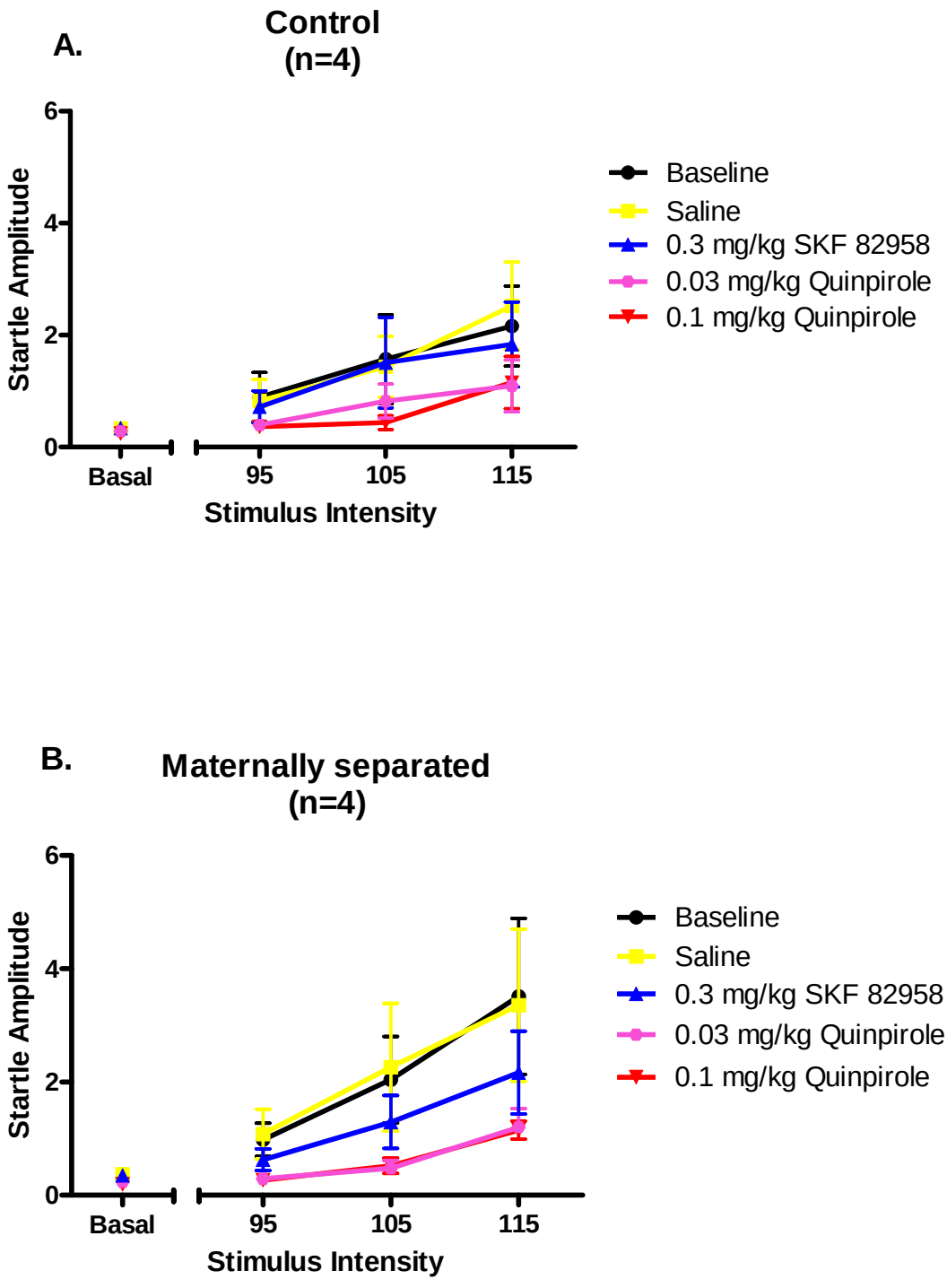

E.
0.3 mg/kg SKF82958
Startle Amplitude
0
1
2
3
4
Basal
95
105
115
Stimulus Intensity
CON (n=4)
MS (n=4)

Figure 15: One way ANOVAs within groups were performed for (**A**) controls and (**B**) maternally separated monkeys. No drug dose was significantly different from baseline or saline startle amplitude levels in controls ($F(4, 10) = 1.639$, $p = 0.24$) or in maternally separated monkeys ($F(4, 10) = 2.24$, $p = 0.13$) but there was a strong trend towards a difference in maternally separated monkeys.


A.
Control
(n=4)
Startle Amplitude
0
2
4
6
Basal
95
105
115
Stimulus Intensity
Baseline
Saline
0.3 mg/kg SKF 82958
0.03 mg/kg Quinpirole
0.1 mg/kg Quinpirole
B.
Maternally separated
(n=4)
Startle Amplitude
0
2
4
6
Basal
95
105
115
Stimulus Intensity
Baseline
Saline
0.3 mg/kg SKF 82958
0.03 mg/kg Quinpirole
0.1 mg/kg Quinpirole

Cortisol following separation challenge

Plasma cortisol was measured under baseline conditions and following a maternal separation challenge when the monkeys were approximately 5.5 months of age (Sanchez et al. 2005). Data from the nine (5 control and 4 maternally separated) monkeys used in the present study were analyzed separately for differences in HPA axis reactivity based on rearing history. There was a significant main effect of separation stress ($F(1, 7) = 121.7$, $p < 0.0001$) but there was no significant main effect of rearing ($F(1, 7) = 1.46$, $p = 0.27$). Plasma cortisol concentration did not differ significantly between groups at baseline ($t(7) = 0.44$, $p = 0.68$) or following a separation challenge ($t(7) = 1.34$, $p = 0.22$), but did follow the trend of the entire group as described by Sanchez et al. (2005) with maternally separated monkeys exhibiting greater separation stress-induced cortisol concentration (Figure 16).

Diurnal rhythm of cortisol

When monkeys were approximately 12 months of age plasma cortisol was measured at 3 time points (AM, PM and NITE) to assess the diurnal rhythm of secretion (Sanchez et al. 2005). Data from the nine monkeys used in the present study were analyzed separately for differences based on rearing history. There was a strong trend towards a main effect of time ($F(2, 14) = 3.12$, $p = 0.08$) but no main effect of rearing ($F(1, 14) = 0.47$, $p = 0.51$) (Figure 17).

Figure 16: Plasma cortisol was measured under baseline conditions and following a maternal separation challenge at approximately 5.5 months of age. Data from the nine monkeys used in the present study were analyzed separately for differences based on rearing history. There was a significant main effect of separation stress ($F(1, 7) = 121.7$, $p < 0.0001$) but there was no significant main effect of rearing ($F(1, 7) = 1.46$, $p = 0.27$). Plasma cortisol concentration did not differ between groups at baseline ($t(7) = 0.44$, $p = 0.68$) or following a separation challenge ($t(7) = 1.34$, $p = 0.22$) but did appear to follow the trend of the maternal separation phenotype described by Sanchez et al. (2005).

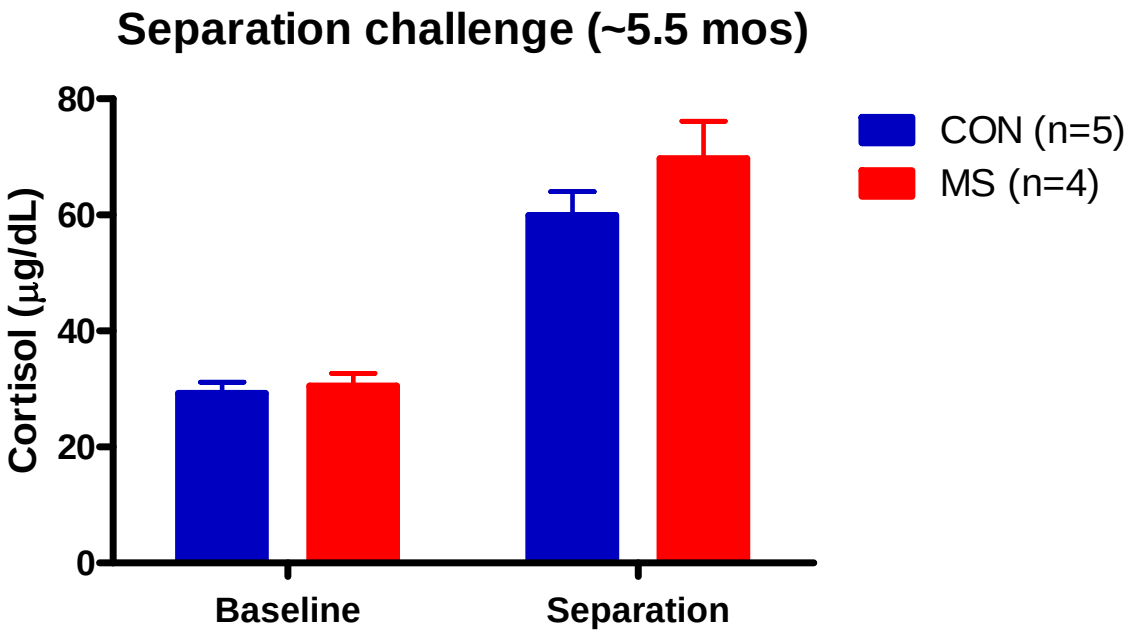
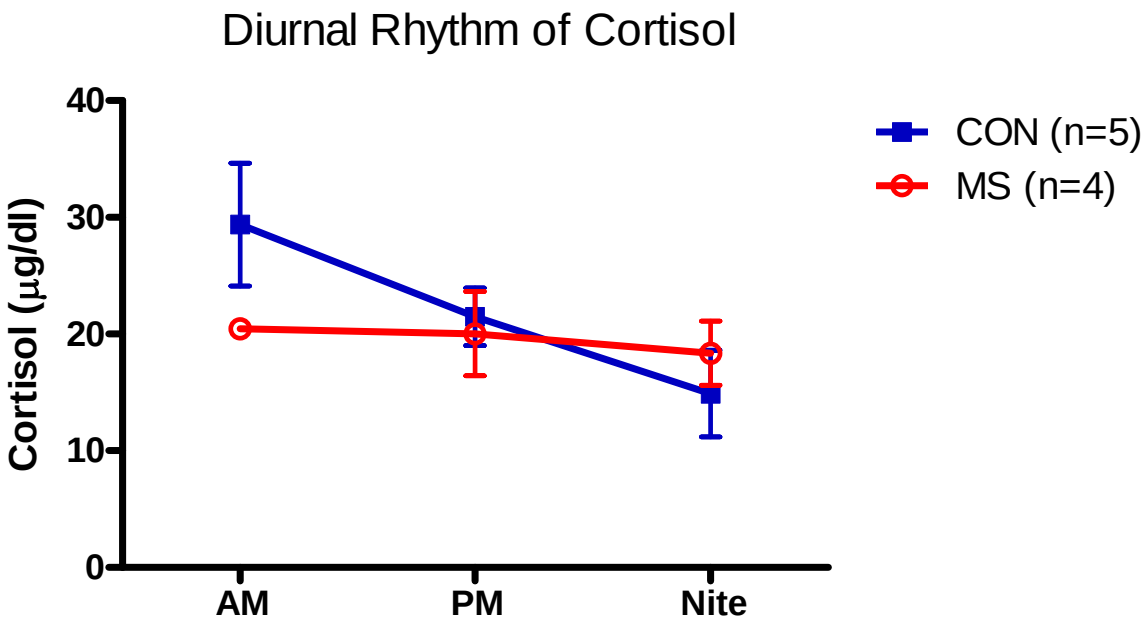

Separation challenge (~5.5 mos)
Cortisol (µg/dL)
80
60
40
20
0
Baseline
Separation
CON (n=5)
MS (n=4)

Figure 17: At 12 months of age, plasma cortisol levels were measured at 3 time points to assess the diurnal pattern of secretion (Sanchez et al. 2005). Data from the nine monkeys used in the present study were analyzed separately from the entire group. There was a strong trend towards a main effect of time ($F(2, 14) = 3.12$, $p = 0.08$) but no main effect of rearing ($F(1, 14) = 0.47$, $p = 0.51$).


Diurnal Rhythm of Cortisol
Cortisol (µg/dl)
40
30
20
10
0
AM
PM
Nite
CON (n=5)
MS (n=4)

There were no significant differences between control and maternally separated monkeys in the AM-PM difference ($t(7) = 1.23$, $p = 0.26$), controls exhibited a slight decrease in plasma cortisol levels from AM-PM (7.91±4.78 μg/dl) while maternally separated monkeys exhibited very little change from AM-PM (0.41±3.22 μg/dl). There was a trend toward a group difference in the AM-NITE difference ($t(7) = 1.77$, $p = 0.12$). Controls tended to show a decrease in cortisol levels (14.51±5.6 μg/dl) from AM to NITE while the maternally separated monkeys exhibited a flattened rhythm with little decrease from AM to NITE (2.08±3.38 μg/dl), which is also in keeping with the phenotype described in Sanchez et al. (2005).

Plasma cortisol and ACTH concentration following cocaine challenge

Plasma cortisol and ACTH concentration were determined prior to and following an i.v. injection of cocaine (1.0 mg/kg). Control and maternally separated monkeys did not differ in baseline plasma cortisol concentration ($t(7) = 0.59$, $p = 0.58$). There was a significant main effect of time ($F(4, 28) = 2.75$, $p = 0.04$) but no main effect of rearing following cocaine injection ($F(1, 28) = 1.80$, $p = 0.22$) (Figure 18).

Although there was a trend towards a group difference in baseline plasma ACTH concentration, this difference did not reach statistical significance ($t(7) = 1.45$, $p = 0.19$). There was no main effect or rearing following cocaine injection ($F(1, 28) = 0.44$, $p = 0.53$) and no main effect of time ($F(4, 28) = 0.17$, $p = 0.95$) for plasma ACTH levels (Figure 19).

Figure 18: Plasma cortisol concentration prior to and following a non-contingent intravenous injection of cocaine (1.0 mg/kg) indicated with the arrow. Control and maternally separated monkeys did not differ significantly in baseline cortisol levels ($t(7) = 0.59$, $p = 0.58$). Following cocaine injection there was no main effect of rearing ($F(1, 28) = 1.80$, $p = 0.22$) but there was a significant main effect of time ($F(4, 28) = 2.75$, $p = 0.04$).

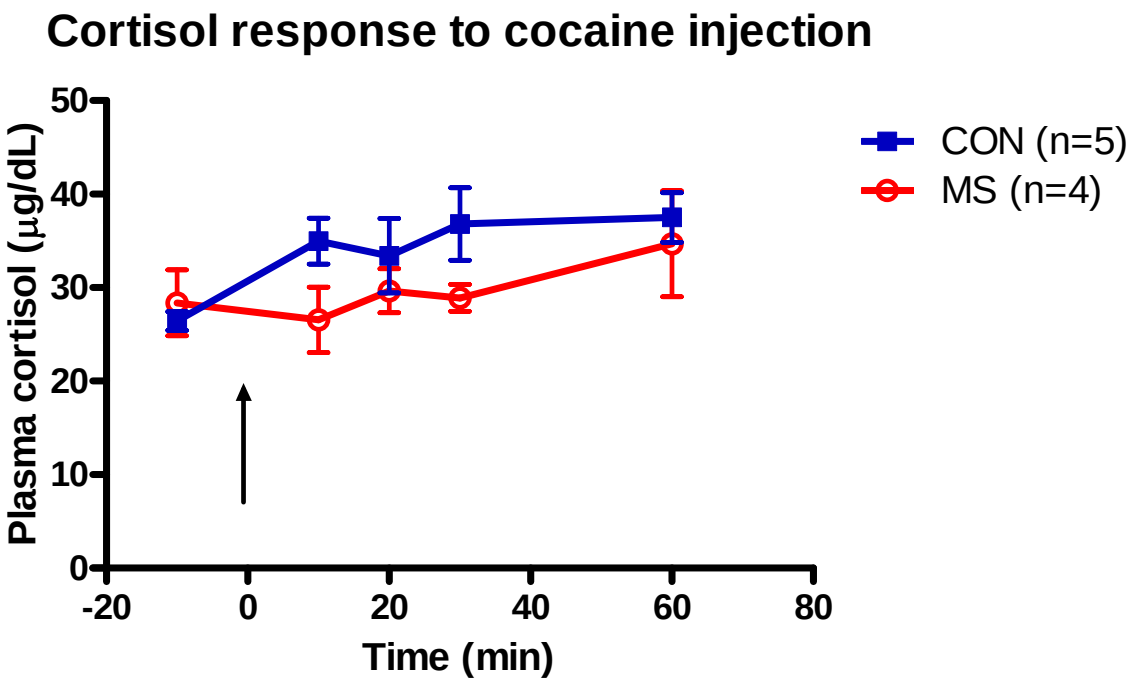
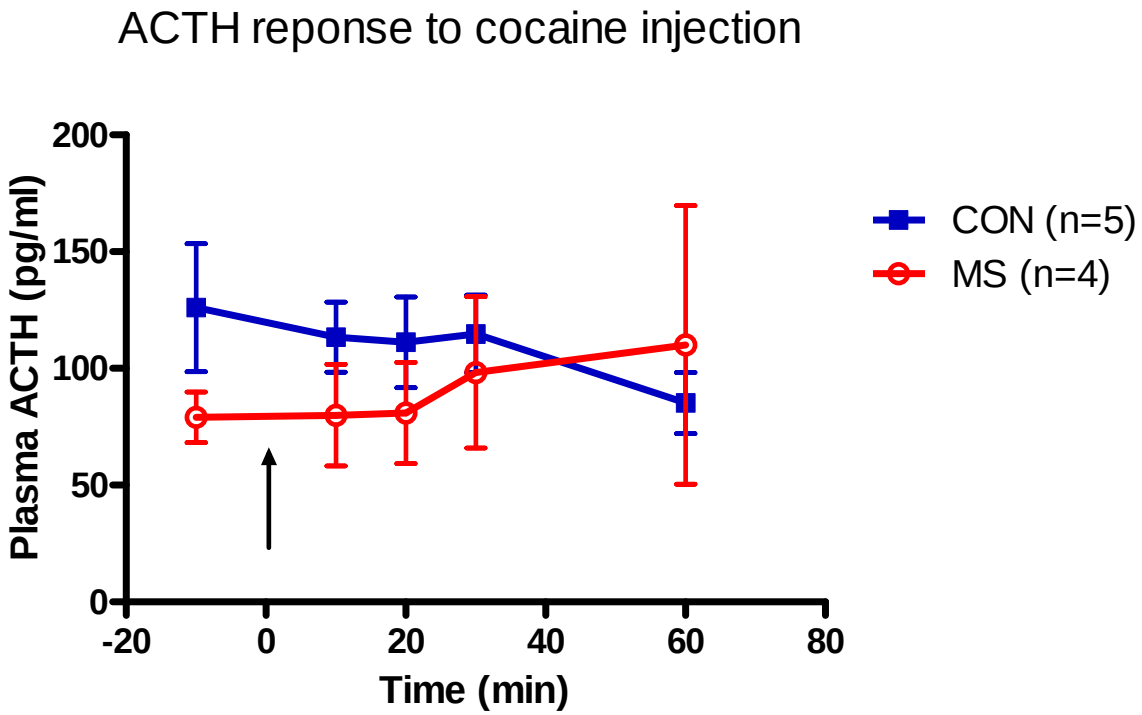

Cortisol response to cocaine injection
Plasma cortisol (µg/dL)
50
40
30
20
10
0
-20
0
20
40
60
80
Time (min)
CON (n=5)
MS (n=4)

Figure 19: Plasma ACTH concentration prior to and following a non-contingent intravenous injection of cocaine (1.0 mg/kg) indicated with the arrow. Control and maternally separated monkeys did not differ significantly in baseline ACTH concentration ($t(7) = 1.45$, $p = 0.19$). Following cocaine injection there was no main effect of rearing ($F(1, 28) = 0.44$, $p = 0.53$) or time ($F(4, 28) = 0.17$, $p = 0.95$).


ACTH reponse to cocaine injection
Plasma ACTH (pg/ml)
200
150
100
50
0
-20
0
20
40
60
80
Time (min)
CON (n=5)
MS (n=4)

Discussion

Early life stress, including maternal separation in infancy, has been shown to have profound effects on the HPA axis and behavioral reactivity. Following acute maternal separation challenges and through the first two years of life, rhesus monkeys exhibited a phenotype of a HPA axis that is basally hypo-functional but hyper-reactive when challenged and increased behavioral reactivity as compared with non-separated controls. The current set of experiments were designed to determine the long-term effects of early life stress on cerebral glucose metabolism, as well as behavioral and HPA axis reactivity in a group of rhesus monkeys. However, the results do not provide support for persistent long-term effects of maternal separation on behavioral and HPA axis reactivity in the nonhuman primate model employed.

Cerebral glucose metabolism

Cerebral glucose metabolism under basal conditions was measured using [^{18}F]fluorodeoxyglucose (FDG) and PET neuroimaging. No differences in cerebral glucose metabolism were found when maternally separated monkeys were compared with controls. Therefore, in the model tested, early life stress in the form of maternal separation did not appear to have an effect on basal cerebral glucose metabolism.

It has been found that chronic cocaine users exhibit decreased glucose metabolism in frontal areas even after 3-4 months of abstinence (Volkow et al. 1992). However, the monkeys used in the present study were 4-5.5 years of age and drug naïve at the time of the scan so chronic cocaine history was not a factor. The monkeys did not engage in any scheduled tasks during the uptake of the radiolabeled FDG. It is possible that differences

between groups might be found once they had a drug history or in response to an acute stimulant injection and this could be explored in future experiments. Following the uptake of FDG, the monkeys were anesthetized with telazol and isoflurane for the imaging session. While previous studies have demonstrated that a drug similar to telazol, ketamine (Langsjo et al. 2003, 2004; Holcomb et al. 2001; Freo and Ori 2004; Honey et al. 2004) and isoflurane (Alkire et al. 1997) produce changes in metabolism or regional cerebral blood flow (rCBF) in a variety of species (rats, monkeys, humans), they all demonstrate that the response within a brain region is relatively constant across subjects (Kalin et al. 2005). Therefore it is unlikely that anesthesia confounded any differences in these monkeys. Additionally, the anesthesia was administered following the uptake of FDG so the differences in glucose metabolism should not have been affected by the anesthesia.

Acoustic startle

Previous studies in rhesus monkeys that underwent maternal separation as infants found that those monkeys exhibited greater acoustic startle reactivity as compared with non-separated controls (Sanchez et al. 2005). These studies were conducted when the monkeys were under 2 years of age and there appear to be no experiments exploring the long-term effects of maternal separation on acoustic startle reactivity. The present study examined acoustic startle reactivity in maternally separated and non-separated control rhesus monkeys under basal conditions and following challenges with psychostimulant drugs and dopamine agonists. While all monkeys exhibited stimulus intensity dependent increases in startle amplitude under baseline conditions, there were no differences

between control and maternally separated groups. This is in contrast with previous findings in a different group of monkeys with the same rearing history. Sanchez et al. (2005) found increased acoustic startle responses in monkeys with early adverse experience when tested at 20-24 months of age and with a protocol and apparatus similar to those used in the present set of experiments. Following pretreatment with saline, cocaine and amphetamine, both groups again exhibited stimulus intensity dependent increases in startle amplitude but there were no differences between groups. Interestingly, neither group exhibited robust increases in startle amplitude following psychostimulant administration. Similarly, the direct dopamine agonists SKF 82958 and quinpirole also failed to produce robust alterations in startle reactivity. SKF 82958 has been found to increase startle amplitude in rodent models, but in the present study pretreatment with the dopamine D_1 agonist resulted in startle amplitudes that were no different from saline and baseline levels in both groups of monkeys. In maternally separated monkeys, SKF 82958 actually resulted in slight decreases in startle amplitude from baseline levels. Both control and maternally separated monkeys did show decreases in startle amplitude from baseline and saline levels following pretreatment with quinpirole, however these reductions did not reach statistical significance. These finding are in contrast with reports of enhancement of startle reactivity following acute cocaine or amphetamine administration in rodent models (Davis 1985) and those showing alterations in startle reactivity with administration of dopamine direct agonists (Davis and Aghajanian 1976; Svensson 1990; Meloni and Davis 1999; Davis 1987; Peng et al. 1990).

It is possible that as the monkeys matured and were further distanced from the maternal separations the acoustic startle reactivity normalized. Alternatively, startle reactivity in the control monkeys could have increased over that period of time. With the exception of two control monkeys that were unable to take part in drug self-administration experiments due to issues involving their vascular catheters, the monkeys had an extensive drug history at the time of the startle experiments. However, there were no differences in startle reactivity when the 2 control monkeys with no drug history were compared with the other control monkeys with a history of drug self-administration. Additionally, previous studies in rodents have found no effect of drug self-administration history on the startle response to tactile (Mansbach et al. 1994; Barros and Miczek 1996 and Mutschler and Miczek 1998) or acoustic (Mansbach et al. 1994) stimuli as compared with a control group or with pre-drug baseline levels. Therefore it does not appear that drug history alone was enough to normalize startle reactivity to acoustic stimuli in this group of rhesus monkeys.

Cortisol response to separation stress and diurnal rhythm

When the data from the nine animals used in the present study were separated and analyzed from the larger data set used by Sanchez et al. (2005) no significant group differences were found in baseline plasma cortisol, plasma cortisol following a separation challenge at 5.5 months or in plasma cortisol concentration across the diurnal rhythm. However, the trends in the data were consistent with the phenotype described in Sanchez et al. (2005) in which maternally separated monkeys exhibited flattened diurnal cortisol rhythm. The group differences in mean plasma cortisol concentration appeared to be

hindered by the small group sizes and as a result, the within group variability may have overshadowed any group differences. The lack of robust group differences in HPA axis function in this subset of monkeys may further explain the lack of robust differences found on the other measures of the current study.

Cortisol and ACTH following cocaine challenge

Plasma cortisol concentration was measured prior to and following an injection of cocaine. While previous studies have demonstrated increases in both plasma cortisol and ACTH in rhesus monkeys in response to stimulant challenges (Sarnyai et al. 1996; Broadbear et al. 1999a,b,c), the present study found only minimal increases in plasma cortisol, but no increase in plasma ACTH following 1.0 mg/kg cocaine injection. There were no significant differences in plasma cortisol or ACTH when maternally separated monkeys were compared with controls. As previously described, all but 2 animals had a somewhat extensive drug history which could potentially lead to a decrease in the cortisol and ACTH following acute cocaine injection. No consistent differences were found between those monkeys with drug history and those without. One of the two monkeys without a history of drug self-administration had the lowest ACTH concentration but the other was not different from other controls. However, these 2 monkeys had the highest cortisol levels of the controls following cocaine, but there were no differences in basal levels (one of the two showed persistent cortisol elevation above all other controls but the other exhibited a transient increase only at the first time point following injection). Individual differences not related to drug history were also seen in the maternally separated group. Two of the monkeys exhibited no change or a very minimal increase

following cocaine, while the other 2 demonstrated a more robust increase (but this persisted beyond the +20 min time point only in one monkey). For ACTH, 2 phenotypes emerged. Three of the 4 maternally separated monkeys exhibited low ACTH levels with only one showing an increase following cocaine administration. However, four of the five control monkeys had somewhat higher levels of ACTH compared to maternally separated monkeys while the 5th control monkey had ACTH levels similar to the maternally separated monkeys. Broadbear et al. (1999b) measured cortisol and ACTH levels following a non-contingent injection of cocaine (1.0 mg/kg) in drug-naïve animals and again in the same animals after they had a drug self-administration experience and found no differences in hormone levels. Therefore, it is unlikely that the drug history impacted the response to the injection of cocaine. Another possible explanation for the minimal increase in hormone response to the drug challenge is that the catheterization of the saphenous vein for the blood draws was so stressful that we reached a ceiling on cortisol and ACTH levels such that the acute administration was not able to increase plasma concentrations. Increases in stress hormones before drug administration have been shown to blunt the effect produced by the drug (Dallman and Jones 1973; Sarnyai et al. 1996). Dallman and Jones (1973) found a negative correlation between baseline cortisol concentration and subsequent changes in ACTH following infusion of 0.8 mg/kg of cocaine, possibly because of down-regulation of HPA axis activity via negative feedback mechanisms. High baseline cortisol levels may down-regulate the activity of the HPA axis by the inhibition of CRF and/or ACTH secretion (Keller-Wood and Dallman 1984) and thus cocaine would be unable to stimulate ACTH and subsequent cortisol secretion. As a result, it may be difficult to separate the contribution of cocaine

to HPA activation from that of the experimental procedure. Monkeys were very accustomed to sitting in the primate chair and measures were taken to habituate them to the catheterization process. It is unlikely that the chair restraint was overly stressful in these monkeys particularly given that an earlier study found similar levels of cortisol in freely moving caged monkeys as compared with chair restrained monkeys (Quabbe et al. 1982). Furthermore, the monkeys did not exhibit any behaviors that would indicate exceptionally high levels of stress following the catheterization.

Intact female rhesus monkeys served as subjects in the present set of experiments. It is possible that variation in menstrual phase had an effect on the response of the HPA axis to the cocaine challenge. However, when plasma cortisol was sampled throughout the menstrual cycle in rhesus monkeys, no consistent variations were found (Leshner et al. 1978). Acute injections of cocaine (0.4 and 0.8 mg/kg) failed to alter ACTH and cortisol levels in ovarectomized female rhesus monkeys (Sarnyai et al. 1995) and this lack of response to cocaine was attributed to the absence of normal levels of gonadal steroids. It is possible that the monkeys used in the present study have altered levels of gonadal steroids and that this is the reason for the lack of response to the cocaine challenge. Gonadal steroid levels were not measured in these monkeys and characterization of this system would be important for future studies. However, the majority of studies reporting increases in cortisol and ACTH in rhesus monkeys used males exclusively and those that used both males and females (Broadbear et al. 1999a, b) found that male monkeys exhibited higher cortisol levels than the female monkeys. Therefore future studies should explore the effects of cocaine on HPA axis function in both male and female rhesus monkeys.

As previously described, in addition to dopamine and CRF modulation, cocaine-induced stimulation of ACTH and corticosterone/cortisol secretion is also mediated to some extent by serotonin (5-HT) neurons in brain, with 5-HT_2 or 5-HT_{1C} receptors being responsible for this effect (Levy et al. 1991). Serotonergic neurons projecting to the PVN innervate CRH-containing cells (Liposits et al. 1987) and are important in the regulation of ACTH secretion from the pituitary gland (Feldman et al. 1987). Depletion of 5-HT, destruction of 5-HT neurons in the brain or blockade of 5-HT_{1c} or 5-HT_2 receptors have all been found to prevent cocaine-induced increases in ACTH and corticosterone/cortisol (Levy et al. 1991; Borowsky and Kuhn 1991). Early life stress has been found to alter serotonergic systems in both rodents and rhesus monkeys (Arborelius and Eklund 2007; Vazquez et al. 2002; Higley et al. 1996a, b). It is possible that the monkeys used in the present study have altered serotonergic function which is contributing to the attenuated cocaine-induced stimulation of cortisol and ACTH. Future studies would benefit from characterization of the serotonergic system in order to address this possibility.

Individual differences in HPA axis response to an acute cocaine challenge have been described in rhesus monkeys. When cocaine (0.8 mg/kg) was administered to a group of rhesus monkeys plasma levels of ACTH and cortisol were increased but only in those monkeys that subsequently demonstrated behavioral stimulation in response to cocaine (Sarnyai et al. 1996). Cocaine actually decreased ACTH release and had no effect on cortisol in the group of monkeys that subsequently showed no behavioral stimulation to cocaine. Prior to the cocaine administration, baseline levels of cortisol were higher in the monkeys that showed no HPA axis or behavioral stimulation of cocaine but there were no differences in baseline ACTH levels (Sarnyai et al. 1996).

Small group sizes and individual differences such as those described above may have contributed to the minimal increases in HPA axis response and the lack of group differences found in the present study.

Overall, significant differences between maternally separated and control monkeys were not observed in basal cerebral glucose metabolism, in baseline acoustic startle reactivity or in acoustic startle reactivity following psychostimulant or dopamine receptor agonist pretreatment. Additionally, significant group differences were not observed in HPA axis reactivity measured when the monkeys were infants or as adults in response to an acute cocaine challenge. The results do not provide support for persistent long-term effects of maternal separation on behavioral and HPA axis reactivity in the nonhuman primate model employed. Rather, these results suggest that the detrimental consequences of maternal separation were not enduring and dissipated as these animals matured into adulthood.

Chapter 5: General Discussion

Previous studies have demonstrated that early life stress, as experienced by animals subjected to maternal separation, has profound effects on the HPA axis and stress reactivity. Prior exposure to early life stress has also been linked to a predisposition to use drugs of abuse. The goal of the current set of experiments was to determine whether early life stress in the rhesus monkey, in the form of maternal separation, influences sensitivity to cocaine or amphetamine and increases the propensity to self-administer psychostimulants. The long-term effects of maternal separation on behavioral and HPA axis reactivity was also examined in this group of rhesus monkeys. While the results do not provide support for early life stress leading to enhanced vulnerability to stimulant use, they do suggest that some alterations resulting from maternal separation did persist into adulthood; leading to differences between groups in sensitivity to psychostimulants. However, these changes do not appear to be related to measures of behavioral or HPA axis reactivity in the nonhuman primate model employed.

All monkeys acquired cocaine self-administration at the same rate, as they all met the acquisition criteria in the minimum amount of time required. Maternally separated monkeys showed reduced behavioral output throughout acquisition of cocaine self-administration as compared with controls. During the maintenance phase of cocaine self-administration, maternally separated monkeys had significantly lower rates of responding for cocaine. However, when saline was substituted for cocaine, both groups of monkeys demonstrated a reduction in responding and met the extinction criteria with no significant group differences in the mean number of days required to meet the criteria for extinction.

When a range of doses of cocaine and amphetamine was substituted for the maintenance dose of cocaine, maternally separated monkeys demonstrated reduced behavioral output and response rates across drug doses for both cocaine and amphetamine, resulting in dose-response functions that were flattened and shifted downward. The doses of cocaine and amphetamine that maintained peak rates of responding were the same in both groups of monkeys with no horizontal shifts observed in dose-response functions. Maternally separated monkeys had significantly lower response rates for cocaine and exhibited a trend towards significantly lower response rates for amphetamine across a range of doses as compared with control monkeys.

Consistent with the low rates of self-administration, when motor activity was measured following drug administration maternally separated monkeys displayed no stimulant-induced increase in locomotor activity. Conversely, control monkeys did exhibit stimulant-induced increases in motor activity but with a great deal of within group variability. As a result, maternally separated monkeys exhibited significantly lower levels of motor activity following acute cocaine injection as compared with control monkeys. Following acute amphetamine injection, maternally separated monkeys demonstrated a strong trend towards significantly lower levels of drug-induced motor activity.

This collection of results is representative of a lack of sensitivity to the reinforcing and motor-activating effects of psychostimulant drugs in maternally separated monkeys as compared with controls. Given that both the reinforcing effects and motor activating effects of psychostimulants have been attributed to dopamine, a set of experiments was designed to examine the dopaminergic system to better elucidate the

mechanisms underlying the blunted effects of psychostimulants in the monkeys exposed to early life stress as infants. Dopamine D_2 receptor binding potential was assessed and no differences were found between control and maternally separated monkeys. Therefore, differences in dopamine D_2 receptor levels cannot explain the differences seen in behavioral output during acquisition of cocaine self-administration, response rates during maintenance and across a range of doses of cocaine and amphetamine or in stimulant-induced increases in locomotor activity.

Similarly, no significant group differences in extracellular dopamine levels following a cocaine or amphetamine challenge were observed. Control and maternally separated monkeys did not differ significantly in stimulant-induced increases in extracellular dopamine in the caudate nucleus as measured with in vivo microdialysis. Both groups exhibited an increase in extracellular dopamine of 300-350% following cocaine administration. Following amphetamine injection however, maternally separated monkeys showed a greater release of extracellular dopamine (700%) as compared with controls but due to limited group sizes and individual variability this difference did not reach statistical significance.

In an attempt to explain these similarities and differences between control and maternally separated monkeys, additional experiments were performed to further characterize potential differences between groups and the long-term effects of rearing history on measures of behavioral and HPA axis function and reactivity. When neuroimaging studies using [^{18}F]FDG were performed, no significant group differences in basal glucose metabolism were found. Additionally, no significant differences in acoustic startle with psychostimulant drug challenges were found. While all monkeys

exhibited stimulus intensity dependent increases in startle amplitude under basal conditions and following pretreatment with saline, cocaine and amphetamine, there were no significant differences in startle reactivity between controls and maternally separated monkeys. This is in contrast to findings in young monkeys exposed to early life stress who exhibited elevated startle amplitude under baseline conditions as compared with controls (Sanchez et al. 2005). Cocaine and amphetamine pretreatment failed to elicit robust increases in startle amplitude in both the control and maternally separated monkeys used in the present study. Likewise, no significant differences in acoustic startle were observed following treatment with dopamine receptor agonists. The direct dopamine D_1 agonist SKF82958 and D_2 agonist quinpirole also failed to produce robust changes in startle reactivity in both groups of monkeys. Direct D_1 agonists have previously been shown to increase startle amplitude while D_2 agonists have been found to decrease startle amplitude. This study found that in control monkeys pretreatment with SKF 82958 resulted in startle amplitudes that were not different from baseline or saline pretreatment levels, however in maternally separated monkeys pretreatment with SKF 82958 actually resulted in a slight decrease in startle amplitude. Following pretreatment with quinpirole, monkeys did exhibit reduced startle reactivity, with the greatest reductions seen in maternally separated monkeys. However, these reductions were not significantly different from baseline and saline levels of startle amplitude. Interestingly, pretreatment with quinpirole caused the monkeys to fall asleep during the startle session, which might partially explain the reduced startle amplitudes.

When the monkeys in the present study were infants (~5.5 months of age), cortisol concentration was assessed under baseline conditions and following a maternal

separation challenge. In order to determine if this subset of monkeys were consistent with the larger group described by Sanchez et al. (2005), data from the nine monkeys used in this study were separated from the larger data set and analyzed for differences in rearing history. While no significant differences based on rearing history were found in this subset, the trends in the data were consistent with the phenotype previously described, in which maternally separated monkeys showed greater elevations in plasma cortisol following the separation stressor. At 12 months of age, the diurnal pattern of cortisol secretion was also determined in these monkeys. Again the data for the nine monkeys used in the current set of experiments were separated from the larger group for analysis. While no significant group differences were found, again, the data were consistent with the maternal separation phenotype, in which maternally separated monkeys exhibited a flattened diurnal cortisol rhythm as compared with control monkeys.

When these monkeys were adults, plasma cortisol and ACTH were measured prior to and following an acute cocaine challenge. While previous studies (Sarnyai et al. 1996; Broadbear et al. 1999a,b,c) have found significant increases in both cortisol and ACTH in response to acute stimulant challenge in rhesus monkeys, the present study found only minimal increases in cortisol and no increases in plasma ACTH following cocaine injection. Additionally, there were no statistically significant differences between control and maternally separated monkeys on measures of plasma cortisol or ACTH concentration. This lack of endocrine response to the cocaine challenge could be a result of elevated baseline levels of cortisol and ACTH due to the stress of the catheterization process. Alterations in the serotonergic system or in levels of gonadal hormones could also have contributed to the results observed.

Collectively, maternally separated monkeys, as compared with controls, appear to have reduced sensitivity to both the reinforcing and motor-activating effects of psychostimulant drugs. This difference could not be explained by measuring dopamine D_2 receptor levels and, due to limited group sizes, could not be determined by measuring extracellular dopamine concentrations. Sensitivity to the reinforcing effects of psychostimulants has often been linked with alterations in stress reactivity but when behavioral and HPA axis reactivity were assessed, no significant group differences were found. When acoustic startle was measured in a group of monkeys with the same rearing history, maternally separated monkeys had elevated startle reactivity as compared with controls. But when acoustic startle was tested in this group of animals as adults, no significant group differences were found and if anything, maternally separated monkeys appeared to have slightly lower startle reactivity compared with controls. Therefore, it appears that behavioral reactivity, as measured here with acoustic startle, normalized in these monkeys. Finally, when HPA axis function was examined maternally separated monkeys appeared to have basally hypo-functional but hyper-reactive HPA axis function as infants but exhibited no significant differences when compared to controls in stimulant-induced cortisol or ACTH release as adults. It is possible that the early effects of the exposure to maternal separation stress may have been robust enough to affect systems beyond the HPA axis, thus resulting in the differences observed between groups in reinforcing and motor activating effects of psychostimulants.

Studies demonstrating a link between early life stress and altered HPA axis function and increased sensitivity to or increased propensity to use psychostimulants have primarily used rodent models. However, the present study utilizes a stress paradigm in a

nonhuman primate model that is quite different from the stressors and testing procedures that are commonly described in the literature derived from rodent studies. Rodents are typically only a few months old when tested for acquisition of drug self-administration and thus, at most, only a few months removed from the stressors. Acute effects of the early life stress and resulting alterations in corticosterone levels are probably playing a major role in the subsequent changes seen in drug self-administration in the rodent. In the present study, the monkeys were 5-6 years removed from the maternal separations when the acquisition of cocaine self-administration sessions began. Therefore, any differences observed in sensitivity to stimulants would be related to enduring changes in neurobiology. There is also a large difference in the maturational state of the HPA axis at birth in rodents as compared with nonhuman primates. In rodents, the HPA axis is much more immature at birth than that of the nonhuman primate. At the time HPA axis function is tested in rodent models it is still undergoing developmental changes so it is difficult to compare with similar measures in the nonhuman primate which typically occur at a time when the HPA axis is much more developed.

Additionally, studies in rodents undergoing maternal separation have yielded conflicting results depending on the timing and duration of the separations as well as the timing and type of testing procedures used. The outcomes appear to be specific to both the developmental manipulation and testing mechanism employed. Therefore, it is difficult to compare findings from one study to the next and given the species differences, difficult to compare these studies in nonhuman primates to those in rodents.

While the majority of studies investigating early life stress and psychostimulants indicate increased sensitivity to and use of psychostimulant drugs, a small group of

studies have found evidence that early life stress actually leads to a dampening of the reward system (Matthews et al. 1996, 1999; Matthews and Robbins 2003). Repeated maternal separations in the rat were associated with reductions in behavioral responding for appetitive stimuli as measured with drug self-administration, intracranial self-stimulation and sucrose preference measures (Matthews et al. 1996). Maternally separated rats also exhibited attenuation in the initiation of cocaine self-administration and rightward (in females) and downward (in males) shifts in dose-response curves for cocaine as compared with control rats (Matthews et al. 1999). It was suggested that these alterations in responding for reinforcing stimuli was indicative of diminished sensitivity to the drugs or an overall blunting of the reward system similar to anhedonia (Matthews et al. 1996, 1999; Matthews and Robbins 2003). Early life stress-induced anhedonia has also been reported in nonhuman primates. A group of marmosets that underwent early parental deprivation (30-120 min/day for the first month of life) demonstrated a reduction, as compared with controls, in operant responses on a progressive-ratio schedule for a banana-flavored milk drink (Pryce et al. 2004). This reduction in responding did not, however, represent a general reduction in consummatory behavior, since both groups consumed similar volumes when the drink was freely available. The authors suggested that this drink reward served as less of an incentive in the parentally deprived animals than it did in the control group of marmosets (Pryce et al. 2004).

In the present study, downward displacements in dose-response functions for both cocaine and amphetamine were seen in maternally separated monkeys as compared with controls. This could represent a reduced sensitivity to the drug, making it less of an incentive. Stressful experiences have previously been shown to reduce the rewarding

value of inter-cranial self-stimulation (ICSS) in the mesocorticolimbic system (Zacharko and Anisman 1991) and induce hyposensitivity to the behavioral effects of amphetamine in rats (Papp et al. 1991). The blunted locomotor stimulant effect seen in maternally separated monkeys supports the idea of diminished sensitivity to the drug.

It has also been suggested that vertical downward shifts in dose-response functions predict a lower motivation to self-administer the drug and a lower predisposition to develop drug use (Piazza et al. 2000). The present set of experiments did not directly examine motivation for the drug and future studies would benefit from testing the animals on a progressive ratio (PR) schedule that could provide more direct evidence of the reinforcing efficacy of cocaine and amphetamine. Reduced motivation for the drug would explain the findings of the self-administration studies but cannot explain the decreased stimulant-induced effects observed on involuntary measures such as locomotor activity.

Suppression or reduction of circulating glucocorticoids may decrease the reinforcing effects of psychostimulants. Adrenalectomy (removal of the adrenal gland where glucocorticoids are released) abolishes acquisition of cocaine self administration over a range of cocaine doses (Goeders and Guerin 1996). This was partially reversed when corticosterone was added to the drinking water suggesting that corticosterone is necessary for the acquisition of cocaine self-administration to occur. Metyrapone pretreatment, which inhibits synthesis of corticosterone, decreased ongoing cocaine self-administration suggesting that corticosterone may also be necessary for the maintenance of self-administration as well (Goeders and Guerin 1996). Adrenalectomy and metyrapone also decreased locomotor response to cocaine (Piazza et al. 1994).

Chronic stress can result in a down-regulation of glucocorticoid receptors (GR) in the PVN and in other brain structures, ultimately resulting in a failure to suppress the hyperactivity of the HPA axis (Makino et al. 2002). GR knockouts (generated with Cre/LoxP system) were able to learn drug self-administration but their dose-response functions were flattened, leading authors to suggest a decreased motivation to self-administer cocaine as compared with wild type animals (Deroche-Gamonet et al. 2003). Administration of a glucocorticoid receptor antagonist (mifepristone) dose-dependently reduced the motivation to self-administer cocaine while working on a PR schedule (Deroche-Gamonet et al. 2003). Using a different glucocorticoid receptor transgenic mouse model that produces about a 50% reduction of GR mRNA levels in the brain also indicated that a reduction in GR levels leads to a reduction in the sensitivity to cocaine (St-Hilaire et al. 2003). Taken together both studies showed that a reduction in GR levels in the brain reduced the behavioral and molecular sensitivity to cocaine.

Early life stress has been shown to induce alterations in the HPA axis including alterations in glucocorticoid negative feedback and decreases in levels of glucocorticoid receptors (Vazquez et al. 2002; Sapolsky et al. 1984a, b; Aisa et al. 2008). It is possible that the maternally separated monkeys involved in the present study have reductions in levels of glucocorticoid receptors which could account for the reductions in responding for cocaine and flattened dose-response functions observed. Future studies would benefit from examination of glucocorticoid receptor levels in both groups of monkeys.

Another possibility is that the monkeys in the present study have altered levels of dopamine transporters. Studies have reported that maternally separated rats have fewer dopamine transporters, particularly in the ventral striatum (Meaney et al. 2002; Brake et

al. 2004). Variations in DAT levels are also strongly linked with variation in both baseline and psychostimulant-induced locomotor activity (Uhl et al. 2002; Sabeti et al. 2002, 2003). Specifically, reductions in DAT levels result in decreased stimulant-induced locomotor activity (Zhaung et al. 2001). Similar to the results seen in the present study, DAT knockout mice were able to acquire and maintain cocaine self-administration but at rates that were lower than wild type animals (Rocha et al. 1998). Conversely, mice that over express DAT, resulting in a 50% increase in functional activity, exhibit behavior that is in opposition to those observed in the present study. These animals show increased stimulant-induced locomotor activity and an enhanced sensitivity to the reinforcing effects of amphetamine as compared with wild type mice (Salahpour et al. 2008). The sensitivity of an animal to the stimulating and reinforcing effects of psychostimulant drugs appear to be directly proportional to the level of DAT (Salahpour et al. 2008). Maternally separated rats have also been shown to exhibit increases in dopamine D_3 receptors in the shell of the nucleus accumbens and increases in D_1 levels in the caudate putamen as compared with handled and non-handled rats, respectively (Brake et al. 2004). The current study examined dopamine D_2 receptor levels and found no significant difference between control and maternally separated monkeys but, D_3 and D_1 levels were not assessed. Given the importance of all of these receptors in the reinforcing effects of psychostimulants, future studies would benefit from further examination of both dopamine transporter and receptor levels.

While the present set of experiments focused on the effects of early life stress and glucocorticoids on the dopaminergic system, it is possible that other neurotransmitter systems were involved. In particular, the serotonergic system and alterations to that

system caused by the chronic stress of maternal separation likely impacted the results observed. Serotonin neurotransmission is involved in both the activation and feedback of the HPA axis throughout the life of an organism and plays a fundamental role in the development of the CNS (Vicentic et al. 2006). Glucocorticoids have been shown to alter several elements of serotonin neurotransmission. Adrenalectomy results in anatomically specific decreases in measures of serotonin metabolism, while exposure to stressors, which raise glucocorticoid levels, results in a corresponding increase in serotonin turnover (Curzon et al. 1972; Van Loon et al. 1981; Chaouloff 1993; Vazquez et al. 2002). Additionally, glucocorticoids may also act to directly modulate serotonin transmission by regulating serotonin receptors (Biegon et al. 1985; Chalmers et al. 1993, 1994).

Models of early life stress have frequently been associated with alterations in the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) in cerebrospinal fluid (CSF). Rhesus monkeys reared with peers only (PR) have exhibited low levels of CSF 5-HIAA as compared with mother reared monkeys (Barr et al. 2004; Higley et al. 1996 a, b). However, earlier studies have demonstrated gender and age specific effects of rearing history on CSF 5-HIAA levels (Higley et al. 1991, 1992). The serotonin transporter has also been implicated in nonhuman primate models of early life stress. There is a naturally occurring polymorphism in both humans and nonhuman primates that results in variations in the serotonin transporter gene promoter. The s allele of the serotonin transporter gene promoter results in diminished transcription relative to the l allele (Barr et al. 2004) and ultimately lower serotonin transporter (SERT) density (Heinz et al. 2001). There appears to be an interaction between the variation in SERT and exposure to

early life stress that influences the activation of the HPA axis response to stress. Accordingly, the effects of the s allele only become evident when the monkey has been exposed to early life stress (Soumi 2006). The SERT gene promoter contains a glucocorticoid response element making it responsive to stress-induced levels of glucocorticoids and this reactivity is particularly evident in carriers of the s allele (Barr et al. 2004).

Alterations in the serotonergic system can also affect the effects of cocaine. In addition to binding to the DAT, cocaine binds to SERT with greater affinity (Wolf and Kuhn 1992). Therefore alterations to the SERT gene and its expression can also impact the effects of cocaine (Malison et al. 1998; Heinz et al. 2000; Huot et al. 2001). In general, activation of the serotonergic system appears to inhibit the effects of cocaine whereas manipulations that decrease serotonergic transmission increase the motor and reinforcing effects of cocaine (Carroll et al. 1990; Loh and Roberts 1990; Morrow and Roth 1996; Herges and Taylor 1999).

When CSF 5-HIAA levels were measured in monkeys with the same rearing history as those in the present study, higher CSF 5-HIAA levels were detected in maternally separated monkeys than in controls during the juvenile period, particularly in the females (Sanchez et al. 2006). This finding is in contrast with the findings in PR monkeys but it is possible that the differences in the maternal separation model employed accounts for the difference in results. Higher levels of serotonin neurotransmission as indicated by the high levels of CSF 5-HIAA could help to explain the diminished reinforcing and motor effects of cocaine and amphetamine observed in the maternally separated monkeys involved in the present study.

Limitations

The present study is not without limitations. Experiments were underpowered due to small group sizes. Given the nature of the experiments and the very specific background of these monkeys the studies drew from a limited population and the addition of more subjects was not a possibility. The limited group sizes were exacerbated by medical issues arising in some monkeys that necessitated their exclusion from certain experiments. Substantial individual differences were seen, particularly in the control group, in a number of the measures investigated in the present group of experiments. It is possible that group differences may have been overshadowed by this individual variability given the small group sizes.

The present set of studies used only female subjects, which did not allow for the evaluation of the effects of gender or the interaction of rearing and gender on sensitivity to psychostimulants. Females were chosen due to the robust endocrine and behavioral responses they displayed following the maternal separation paradigm (Sanchez et al. 2005). If group differences in drug sensitivity existed based on rearing history it was expected that they would be most evident in those monkeys that exhibited the greatest effects of the maternal separations. The females used in the present study were intact but their menstrual cycles were not monitored. Therefore, it is possible that variations in menstrual phase had an effect on the measures examined. The effects of menstrual phase on some measures have shown conflicting results.

The manner in which the animals were reared calls into question the appropriateness of the control group to serve as true controls. The entire social group was disrupted during the maternal separations and the control and maternally separated

monkeys were later pair housed for a period of time. These factors may have adversely affected the control monkeys.

Closing remarks

Clinical reports appear to indicate a strong link between exposure to negative environmental factors or stressors early in life and propensity to abuse drugs. However, these studies are typically correlational and based on retrospective reports. The present study utilized an animal model of exposure to early life stress in order to determine if early life stress would in fact increase sensitivity to and propensity to use drugs and to provide a mechanistic basis for this effect. While there are factors from the human drug abuse condition that cannot be modeled in animals, this animal model did demonstrate differences in abuse related behaviors based on rearing history. But the expected outcome was not observed. Instead, this study found that monkeys exposed to early life stress were actually less sensitive to both the reinforcing and motor activating effects of psychostimulant drugs.

While these effects are in contrast with the expectations of the clinical reports, they do provide an opportunity to explore targets for treatments. It is clear that the group differences in sensitivity to psychostimulants were not directly related to dopamine D_2 receptor availability, which has been closely linked to both the reinforcing effects (Volkow et al. 1999; Morgan et al. 2002) and motor stimulating effects (Baker et al. 1996) of these drugs. Moreover, in adulthood, measures of HPA axis function and behavioral reactivity, previously associated with the propensity to self-administer drugs of abuse, could not account for the group differences observed. Subsequent studies that

can elucidate the mechanistic basis of the reduced sensitivity to psychostimulants observed in the maternally separated monkeys may be useful in determining targets for treatment strategies.

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