Early Rearing Experience, Hypothalamic-Pituitary-Adrenal (HPA) Activity, and Serotonin Transporter Genotype: Influences on the Development of Anxiety in Infant Rhesus Monkeys (Macaca mulatta)

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EARLY REARING EXPERIENCE, HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) ACTIVITY, AND SEROTONIN TRANSPORTER GENOTYPE: INFLUENCES ON THE DEVELOPMENT OF ANXIETY IN INFANT RHESUS MONKEYS (MACACA MULATTA)

A Dissertation Presented

by

AMANDA MICHELLE DETTMER

Submitted to the Graduate School of the University of Massachusetts in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2009

Neuroscience and Behavior Program
EARLY REARING EXPERIENCE, HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) ACTIVITY, AND SEROTONIN TRANSPORTER GENOTYPE: INFLUENCES ON THE DEVELOPMENT OF ANXIETY IN INFANT Rhesus Monkeys (Macaca Mulatta)

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DEDICTATION

To Gerry Ruppenthal, my first mentor and the one responsible for getting me into all this monkey business.
ACKNOWLEDGEMENTS

I must thank my advisor, Melinda Novak, for her mentorship and advice throughout this project, and most of all for granting me the level of independence I craved and thrived upon. I thank Stephen Suomi for providing the setting and funding for my research for the last two and a half years. Angela Ruggiero saved my hide more times than I can count, and there is so much of my data I wouldn’t have without her. I am indebted to Matt Novak for his endless support, friendship, and advice, especially in the last two years, and for the regular sustenance he provided. I promise to pay it forward. I thank all those who made the technical parts of my dissertation possible: to Mat Davenport for teaching me the hair cortisol assay; to Brian Kelly, Kaushal Jani, Karen Stonemetz, and Elizabeth Henchey for help with cortisol assays; to Nicole Bowling, Liz Mallott, Dan Hipp, Annika Paukner, Helen-Marie Graves, Elizabeth Kerschner, and Lisa Darcey for cortisol sample and behavioral data collection; to Sever Consuel for teaching me DNA extraction; to the veterinary and animal care staff at NIH, especially John Hackley and Dave Brown for their constant help and communication. I owe many thanks to Aline Sayer, my committee member who made multivariate statistics not only understandable, but fun, and who graciously gave so much of her time and advice to me during the analysis and write-up of this project. I am grateful to Jerry Meyer who provided the lab space and equipment for the cortisol assays, and to Gordon Wyse for making neurobiology intriguing and exciting. I thank all my committee members for their dedication to my project.
I am forever grateful for my best friend and husband, James, who has seen me through this entire project from start to finish, through the best and worst of it all, and who always knew just the right words to say and just the right type of encouragement to give when I needed it most. I really wouldn’t have been here without my parents, Bill and Diane, whom I thank for 40 years of dedication and commitment to each other and to our family. I’ve never been in doubt of their support for all my endeavors, and for that I am forever grateful. I thank my sister, who often provided the respite I needed from grad school, and who understood and supported her poor sister’s budget more times than I can count. I thank my second set of parents, Michael and Jeanette whose home was a second home for me; they’ll never know how much I appreciate their warmth, love, hospitality, and support in all the times I had to travel back and forth between NIH and UMass. I am indebted to my dearest and longest friends, Jilly, Jakey, Katie, the 1204 girls, for reminding me that there is more to life than research. I thank Nancy Luce for making my transition to Massachusetts and the start of my graduate career such a welcoming and happy experience. I couldn’t have made it through the program without my fellow partners in crime: Ashvin Shah, Eliza Nelson, Lori Astheimer, and several others. Thanks for some good times! Finally, I need to thank my pup Molly, who has provided me with more joy and love then I ever thought possible, and who more than once was the only thing that could provide the sanity breaks I needed.
ABSTRACT

EARLY REARING EXPERIENCE, HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) ACTIVITY, AND SEROTONIN TRANSPORTER GENOTYPE: INFLUENCES ON THE DEVELOPMENT OF ANXIETY IN INFANT Rhesus MONKEYS (Macaca mulatta)

MAY 2009

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Directed by: Doctor Melinda A. Novak

A gene x environment interaction exists in the expression of anxiety for both human and nonhuman primates, such that individuals who are carriers of the (s) allele of the serotonin transporter genotype (5-HTTLPR) and exposed to early life stress are more at risk for exhibiting anxiety. The hypothalamic-pituitary-adrenal (HPA) axis has also been implicated in anxiety disorders but the relationship between early life/genotype, HPA activity, and anxiety is not well understood. Further, studies linking the HPA axis to anxiety have relied on “point” samples (blood and salivary cortisol) which reflect moments in time rather than long-term activity. The purpose of this dissertation was three fold: 1) to examine anxious behavior in monkeys with different 5-HTTLPR genotypes and rearing environments across the first two years of life, 2) to compare long-term HPA activity (as measured with hair cortisol) with acute HPA activity (as measured with salivary cortisol) in the same period, and 3) to determine which measure of HPA activity predicts anxiety and/or mediates the rearing/genotype influences on anxious behavior. Infant rhesus monkeys (Macaca mulatta, N=61) were
mother-peer-reared (MPR, n=21), peer-reared (PR, n=20), or surrogate-peer-reared (SPR, n=20) for 8 months, then all relocated into a large social housing situation for the next 18 months. Monkeys were genotyped for 5-HTTLPR and hair and saliva samples were collected for cortisol analysis at months 6, 12, 18, and 24. Behavior was recorded twice per week per subject from 2-24 months and analyzed for the duration of anxiety, social play, and grooming. Regression analysis established predictors of these behaviors. Rearing condition and sex were significant predictors of anxiety across the two years, and HPA activity added significant predictive power in the first six months only. Mediation of the rearing/anxiety relationship by the HPA axis was not evident. Interestingly, hair (but not salivary) cortisol early in life was positively correlated with later anxious behavior. These findings demonstrate the detrimental effects of adverse early life experience on behavioral development and shed light on the interplay between environment, adrenocortical activity, and anxiety. They further demonstrate the usefulness of a long-term measure of HPA activity in predicting later behavior.
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CHAPTER 1

INTRODUCTION

1.1 Anxiety Disorders

Anxiety disorders are the most common form of mental illness in the U.S., occurring in 18% of adults with women having a higher prevalence rate than men. There are many categories of anxiety disorders, including generalized anxiety disorder, obsessive compulsive disorder (OCD), posttraumatic stress disorder (PTSD), and social anxiety disorder or social phobia. Social anxiety is the most common type of anxiety disorder, with approximately 15 million (or 6.8%) U.S. adults affected (National Institutes of Mental Health, 2006). The onset of social anxiety usually occurs in childhood or early adolescence and is characterized in humans by extreme anxiety about being judged by peers and excessive fear and/or avoidance of social situations in which embarrassment or judgment may occur (Mathew et al., 2001).

Many nonhuman primate species are also dependent on social relationships and can exhibit social anxiety which is characterized by behavioral withdrawal, increased distress vocalizations, and higher incidence of locomotor stereotypies (Suomi, 1991). In both human and non-human primates, behavioral anxiety has been correlated with early life experience as well as neuroendocrine and neurotransmitter activity (Heim and Nemeroff, 2001; Higley et al., 1991b; Shannon et al., 1998). In nonhuman primates, early life experience has been most thoroughly studied through the implementation of different rearing conditions in infancy.
1.2 Experimental Rearing Conditions in Monkeys

Nonhuman primate models of human development have the advantage over rodent models of sharing more of the same endocrine and neural substrates with humans (Tamashiro et al., 2005). Additionally, rhesus monkey society includes a more complex social structure while also exhibiting stable individual differences in behaviors (Suomi, 2005; Suomi et al., 1996). Thus infant monkeys exposed to different rearing environments represent excellent models of early-life stress in humans.

Studies comparing early rearing environments in monkeys typically rely on three experimental groups: mother-reared (MR), peer-reared (PR), and surrogate-peer-reared (SPR) monkeys. MR can take two forms: mother-only rearing, in which infants are reared with only their mothers in standard laboratory housing (see Capitanio et al., 2005), or mother-peer-rearing (MPR), in which infants are raised with their mothers in a large harem group comprised of multiple females and one or two males, as well as other juveniles and infants (see Capitanio et al., 2005; Sackett et al., 2002; Shannon et al., 1998).

Because mother-only rearing yields infants who are more aggressive than MPR infants (Harlow & Harlow, 1962), the MPR condition is considered more representative of free-ranging monkeys. In support of this view, MPR monkeys develop species-typical patterns of affiliation, exploration, and play (Suomi, 1991) and complex social interactions as characterized by increased interactions in trios and larger subgroups (Capitanio, 1985). Thus, the MPR condition has been characterized as the normative rearing condition in the laboratory setting.
The two most common methods of nursery-rearing (NR) in infant monkeys are peer-rearing (PR) and surrogate-and limited peer-rearing (SPR), with PR being the most common. For both types of rearing, infants are removed from their mothers within 1-3 days post-partum. After a period of housing in individual incubators, PR infants are then reared together 24 hours per day whereas SPR reared infants are reared with inanimate surrogates 24 hours per day and given brief daily physical interaction with other infants.

However, the specific methods for nursery rearing are not standardized across laboratories. In peer-rearing (PR), both the onset of social experience and the number of partners has been shown to vary widely. At the Yerkes National Primate Research Center at Emory University in Atlanta, PR monkeys were housed individually for the first 45-60 days of life and then paired with one other agemate for most of the day, except for 4 to 6 hours (between 10am and 4pm) when they were separated for individual feeding and bottle training. At three months of age they were paired together 24 hours per day (Winslow et al., 2003). At the Harlow Primate Lab at the University of Wisconsin, PR monkeys were reared in single cages for the first month of life, during which time they were given daily 30-min socialization with two other peers. At six weeks of age, these peer groups of three were housed together 24 hours per day for the next six months of life (Clarke, 1993). At the California National Primate Research Center at the University of California, Davis, Capitanio et al. (2005), reared PR monkeys individually for the first 30 days of life, then placed them into dyads and allowed them to interact between 6 and 24 hours per day. At the Laboratory of Comparative Ethology at the National Institutes of Health, PR monkeys were housed individually for the first
37 days of life, and then placed into groups of four, 24 hours per day, for the duration of their 6-8 month stay in the nursery (Shannon et al., 1998)

Despite differing protocols, PR infant macaques have repeatedly been shown to exhibit under-developed species-typical behaviors such as social play, grooming, and exploration. They also develop abnormal behaviors like locomotor stereotypies, as well as hyperemotional behaviors including high levels of clinging, fear, and social withdrawal, behaviors which persist later in life (Harlow, 1963; Chamove et al., 1973; Capitanio, 1986; Ruppenthal et al., 1991; Champoux et al., 2002).

SPR is less common than peer-rearing, and is only regularly practiced in two laboratories (the University of Washington and the Laboratory of Comparative Ethology at NIH; see Sackett et al., 2002 and Shannon et al., 1998). SPR monkeys are reared in single cages with an inanimate cloth-covered surrogate mother and are provided daily social interaction with peers lasting 30-120 minutes per day. In contrast to their PR counterparts, SPR monkeys given just 30 minutes of daily social contact show a behavioral repertoire that is more equivalent to that of mother-reared infants (Sackett, 1982) and involves less clinging and more exploratory behavior than peer-reared infants (Ruppenthal et al., 1991). SPR animals show no difference compared to MPR animals in survival or reproductive outcome, while PR animals exhibit less adequate mothering than MR animals (Ruppenthal et al., 1976; Sackett et al., 2002).

In addition to affecting behavior, early rearing experience has also been shown to alter the neuroendocrine activity that regulates stress responsivity in nonhuman primates, specifically the hypothalamic-pituitary-adrenal (HPA) axis.
1.3 The HPA Axis

The hypothalamic-pituitary-adrenal (HPA) axis represents the body’s primary neuroendocrine stress response system. As such, it is responsible for synchronizing the body’s responses to perceived or actual stress. During stress, the hypothalamus in the forebrain releases corticotropin releasing factor (CRF), which stimulates the anterior pituitary gland to release adrenocorticotropic hormone (ACTH) into the blood. ACTH in turn targets the adrenal cortex to generate the release of the glucocorticoid cortisol, the primary stress hormone in primates. Cortisol feeds back negatively to the hypothalamus and anterior pituitary to inhibit further release of CRF and ACTH respectively, thereby placing a limit on the duration of the stress response. This HPA activity stimulates the metabolic, cardiovascular, respiratory, and behavioral responses that allow an organism to adapt to stress (Fig. 1), while limiting the duration of the response (Charmandari et al., 2003).

The stress response is meant to be short in duration by temporarily suppressing vegetative functions and increasing metabolism, thereby ensuring survival. Metabolic changes such as heightened glucogenesis and lypolysis act in concordance with behavioral adaptations to stress, including increased arousal, improved cognition, heightened analgesia, and inhibition of appetite, feeding, reproduction, and immunity. HPA axis activation also produces simultaneous physical adaptations that allow energy to be redirected. Oxygen and nutrients are sent to stressed body sites, and cardiovascular tone and respiration are increased (Chrousos, 1996; Charmandari et al., 2003). During stress, cortisol feeds back in a negative fashion on the HPA axis to impose
necessary restraining forces on the above adaptations, which prevent hyper-responsivity of the stress system.

Figure 1. The hypothalamic-pituitary-adrenal (HPA) axis.

However, some stressful experiences may be long-lasting or severe enough to impair the individual’s ability to adapt to the stress, thereby producing chronic activation of the stress response system. Chronic activation of the HPA axis in which there is prolonged exposure to CRF and glucocorticoids is implicated in the development of many diseases and disorders. Such exposure can lead to increased or decreased sensitivity (McEwen and Sapolsky, 1995), with some individuals exhibiting hyper-secretion of cortisol and some exhibiting hypo-secretion of cortisol in response to stress. Numerous animal and human studies have linked a hypo-responsive HPA axis to disorders such as post-traumatic stress disorder (PTSD) and seasonal depression, and a hyper-responsive HPA axis, both long-term and short-term, to the eventual
development of several anxious behaviors and anxiety-related disorders (Charmandari et al., 2003; Yehuda et al., 1996; Sanchez et al., 2001). In primates, signs of anxiety include stereotypic behaviors (Sanchez et al., 2001), chronic and excessive fear (Levine et al., 1956; Gunnar et al., 1981; Suomi, 1991; Chrousos and Gold, 1999), inhibition and social withdrawal (Higley et al., 1991b; Suomi, 1991), and panic attacks (Gold et al., 1988). Anxiety is often associated with other deleterious conditions such as excessive alcohol consumption (Wand and Dobs, 1991; Higley et al., 1991a).

Much research in animals and humans has demonstrated the detrimental yet often preventable role of adverse early experiences on dysregulated HPA activity and the development of anxious behaviors (Levine, 2000; Heim and Nemeroff, 2001; Charmandari et al., 2003; Mathew et al., 2001; Sanchez et al., 2001). Neglect and abuse early in life produce abnormal behavioral responses and alter the neurochemistry and organization of particular brain regions associated with stress responsivity; particular brain alterations include overall increased levels of glucocorticoids, decreased glucocorticoid receptor binding in the hippocampus, hypothalamus, and frontal cortex, and enhanced CRF gene expression in the amygdala (Francis et al., 1999; Meaney, 2001). Most studied, however, are early life effects on the activity and responsivity of the HPA axis.
1.3.1 Early Experience Effects on HPA Activity

Exposure to early life stress has proven detrimental to the normal development of the HPA axis in many species including rodents, monkeys, and humans (Plotsky and Meaney, 1993; Shannon et al., 1998; Heim and Nemeroff, 2001; Heim et al., 2002; Leucken and Lemery, 2004). Altered HPA responsivity to stress persists into adulthood in all these groups. Rat pups aged 2-14 d and exposed to 180 minutes of maternal separation exhibited significantly more plasma corticosterone (the rodent glucocorticoid) in response to stress and more CRF mRNA as adults than those left undisturbed (Plotsky and Meaney, 1993). Bonnet macaques (Macaca radiata) reared as infants under their mothers’ variable foraging demand (VFD) condition, which disturbs the mother-infant attachment (Rosenblum and Andrews, 1994; Rosenblum et al., 1994), demonstrated ongoing anxious behaviors and persistent elevated CSF concentrations of CRF as juveniles and adults (Coplan et al., 1996; Coplan et al., 2001). Adult women with a history of child abuse exhibited higher plasma ACTH concentrations in response to a psychosocial stressor compared to controls (Heim et al., 2000).

The relationship between infant rearing environments and HPA activity in rhesus monkeys is not as straightforward, particularly for the PR condition. Initially it appeared that PR monkeys showed reduced reactivity. Clarke (1993) reported that PR monkeys had lower ACTH and similar basal plasma cortisol levels to MR monkeys at 1-6 months of age, and significantly lower stress reactivity, as measured by ACTH and cortisol, after a mild stress. However, subsequent studies found the reverse, namely that PR monkeys had lower basal plasma cortisol levels than MPR monkeys at 2 months of
age (Capitanio et al., 2005; Shannon et al., 1998), and larger cortisol increases after repeated stress (Higley et al., 1992). Finally, it should be noted that in one study (Winslow et al. 2003), CSF basal or stress-related cortisol did not differ between PR and MPR monkeys. The inconsistency in HPA axis activity in PR monkeys may in part reflect differing protocols across laboratories.

Less is known about the effects of SPR on HPA axis activity, however, the existing data are all in agreement. SPR infants exhibited lower levels of plasma cortisol than MPR and PR infants after exposure to moderate stress (Davenport et al., 2003; Shannon et al., 1998; Meyer et al., 1975). Similarly, juvenile SPR infants exhibited lower salivary cortisol than MPR juveniles after moderate stress (Davenport et al., 2003). As these studies utilized plasma and salivary cortisol, which represent “point” samples (Table 1), little is known about long-term HPA axis activity of SPR infants. These findings underscore the added value of a long-term measure of HPA axis activity in monkeys exposed to various rearing conditions.

While early rearing effects on HPA activity have been well-documented at least in MR/MPR and PR monkeys, less is known about how the serotonergic system, which influences HPA activity, may alter adrenocortical functioning.

1.3.2 Serotonergic Effects on HPA Activity

The serotonergic system influences activation and feedback control of the HPA axis in two chief ways. First, circulating serotonin (5HT) has been shown to act on the adrenal glands and possibly the anterior pituitary to stimulate cortisol and ACTH release (Dinan, 1996). In addition, serotonin precursors (e.g. 5-hydroxytryptophan
and 1-tryptophan) and drugs that stimulate 5HT (e.g. fenfluramine) both stimulate ACTH release via increased CRH release. Second, allelic variation in genetic expression of the serotonin transporter is associated with variations in HPA activity. Serotonin transporter (SERT) knockout mice (heterozygous or homozygous) demonstrated overall reduced basal HPA activity, reduced CRF expression in the paraventricular nucleus (PVN) and hypothalamus (HT), and reduced glucocorticoid receptor (GR) expression in the HT, pituitary, and adrenal cortex (Jiang et al., 2008). The reduced GR expression may have also led SERT knockout mice to exhibit heightened HPA reactivity and anxiety-like behavior after the stress of the elevated plus-maze, since GR functioning most directly inhibits corticosterone secretion (Jiang et al., 2008; Holmes et al., 2003).

Regulation of the HPA axis by 5HT is important because humans and animals exhibiting anxiety-related behaviors have been shown to exhibit abnormal serotonergic activity in conjunction with altered HPA axis activity (Heim and Nemeroff, 2001; Mathew et al., 2001; Barr et al., 2004). In one study, infants were exposed to repeated separations over a four week period, and observed for withdrawal behaviors and examined for plasma cortisol and CSF concentrations of serotonin before and during the separations. Based on behavioral observations, infants were described as either high-withdrawal or low-withdrawal, where high-withdrawal infants exhibited greater absence of social behaviors, directed movement, and exploration of objects in the environment. Whereas low-withdrawal infants physiologically adapted to the procedure, high-withdrawal infants maintained chronic elevated levels of both plasma
cortisol and cerebrospinal fluid (CSF) serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA), the serotonin metabolite (Erickson et al., 2005). In a study with adult cynomologus macaques (Macaca fascicularis), monkeys subjected to subordination stress (i.e., stress imposed by more dominant animals) exhibited impaired serotonergic functioning in the form of a blunted prolactin response in conjunction with hyperactivity of the HPA axis (Shively, 1998). Additional support for the interaction of the HPA axis and serotonergic system comes from a neuroimaging study in which peer-reared monkeys, who previously exhibited elevated plasma cortisol after separation stress (Fahlke et al., 2000), demonstrated significantly decreased serotonin transporter binding potential compared to mother-reared monkeys in PET images of the raphé nuclei, which contain the majority of the serotonergic projections to the forebrain (Ichise et al., 2006; Heim and Nemeroff, 2001).

In humans, a polymorphism in the transcriptional control region of the serotonin transporter length polymorphic region (5-HTTLPR) is directly implicated in the development of anxiety-related disorders, and this relationship is likely moderated by alterations in HPA activity in individuals with differing genotypes (Gotlib et al., 2008; Lesch et al., 1996; Dinan, 1996). The short (s) allele confers reduced transcription of the transporter, which in turn leads to less reuptake of 5HT and increased 5HT signaling; this amplified serotonergic neurotransmission is anxiogenic in humans and animals (Lesch et al., 1996; Reimold et al., 2008). The 5-HTTLPR genotypes of 505 human individuals examined and found to be distributed according to the Hardy-Weinberg equilibrium, with 32% l/l, 49% l/s, and 19% s/s, and individuals heterozygous or
homozygous for the short (s) allele (i.e., (s) allele carriers) were more likely than l/l individuals to exhibit anxious behaviors (Lesch et al., 1996). Ambruster et al. (2008) found that carriers of the (s) allele exhibited increased activation of the amygdala in response to stress or fearful events. Further, (s) carriers have showed a higher incidence of depression, alcoholism, and social withdrawal (Hariri et al., 2002; Lichtermann et al., 2000). Although individuals heterozygous for 5-HTTLPR regularly exhibit anxious behaviors, some studies suggest that two copies of the (s) allele are required for dysregulated HPA activity and feedback regulation, though this relationship may only be present in females. Two studies have demonstrated that s/s girls exhibit higher basal and waking cortisol concentrations than either l/l or l/s girls (Wust et al., 2009; Chen et al., 2009). In another study of adolescent girls exposed to an emotionally stressful interview, only s/s individuals showed a heightened and prolonged salivary cortisol stress response compared to l/l or l/s girls (Gotlib et al., 2008). Finally, a gene by environment (G x E) interaction exists in human carriers of the (s) allele, such that l/s or s/s humans who experienced early life adversity (e.g. neglect, abuse) went on to develop depression and bulimia-spectrum disorders, which included anxiety disorder (Caspi et al., 2003; Richardson et al., 2008).

A homolog of the 5-HTTLPR, the rhesus serotonin transporter-linked polymorphic region (rh5-HTTLPR), was identified in rhesus monkeys over ten years ago (Lesch et al., 1997), and the monkeys in this study exhibited similar allele and genotype frequencies of their 5-HTTLPR as humans. A similar G x E interaction to that of humans has also been reported for rhesus monkeys exposed to early life adversity in the form of...
Peer rearing. Peer-reared juveniles who were carriers of the (s) allele for the rh5-HTTLPR displayed higher ACTH and lower cortisol in response to stress than peer-reared monkeys homozygous for the (l) allele or mother-peer-reared monkeys with either genotype (Barr et al., 2004). Altered serotonergic functioning persisted in adult monkeys with early adverse rearing experiences (Rosenblum et al., 1994), presumably due in part to this G x E interaction. Peer-reared rhesus monkeys carrying the (s) allele were also more sensitive to alcohol and displayed behaviors similar to those found in anxiety-related disorders (Barr et al., 2003a; Barr et al., 2003b; Champoux et al., 2002).

While these studies just described have repeatedly demonstrated a link between abnormal HPA and serotonergic responses to stress, as well as the occurrence of anxiety-like behaviors, the majority of these studies have relied on short-term measures of HPA axis activity. Researchers have primarily relied on blood plasma cortisol as a measure of HPA activity, with secondary reliance on other cortisol concentrations such as those found in saliva, urine, and occasionally feces. A reliable measure of long-term HPA activity that eliminates the need for multiple, repeated sampling (such as occurs with blood sampling) would provide valuable insight into truly chronic HPA activity and associated risks for developing anxiety-related disorders.

1.4 Measuring HPA Activity

Studies measuring HPA activity or reactivity rely on cortisol concentrations from various matrices, depending on the desired measure or outcome (Table 1). Cortisol is most commonly analyzed in blood plasma or serum, yet measurements may also be
obtained in urine, feces, saliva, and CSF. Each of these approaches may be used to
measure cortisol under baseline conditions or in response to a stressor, and each has its
own merits and limitations. Blood plasma, saliva, and CSF samples reflect cortisol
values over a matter of minutes (i.e., “point” samples). Samples from these fluid
compartments are characterized by high circadian variability and low long-term
stability, and generally require freezing of the sample prior to analysis. Blood samples
provide a measure of total cortisol (i.e. the protein-bound and unbound, biologically
active portions) whereas saliva and CSF provide a measure of the “free” biologically
active cortisol (Mendel, 1989). These three measures vary in their invasiveness, with
saliva sampling being minimally invasive, blood sampling being modestly invasive, and
CSF sampling being very invasive (Kirschbaum and Hellhammer, 1994; Lutz et al.,
2000). When working with animals, all of these “point” measures require capture,
restraint, and sometimes needle puncture, processes which may confound the results
regardless of whether the goal is to obtain baseline or stress-response cortisol values.
Other variables that may affect the results of blood plasma, salivary, or CSF cortisol are
the environment, time of day, and food intake. Point samples are essential when
assessing an individual’s response to an acute stressor.

In contrast, urinary or fecal cortisol values represent a longer time frame (up to
24 hours as opposed to minutes) and are referred to as “state” samples. Like plasma,
saliva and CSF samples, urine and fecal samples are characterized by circadian
variability and require freezing for storage. The advantages to measuring cortisol in
urine or feces include reduced invasiveness of the procedure and moderate long-term
stability of samples. However, a major disadvantage is the potential for cross-contamination either from other animals when housed socially or from contamination with other fluid compartments in a single animal (e.g., blood contaminating urine or feces contaminating urine). A secondary problem is that neither urinary nor fecal samples are useful for assessing short-term acute stressors. To obtain a chronic estimate of cortisol, repeated sampling is necessary with all five matrices just described.

For investigators interested in studying HPA activity over the long term, sample collection can be cumbersome and results vulnerable to confounding variables. A reliable method of measuring chronic endogenous glucocorticoids long-term is therefore desirable. To this end, our laboratory recently developed and validated a novel method for assessing chronic HPA activity by measuring cortisol in the matrix of hair in adult rhesus monkeys. Moreover, hair cortisol has been shown to provide a reliable measurement of HPA activity in response to a long-term stressor in rhesus monkeys (Davenport et al., 2006). Hair sampling provides an integrated measure of cortisol over several months (i.e. “chronic” sample); the cortisol obtained reflects the free portion; and the procedure to obtain the hair sample is minimally invasive. Unlike the “point” or “state” samples, hair cortisol sampling is unaffected by circadian variation; the hair samples have high long-term stability; and the samples can be stored at room temperature rather than frozen prior to analysis. Less frequent sampling is required to garner long-term HPA activity profiles; thus hair provides an excellent matrix for characterizing an individual’s true hormonal “phenotype” (Davenport et al., 2006), a useful measure for studying long-term effects of experimental conditions and
determining the relationship between HPA activity and the development of anxiety-related disorders.

Research is lacking in the comparison between short-term and long-term measures of HPA activity with respect to the development or expression of anxious behaviors. A direct comparison between a “point” measure of cortisol, such as salivary cortisol, and a “chronic” measure, such as hair cortisol, would provide valuable information as to whether an individual’s immediate physiological reaction to stress or its long-term physiological reaction is a better indicator for anxiety. This dissertation focuses on such a comparison by examining the genetic and early-life contributions to the development of anxious behaviors in young monkeys, and whether these contributions are better mediated by short-term cortisol measures found in saliva or long-term measures found in hair (see “Methods,” Chapter 2).

Table 1. Comparison of various matrices for measuring cortisol concentrations.

<table>
<thead>
<tr>
<th>Sample Medium</th>
<th>Sample Type</th>
<th>Time Frame</th>
<th>Steroids</th>
<th>Invasiveness</th>
<th>Circadian Variability</th>
<th>Stability</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>Point</td>
<td>Minutes</td>
<td>Total</td>
<td>Modest</td>
<td>High</td>
<td>Low</td>
<td>Freeze</td>
</tr>
<tr>
<td>Saliva</td>
<td>Point</td>
<td>Minutes</td>
<td>Free</td>
<td>Minimal</td>
<td>High</td>
<td>Low</td>
<td>Freeze</td>
</tr>
<tr>
<td>Urine</td>
<td>State</td>
<td>Hours/day</td>
<td>Free</td>
<td>None</td>
<td>High</td>
<td>Medium</td>
<td>Freeze</td>
</tr>
<tr>
<td>Feces</td>
<td>State</td>
<td>Hours/day</td>
<td>Free</td>
<td>None</td>
<td>High</td>
<td>Medium</td>
<td>Freeze</td>
</tr>
<tr>
<td>CSF</td>
<td>Point</td>
<td>Minutes</td>
<td>Free</td>
<td>Very</td>
<td>High</td>
<td>Low</td>
<td>Freeze</td>
</tr>
<tr>
<td>Hair</td>
<td>Chronic</td>
<td>Months</td>
<td>Free</td>
<td>Minimal</td>
<td>Low</td>
<td>High</td>
<td>Room Temp</td>
</tr>
</tbody>
</table>

1.5 Objectives

Nonhuman primates serve as a valuable model for child development, and procedures for examining early life disturbances on future outcomes are well-
established. Virtually all studies examining early rearing effects on HPA development and reactivity in monkeys have relied on short-term concentrations in blood or saliva, and most have also relied on the PR method of nursery rearing. Hair cortisol has never been employed in studies with infant monkeys, yet it represents an ideal medium for characterizing adrenocortical “phenotype” and for measuring the risk of developing future anxiety-related behaviors for reasons outlined above. Additionally, to my knowledge no research has examined the interaction between SPR and serotonergic activity. Such research, in conjunction with the examination of chronic HPA axis activity, would provide valuable insight into the possible development of anxiety-related disorders related to this type of rearing.

The focus of this dissertation extends beyond an understanding of genetic and early life stress effects on anxiety to include an understanding of the physiology associated with the relationships between these variables. With the following experiments, I aimed to answer the following questions:

1. Does chronic HPA activity, as measured in hair, mediate the effects of rearing condition and serotonin transporter genotype on the development of anxiety?

2. Does acute HPA activity, as measured in saliva, mediate the effects of rearing condition and serotonin transporter genotype on the development of anxiety?

3. Is the cortisol measured in saliva correlated with that measured in hair across the first two years of life?
And, ultimately,

4. Which measure of HPA activity is a better predictor of the development of anxiety in differently-reared monkeys expressing different serotonin transporter genotypes?

I hypothesized that early rearing experience in infant monkeys, specifically the type of nursery rearing experienced, combined with serotonin transporter genotype would relate to the expression of physiological and behavioral traits observed in anxiety-related disorders. I predicted that the PR infants who were carriers of the (s) allele for the \( rh5-HTTLPR \) would exhibit the most anxious behaviors, and that this relationship would be mediated by their hair cortisol values. Based on limited findings in SPR infant that indicate that their behavioral profiles are more similar to that of MPR infants (Sackett et al., 2002; Strand and Novak, 2005), I predicted that SPR infants would behaviorally resemble MPR infants and that their rearing-genetic-behavioral relationship would be less mediated by hair cortisol. Further, I predicted that hair cortisol would be a superior predictor of anxiety over salivary cortisol, and that the salivary and hair cortisol concentrations taken at each 6-month interval would be weakly correlated, if at all. As such I aimed to demonstrate the efficacy long-term HPA activity as measured by hair cortisol in predicting and mediating the relationship between early life experience, \( rh5-HTTLPR \), and the development of anxiety in young rhesus monkeys.
CHAPTER 2

METHODS

2.1 Subjects and Rearing

Subjects were raised at the Laboratory for Comparative Ethology (LCE) of the Eunice Kennedy Shriver National Institutes of Child Health and Human Development at the National Institutes of Health Animal Center in Poolesville, MD. Subjects included infants born in 2006 (n=25) and 2007 (n=36), for a total sample size of N=61 (33 males, 28 females). Each infant was assigned to one of three rearing conditions: mother-peer-reared (MPR, n=21), peer-reared (PR, n=20), or surrogate peer-reared (SPR, n=20). Infants were reared according to the standard protocol at the LCE as described in detail by Shannon et al (1998).

2.1.1 Mother-Peer-Rearing

MPR infants were raised with their biological mothers in large social groups containing 2 adult males, 6-8 adult females, and other infant offspring. Each group lived in indoor-outdoor pens constructed of galvanized steel mesh connected by guillotine doors, the floors of which were covered with wood chips. The indoor pen measured 2.44 x 3.05 x 2.21 m, and the outdoor pen measured 2.44 x 3.0 x 2.44 m. Animals were given free access between indoor and outdoor pens except when confined to one half for cleaning, laboratory protocol procedures, or inclement weather (e.g., 4°C or below). Inside lighting was maintained on a 12:12 cycle (0700-1900). Animals were fed Purina High Protein Monkey Chow (#5038) and received water ad libitum. Supplemental fruit was provided three times each week, with other foraging treats such as peanuts or
sunflower seeds presented daily. Infants remained in these groups for the first six to eight months of life, at which time they were relocated to a large, mixed-rearing social group with other infants of similar ages.

2.1.2 Peer-Rearing and Surrogate Peer-Rearing

Infants were assigned to either the PR or the SPR condition at birth; however, PR and SPR infants were treated nearly identically for the first 37 days of life. Nursery-reared infants were separated from their mothers within 1-3 d postpartum and reared in the neonatal nursery facility according to the procedure described by Ruppenthal (1979) and Shannon et al (1998). From days 1-15 of life, infants were individually housed in plastic cage incubators measuring 51 x 38 x 43 cm. The internal temperature was maintained at ~ 27°C.

Both groups’ incubators were furnished with inanimate, cloth-covered “surrogate mothers” as described previously (Shannon et al., 1998; Dettmer et al., 2008). All surrogates were covered with a heating pad and fleece fabric to provide warmth and contact comfort. During the first 15 days of life, infants were able to see and hear, but not touch, other infants.

From day 15-37, nursery-reared infants were moved to a larger housing room into individual wire mesh cages measuring 64 x 61 x 76 cm. The infants retained their surrogates, without the heating pad, until they left the nursery at approximately 8 months of age. For this three week period after removal from the incubators, infants continued to have visual, auditory, and olfactory, but not tactile, contact with each other.
Lighting was maintained on a 14:10 cycle (0700-2100), and room temperature was maintained at 22-26°C with humidity kept at 50-55%.

LCE protocol dictated that social groups were formed when the youngest animal of each designated group turned 37 days old. At this time, PR infants were placed into permanent 24 h groups of four similar-aged infants. To accommodate 4 infants, the PR groups were moved into large cages measuring 71 x 81 x 152 cm. SPR infants were assigned to four-member permanent groups, but received 2 h of peer contact per day as opposed to 24 h per day. Daily contact occurred in large cages measuring 71 x 81 x 152 cm with a surrogate present. For the remainder of the day, SPR infants lived in their individual cages with their surrogates. Both PR and SPR infants underwent a battery of cognitive testing 2-3 times per week (see Ruppenthal and Sackett, 1992).

All nursery infants were fed a 50:50 mixture of Similac (Ross Laboratories, Columbus, OH), and Primilac (Bio-Serv, Frenchtown, NJ) formulas. They were hand-fed until they could independently feed, at which time formula was provided ad libitum through 4 months of age. At 4 months, the infants were placed on a ration of 300 mL/d of formula. At 5 months, they were fed a ration of 200 mL/d and then weaned entirely at 6 months. Purina High Protein monkey chow (#5038) and water were provided ad libitum when infants reach 1 month of age.

2.1.3 Mixed-Rearing Social Housing

At approximately 8 months of age, infants from all three rearing conditions in each birth cohort were relocated to a different building at the NIH Animal Center and were placed together into a larger group for the next two years. Each birth cohort
contained approximately 60 animals in total. To accommodate the 60 animals, subjects were housed in one of two conditions and rotated between the two conditions for husbandry purposes: two indoor enclosures or a combined indoor/outdoor enclosure. The indoor enclosures each measured 7.3 x 3.4 x 3.7 m and were equipped with perches, barrels, swings, and wood shavings. The outdoor enclosure was a circular corn-crib enclosure measuring 5.03 m in diameter by 5.49 m high. All subjects had free access between the indoor and outdoor housing areas, except when they were partitioned to either side for routine cleaning or for social behavior observations, or to the inside during inclement weather (e.g., 4°C or below). Water was provided ad libitum and monkeys were fed and provided enrichment as described in section 2.1.1.

2.2 Measuring Long-Term HPA Axis Activity

To assess long-term HPA axis activity in these differently-reared monkeys, I measured cortisol concentrations in hair samples collected every six months at the LCE. Hair cortisol analyses were conducted via enzyme immunoassay (EIA) in the Neurochemistry Laboratory at the University of Massachusetts, Amherst, MA (UMass) according to procedures established by Davenport et al (2006).

2.2.1 Hair Sampling/Assay

Hair samples were collected from all infants at day 14 and months 6, 12, 18, and 24 of life using pet grooming clippers. Sample collection occurred every 6 months (Fig. 2) to allow for sufficient re-growth of hair. Hair was shaved at the posterior vertex region of the neck. Approximately 250mg of hair was washed twice with isopropyl alcohol and powdered with a Retsch ball mill (mixer mill MM200; 10mL stainless steel
grinding jars; single 12mm stainless steel grinding balls) at 30Hz for 5 min before steroid extraction for 24 h by 1 mL methanol. 600μL of extracted cortisol was then dried down under nitrogen gas, reconstituted with 400μL assay diluent, and analyzed according to the protocol included with the EIA kit (Salimetrics, State College, PA). Resulting values were converted from μg/dL to pg/mg for data analysis (Davenport et al., 2006).

2.2.2 Long-Term HPA Axis Response to a Major Social Challenge

At approximately 8 months of age, all infants in a cohort (birth year 2006 or 2007) representing each of the three rearing conditions were removed from their housing situations and placed together into a single social group comprised of approximately 60 animals. This represented a major social challenge in the lives of the young rhesus monkeys, as they had to adapt to a new environment, new peers, and establish a new social hierarchy. To assess chronic physiological responses to this major challenge, I compared the cortisol in hair measured across the first 6 months of life with that measured in the next 6 months of life and beyond. Hair samples were collected at 6 months of age prior to mixed-group formation, and then again at approximately months 12, 18 and 24. The change in cortisol concentrations across these time points were thought to reflect long-term responses to a major challenge, as hair has previously been used to measure long-term stress reactions in adult rhesus monkeys (Davenport et al., 2006; Davenport et al., 2008).
2.3 Measuring Acute HPA Axis Activity

To assess acute HPA reactivity (i.e., immediate responses to a mild stressor) in differently-reared monkeys, I measured salivary cortisol responses in two contexts: 1) 10 minutes before and immediately after a 20-minute social separation at six months of age, and 2) in six-month intervals at months 12, 18, and 24, after the major life stress of large group formation which was imposed at approximately 8 months of age (Fig. 2). Saliva sample collection occurred at the LCE, while salivary analysis occurred via EIA at the Neurochemistry Lab at UMass.

2.3.1 Saliva Sampling/Assay

For the social separation challenge, I collected both baseline (i.e., immediately upon removal from the social group) and post-challenge (i.e., immediately after the 20-minute separation ended) saliva samples. Due to logistical difficulties in the capture and sedation at 12, 18, and 24 months, baseline saliva samples were not collected until at least 20 minutes after the start of the challenge (defined as the time when researchers entered the room). Because these did not represent true baseline samples, they were excluded from analysis. Post-challenge samples were collected while the subjects were sedated (10mg/kg ketamine HCl, IM), at least 30 minutes after the start of the challenge (ranging from 30min 15 sec to 50min). A ½-in. long braided cotton dental rope (Richmond Dental, Charlotte, NC) was placed bilaterally into each animal’s cheek pouch until the rope was saturated with saliva. The ropes were then carefully removed, placed in Salivette® tubes (Sarstedt Inc., Newton, NC) and centrifuged at 2,700 rpm for 20 minutes. Samples were aliquotted and stored at -80°C until assay, which occurred using
the same EIA kit as for the hair assays. If a subject did not yield enough saliva for analysis with the EIA kit, the sample was diluted with assay diluent to produce a 50:50 mixture of saliva:diluent for use in analysis.

2.4 Serotonin Transporter Promoter Region Genotyping

To examine the propensity for these monkeys to develop anxiety-like behaviors, I examined the interaction between their serotonin transporter promoter region length polymorphism (rh5-HTTLPR) and rearing environment, and associated these variables with HPA axis activity and social behavior. Genotyping of infant DNA samples occurred either at the California National Primate Research Center as described by Kinnally et al. (2008) or at the New England Primate Research Center as described by Vallender et al. (2008) via polymerase chain reaction (PCR) analysis. DNA was isolated using standard extraction methods from whole blood collected from the femoral vein under ketamine anesthesia (10 mg/kg ketamine HCl, IM). Blood collection occurred during routine health exams within the first 18 months of life. For this study, the rh5-HTTLPR genotypes were distributed as follows: 47 l/l, 12 l/s, and 2 s/s. Because the frequency of the s/s genotype was so low, l/s and s/s individuals were combined together in all analyses.

2.5 Social Behavior

Because a measure of anxiety was crucial to the tests of my hypotheses, I examined anxiety-related, play, and grooming behaviors across the first two years of life. In addition to exhibiting more anxiety, I predicted that more anxious monkeys would spend less time playing and grooming. My ethogram initially included 24 behaviors (for
a complete ethogram, see Appendix A), and twice each week I employed a 5-minute focal-animal sampling procedure to record the frequencies and durations of all behaviors using a computerized data acquisition program (JWatcher; Blumstein, Daniel, & Evans, 2006). Although data were obtained for the 24 categories of behavior, I created a general anxiety score in which the duration of the following categories was summed: clinging, fear vocalizations, huddling, self-rocking, self-clasping, self-biting, and scratching. The general category of “play” included the sum of the durations of contact and non-contact play that was both initiated and received. The general category of “grooming” was defined only by the time an individual spent grooming another individual. Only durations of these behaviors were considered for analysis, as these behaviors are characterized as “state” behaviors (occurring over several seconds or minutes) as opposed to “event” behaviors (i.e., “yes” or “no”).

Behavior was recorded both while subjects were in their MPR, PR, or SPR groups and in the mixed-rearing social groups. Each monkey was observed from day 37, when social groups were formed in the nursery, to approximately month 8 in its individual social situation twice per week as described above, for an average of 18.6 observations per subject. From month 8 onward, when all three rearing conditions were combined into a large mixed-rearing social group, each infant was observed twice per week in either the indoor or outdoor portion of the housing area described above, weather-dependent. An average of 58 observations per subject were recorded. All observations were balanced across morning and afternoon sessions, and the order of observation of individuals was randomized.
Inter-observer reliability was achieved when both observers scored 5 subjects across three separate sessions in a row with 90% agreement ($r^2=0.90$) or a kappa score of $\kappa=0.60$ or higher.

2.6 Statistical Analyses

Prior to all analyses, I examined the hair and salivary cortisol data for normal distribution using the Shapiro-Wilk Normality Test. If any of these variables violated the assumption of normality, I employed Tukey’s ladder of transformations to identify the best transformation for the data (likely a log transformation) prior to using the variable in any analyses (Tukey, 1977).

2.6.1 Rearing Differences in Behavior and HPA Activity

I examined group differences in total anxiety (defined in section 2.5), play, and grooming behaviors. I performed a mixed-design analysis of variance (ANOVA) with the duration of each behavior as the dependent variable, age as the within-subjects variable, and rearing condition, rh5-HTTLPR genotype, and sex as between-subjects variables. I also analyzed component categories of anxiety (total self-directed behaviors, fear vocalizations, and scratching). Because these analyses did not add much beyond what was obtained by analyzing total anxiety, these analyses are not reported below but are included in Appendix B.

I used the same mixed-design ANOVA to examine group differences in both hair and salivary cortisol across the first two years of life, with age as the within-subjects factor and rearing condition, rh5-HTTLPR genotype, and sex as between-subjects variables.
2.6.2 Mediation by HPA Axis Activity

For behavioral data at each six-month interval (see Fig. 2), I employed a mediational model (Baron & Kenny, 1986), which relies on regression analysis, to examine the extent to which hair or salivary cortisol in that interval influenced the relationship between rearing condition, serotonin genotype, and anxiety. Initial analyses determined how well rearing and genotype individually, as well as the interaction between the two, predicted anxiety. I retained the significant predictors (coefficients) from the first model as follows:

\[
\text{M1: Anxiety} = \beta_0 + \beta_1(\text{Sex}) + \beta_2(\text{Genotype}) + \beta_3(\text{PR}) + \beta_4(\text{SPR}) + \beta_5(\text{PR}\times\text{Genotype}) + \beta_6(\text{SPR}\times\text{Genotype})
\]

Following these analyses, I examined the extent to which hair cortisol added significant predictive power to the model using the \(\Delta R^2\) test. The second model appeared as follows, retaining only the significant coefficients:

\[
\text{M2: Anxiety} = \beta_0 + \beta_1(\text{Sex}) + \beta_2(\text{Genotype}) + \beta_3(\text{PR}) + \beta_4(\text{SPR}) + \beta_5(\text{PR}\times\text{Genotype}) + \beta_6(\text{SPR}\times\text{Genotype}) + \beta_7(\text{Hair})
\]

If the coefficients for rearing condition between M1 and M2 decreased after the addition of the significant hair cortisol predictor, I then utilized the Sobel test to examine potential mediation of the rearing or rearing x genotype and anxiety relationship by cortisol (Baron & Kenny, 1986). If the coefficients between M1 and M2 increased after the addition of the significant hair cortisol predictor, I retained the final model and reported the changes in rearing or rearing x genotype effects after controlling for hair cortisol. If hair cortisol was not a significant predictor (i.e., the \(\Delta R^2\) test was not
significant), I reported the regression model for the behavior as it appeared in M1. The same statistical methods were employed for salivary cortisol, substituting salivary cortisol for hair cortisol in the steps outlined above.

For the regression analyses I created “dummy” variables, as is standard with these analyses, to compare PR and SPR infants to MPR infants. These “dummy” variables were called “peerrear” and “surrear,” and infants were coded as 1 (yes) or 0 (no) for each of these variables. Thus, MPR infants were coded as 0 for both; PR infants were coded as 1 for “peerrear” and 0 for “surrear;” and SPR infants were coded as 0 for “peerrear” and 1 for “surrear.” In this way, the regression models allowed for direct comparison between each of the three rearing groups. Sex and genotype were also coded as dummy variables: males were coded as 0 and females as 1, and l/l infants were coded as 0 and (s) carriers as 1. Thus, each model compared the value of a 1 to that of a 0 for each variable.

2.6.3 Correlation Between HPA Axis Measures and Anxiety

To test for relationships between acute (i.e., salivary) and long-term (i.e., hair) cortisol concentrations at each six-month interval, I employed Pearson’s partial correlation, which controlled for genotype and rearing condition. I used the same correlation to determine the relationships between cortisol measurements (salivary or hair concentrations) and average durations of total anxiety at months 6, 12, 18, and 24.

I compared the outcomes of the models in the above analyses to determine which variable (hair or salivary cortisol) provided a stronger predictor of the development of anxiety. For all results, I reported significant findings only (α=0.05); if group differences
or predictors for regression models were not significant, I did not report their test statistics or significance values.

Figure 2. Timeline of experiments.
CHAPTER 3
RESULTS PART 1

The results in this section contain mixed-design ANOVAs described in section 2.6.1. Data presented here reveal rearing, genetic, and sex differences, as well as any interactions, in total anxiety, play, and grooming. Additionally, effects of age and any age x group interactions are presented. In Figures 1-10, the yellow arrow indicates the timing of housing relocation (i.e., the major stressor). A table of descriptive statistics for anxiety, play, grooming, and the two cortisol measures is presented in Appendix C.

3.1 Rearing Differences in Anxiety

The mixed-design ANOVA revealed a significant between-subjects effect of rearing on total anxiety in the first two years of life ($F_{(2,4670)}=11.08; p<0.001; \text{Fig. 3}$).

Overall, PR infants exhibited more anxiety than either MPR or SPR infants, who did not differ from each other (PR: $\bar{x} \pm S.E.=49.59 \pm 2.69 \text{sec}$; MPR: $\bar{x}=34.50 \pm 2.29 \text{sec}$; SPR: $\bar{x}=39.79 \pm 2.23 \text{sec}$). Main effects of sex and genotype were not present.

![Figure 3. Total anxiety by rearing condition in the first two years of life; **$p<0.001$.](image-url)
Significant within-subjects effects of age (F(3,14010)=169.06; p<0.001), age x rear (F(6,14010)=6.62; p<0.001), age x sex (F(3,14010)=4.28; p=0.005), and age x sex x rear (F(6,14010)=2.15; p=0.045) on anxiety were revealed across the first two years of life (Fig. 4a-d). The age effect followed linear (F(1,4670)=121.10; p<0.001), quadratic (F(1,4670)=189.96; p<0.001), and cubic (F(1,4670)=167.59; p<0.001) trends. Anxiety increased from months 6-12 and 12-18, and then decreased between months 18-24 to levels near those at in the first six months of life. The age x rear effect followed linear (F(2,4670)=7.01; p=0.001), quadratic (F(2,4670)=10.59; p<0.001), and cubic (F(2,4670)=4.12; p=0.02) trends. PR infants exhibited more anxiety at between months 6-12 and 12-18 than MPR and SPR infants, and from months 12-18 SPR infants exhibited more anxiety than MPR infants. Between months 18-24, the groups were virtually indistinguishable. The age x sex interaction followed a quadratic trend (F(1,4670)=10.03; p=0.002). Females demonstrated more anxiety than males in the first twelve months of life, whereas males tended to exhibit more anxiety between months 12-18. Between months 18-24, females again exhibited more anxiety than males. Finally, SPR females exhibited more anxiety than any other sex/rearing group in the first six months of life, whereas PR females did so between months 6-12. Between months 12-18, PR and SPR males as well as PR females displayed more anxiety than either MPR males or females, or SPR females.

The mixed-design ANOVA revealed significant between-subjects effects of rearing (F(2,2525)=33.99; p<0.001), sex (F(1,2525)=17.55; p<0.001), and genotype (F(1,2525)=9.25; p=0.002) on total time spent in play in the first two years of life (Fig. 5a-c). SPR infants
played more than PR or MPR infants, who did not differ from each other (SPR: $\bar{x} = 48.57 \pm 1.51$ sec; PR: $\bar{x} = 32.33 \pm 1.35$ sec; MPR: $\bar{x} = 29.68 \pm 1.28$ sec). Males played more than females ($\bar{x} = 41.94 \pm 1.17$ vs. $32.66 \pm 1.15$ sec), and (s) carriers for rh5-HTTLPR played more than l/l infants ($\bar{x} = 38.86 \pm 1.79$ vs. $37.36 \pm 0.94$ sec).

Figure 4. Longitudinal anxiety by (a) age, $p<0.001$; (b) rearing condition, $p<0.001$; (c) sex, $p<0.01$; and (d) sex x rearing condition, $p<0.05$. 
Significant within-subjects effects of age \((F_{(3,7575)=59.35; p<0.001})\) and age x rearing \((F_{(6,7575)=18.35; p<0.001})\) were revealed for time spent in play (Fig. 6a-b). The age effect followed linear \((F_{(1,2525)=11.33; p=0.001})\), quadratic \((F_{(1,2525)=150.01; p<0.001})\), and cubic \((F_{(1,2525)=29.92; p<0.001})\) trends. Total play decreased with age through month 18, then rose slightly from months 18-24. The age x rear effect followed linear \((F_{(2,2525)=25.96; p<0.001})\) and quadratic \((F_{(2,2525)=18.03; p<0.001})\) trends. In the first six months of life, SPR infants played more than PR infants, who played more than MPR infants, and from months 6-12 SPR infants played more than PR/MPR infants, who did not differ from each other.

The mixed-design ANOVA revealed a significant between-subjects effect of sex \((F_{(1,829)=5.48; p=0.02})\) and genotype \((F_{(1,829)=4.02; p=0.045})\) on total time spent grooming (Fig. 7a-b). Females spent more time grooming than males \((\bar{x} =39.53\pm2.75 \text{ vs. } 24.17\pm1.81 \text{ sec})\), while infants with the l/l genotype spent more time grooming than \((s)\) carriers for
rh5-HTTLPR (\(\bar{x}=32.77\pm1.91\) vs. 27.00±3.09 sec). Significant within-subjects effects of age (\(F(3,2487)=20.21; p<0.001\)) and age x sex (\(F(3,2487)=3.807; p=0.04\)) were also revealed for grooming (Fig. 8a-b). Grooming continually increased with age, and females groomed more than males continually after month 6. The age effect followed linear (\(F(1,829)=44.52; p<0.001\)) and cubic (\(F(1,829)=16.04; p<0.001\)) trends and the age x sex interaction followed a linear trend (\(F(1,829)=5.65; p=0.02\)).

Figure 6. Longitudinal duration of play by (a) age, \(p<0.001\); and (b) rearing, \(p<0.001\).

Figure 7. Total grooming by (a) sex, \(p<0.05\); and (b) genotype, \(p<0.05\).
Figure 8. Longitudinal duration of grooming by (a) age, \( p<0.001 \); and (b) sex, \( p<0.05 \).

3.2 Rearing Differences in HPA Activity

The mixed-design ANOVA revealed a significant effect of age (\( F_{(3,39)}=5.95; p=0.001 \)) and a significant age x rearing effect (\( F_{(6,39)}=3.36; p=0.003 \)) for hair cortisol concentrations across the first two years of life (Figure 9a-b). Hair cortisol concentrations declined between months 12-24, and at month 18, both PR and SPR infants had higher hair cortisol than MPR infants. The age effect followed linear (\( F_{(1,39)}=7.93; p<0.01 \)) and quadratic (\( F_{(1,39)}=7.31; p=0.10 \)) trends, as did the age x rear effect (linear: \( F_{(2,39)}=4.61; p=0.016 \); quadratic: \( F_{(2,39)}=4.98; p=0.012 \)).

The mixed-design ANOVA revealed a significant effect of age (\( F_{(3,49)}=4.25; p=0.007 \)) on salivary cortisol across the first two years of life, but no other main effects or
age x group interactions were present (Figure 10). Salivary cortisol concentrations peaked at month 12, decreased at month 18, and increased again at month 24. The age effect followed linear ($F(1, 49) = 5.67; p = 0.02$) and cubic ($F(1, 49) = 6.55; p = 0.014$) trends.

![Figure 9](image9.png)

**Figure 9.** Longitudinal hair cortisol concentrations by (a) age, $p < 0.01$; and (b) rearing condition, $p < 0.01$.

![Figure 10](image10.png)

**Figure 10.** Longitudinal salivary cortisol concentrations by age; $p < 0.01$. 

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CHAPTER 4

RESULTS PART 2

The results in this section contain the regression models and mediation tests described in section 2.6.2. Data presented here reveal the predictive power of rearing, genotype, and sex on behavior in each six-month interval (anxiety, play, and grooming). The data then show whether hair or salivary cortisol in the concurrent six-month block added significant predictive power for the behaviors (i.e., whether hair cortisol at month 6 predicted behavior at month 6, etc.). Mediation test were performed if the addition of hair or salivary cortisol met the requirements for mediation (see section 2.6.2 and Baron and Kenny, 1986). The end of the chapter reveals correlational data for hair and salivary cortisol, and HPA activity and anxiety, across the first two years of life.

4.1 Hair Cortisol and Anxiety

Hair cortisol concentrations at months 6, 18, and 24 were not normally distributed (month 6: W=0.911, p=0.001; month 18: W=0.806, p<0.001; month 24: W=0.958, p=0.004). These variables were transformed using Tukey’s ladder of transformations so that they were normally distributed (Tukey, 1977). Month 6 hair cortisol was transformed into log_{10}(m6hair); month 18 hair cortisol was transformed into (m18hair)^{-1/2}; month 24 hair was transformed into \sqrt{m24hair}. Month 12 hair cortisol was retained for analysis (m12hair).
4.1.1 Months 0-6

4.1.1.1 Hair Cortisol

Subjects did not differ by rearing, genotype, or sex in hair cortisol concentrations at month 6. No significant interactions were found.

4.1.1.2 Anxiety

Initial regression analysis revealed that sex, PR, and SPR were significant predictors of total duration of anxiety \( (R^2=0.145; p<0.001) \), but that \( rh5-HTTLPR \) genotype was not. The rearing x genotype interactions were not significant predictors of anxiety and so were not included in the model. Therefore the final first model, M1, appeared as follows:

\[
M1: \text{DurAnxiety} = 79.24 + 11.02\text{SEX} - 52.70\text{PR} - 59.57\text{SPR}
\]

The addition of \( \log_{10}(m6hair) \) to the model significantly changed the model fit \( (\Delta R^2=0.004; p=0.017) \), and was a significant predictor of anxiety \( (p=0.017) \). Thus the final second model, M2, appeared as follows (Fig. 11):

\[
M2: \text{DurAnxiety} = 4.33 + 10.12\text{SEX} - 56.08\text{PR} - 58.30\text{SPR} + 34.17\text{HAIR}
\]

The addition of \( \log_{10}(m6hair) \) increased the coefficient for SPR. Thus, after controlling for hair cortisol the differences in anxiety between SPR and MPR increased. The addition of \( \log_{10}(m6hair) \) decreased the coefficients for PR and MPR, and Sobel tests were conducted to test for mediation. Sobel tests revealed that hair cortisol significantly mediated the MPR→anxiety relationship \( (z=3.54; p<0.001) \), but not the PR→anxiety relationship.
As described in section 3.1.1.1, the increased clinging by MPR infants explained the group differences in anxiety. Thus, I also determined the regression equation for total anxiety minus clinging (i.e., including self-directed behaviors, huddling, and fear vocalizations; dependent variable = DurAnx1). Only sex and genotype were significant predictors for DurAnx1 ($R^2=0.013; p=0.001$), and the model appeared as follows (Fig. 12):

$$M1: DurAnx1 = 4.12 + 5.01SEX + 4.45GEN$$
Figure 12. The model for duration of anxiety (minus clinging) in the first six months of life.

Sex, PR, SPR, and genotype were significant predictors of initiate play ($R^2=0.347; p<0.000$). The SPR x genotype interaction added significant predictive power while at the same time negating the significance of the genotype coefficient ($\Delta R^2=0.007; p=0.004$), so it was retained for the model, which appeared as follows (Fig. 13):

$$M1: \text{DurPlay} = 15.89 - 8.39SEX + 24.39PR + 62.27SPR - 1.38PRxGEN + 26.63SPRxGEN$$

The addition of $\log_{10}(m6hair)$ did not add significant predictive power to the model so it was not retained.

PR and SPR were the only significant predictors of grooming ($R^2=0.095; p=0.006$). The addition of $\log_{10}(m6hair)$ did not add significant predictive power to the model and thus was not retained. The final model appeared as follows (Fig. 14):

$$M1: \text{DurGroom} = 1.80 - 0.79PR - 1.02SPR$$
Figure 13. The model for duration of play in the first six months of life.

Figure 14. The model for duration of grooming in the first six months of life.
A summary of the predictors of total anxiety, anxiety not including clinging, play, and grooming in the first six months of life is presented in Table 2.

Table 2. Predictors of behaviors in the first six months of life.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Total Anxiety</th>
<th>Anxiety (minus cling)</th>
<th>Play</th>
<th>Grooming</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SPR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PRxGen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPRxGen</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Hair</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saliva</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

4.1.2 Months 6-12

4.1.2.1 2006 Cohort

4.1.2.1.1 Hair Cortisol

Subjects in the 2006 cohort differed in month 12 hair cortisol concentrations by rearing condition only ($F_{(2,22)}=5.366; p=0.013$). Tukey’s post-hoc analysis revealed that PR infants exhibited lower hair cortisol than SPR infants, but that neither group differed from MPR infants (MPR: $\bar{x} = 196.24 \pm 12.92$ pg/mg; PR: $\bar{x} = 162.66 \pm 14.81$ pg/mg; SPR: $\bar{x} = 239.37 \pm 20.85$ pg/mg; Fig. 15).
4.1.2.1.2 Anxiety

As stated in section 3.1.2.1.1, genotype x rearing interactions were not investigated because there were not enough l/s or s/s infants in each rearing group for this cohort. Initial regression analysis revealed that only PR and SPR, but not sex or genotype, were significant predictors of anxiety in the first ten days after housing relocation ($R^2=0.439; p=0.004$). The addition of m12hair did not add significant predictive power to the model, which appeared as follows (Fig. 16):

$$M1: DurAnxiety = 62.25 + 70.49PR + 74.23SPR$$

4.1.2.2 2007 Cohort

4.1.2.2.1 Hair Cortisol

Subjects in the 2007 cohort differed in month 12 hair cortisol concentrations by rearing only ($F_{(2,33)}=5.07; p=0.01$). PR infants exhibited higher hair cortisol than MPR
infants, but PR/SPR and MPR/SPR infants did not differ (MPR: $\bar{x} = 155.90 \pm 14.41$ pg/mg; PR: $\bar{x} = 213.48 \pm 8.86$ pg/mg; SPR: $\bar{x} = 181.85 \pm 13.60$ pg/mg; Fig. 17).

**Figure 16.** The model for duration of anxiety for the 2006 cohort from months 6-12.

![Duration of Anxiety](image)

**Figure 17.** Rearing group differences in month 12 hair cortisol in the 2007 cohort.

* $p < 0.05$

### 4.1.2.2.2 Anxiety

For the 2007 cohort, PR was the only significant predictor variable for total anxiety between months 6-12 ($R^2 = 0.014; p = 0.026$). The addition of m12hair did not add
significant predictive power and thus was not retained in the final model, which appeared as follows (Fig. 18):

\[ M1: \text{DurAnxiety} = 21.95 + 14.16PR - 1.94SPR \]

Figure 18. The model for duration of anxiety for the 2007 cohort from months 6-12.

Sex and SPR were the only significant predictors of play \( (R^2=0.067; p<0.001) \), with m12hair adding no significant predictive power. The model for initiate play appeared as follows (Fig. 19):

\[ M1: \text{DurPlay} = 16.05 - 7.25SEX + 0.66PR + 14.998SPR \]

SPR was a significant predictor of grooming, and the model appeared as follows:

\[ M1: \text{DurGroom} = 4.01 - 2.00PR - 2.96SPR \]

The addition of m12hair added significant predictive power \( (\Delta R^2=0.009; p=0.042) \), and the final model appeared as follows (Fig. 20):

\[ M2: \text{DurGroom} = 0.370 - 3.32PR - 3.71SPR + 0.02HAIR \]
Figure 19. The model for duration of play for the 2007 cohort from months 6-12.

Figure 20. The model for duration of grooming regressed onto hair cortisol for the 2007 cohort from months 6-12.
Adding m12hair to the model increased the coefficient for MPR from M1 to M2; thus, after controlling for hair cortisol the differences between MPR and the other rearing conditions in initiate groom increased and no Sobel tests were performed. Adding m12hair to the model decreased the coefficients for both PR and SPR from M1 to M2, and Sobel tests revealed that month 12 hair cortisol was a significant mediator of both the PR→groom (z=-2.38; p=0.02) and the SPR→groom (z=-2.94; p=0.003) relationships.

A summary of predictors of total anxiety, play, and grooming from months 6-12 for the 2007 cohort is presented in Table 3.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Total Anxiety</th>
<th>Play</th>
<th>Grooming</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPR</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PRxGen</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SPRxGen</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Hair</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Saliva</td>
<td></td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>
4.1.3 Months 12-18

4.1.3.1 Hair Cortisol

Subjects differed in month 18 hair cortisol concentrations by rearing condition only ($F_{(2,61)}=7.242; p=0.002$). MPR infants had lower hair cortisol than PR and SPR infants, but PR/SPR did not differ from each other (MPR: $\bar{x}=122.61\pm5.09$ pg/mg; PR: $\bar{x}=168.10\pm11.25$ pg/mg; SPR: $\bar{x}=198.54\pm20.02$ pg/mg; Fig. 21). No other group differences or interactions were found.

![Month 18 Hair Cortisol](image)

Figure 21. Rearing group differences in hair cortisol concentrations at month 18.

**$p<0.01$**

4.1.3.2 Anxiety

Regression analysis revealed that PR was the only significant predictor of total anxiety ($R^2=0.005; p=0.023$). The addition of $(m18\text{hair})^{1/2}$ (the transformed value of $m18\text{hair}$ for normality) did not add significant predictive value to the model which appeared as follows (Fig. 22):
M1: DurAnxiety = 41.63 + 27.40PR + 10.92SPR

Sex, PR, and SPR were significant predictors of duration of play (R²=0.022; p<0.001). The addition of (m18hair)^{1/2} did not add significant predictive power to the model, which appeared as follows (Fig. 23):

M1: DurPlay = 15.45 – 6.36SEX – 3.72PR – 3.38SPR

Figure 22. The model for duration of anxiety from months 12-18.

Figure 23. The model for duration of play from months 12-18.
SPR was the only significant predictor for duration of grooming \((R^2=0.003;\ p=0.027)\). Adding \((m18\text{hair})^{1/2}\) did not add significant predictive power to the model, which appeared as follows (Fig. 24):

\[
M1: \text{DurGroom} = 8.52 - 1.13 \text{PR} - 3.42 \text{SPR}
\]

![Graph showing duration of grooming from months 12-18.](image)

**Figure 24.** The model for duration of grooming from months 12-18.

A summary of predictors of total anxiety, play, and grooming from months 12-18 is presented in Table 4.

**Table 4. Predictors of behaviors from months 12-18.**

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Total Anxiety</th>
<th>Play</th>
<th>Grooming</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>SPR</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>PRxGen</td>
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<tr>
<td>SPRxGen</td>
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<td>Hair</td>
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<tr>
<td>Saliva</td>
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</tr>
</tbody>
</table>

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4.1.4 Months 18-24

4.1.4.1 Hair Cortisol

Subjects did not differ by rearing condition, genotype, or sex in month 24 hair cortisol concentrations. No significant interactions were revealed.

4.1.4.2 Anxiety

Regression analysis revealed that sex was the only significant predictor of total anxiety ($R^2=0.004; p=0.02$). The addition of $\sqrt{m24hair}$ did not add significant predictive value to the model which appeared as follows (Fig. 25):

$$M1: \text{DurAnxiety} = 8.815 + 4.311SEX$$

![Anxiety Graph](image)

**Figure 25.** The model for duration of anxiety from months 18-24.

Sex and genotype were significant predictors of play ($R^2=0.046; p<0.001$). The PR x genotype and the SPR x genotype interactions added significant predictive power to the model ($\Delta R^2=0.004; p=0.02$). The addition of $\sqrt{m24hair}$ did not add significant predictive power to the model. The final model appeared as follows (Fig. 26):

$$M1: \text{DurPlay} = 23.38 – 11.01SEX + 15.34GEN – 10.00PRxGEN – 11.40SPRxGEN$$
Sex and genotype were the significant predictors of grooming ($R^2=0.01; p=0.001$), and the addition of $\sqrt{m24\text{hair}}$ did not add significant predictive power. The final model appeared as follows (Fig. 27):

$$M1: \text{DurInitGroom} = 6.29 + 4.92\text{SEX} - 4.30\text{GEN}$$

A summary of predictors of total anxiety, play, and grooming from months 18-24 is presented in Table 5.
Table 5. Predictors of behaviors from months 18-24.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Total Anxiety</th>
<th>Play</th>
<th>Grooming</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Genotype</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>PR</td>
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<tr>
<td>SPR</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>PRxGen</td>
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<tr>
<td>SPRxGen</td>
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<tr>
<td>Saliva</td>
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<td>+</td>
</tr>
</tbody>
</table>

4.2 Salivary Cortisol and Anxiety

Salivary cortisol concentrations at months 6, 12, 18, and 24 were not normally distributed (month 6 pre-challenge saliva: W=0.883, p<0.001; month 6 post-challenge saliva: W=0.736; p<0.001; month 12 saliva: W=0.750; p<0.001; month 18: W=0.883, p<0.001; month 24: W=0.896; p<0.001). These variables were transformed to the natural log of each variable so that they were normally distributed. The transformed variables were ln_m6presal, ln_m6postsal, ln_m12sal, ln_m18sal, and ln_m24sal. In examining anxiety in the first six months of life, I examined both pre-challenge and post-challenge salivary cortisol as predictors and mediators of behaviors.

4.2.1 Months 0-6

4.2.1.1 Salivary Cortisol

Subjects did not differ by rearing, genotype, or sex in pre-challenge or post-challenge salivary cortisol concentrations at month 6.
4.2.1.2 Anxiety

Initial regression analysis revealed that sex, PR, and SPR were significant predictors of total duration of anxiety (\(R^2=0.144\); \(p<0.001\)), but that rh5-HTTLPR genotype was not. The rearing x genotype interactions were not significant predictors of anxiety and so were not included in the model. The first model appeared as follows:

M1: \(\text{DurAnxiety} = 79.61 + 10.34\text{SEX} – 51.48\text{PR} – 59.60\text{SPR}\)

The addition of ln6presal significantly changed the model fit (\(\Delta R^2=0.005\); \(p=0.017\)). Thus the final model appeared as follows (Fig. 28):

M2: \(\text{DurAnxiety} = 90.23 + 9.11\text{SEX} – 54.38\text{PR} – 59.99\text{SPR} + 9.07\text{PRESAL}\)

Adding ln6presal to the model did not change the coefficients for either MPR or SPR between M1 and M2, but did decrease the coefficient for PR. Sobel tests revealed that ln6presal did not mediate the PR\(\rightarrow\)anxiety relationship. The addition of ln6postsal did not add significant predictive power to the model.

![Anxiety model](image)

Figure 28. The model for duration of anxiety regressed onto pre-challenge salivary cortisol in the first six months of life.
Sex and genotype were significant predictors for anxiety minus clinging (dependent variable = DurAnx1; $R^2=0.013; p=0.001$), and the first model appeared as follows:

$$M1: DurAnx1 = 4.03 + 5.21SEX + 3.79GEN$$

The addition of ln(m6presal) added significant predictive power to the model ($\Delta R^2=0.005; p=0.016$). The final model appeared as follows (Fig. 29):

$$M2: DurAnx1 = 7.97 + 4.68SEX + 2.65GEN + 3.41PRESAL$$

Because there were no rearing conditions predicted DurAnx1, Sobel tests were not performed.

The final first model for duration of play was identical to that in section 4.1.1.2. The addition of ln(m6presal) added significant predictive power to the model ($\Delta R^2=0.004; p=0.02$), which appeared as follows (Fig. 30):

$$M2: DurPlay = 9.99 - 7.83SEX + 25.30PR + 61.93SPR + 1.96PRxGEN + 29.27SPRxGEN - 5.50PRESAL$$

![Figure 29. The model for duration of anxiety (minus clinging) regressed onto pre-challenge salivary cortisol in the first six months of life.](image-url)
The addition of ln6postsal also added significant predictive power to the model for play ($\Delta R^2=0.003; p=0.03$). The final model for post-challenge salivary cortisol appeared as follows (Fig. 31):

$$M2: \text{DurPlay} = 15.02 - 9.68 \text{SEX} + 23.32 \text{PR} + 63.45 \text{SPR} + 3.00 \text{PRxGEN} + 30.78 \text{SPRxGEN} - 5.76 \text{POSTSAL}$$

Similar to the hair cortisol results, PR and SPR were the only significant predictors of grooming and the addition of salivary cortisol did not add significant predictive power. The final model appeared as in section 4.1.1.2 (see Fig. 14).

---

**Figure 30.** The model for duration of play regressed onto pre-challenge salivary cortisol in the first six months of life.
Figure 31. The model for duration of play regressed onto post-challenge cortisol in the first six months of life.

4.2.2 Months 6-12

4.2.2.1 2006 Cohort

4.2.2.1.1 Salivary Cortisol

Subjects in the 2006 cohort did not differ in ln(m12sal) by rearing, sex, or genotype. No significant interactions were present.

4.2.2.1.2 Anxiety

Like with the hair cortisol results, PR and SPR, but not sex or genotype, were significant predictors of anxiety in the first ten days after housing relocation. The addition of ln(m6postsal) did not add significant predictive power to the model, which appeared as in section 4.1.2.1.2 (see Fig. 16).
4.2.2.2 2007 Cohort

4.2.2.2.1 Salivary Cortisol

Subjects in the 2007 cohort did not differ by rearing, sex, or genotype in ln12sal. No interactions were found.

4.2.2.2.2 Anxiety

As with the hair cortisol results, PR was the only significant predictor variable for total anxiety between months 6-12 and the addition of ln12sal did not add significant predictive power. The final model appeared as in section 4.1.2.2.2 (see Fig. 18).

Sex and SPR were significant predictors of initiate play ($R^2=0.058; p<0.001$). The first model for initiate play appeared as in section 4.1.2.2.2. The addition of ln12sal added significant predictive power to the model ($\Delta R^2=0.008; p=0.04$), and the final model appeared as follows (Fig. 32):

$$M2: \text{DurPlay} = 13.90 - 6.37SEX + 1.13PR + 14.30SPR + 3.38SAL$$

Because the addition of ln12sal either increased or did not change the coefficients for PR, SPR, and MPR between M1 and M2, mediation did not occur and Sobel tests were not performed.
Figure 32. The model for duration of play regressed onto salivary cortisol for the 2007 cohort from months 6-12.

SPR was the only significant predictor of grooming, and the final model appeared as follows (Fig. 33):

\[
M1: \text{DurGroom} = 3.97 - 1.73PR - 2.92\text{SPR}
\]

Figure 33. The model for duration of grooming for the 2007 cohort from months 6-12.
4.2.3 Months 12-18

4.2.3.1 Salivary Cortisol

Subjects did not differ by rearing, sex, or genotype in lnm18sal. No interactions were found.

4.2.3.2 Anxiety

Regression analysis revealed that the model for total anxiety was the same as in section 4.1.3.2. The addition of lnm18sal did not add significant predictive power.

Similar to the results for hair cortisol, sex, PR, and SPR were significant predictors of duration of play, and the addition of lnm18sal did not add significant predictive power to the model which appeared in section 4.1.3.2 (see Fig. 23).

Like with the hair cortisol results, SPR was the only significant predictor for duration of grooming and adding lnm18sal did not add significant predictive power to the model. The final model appeared as in section 4.2.3.2 (see Fig. 24):

\[
M1: \text{DurGroom} = 8.52 - 1.13PR - 3.42SPR
\]

4.2.4 Months 18-24

4.2.4.1 Salivary Cortisol

Subjects did not differ by rearing, genotype, or sex in lnm24sal. No interactions were revealed.

4.2.4.2 Anxiety

The regression equations for anxiety and initiate play were identical to those reported in the hair cortisol section (see section 4.1.4.2 for equations and figures). The
addition of ln24sal did not add significant predictive power to the model for these behaviors.

For grooming, sex and genotype were significant predictors ($R^2=0.01; p=0.001$). The first model appeared as follows:

$$M1: \text{DurGroom} = 6.29 + 4.92\text{SEX} – 4.30\text{GEN}$$

The addition of ln24sal added significant predictive power to the model ($\Delta R^2=0.005; p=0.007$). The final model appeared as follows (Fig. 34):

$$M2: \text{DurGroom} = 5.77 + 5.25\text{SEX} – 4.84\text{GEN} + 2.53\text{SAL}$$

Because no rearing conditions predicted grooming, Sobel tests were not performed.

A summary of the predictors for total anxiety, play, and grooming across the first two years of life is presented in Table 6.

---

**Initiate Groom**

![Graph showing duration of grooming from months 18-24.](image)

**Figure 34.** The model for duration of grooming from months 18-24.
Table 6. Predictors of behaviors across the first two years of life (by month).

<table>
<thead>
<tr>
<th>Age/Pred</th>
<th>Anxiety</th>
<th>Play</th>
<th>Grooming</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 12 18 24</td>
<td>6 12 18 24</td>
<td>6 12 18 24</td>
</tr>
<tr>
<td>Sex</td>
<td>+ + + + + +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gene</td>
<td>+</td>
<td>+</td>
<td>+ +</td>
</tr>
<tr>
<td>PR</td>
<td>+ +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SPR</td>
<td>+ + +</td>
<td>+</td>
<td>+ + +</td>
</tr>
<tr>
<td>PRxG</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>SPRxG</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Hair</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Sal</td>
<td>+</td>
<td>+ +</td>
<td></td>
</tr>
</tbody>
</table>

4.3 Correlational Measures of HPA Activity and Anxiety

After controlling for rearing and genotype, Pearson’s partial correlations revealed significant positive correlations between several hair cortisol measures and between several salivary cortisol measures (Table 7). Overall, hair and salivary cortisol concentrations were not strongly correlated with each other.

Pearson’s partial correlation revealed that hair cortisol at month 6 was positively correlated with mean duration of anxiety at months 12 and 18, and month 12 hair cortisol was positively correlated with mean anxiety at months 12 and 24. Mean anxiety at month 6 was also positively correlated with mean anxiety at month 24, and mean anxiety at month 12 was positively correlated with mean anxiety at month 18 (Table 8). Partial correlations for salivary cortisol and anxiety revealed a positive correlation between anxiety at month 12 and salivary cortisol at months 12, 18, and 24 (Table 9).
Table 7. Partial correlations for hair and salivary cortisol at each six-month interval.

<table>
<thead>
<tr>
<th>Control Variables</th>
<th>M6 HAIR</th>
<th>M12 HAIR</th>
<th>M18 HAIR</th>
<th>M24 HAIR</th>
<th>M6 SAL</th>
<th>M12 SAL</th>
<th>M18 SAL</th>
<th>M24 SAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>rear &amp; httpr</td>
<td>1.000</td>
<td>.239</td>
<td>.471**</td>
<td>.134</td>
<td>-.046</td>
<td>-.288</td>
<td>-.307</td>
<td>-.221</td>
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<tr>
<td>M6HAIR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M12HAIR</td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M24HAIR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M6SAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M12SAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M18SAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>M24SAL</td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

Correlation is significant at 0.01 level**.
Correlation is significant at 0.05 level*.

Table 8. Partial correlations for hair cortisol and duration of anxiety at each six-month interval.

<table>
<thead>
<tr>
<th>Control Variables</th>
<th>M6ANX</th>
<th>M12ANX</th>
<th>M18ANX</th>
<th>M24ANX</th>
</tr>
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<tbody>
<tr>
<td>rear &amp; httpr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M6HAIR</td>
<td>.118</td>
<td>.420**</td>
<td>.362*</td>
<td>.339*</td>
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<td>M12HAIR</td>
<td>.085</td>
<td>.380*</td>
<td>.156</td>
<td>.321*</td>
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<td>M18HAIR</td>
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<td>.772**</td>
<td>.253</td>
<td>.331*</td>
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<tr>
<td>M24HAIR</td>
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<td>.283</td>
<td>.011</td>
<td>.030</td>
</tr>
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<td>M6ANX</td>
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<td>.025</td>
<td>.307</td>
</tr>
<tr>
<td>M12ANX</td>
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<td>.370*</td>
<td>.206</td>
<td></td>
</tr>
<tr>
<td>M18ANX</td>
<td>1.000</td>
<td>.252</td>
<td>.206</td>
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</tr>
<tr>
<td>M24ANX</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**. Correlation is significant at 0.01 level
*. Correlation is significant at 0.05 level
Table 9. Partial correlations for salivary cortisol and duration of anxiety at each six-month interval.

<table>
<thead>
<tr>
<th></th>
<th>M6ANX</th>
<th>M12ANX</th>
<th>M18ANX</th>
<th>M24ANX</th>
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<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rear &amp;</td>
<td>.182</td>
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<td>-.152</td>
<td>.113</td>
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<tr>
<td>httlpr</td>
<td>-.097</td>
<td>-.475**</td>
<td>-.269</td>
<td>-.133</td>
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<td>M12ANX</td>
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<td>-.495**</td>
<td>-.204</td>
<td>-.204</td>
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<tr>
<td>M18ANX</td>
<td>.203</td>
<td>-.559**</td>
<td>-.219</td>
<td>-.127</td>
</tr>
<tr>
<td>M24ANX</td>
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<td>.041</td>
<td>.027</td>
<td>.168</td>
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<tr>
<td>M6SAL</td>
<td></td>
<td>1.000</td>
<td>.455**</td>
<td>.309*</td>
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<td>M12SAL</td>
<td></td>
<td></td>
<td>.455**</td>
<td>.309*</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>.172</td>
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<td>M24SAL</td>
<td></td>
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</tbody>
</table>

* · Correlation is significant at 0.05 level
** · Correlation is significant at 0.01 level
CHAPTER 5
DISCUSSION

This dissertation examined the influences of early life experience, serotonin transporter (5-HTTLPR) genotype, and hypothalamic-pituitary-adrenocortical (HPA) activity on the expression of anxiety over the first two years of life in rhesus macaques. Because in humans the onset of social anxiety occurs in childhood or adolescence (NIMH, 2006), and because rhesus monkeys and humans share many biological, physiological, and social characteristics (Tamashiro et al., 2005; Suomi et al., 2005; Rogers et al., 2006), studying the development of anxiety in rhesus monkeys in the first two years of life served as a valuable nonhuman primate model of human child development. I sought to determine whether rearing condition and/or rh5-HTTLPR genotype predicted anxiety, and further whether short-term or long-term HPA activity could explain, or mediate, the relationship between rearing and/or genotype and anxiety. For the first time, long-term HPA activity was assessed and studied continually across development as a measure of adrenocortical “phenotype” in young monkeys by measuring cortisol concentrations found in hair. These hair cortisol concentrations, taken every six months, were compared to short-term HPA activity as measured by salivary cortisol taken at the same time points and both measures were related to behavior, which was also studied continually for the first two years of life. In sum, this study represented the first continual, longitudinal study of infant monkeys with respect to HPA activity and anxious behavior.
5.1 Anxiety

The data presented here indicate that rearing condition (specifically peer-rearing) and sex are the most salient predictors of anxiety in the early development of young rhesus monkeys, while rh5-HTTLPR genotype and current HPA activity are not significant predictors. PR infant monkeys are more prone to develop anxiety than either SPR or MPR infants, and it appears as though SPR monkeys more closely resemble MPR monkeys in terms of behavior. PR infants exhibited the most total anxious behavior in the first two years of life, while MPR and SPR infants did not differ from each other (Fig. 3). Though these findings are the first reported for SPR infants, they are in line with previous research demonstrating elevated anxiety in PR infants (Captianio, 1985; Higley et al., 1991a; Ruppenthal et al., 1991; Barr et al., 2003b). PR monkeys exhibited the most anxious behaviors after a major life stressor, both in the short-term and many months after exposure to the stress. In this study, all monkeys were subjected to the major stress of housing relocation and social group reformation at roughly 8 months of age, but it was PR monkeys who exhibited elevated anxiety in the 3-4 months immediately after the stressor while SPR and MPR infants did not differ from each other (Fig. 4b). Further, though all infants continued to show an increase in anxiety in the following six months (i.e., from months 12-18), PR monkeys exhibited the most anxiety of all three rearing groups while SPR monkeys fell between the levels of PR and MPR monkeys (Fig. 4b). Additionally, the PR condition predicted the amount of anxious behavior from months 6-12 and 12-18 (Figs. 18 and 22; Table 6) while the SPR condition did not predict anxiety at any time point. These data imply that PR monkeys are vulnerable to prolonged
behavioral disturbances in response to stress, while SPR monkeys are relatively protected from such disturbances. Interestingly, By 24 months of age, all three rearing groups were indistinguishable from each other in total anxious behavior and levels at this age were similar to those observed in the first six months of life (Fig. 4b); rearing condition also did not predict anxiety at this age (Fig. 25; Table 6). Taken together, these findings support past research indicating that early life adversity imposes long-lasting behavioral effects after a major stressor (Captianio, 1985; Higley et al., 1991a; Chrousos and Gold, 1999; Heim and Nemeroff, 2001) and suggest that when nursery-rearing is necessary, the SPR condition produces optimal behavioral development.

The data presented here also support previous findings that females are more prone to anxiety disorders than males. Unlike the rearing condition effects, sex differences in anxiety were present in the first six months of life such that females spent more time in anxious behavior (Fig. 4c), suggesting an early propensity for females to develop anxiety. This idea is consistent with human literature in which young girls demonstrate more anxiety than boys (Reardon et al., 2009; Greca and Lopez, 2004). Female monkeys also exhibited more anxiety than males from months 18-24, though during the periods of stress (i.e. months 8-18) sex differences were not revealed (Fig. 4c) and sex was not a significant predictor of anxiety (Table 6). However, closer inspection of the data revealed that female PR monkeys exhibited the most anxious behavior soon after the stressor and were among the most anxious in the following six months (Fig. 4d). Together, these data imply that in relatively stable environments, young female monkeys are more prone to anxiety than males, a finding that has been well-established.
in the human literature. They further support the numerous studies that have found
that early-life adversity is particularly detrimental to girls and women (NIMH, 2006;
Richardson et al., 2008; Heim et al., 2000; Heim and Nemeroff, 2001; Heim et al., 2002;
Barr et al., 2004; Higely et al., 1991b).

Serotonin transporter genotype was not a significant factor in anxiety in these
young monkeys. The polymorphism predicted anxious behavior only in the first six
months of life (Fig. 12; Table 6) though significant group differences were not revealed
by ANOVA at any time point. It is likely that the lack of a genetic effect in this study
resulted from a small sample size. Despite the fact that this study contained over 60
subjects, only 14 of those (or 22%) were carriers of the (s) allele for rh5-HTTLPR. This
frequency is well below that in a study by Lesch et al. (1997) which contained 154 rhesus
monkeys, 34% of which were carriers of the (s) allele. Further, only two infants were
homozygous for the (s) allele, and as some human studies indicate that the s/s genotype
is even more detrimental than the l/s genotype with respect to anxiety, it is unfortunate
that I could not study the effects of this genotype in more detail. As another study of
over 100 infant monkeys previously found heightened anxious temperament in (s)
carriers of rh5-HTTLPR (Champoux et al., 2002), it is likely that with more subjects rh5-
HTTLPR genetic effects on anxiety would have been revealed. However, it is also
possible that the effects of the rh5-HTTLPR polymorphism may not appear until later in
development, as several studies with humans have not found such effects in early
childhood but rather in teenage children (Jorm et al., 2000; Ebstein and Auerbach, 2002;
Ebstein et al. 1998; Auerbach et al., 1999).
Collectively, the data presented in this dissertation implicate the PR condition and sex as the most salient factors for the development of anxious behaviors in young monkeys. These findings suggest that the SPR condition yields more normative behavioral development in instances when nursery of young primates is necessary to study nonhuman primate models of human development. They further underscore the need for effective therapeutic treatments for young girls exposed to neglect, abuse, or other impoverished early-life experiences.

5.2 Social Behaviors

The data presented here do not provide strong evidence for a relationship between the expression of anxious behaviors and that of social behaviors in young monkeys. The most anxious monkeys, i.e., PR and female monkeys, showed varying trajectories for play and grooming behaviors that were not consistent with their patterns of anxious behavior.

The pattern of play in PR monkeys across development lent mild support to my prediction that anxious monkeys would exhibit less social behavior. Across the first 18 months of life PR monkeys consistently decreased the amount of time spent in play while increasing the amount of time spent in anxious behavior (Figs. 4b and 6b). However, while they regularly exhibited more anxiety than either MPR or SPR infants, PR infants exhibited middle ranges of play compared to MPR/SPR infants or did not differ from MPR infants. With respect to grooming, PR infants only differed from MPR infants at month 6, when they groomed less (but were playing more; see Figs. 13 and 14). Further, when the PR condition predicted anxiety (months 6-12 and 12-18), it did not
reliably predict play or grooming (Table 6). These data indicate that PR infants are less able to regulate their patterns of anxious and social behaviors, particularly in response to a major life stressor. However, for SPR infants, who exhibited levels of anxious behaviors more similar to those of MPR infants, rearing condition did not predict anxiety but did predict both social play and grooming across the first 18 months of life (Table 6). SPR infants regularly played more and groomed less than MPR infants; these data also lend mild support to my prediction that less anxious monkeys would exhibit more social behaviors. Additionally, SPR infants appear to be able to regulate their patterns of anxious and social behaviors better than PR infants.

Across the first two years of life, female monkeys consistently played less and groomed more than males (Figs. 5 and 7). These findings are not novel and are likely not related to anxiety. Sex differences in social play and social bonding behaviors such as those reported above are a hallmark of many mammalian species including rhesus monkeys (Suomi, 2005; for a review see Meaney et al., 1985). Further, the patterns of prediction of anxiety, play, and grooming by sex were not consistent with the notion that more anxious monkeys spend less time in social behaviors (Table 6). Sex predicted anxiety only in the first six months and from months 18-24, while it predicted play at every time point and predicted grooming only in the last six months.

Taken together, the data presented here comparing anxious and social behaviors indicate that in young rhesus monkeys anxiety overall is not strongly related to the expression of some social behaviors. However, it appears as though there is more of dysregulation of the anxiety/social behavior relationship for PR monkeys than either
SPR or MPR monkeys, a finding which supports the more normative behavioral development of SPR monkeys.

5.3 HPA Axis Activity and Anxiety

Overall, evidence for mediation of the rearing→anxiety relationship by HPA activity was not revealed. Hair cortisol obtained at month 6 predicted the duration of total anxiety (including clinging) in the first six months of life (Fig. 11), but only mediated the relationship between MPR and anxiety (i.e. not between PR or SPR and anxiety). Because MPR infants exhibited significantly more clinging (due in large part to the mother’s control), the model for anxiety in the first six months was corrected for clinging. After correction, neither rearing condition nor hair cortisol predicted anxiety (Fig. 12). Since concurrent hair cortisol did not predict current anxiety at any later time (i.e., month 12 hair cortisol did not predict anxiety from months 6-12, etc.; Table 6), I concluded that hair cortisol did not mediate the relationship between rearing condition and anxiety.

Likewise, though baseline salivary cortisol predicted both measures of anxiety in the first six months of life (anxiety including clinging and anxiety without clinging; Figs. 28 and 29), Sobel tests revealed no mediation by salivary cortisol. Concurrent salivary cortisol also did not predict current anxiety at any later age (Table 6). Thus, overall, it appears that in infant monkeys HPA activity measured either acutely or long-term does not mediate the rearing→anxiety relationship when current cortisol values are examined with current anxious behaviors.
Despite the lack of mediation of the rearing→anxiety relationship by concurrent HPA activity, an interesting trend developed with respect to earlier long-term HPA activity and later anxiety. Hair cortisol in the first six months of life was positively correlated with average anxiety at every time point thereafter (i.e., months 12-24; Table 8). Similarly, hair cortisol measured between months 6-12 was positively correlated with average anxiety at between months 6-12 and 18-24, and hair cortisol measured between months 12-18 was positively correlated with average anxiety between months 6-12 and 18-24 (Table 8). These findings are in line with recent studies utilizing hair cortisol, in which higher cortisol concentrations are related to health problems in the predicted direction: hair cortisol was higher in adults with chronic pain than in controls (Van Uum et al., 2008), and hair cortisol was also associated with increased hospitalization and treatment procedures in infants studied in a neonatal intensive care unit (Yamada et al., 2007). In contrast, in this study the only relationship between anxiety and salivary cortisol occurred for average anxiety between months 6-12, which was positively correlated with salivary cortisol measured at months 12, 18, and 24 (Table 9). These findings indicate an exciting role for hair cortisol in predicting future anxious behavior.

These are the first data linking hair cortisol with behavior at any age in nonhuman primates, and these data demonstrate the differences in enduring correlations between short- or long-term HPA activity and anxiety. My prediction that hair cortisol would serve as a superior predictor of anxiety was upheld: the data here support the notion that hair cortisol, or adrenocortical “phenotype,” is a superior
predictor for future anxiety over salivary cortisol (or “point” measures of HPA activity). Further, these findings suggest that early adrenocortical “phenotype” predicts the expression of anxious behaviors after a major life stressor is imposed, and that more anxious individuals will also later exhibit higher chronic cortisol levels.

Acute and long-term HPA profiles were not strongly related in these young monkeys. As presented in Table 7, hair and salivary cortisol were not significantly correlated with each other across development. However, hair cortisol and salivary cortisol values independently showed strong positive correlations at progressive time points. That is, hair cortisol at month 6 was correlated with hair cortisol at month 18; hair cortisol at month 12 was correlated with hair cortisol at months 18 and 24; and month 18 hair cortisol was correlated with month 24 hair cortisol (Table 8). These findings suggest strong stability in an individual monkey’s adrenocortical “phenotype” during the first two years of development. A similar relationship existed for salivary cortisol: month 6 values were correlated with month 18 values; month 12 values were correlated with month 18 and 24 values, and month 18 values were correlated with month 24 values (Table 9). Thus, despite the fact that short-term and long-term HPA concentrations were not correlated across development, there is strong evidence for the stability of each of these systems in the first two years of life. The fact that cortisol values at months 6 and 12 were not correlated, but that month 6 values were correlated with values at other later time points, suggests that the imposition of a major social stressor disrupts an individual’s adrenocortical stability over the short term, but that
after acclimation to a new social environment over a period of many months the
adrenocortical stability is reestablished.

5.4 Conclusions

Overall, it appears that early life experience and sex are the strongest predictors
of anxious behavior in early development in rhesus monkeys, and that 5-HTTLPR
genotype may not play a significant predictive role in the development of anxiety in the
first years of life. In translating to early childhood development, it is likely that a child’s
upbringing imparts a greater influence on the development of anxious behaviors than
certain genetic factors and concurrent pituitary-adrenocortical activity, particularly for
females. This notion underscores the need for effective interventions and therapies for
children exposed to impoverished rearing environments.

Current HPA activity, either long- or short-term, does not appear to be a
significant predictor of current levels of anxious behaviors and HPA activity does not
appear to mediate the relationship between early life experience and anxious behavior.
However, an integrated measure of HPA activity such as hair cortisol measured early in
life is useful in predicting later anxious behavior. Thus, health care providers interested
in the treatment and prevention of anxiety-related disorders in children and adolescents
should consider an individual’s early life history and sex as predictors for these
disorders; they may also consider a child’s early adrenocortical “phenotype” as a useful
tool in early prediction and prevention. Such a tool may provide valuable information
for the development of treatment programs for the most common form of mental illness
in the U.S.
### APPENDIX A

#### SOCIAL BEHAVIOR ETHOGRAM

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Social Contact</td>
<td>Two or more animals within a limb’s length of each other*.</td>
</tr>
<tr>
<td>Contact Play</td>
<td>Two or more infants engaging in play characterized by wrestling, rough-and-tumble, grabbing, rolling, etc. Does not include brief swats at each other during which contact is made. Generally includes open-mouth play face, ear-twitches, raised eyebrows, and mock biting. Can be initiated or received*.</td>
</tr>
<tr>
<td>Non-Contact Play</td>
<td>Two or more infants engaging in play characterized by chasing, swatting, bouncing, etc. Generally includes open-mouth play face, ear twitches, raised eyebrows, lunging, jumping, and swatting. Can be initiated or received*.</td>
</tr>
<tr>
<td>Mount</td>
<td>One infant clasping another with its hands and/or feet and thrusting its pelvis. Can include mounting of any part of the body. Can be initiated or received*.</td>
</tr>
<tr>
<td>Aggression</td>
<td>Social interaction characterized by open-mouth threat, contracted brow, lunging, chasing, or biting. Differs from play in brow movements and intensity. Can be contact or non-contact, initiated or received*.</td>
</tr>
<tr>
<td>Submit</td>
<td>Social interaction characterized by fear-grimace with or without lip-smacking, shrieking, raised tail &amp; presented rump, or running away. Usually occurs in response to aggression, but sometimes spontaneous. Can be initiated or received*.</td>
</tr>
<tr>
<td>Passive/Huddle</td>
<td>Sleep or a completely inactive state. Does not include visual explore.</td>
</tr>
<tr>
<td>Cling</td>
<td>Two or more subjects in ventral contact with another animal AND clasping of the hands OR feet to another animal. If there is no ventral contact/clasping, this is considered huddling and is scored as social contact*.</td>
</tr>
<tr>
<td>Locomotion</td>
<td>Movement from point A to point B. Requires more than 2 steps (one with each foot). Does not include the shuffling that occurs while foraging. Differs from movement play in intensity (less intense).</td>
</tr>
<tr>
<td>Visual Explore</td>
<td>Active visual inspection of the environment or another animal. Differs from passive; eyes must be scanning or intently looking at an object/animal.</td>
</tr>
<tr>
<td>Tac/Oral</td>
<td>Active exploration of the environment with the fingers and/or mouth. Differs from social grooming in that no other animal is involved (but it CAN occur in social contact).</td>
</tr>
<tr>
<td>Hoot/Scream</td>
<td>“Hooo” call or shrieking; can be in social contact or alone.</td>
</tr>
<tr>
<td>Social Groom</td>
<td>One animal using its hands/fingers or mouth to pick, pat, or smooth the hair or body of another. If lip-smacking occurs while grooming, do not enter “submit.” Can be initiated or received*.</td>
</tr>
<tr>
<td>Rock</td>
<td>Self-rocking or rocking on surrogate or with peers. Characterized by back-and-forth motion of the body.</td>
</tr>
<tr>
<td>Toy Play</td>
<td>Hanging, swinging, running with, throwing, or other active interaction with a toy or object in the environment. Differs from tac/oral in intensity (more intense).</td>
</tr>
<tr>
<td>Movement Play</td>
<td>Jumping, bouncing, cage-shaking, twirling, or other playful motions that occur alone. Differs from locomotion in intensity (more intense)</td>
</tr>
<tr>
<td>Self Groom</td>
<td>An animal using its own hands/fingers or mouth to pick, pat, or smooth its own hair or body (excluding anogenital region, which is coded as “self sex”).</td>
</tr>
<tr>
<td>Scratch</td>
<td>An animal moving its hands/fingers or feet in a rapid back-and-forth or up-and-down motion on its own skin or hair.</td>
</tr>
<tr>
<td>Self Stereotypy</td>
<td>Repetitive (&gt;3 sec) self-directed behaviors including saluting, limb-flailing, self-biting, etc. Does not include self-rocking (which is coded as “rock”).</td>
</tr>
<tr>
<td>Locomotor Stereotypy</td>
<td>Repetitive (&gt;3 sec) behavior generally comprised of pacing (walking or running) in the same pattern. May also include repetitive swinging from cage.</td>
</tr>
<tr>
<td>Self Mouth</td>
<td>Any digit (finger/toe) or appendage part in the infant’s own mouth.</td>
</tr>
<tr>
<td>Self Clasp</td>
<td>Fingers and/or toes clasped around any part of the infant’s own body. Often occurs with self-rocking.</td>
</tr>
<tr>
<td>Self Sex</td>
<td>Manual inspection or self-mouthing of the anogenital region; also includes masturbation. Coded as Mr. Perv.</td>
</tr>
<tr>
<td>Self Bite</td>
<td>Forceful placement of the teeth around any limb, usually the wrist/arm or ankle/leg.</td>
</tr>
</tbody>
</table>

*Requires a modifier in the JWatcher code: mom, peer(s), other, or 2+ animals
APPENDIX B

COMPONENT CATEGORIES OF ANXIETY

Self-Directed Behavior

Months 0-6

ANOVAs revealed a significant main effect of rearing on total duration of self-directed behavior ($F_{(2,1125)}=3.96; p=0.02$). PR infants exhibited more self-directed behavior than SPR infants ($\bar{x}=4.84\pm0.60$ sec vs. $2.90\pm0.63$ sec), but PR/MPR and SPR/MPR infants did not differ from each other. A significant rearing x genotype interaction was present for self-directed behavior ($F_{(5,1125)}=2.37; p=0.04$). MPR l/s or s/s infants exhibited more self-directed behavior than MPR l/l infants ($\bar{x}=4.35\pm0.83$ vs. $2.62\pm0.57$ sec).

Significant sex differences were revealed for self-directed behaviors ($F_{(1,1125)}=4.35; p=0.04$). Females exhibited more self-directed behaviors than males ($\bar{x}=4.19\pm0.52$ vs. $2.98\pm0.30$ sec).

Regression analysis revealed that only sex and PR were significant predictors ($R^2=0.011; p=0.005$). There were no significant rearing x genotype interactions, so the first model appeared as follows:

$M1: \text{DurSelfDir} = 2.57 + 1.31\text{SEX} + 1.66\text{PR} - 0.32\text{SPR}$

The addition of $\log_{10}(m6\text{hair})$ added significant predictive power to the model ($\Delta R^2=0.005; p=0.016$) and thus was retained for the final model, $M2$ (Fig. 35):

$M2: \text{DurSelfDir} = -8.41 + 1.21\text{SEX} + 1.28\text{PR} - 0.07\text{SPR} + 4.97\text{HAIR}$
Figure 35. The model for self-directed behavior regressed onto hair cortisol in the first six months of life.

The addition of log10(m6hair) increased the coefficient for SPR from M1 to M2; thus, after controlling for hair cortisol, the differences between MPR and SPR in self-directed behavior increased and no Sobel tests were performed. Adding log10(m6hair) decreased the coefficients for PR and MPR, so Sobel tests were performed to test for mediation. Hair cortisol did not mediate either the PR→self-directed or the MPR→self-directed relationships.

Regression analysis revealed that neither ln(m6presal) nor ln(m6postsal) added significant predictive power to the model for self-directed behavior, so no further tests were performed.
**Months 6-12**

**2006 Cohort**

ANOVA revealed a significant main effect of rearing only for self-directed behavior \( F_{(2,22)} = 6.62; p = 0.01 \). PR and SPR infants both demonstrated more self-directed behaviors than MPR infants (MPR: \( \bar{x} = 2.93 \pm 1.10 \) sec; PR: \( \bar{x} = 60.48 \pm 13.48 \) sec; SPR: \( \bar{x} = 51.56 \pm 16.20 \) sec). PR and SPR were the only significant predictors for self-directed behavior in the 2006 cohort \( R^2 = 0.398; p = 0.006 \). Neither the addition of m12hair nor lnm12sal added significant predictive power so it was not retained in the model, which appeared as follows (Fig. 36).

\[
M1: \text{DurSelfDir} = 2.94 + 57.54PR + 48.63SPR
\]

![Figure 36. The model for duration of self-directed behavior for the 2006 cohort from months 6-12.](image)
2007 Cohort

No group differences, interactions, or predictors were revealed for self-directed behaviors for the 2007 cohort, so no further analyses were performed.

Months 12-18

No significant group, interaction effects, or predictors for self-directed behavior were revealed at this age, so mediation tests were not performed.

Months 18-24

ANOVA revealed a significant main effect of rearing for self-directed behavior ($F(2,1385)=12.79; \ p<0.001$) SPR infants exhibited more self-directed behavior than both PR and MPR infants (SPR: $\bar{x} = 5.70 \pm 0.65$ sec; PR: $\bar{x} = 3.38 \pm 0.35$ sec; MPR: $\bar{x} = 2.45 \pm 0.22$ sec), who did not differ from each other. SPR was the only significant predictor of duration of self-directed behavior ($R^2 = 0.018; \ p<0.001$). Neither the addition of $\sqrt{m24hair}$ nor ln$m24sal$ added significant predictive power, and the final model appeared as follows (Fig. 37):

$$M1: DurSelfDir = 2.45 + 0.94PR + 3.27SPR$$

![Figure 37. The model for duration of self-directed behavior from months 18-24.](image)
Fear Vocalizations

Months 0-6

ANOVA revealed significant main effects of rearing \((F_{(2,1125)}=10.32; p<0.001)\) and sex \((F_{(1,1125)}=9.26; p=0.002)\) on total duration of fear vocalizations. SPR infants vocalized more than either PR or MPR infants (SPR: \(\bar{x} = 5.98\pm1.28\) sec; PR: \(\bar{x} = 0.68\pm0.25\) sec; MPR: \(\bar{x} = 1.43\pm0.69\) sec), but PR/MPR infants did not differ from each other. Females vocalized more than males (\(\bar{x} = 4.42\pm0.97\) vs. \(1.29\pm0.46\) sec).

A significant rearing \(\times\) genotype interaction was present for fear vocalizations \((F_{(5,1125)}=5.37; p<0.001)\) such that both MPR and SPR \((s)\) carriers vocalized more than MPR or SPR \(l/l\) infants (MPR: \(4.05\pm1.45\) vs. \(0.20\pm0.99\) sec; SPR: \(9.07\pm2.81\) vs. \(5.65\pm0.93\) sec).

Regression analysis revealed that sex, genotype, and SPR were significant predictors of fear vocalizations \((R^2=0.03; p<0.001)\). No interactions were present, and \(\log_{10}(m6hair)\) did not add significant predictive power to the model. The addition of \(\ln m6postsal\) did add significant predictive power to the model \((\Delta R^2=0.014; p<0.001)\), and the final model appeared as follows (Figs. 38 and 39):

\[
M2: DurFearVoc = 0.26 + 3.35SEx + 2.05Gen - 0.86PR + 3.85SPR + 3.72POSTSAL
\]

Adding \(\ln m6postsal\) to the model increased the coefficient for MPR and decreased the coefficients for both PR and SPR between M1 and M2. Sobel tests revealed that \(\ln m6postsal\) mediated the relationship between SPR and fear vocalizations only \((z=2.99; p=0.003)\).
Figure 38. The model for duration of fear vocalizations regressed onto post-challenge salivary cortisol for males in the first six months of life.

Figure 39. The model for duration of fear vocalizations regressed onto post-challenge salivary cortisol for females in the first six months of life.
**Months 6-12**

No group differences or interactions were revealed for fear vocalizations for the 2007 cohort. There were no significant predictors for fear vocalizations, so no further analyses were performed.

**Months 12-18**

ANOVAs revealed only a significant effect of sex on fear vocalizations ($F_{(1,1626)}=4.65; p=0.03$). Females vocalized more than males (vocalizations: $\bar{x}=1.92\pm0.40$ vs. $0.95\pm0.27$ sec; play: $3.88\pm0.43$ vs. $7.08\pm0.55$ sec). Regression analysis revealed that sex was the only significant predictor of duration of fear vocalizations ($R^2=0.003; p=0.037$). Neither the addition of $(m18hair)^{1/2}$ nor $\ln m18sal$ added significant predictive power, and the model appeared as follows (Fig. 40):

$$M1: DurFearVoc = 0.95 + 0.97\text{SEX}$$

![Fear Vocalizations](image)

**Figure 40.** The model for duration of fear vocalizations from months 12-18.
Months 18-24

ANOVA revealed no significant group effects or interactions for fear vocalizations at this age. Regression analysis revealed no significant predictors of fear vocalizations, so no further analyses were performed.

Scratching

Months 0-6

ANOVA revealed a significant main effect of rearing on total duration of scratching ($F_{(2,1125)}=27.83; p<0.001$). SPR infants scratched less than PR and MPR infants, who did not differ from each other (SPR: $\bar{x} =1.03\pm0.11$ sec; PR: $\bar{x} =4.14\pm0.46$ sec; MPR: $\bar{x} =3.17\pm0.28$). A significant main effect of *rh5-HTTLPR* genotype was found for scratching ($F_{(2,1125)}=10.87; p=0.001$) whereby infants with the (s) allele for *rh5-HTTLPR* scratched more than l/l infants ($\bar{x} =3.67\pm1.45$ vs. $2.46\pm0.51$ sec). A significant rearing x genotype interactions was present for scratching ($F_{(5,1125)}=13.05; p<0.001$). MPR (s) carriers scratched more than their l/l counterparts ($\bar{x} =4.35\pm0.48$ vs. $1.00\pm0.33$ sec).

Regression analysis revealed that PR and SPR were significant predictors for scratching ($R^2=0.047; p<0.001$). Neither genotype x rearing interactions, log$_{10}$(m6hair), nor salivary cortisol added significant predictive power to the model and so were not retained. Thus the final model was (Fig. 41):

$$M1: DurScratch = 3.17 + 0.93PR - 2.14SPR$$
Months 6-12

ANOVA revealed a significant main effect of rearing on scratching ($F_{(2,506)}=4.38; p=0.01$). SPR infants scratched less than either PR or MPR infants (SPR: $\bar{x}=1.49\pm0.20$ sec; PR: $\bar{x}=2.93\pm0.56$ sec; MPR: $\bar{x}=3.07\pm0.52$ sec), but PR/MPR infants did not differ.

Significant effects of $rh-5HTTLPR$ genotype were found for scratching ($F_{(1,506)}=4.72; p=0.03$) such that infants with the $l/s$ or $s/s$ genotype scratched more than $l/l$ infants ($\bar{x}=3.38\pm0.73$ vs. $2.12\pm0.23$ sec).

Regression analysis revealed that SPR was the only significant predictor for scratching ($R^2=0.017; p=0.01$), and neither m12hair nor lnm12sal added significant predictive power. The model for scratching appeared as follows (Fig. 42):

$$M1: DurScratch = 3.07 - 0.14PR - 1.59SPR$$
Months 12-18

ANOVA revealed no significant group effects or interactions for scratching at this age. Regression analysis revealed no significant predictors, so mediation tests were not performed.

Months 18-24

ANOVA revealed a significant main effect of rearing on scratching ($F_{(2,1385)}=6.37; p=0.002$). SPR infants scratched more than MPR infants ($\bar{x}=3.44\pm0.21$ vs. $2.39\pm0.22$ sec). PR infants did not differ from MPR or SPR infants in scratching behaviors.

A significant effect of sex was revealed for scratching ($F_{(1,1385)}=8.42; p=0.004$). Females scratched more than males ($\bar{x}=3.28\pm0.19$ vs. $2.55\pm0.16$ sec).

Regression analysis revealed that sex and SPR were the significant predictors of duration of scratching ($R^2=0.016; p<0.001$). Neither the addition of $\sqrt{m24hair}$ nor
Inm24sal added significant predictive power to the model, which appeared as follows (Fig. 43):

M1: DurScratch = 1.91 + 0.80SEX + 0.59PR + 1.12SPR

Figure 43. The model for duration of scratching from months 18-24.
**APPENDIX C**

**DESCRIPTIVE STATISTICS**

<table>
<thead>
<tr>
<th></th>
<th>Month 6</th>
<th>Month 12</th>
<th>Month 18</th>
<th>Month 24</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hair Cortisol</strong></td>
<td>179.60±8.60</td>
<td>190.15±6.62</td>
<td>162.42±8.61</td>
<td>128.82±5.89</td>
</tr>
<tr>
<td>(pg/mg)</td>
<td>(1.30a, 1.20b)</td>
<td>(0.37a, 0.72b)</td>
<td>(0.35a, 0.70b)</td>
<td>(0.35a, 0.70b)</td>
</tr>
<tr>
<td><strong>Salivary Cortisol</strong></td>
<td>.89±0.08</td>
<td>1.54±0.17</td>
<td>1.28±0.12</td>
<td>1.63±0.14</td>
</tr>
<tr>
<td>(ug/dL)</td>
<td>(3.02a, 13.17b)</td>
<td>(1.31a, 0.54b)</td>
<td>(1.21a, 0.89b)</td>
<td>(0.76a, -0.08b)</td>
</tr>
<tr>
<td><strong>Anxiety (sec)</strong></td>
<td>6.16±0.63</td>
<td>25.47±2.60</td>
<td>20.03±1.28</td>
<td>11.20±0.92</td>
</tr>
<tr>
<td><strong>Play (sec)</strong></td>
<td>41.17±1.49</td>
<td>21.18±1.54</td>
<td>10.40±0.68</td>
<td>19.24±0.94</td>
</tr>
<tr>
<td><strong>Grooming (sec)</strong></td>
<td>1.27±0.16</td>
<td>2.36±0.45</td>
<td>7.67±0.78</td>
<td>8.13±0.84</td>
</tr>
</tbody>
</table>

^aSkew
^bKurtosis
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