Behavioral and Gene Expression Changes Following Early Social Bond Disruption in the Rhesus Monkey

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The effects of early life stressors on the developing infant can be severe. These effects include, but are not limited to, disordered attachment behavior in childhood, behavioral problems throughout childhood and adolescence, and increased risk for developing psychiatric illnesses later in life. Recent work in monkey models of early life stress suggests that the timing of the stressor is of paramount importance in determining the exact nature of the long-term behavioral disturbances. In monkeys, when stress is experienced at one-week of age, a pattern of decreased social affiliation and increased anxious behaviors develops that is not unlike children with the inhibited form of reactive attachment disorder. Conversely, if the stress is experienced at one-month of age, a pattern of increased social affiliation and increased anxious behaviors develops that resembles children with the disinhibited form of reactive attachment disorder. The work presented in this thesis describes two more features in this monkey model that depend on the timing of the early life stress. First, analysis of the acute behavioral response to the early life stress indicates that infants experiencing this stress at one-week of age immediately respond by increasing their level of nonsocial contact comforting behaviors, while infants experiencing the stress at one-month of age immediately respond by increasing their level of social comforting behaviors. It will be shown that the levels of each of these behaviors can predict aspects of the longer term behavior when measured between two and three months of age in their social rearing

group environments. Next, it will be shown that gene expression in the amygdala, a brain region that controls socioemotional behaviors, also critically depends on the age at which early life stress is experienced. Furthermore, the expression of Guanylate Cyclase 1 α 3 in this brain region will be shown to correlate with certain differing aspects of behavior again measured between two and three months of age. Collectively, these studies may offer clues in determining very early which children are at risk for psychopathology following early life stress, and represent early work in determining a potential therapeutic target for the risks that ensue.

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1. Introduction

1.1. Overview

Over the past several decades, clinical psychiatrists have become increasingly aware that disturbances early in life have profound effects on behavioral development and represent a major risk factor for the development of psychopathology later in life. Disturbances may take many forms, but those that are most often linked with later psychopathology include childhood sexual, physical or emotional abuse, child neglect, toxic stress exposure, and parental loss due to death or permanent separation. The long-term effects that follow these early life adverse experiences include major depression, post-traumatic stress disorder, attention-deficit/hyperactivity disorder, anxiety, suicide attempts, schizophrenia, and substance abuse. It appears that early adverse experience interacts with both a genetic diathesis and current stress to contribute to the expression of mental illnesses. It has been offered as an explanation that the early adverse experiences tune the LHPA axis stress response to predispose later stressful experiences to have a magnified effect. Clinical and preclinical studies support the dysregulation of this system in those that have experienced significant early life stress. Indeed, the field of neuroscience has informed, and been informed by, these clinical observations. Seminal work in animal models of early life experience by Harlow et al. (1964; 1971) in monkeys, and Levine et al. (1959; 1966) in rodents has been followed by 60 years of intense research on this subject. A recent model of early life stress in monkeys has highlighted the importance of the timing of the stressor with respect to the development of later social and anxious behaviors (Cameron, 2001). In this thesis, I will examine the acute behavioral reaction to early life separation in this model and the differential expression of genes in the amygdala that result from this early life stressor. In the sections that follow in this introduction, I will review the clinical and preclinical data regarding

the short- and long-term effects of early life stress, introduce and describe the advantages of our monkey model of early life stress, and implicate the amygdala as a potential mediator of some of the long-term behavioral sequelae of early life stress. Chapter two will examine the acute reaction to the stress of maternal bond disruption early in life in the aforementioned monkey model, and whether this acute reaction is dependent on the timing of the stressor. We will also verify that the previously described long-term behavioral phenotype observed in a mixed-sex cohort of monkeys (Cameron, 2001) is evident in the current all-female cohort. Further, this chapter will begin to examine the question of whether elements of the acute response to early maternal bond disruption can predict the longer-term behavioral phenotype. The discussion of these data will include the potential clinical significance of early detection in children most affected by early adverse experience. In chapter three, the involvement of the amygdala, as assayed by gene expression, in this paradigm will be examined. There, we will show that Guanylate Cyclase 1, α 3 subunit (GUCY1A3), is decreased in monkeys that have experienced early life maternal bond disruption; that this decrease is dependent on the timing of the disruption; and that it is correlated with certain species atypical behavioral traits at the level of the individual monkeys. The possible functional role of decreased GUCY1A3 in the amygdala will be discussed. Chapter four will summarize the data and offer a model for the importance of this and future work in clinical populations. Finally, in the appendix, I will briefly examine some of the technical difficulties of performing the gene expression analysis in our studies.

1.2. Later Consequences of Early Life Stress Experiences in Humans

Beginning around the turn of the 20th century, psychoanalysts began emphasizing the role of early untoward experiences in contributing to adult psychopathology. Freud suggested that

these experiences acted via organic pathways in the nervous system to create a propensity for the later expression of mental illness. As a specific example he hypothesized that the early loss of a loved object rendered the individual vulnerable to depression later in life when loss is again experienced (Freud, 1917). The dynamic processes by which these experiences predispose the individual to later mental illness were a subject of continued interest (Abraham, 1927), and these ideas were conceptualized in the framework of attachment theory (Bowlby, 1961). As such, the idea that early parental loss (EPL) or adverse early rearing environment contributes to adult psychopathology has been an area of intense study.

The idea that EPL is associated with mental illness has been examined recently by Agid et al. (1999) in a study that recruited patients with major depressive disorder (MDD), bipolar disorder (BPD), and schizophrenia (SCZ) from inpatient psychiatric units in Israel and compared them with healthy controls randomly recruited from neighborhood community centers in the same area. Subjects were matched for age, gender, and ethnic origin. This study found that EPL increases the likelihood of developing MDD by a factor of 3.8 in this population. Furthermore, the loss due to separation was much more robust than the loss due to death, as was loss before age nine compared to loss between ages nine and sixteen. SCZ was also associated with a significantly increased likelihood of EPL, particularly before the age of 9, and this was more evident due to death of the parent, and when the parent involved was the mother. The increase likelihood of EPL in BPD did not meet statistical significance after corrections for multiple comparisons. In a study of female twins, Kendler et al. (1992) also suggested that EPL increased an individual's vulnerability to several psychiatric illnesses. In this study, MDD and generalized anxiety disorder were associated with separation but not parental death, panic disorder was associated with parental death, and maternal separation, and phobic disorders were associated

with parental death but not parental separation. The fact that EPL due to separation is a stronger predictor of later mental illness than EPL due to death in many studies (reviewed in Tennant, 1988) deserves further consideration. It has been proposed that this frequently noted association may indicate that EPL due to separation is merely a consequence of other pathogenic factors in the family environment that actually drive the association.

Consistent with the latter hypothesis, several investigations have examined the relationship between parenting style and later psychopathology. Breier et al.(1988) examined EPL and the subsequent parental rearing style on the development of affective disorders and SCZ. Although in this study, the ability of EPL alone to predict psychopathology did not reach statistical significance, EPL followed by an unsupportive relationship with the remaining parent was a strong predictor of the occurrence of mental illness later in life. This finding was in agreement with an earlier study which concluded that loss of a mother before the age of 17 in women over the age of 18 was associated with a current episode of MDD (Harris et al., 1986). The increased likelihood of current MDD being associated with paternal loss did not reach statistical significance. Examination of the familial environment suggested that it was the persistent neglect following these adverse life events that contributed to the adult MDD. This neglect was more often associated with maternal loss than paternal loss, presumably explaining the outcome difference that depended on parental sex. These clinical studies underscore the importance of the social environment after a major stress event in contributing to the ultimate risk for mental illness. Preclinical studies in monkeys (Hinde and Davies, 1972; Schlottmann and Seay, 1972; Hinde et al., 1978) and rodents (Francis et al., 1999; Bredy et al., 2004), too, are consistent with the idea that the post-separation social environment is an important factor for mediating the ultimate outcomes following chronic or acute maternal separations. Other studies

in humans underscore the importance of the familial environment even in the absence of EPL. Persons with MDD recruited from an outpatient clinic are more likely to rate their parents as having provided insufficient care, or as having been overprotective (Parker, 1983). The relative risk for developing MDD in persons reporting this characteristic in one or both parents (low care-high protection) was 3.4. Other studies from the same author suggest that the relative risks of this characterization for social phobia, anxiety disorders, agoraphobia, and schizophrenia were 9.0, 4.3, 3.3 and 2.1, respectively (Parker, 1984). Taken together, these studies support the notion that chronic parenting styles in the presence or absence of EPL can have a lasting impact on later psychological well-being.

Abusive early relationships are also associated with the later development of several types of psychiatric illnesses. Abuse can take many forms including physical abuse (often called child violence), psychological abuse, maltreatment, and sexual abuse. Child abuse is an important and prevalent problem in the U.S.A. and elsewhere. It was estimated in 1993 that between 2.3% and 4.2% percent of children in the U.S.A. were abused, the range reflecting whether abuse implied demonstrable harm or only danger of being harmed as reported by community professionals (the latter includes more suspected psychological abuse) (Sedlak and Broadhurst, 1996). It should be noted that both child-oriented and parent-oriented risk factors have been identified as occurring more often in the context of child abuse (Larrance and Twentyman, 1983; Pianta et al., 1989). A large enough corpus dedicated to the later consequences of early child abuse now exists such that different forms of abuse are being associated with different outcomes. For example several studies support the assertion that physical abuse preferentially leads to PTSD (post traumatic stress disorder) in one-quarter to one-half of all victims (Kiser et al., 1991; Famularo et al., 1994), and further that the risk of

PTSD increases when the abuse is severe or long-lasting (Kiser et al., 1991). These types of associations are of paramount importance when considering the utility of early psychological intervention. This is especially true for PTSD since certain populations have been shown to respond from these treatments (Frueh et al., 1996). In a sample of British, adult, working-class women, 64% of those who reported sexual abuse before the age of 17 were treated for MDD during the three year study period (Bifulco et al., 1991). In a U.S. study of women recruited from community primary care settings, those who reported early physical or sexual abuse had higher scores for depression, anxiety, and low self-esteem as assessed by a the psychological symptoms checklist and also had more frequent suicide attempts than non-abused women (McCauley et al., 1997). Although abundant epidemiologic evidence exists linking these adverse early life experiences to later psychopathology, evidence of the neural underpinnings of this link is not as abundant.

Studies assessing changes in brain function in children following early adverse experiences implicate many of the brain systems involved in stress response and sensitivity. Several studies that examined salivary cortisol in children of different ages who have experienced early life stress report a flattening of the normal diurnal rhythm, with an elevation in the nighttime cortisol levels (Carlson and Earls, 1997; Gunnar and Vazquez, 2001). The lack of decline in the evening has been attributed to either higher levels of stress during the day, or rather an increased sensitivity to normal levels of daily stress (Heim and Nemeroff, 2001). In another study in children with PTSD and a history of past maltreatment, the 24-hour output of cortisol in the urine was correlated with the duration of the precipitating trauma, and a severity of the current PTSD symptoms (De Bellis et al., 1999). In a previous study by this group, in response to challenge with sheep CRF (cortisol releasing factor), self-reported sexually abused

girls responded with a lower ACTH (adrenocorticotropic hormone) response compared to control girls (De Bellis et al., 1994). They also showed lower basal levels of this hormone. Interestingly, plasma cortisol response to CRF challenge and 24-hour urinary output of cortisol were not different from controls. Taken together these results suggest that the pituitary CRF receptors have been downregulated in the face of hypothalamic CRF overexcretion. In another study, Kaufman et al. (1997) reported enhanced ACTH response to CRF challenge in depressed children aged 7-13 who also report a history of abuse, although this finding was limited only to those children with ongoing adversity. This study, too, reported normal cortisol output among all groups. A few studies have examined the ability for the synthetic corticosteroid dexamethasone to suppress the release of cortisol following early life adversity in children. This ability is thought to be mediated by the low affinity GR's (glucocorticoid receptor) which bind both synthetic and endogenous corticosteroids, at several sites in the limbic portion of the central nervous system. Thirty-nine percent of bereaving children, one month following the death of a parent, showed a decreased suppression of cortisol following dexamethasone administration; importantly, those that showed this decreased suppression reported more symptoms of depression than those who suppressed normally (Weller et al., 1990). Nevertheless, this test yielded opposite results in a study of children who had experienced an earthquake five years prior. In this study lower baseline morning cortisol and an increased suppression following dexamethasone administration were seen in more affected children, and these differences were correlated with the severity of intrusive PTSD symptoms. In total, these studies describe a variable response to early life stress in children; one that possibly varies with respect to the type of stress, age when subjected, duration of stress, and present stress levels. Furthermore these effects may represent an intermediate phenotype in the trajectory toward adult psychopathology.

Other related measures have also been examined in children following early adversity. In the same study that measured urinary cortisol in children with PTSD and a history of maltreatment (De Bellis et al., 1999), elevations were also observed in urinary norepinephrine, epinephrine, and dopamine. Abused children with PTSD have also been shown to exhibit increased heart rate and blood pressure at rest and following orthostatic challenge (Perry, 1994). The elevation of catecholamine secretion was not only observed when the development of PTSD followed the abuse. Similar elevations were seen in sexually abused girls without symptoms of PTSD (De Bellis et al., 1997), and in children whose parents had marital discord (Gottman and Katz, 1989). Interestingly, decreases in norepinephrine (NE) and dopamine-β-hydroxylase (the rate limiting enzyme for NE synthesis) were observed in children who were neglected in infancy (Rogeness, 1991). Worth noting here, the latter finding is consistent with a related finding in monkeys (decreased cerebrospinal fluid NE) who are deprived of their mothers early in life (Kraemer et al., 1989). The opposing direction of changes in these studies again emphasizes the fact that both the type and timing of the early adverse experience may determine the resulting neurochemical changes.

Some retrospective studies in adults have examined the long-term consequences of early life stress. In women with PTSD and a history of sexual abuse as a child, significantly elevated daily levels of norepinephrine, epinephrine, dopamine, and cortisol in the urine were observed (Lemieux and Coe, 1995). Increased plasma cortisol was also seen in adults with psychopathology who had experienced the death of a parent in childhood compared to healthy adults who had experienced the same loss (Breier et al., 1988). Interestingly, both the cortisol and ACTH levels were significantly correlated with a score that evaluated the home life and personal adaptation following parental loss. Of note, cortisol suppression after dexamethasone

treatment was normal in this group suggesting that the negative feedback mechanisms were intact. In another study limited to adult women with a childhood history of severe sexual abuse, increased suppression of salivary cortisol following dexamethasone administration was observed (Stein et al., 1997). Heim et al. (2000) similarly examined the relationship between early life abuse, current MDD diagnosis, and current stress response to a psychosocial stressor (public speaking) in adult women. Women who suffered early abuse showed a greater plasma ACTH level following the stress, but only those women with a concurrent diagnosis of MDD also had an increased plasma cortisol. Another report by this group examined the pituitary responsiveness to CRF administration in these women (Heim et al., 2001). Abused women without depression showed an increased ACTH response and normal cortisol response to challenge. Interestingly, though, abused women with concurrent MDD (and nonabused women with MDD) showed a blunted response to CRF challenge. In another study of stress sensitivity, Luecken et al. (1998) examined the relationship between the childhood loss of a parent, rearing quality, and current response to two laboratory stressors: 1) a video clip depicting parental loss, and 2) an impromptu speech. Adults with poor quality family rearing showed increased cortisol during the movie presentation, and adults who had experienced loss showed an increased cortisol during the impromptu speech stressor. Again, discordant results may be explained by differences in the type of early life stress, loss versus abuse, and/or by different subgroups of depression.

In this brief review of the effects of early life stress in humans, I have underscored several major points with the studies that were included. The ultimate effects appear to depend on many different features; among these are: 1) the initial timing of the stressor, 2) the initial type of the stressor, 3) the presence or absence of concurrent psychopathology, and 4) the particular aspect of the response measured. In order to tease apart this very intricate scheme of

interrelated variables, researchers must look toward prospective studies of early life stress in both animals and humans. The ability to prospectively follow a clinical sample of adequate size and for a long enough duration to examine these variables is limited both by ethics and practicality. For this reason, laboratory controlled animal studies of early life stress are indispensable. Although it is difficult to claim, based on behavioral observations, that animals have many of the implicated psychopathologies; it is, on the other hand, easy to examine these behaviors and suggest that some features of human psychopathology are apparent. Furthermore these animal studies provide the ability to invasively examine the brain substrates following early life stress at the cellular level. This ability may or may not ever be achieved with functional imaging studies in humans. In the several sections that follow, after a brief foray describing attachment, I will review the animal data concerning early life stress, with a particular emphasis on the most potent stressor in this regard, maternal separation. Based on these and other data, the amygdala is a likely site governing some of the effects of early life stress. Hence, a brief review of the amygdala and its contribution to socioemotional behaviors will then be offered. Following this review, I will describe the monkey model of early life stress that will be the subject of the rest of this thesis.

1.3. Attachment: Theory and Research

Attachment theory was posed by Bowlby to explain the development of relationships throughout the life of an individual, and how they may be tuned by the initial bonding experience with the primary caregiver, most often the child's mother (Bowlby, 1969; Bowlby, 1982). In this view, typical attachment behaviors are guided by the child's ability to attain comfort from his or her mother in times of fright, fatigue, or sickness. Attachment behaviors were defined as those

behaviors in which a person engages to assuage these negative feelings by obtaining proximity to the attachment figure. According to this theory, the early mother infant bond was posited to act as the basis of all future social relationships. If the early mother infant bond was characterized by secure attachment, then the child's level of distress was maintained at low levels in a homeostatic-like effect. In relationships that are characterized in this way, the infant uses the caregiver as a secure base from which to explore the environment. If, however, separations from the primary caregiver were repeated and enduring, then the fear responses engaged by these separations were also enduring. Bowlby (1982) postulated that the exaggeration of the fear-response elicited by separation may be a core feature in those with attachment disorders. In this light, he formulated ideas about the coping mechanisms that a child uses after long periods of separation from his or her caregiver. A typical clinical situation that results in prolonged separation is when the child is in the hospital or a residential nursery unvisited. Upon reunion the child immediately treats the caregiver as a stranger, but then over a period of hours or days the child becomes intensely clinging and anxious of the possibility of future separations. In this clinical situation, the anxious feelings are often manifested by increased anger toward the caregiver. The period of time that the child does not seek comfort was coined as the detachment phase. Indeed current research reports are consistent with this theory. Infants who are isolated for protracted periods of time in hospitals or in orphanages show elements of detachment upon reunion or adoption (O'Connor, 1999). Repeated exposures to separation have been observed to have a multiplicative effect in both clinical and preclinical studies (Brown and Harris, 1978). In rhesus monkeys, infants who are separated multiple times during the first 6 months of life display differences in behavior compared to infants that are separated a single time (Spencer-Booth and Hinde, 1971). It also appears that adverse early attachment experiences as an infant

predispose an individual to have adverse attachment experiences as a parent. People who are raised in homes self-reported as unhappy or disrupted are more likely to have illegitimate children, become teenage mothers, to make unhappy marriages, and to divorce (Rutter, 1979). Examples of non-genomic transmission of early adverse life experiences are also evident in rodent studies. This method of transmission will be considered in detail in other parts of this introduction, but briefly, rats that are foster reared by mothers who exhibit low levels of intimate maternal care, themselves exhibit low levels of intimate maternal care as adults (Francis et al., 1999). These theories have been accepted by clinical psychiatrists, and recently (~1980) the disorders that are thought to occur following pathological early life caregiver-infant relationships have been added to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV).

As defined in the DSM-IV, the core feature of Reactive Attachment disorder is a "markedly disturbed and developmentally inappropriate social relatedness in most contexts that begins before age 5 years and is associated with grossly pathological care" (2000). Pathological care usually takes the form of extreme neglect or abuse, but may stem from prolonged hospitalizations of the child, parental inexperience, or extreme poverty. A child may show either the inhibited form or disinhibited form of reactive attachment disorder. In the inhibited type the child fails to initiate and to respond to social interactions in a pattern consistent with normal development; this form may manifest as resistance to comforting attempts from the caregiver, hypervigilance in social situations, or ambivalence to social interaction. In the disinhibited type, a child displays indiscriminate sociability and a lack of selectivity in choosing attachment figures. In a recent study, some children who were adopted out of orphanages in Romania were found to exhibit this type of attachment disorder (O'Connor, 1999).

Recent studies in rodents suggest that there are neural systems dedicated to attachment behaviors. For example it was noted that administration of μ-opioid agonists decreased the ultrasonic vocalization (UV's) that pups emit when separated from their dams (Carden et al., 1991). The nature of this decrease was further elucidated in a study of the μ-opioid receptor null mouse (Moles et al., 2004). Null mice emitted fewer calls during periods of separation on postnatal day's (PND's) 4, 8, and 12 compared to wild-type controls. These effects were shown to be specific to the maternal separation stress, since other stressful stimuli, male-odor and cold stress, elicited the same number of UV's in null mice versus controls. Interestingly these mice also showed an indiscriminate form of attachment since they were shown to have less preference for their own nest compared to another dam's nest.

In rhesus monkeys that experience social bond disruption at different times early in development, patterns of behavior analogous to both forms of reactive attachment disorder in children may develop depending on the exact timing of the disruption. In monkeys that experience the bond disruption at one-week of age, decreased social behavior and increased self-directed behaviors develop later in life (Cameron, 2001). One-week separated animals are active, learn to bottle-feed rapidly, however never engage in species-typical social interaction in adolescence and adulthood. These behaviors appear to resemble the inhibited form of reactive attachment disorder. In monkeys that experience social bond disruption at one-month of age, increased social behaviors and increases in certain anxious behaviors are evident later in life. These monkeys will form indiscriminate attachments with other monkeys, spending greater time in ventral contact with other monkeys; these behaviors are long-lasting and evident past puberty. This constellation of behaviors is reminiscent of the indiscriminate form of reactive attachment

disorder. Chapter two of this thesis will examine the early manifestations of maternal-infant social bond disruption and ask whether they are predictive of later attachment responses.

1.4. Early Life Experience in Rodents

The most well-studied model of early life stress in rodents is the variable handling paradigm, first introduced by Levine (1957) and extensively studied later by Meaney, Plotsky, et al. (reviewed in Sanchez et al., 2001; and Pryce and Feldon, 2003). These studies were guided by the initial observations that infant rats that were exposed to brief periods of stimulation between birth and weaning show differences in HPA axis activation to stress in adulthood. It was hypothesized that the early experience of a mild stressor may permanently affect either the CNS or adrenal response to stress, in such a way that activation patterns are altered by subsequent stressors in the long term. Interestingly, these early studies describe an increased adrenal corticosterone response to acutely noxious stimuli, such as foot shock or cold stress, in adult animals that were handled for three minutes per day for the first 16 days of life (Levine, 1957, 1962). Later studies of the behavioral response showed that handled animals were more active and exploratory, and defecated less in the open field test (Levine 1966). Both of these measures are classically thought to relate to decreased anxiety in rodents. This interpretation stems largely from the fact that anxiolytic drugs, such as benzodiazepine receptor agonists, increase exploration in behavioral tests including the open field test, in doses which are clinically meaningful (Crawley, 1985); while anxiogenic drugs, such as pentylenetetrazol, decrease these behaviors (Pellow and File, 1984). In the aforementioned open field test, handled animals were also observed to release significantly less corticosteroids from the adrenals, suggesting that they did not react to the open field with as much stress as the nonhandled

controls. This was one of the earliest examples of neonatal handling that led to a blunted stress response in adulthood. Interestingly, though, less noxious stimuli such as a novel environment or transporting the animals had been anecdotally observed in earlier studies to have the same effect: handled animals did not respond with an observable stress reaction, while nonhandled animals responded with an observable stress reaction and increased autonomic reactivity (Levine et al., 1956).

These early studies in rats also began to examine the issue of whether the timing of the early-life stress is important for developing certain behavioral and neuroendocrine phenotypes. With respect to adrenal activation following acute cold stress, the periods of handling that were associated with the most activation were PND 2-13 and PND 2-5, while the periods PND 6-9 and PND 10-13 led to increases that were not significantly different from nonhandled controls (Levine and Lewis, 1959). Another study examined whether preweaning versus postweaning handling (three minutes per day) affected the stress response to electric foot shocks of varying duration (Ader, 1970). In this experiment, plasma corticosterone levels were measured repeatedly following transfer to the experimental apparatus and acute foot shock. Initial corticosterone response was similar between nonhandled, handled preweaning (PND 1-21), and handled postweaning (PND 22-42), however both handled groups returned to baseline levels more rapidly than the nonhandled group. These experiments seem to be contradictory since the former implicates the first 5 days of the postnatal period as more robustly altering the stress response than the next 8 days, while the latter suggests that handling effects are the same whether they are performed during weeks 1, 2 and 3, or during weeks 4, 5, and 6. It is unclear why Ader (1970) did not see a difference in early versus late handled groups, especially since the handling durations were the same, and in previous studies, cold stress and foot shock stress

produced similar physiological responses. It has been proposed that differences between laboratories with respect to daily care and handling may contribute to some of the experimental variation observed in otherwise similar testing conditions (Lehmann and Feldon, 2000).

As well as providing engaging data on the importance of the postnatal social experience in shaping the adult response to novel and noxious stimuli, these early studies also revealed a potential confounder when interpreting postnatal manipulation studies - what is the appropriate control group (Levine, 1960)? One interpretation is that nonhandled rats receive lower than normal amounts of stimulation, and they are therefore less prepared to mount an appropriate stress response, as indicated by the protracted period of increased corticosteroids observed following foot shock stress (Ader, 1970) and blunted response to noxious stimuli (Levine et al., 1957; Levine, 1962). These interpretations have been studied extensively due to renewed interest in this model, and its effects on neurobehavioral development, over the last 20 years.

The more recent studies stem from the idea that maternal separation on the order of minutes (subsequently denoted as EH for early handling) represents species-typical time periods between bouts of maternal licking and grooming in the wild (Leon et al., 1978; Jans and Woodside, 1990; Caldji et al., 1998), and therefore may be adaptive for the animal, while maternal separation on the order of hours (subsequently denoted as MS) is species-atypical and is therefore maladaptive. The variable data on nonhandled (subsequently denoted as NH) animals has prompted some investigators to consider the NH group as an experimental group instead of a control group (Pryce and Feldon, 2003). This notion is supported in studies of latent inhibition: the decreased association of a neutral stimulus such as a tone or light to an unconditioned stimulus such as a foot shock following a previously nonreinforced exposure. In rats grouped by both early handling paradigm and sex, NH males did not develop latent inhibition unlike females

(regardless of treatment) and EH males (Weiner et al., 1985). These data also highlight the fact that sex differences in behavior can be elicited by early life handling paradigms. Other groups, most notably Meaney, Plotsky et al., continue to treat EH as an experimental intervention that leads to anxiolytic and stress-coping effects relative to the NH controls and MS experimentals. In the paragraphs that follow in this section, the more recent data in rodents including data from groups with each type of interpretation will be reviewed.

Meaney et al. (1985) hypothesized that the expedited return to baseline levels of adrenal corticosteroids in handled animals exposed to foot shock stress as adults (Ader, 1970) was in part mediated by a handling dependent increase in the central negative feedback system to circulating glucocorticoids. As such, glucocorticoid receptor binding, the neural substrate for reactive feedback, was found, following a 20-minute acute restraint stress, to be increased in the hippocampus of adult rats handled for 15 minutes per day for the first 3 weeks of life. Subsequently, the critical window of EH for establishing superior reactive feedback to stress was reduced to PND 2-14, when decreases in Cortisol Releasing Factor (CRF) mRNA in the hypothalamus, CRF protein in the median eminence and plasma corticosterone were still observed following the same stress paradigm (Plotsky and Meaney, 1993). The proportion of CRF remaining in the median eminence was lower in NH rats suggesting that the amount of CRF released was increased. Importantly, MS for 3 hours had opposite effects compared to EH rats in these same measures. These data are compelling in that they provide a neural substrate for the prolonged stress response seen in NH rats, however by themselves they do not explain the more rapid and efficient corticosterone response seen in handled rats following more noxious stimuli in some studies (Levine, 1962). It has been proposed, but not tested in EH versus NH rats, that differences in CRF receptor expression in the pituitary, or elsewhere in the brain, may mediate

some of these observed discrepancies. This hypothesis is particularly noteworthy in light of two studies: 1) in adult rats that are handled for 15 min / day during PND 2-8, increases in CRF mRNA are seen in both the Central Nucleus of the Amygdala and the Bed Nucleus of the Stria Terminalis (Fenoglio et al., 2004), and 2) in adult rats that have experienced MS for 8 hours every other day during PND 2-8, oral administration of the CRF_1 antagonist DMP696 reduces anxiety-like behavior in both a social interaction assay and the elevated plus maze (Maciag et al., 2002).

The involvement of the amygdala, touched upon briefly in the preceding paragraph (Fenoglio et al., 2004) has been studied in more detail. The impetus for these studies stems from the known function of the amygdala as a regulator of the behavioral, autonomic, and endocrine responses to environmental challenge (LeDoux, 2000). Caldji et al. (2000b) examined the behavioral responses and amygdala GABAa and CBZ (central benzodiazepine) receptors in male rats that had been handled for 15-minutes / day, PND 1-14. These rats exhibited a decreased latency to eat in a novel environment, visited the food more frequently, and spent more total time eating than both nonhandled rats and rats that had been MS for 180 minutes / day. Additionally, in other tests of anxious behavior, they spent more time examining the center of an open-field and displayed a decreased response to auditory startle. Another cohort of similarly treated rats showed increased binding of benzodiazepines in the central and lateral nuclei of the amygdala in 15-minute handled rats versus NH or 180-minute MS rats. Finally, mRNA for the γ2 subunit of the GABAa receptor was increased in the same group in the central, lateral, and basolateral nuclei of the amygdala. The amygdala has been previously shown to control fearful and anxious behaviors directly through lesions studies (reviewed in Davis, 1992); these studies will be examined in detail in section 1.8. Furthermore, the amygdala has been implicated in the control

of the local concentration of CRF in the locus coeruleus (LC) (Koegler-Muly et al., 1993). In this study lesions of the CeA were shown to significantly decrease the concentration of CRF in the LC. Since early-life stress has also been observed to impact LC CRF concentration (Ladd et al., 1996) and the amygdaloid-CRF system controls behavioral responses to previously conditioned stressful stimuli (Hitchcock and Davis, 1986; Liang et al., 1992), the studies by Caldji et al. (2000b) may provide the neurochemical link between early-life stress, the amygdala, and CRF responses in the brain.

Exactly how the early-life stress of social bond disruption in rats is transduced into long-term differences in physiology and behavior has also been investigated. The importance of the mother's reaction to the separated pup has been offered as an explanation for these effects (Denenberg, 1964), and Kalinichev et al. (2000) has reported on the persistent effects of this MS paradigm in these dams. These investigations were prompted by the initial observations that EH stimulates an increase in maternal behavior, especially pup-licking, that lasts beyond the immediate reunion period (Lee and Williams, 1974; Liu et al., 1997). Liu et al. (1997) described differences in licking, grooming and arched-back nursing (LG-ABN) throughout the first 10-days of life to EH, rats versus NH rats. Increased LG-ABN was observed in the EH rats with no differences in total nursing bouts between these groups. To examine whether LG-ABN affects the long-term measures in the stress axis that were observed for EH and NH rats, groups of rats were segregated on the basis of normal variation in LG-ABN behavior in the absence of differences in early handling. Adult rats whose mothers were "high LG-ABN" showed decreased plasma ACTH and corticosterone during and following 20 minutes of restraint stress compared to adult rats whose mothers were "low LG-ABN". The peak corticosteroid response was significantly negatively correlated with the amount of LG-ABN. Interestingly, CRF mRNA

expression was decreased in the parvocellular neurons of the hypothalamic paraventricular nucleus and glucocorticoid receptor mRNA expression was increased in the hippocampus of adults whose mothers were "high LG-ABN" compared to "low LG-ABN". Recently, decreased methylation of the neuron specific promoter site of the GR gene has been offered as an explanation of the glucocorticoid receptor level differences (Weaver et al., 2002). Further experiments that capitalized on the normal variation of maternal behavior with respect to nursing style showed that adult offspring of "high LG-ABN" mothers spent more time exploring the inner circle in the open-field test, and decreased latency to eat in a novel environment (Caldji et al., 1998). Additionally, central benzodiazepine receptor binding by flunitrazepam was increased in the central, lateral, and basolateral nuclei of the amygdala. Among individual animals, the frequency of LG-ABN was significantly positively correlated with both open-field exploration and CE (Central nucleus) flunitrazepam binding. A functional link between the increased CBZ binding in the amygdala and reaction to anxiogenic stimuli was further studied in adult offspring of high or low LG-ABN mothers (Fries et al., 2004). The offspring of high LG-ABN mothers showed a significantly decreased latency to first eating and spent more total time eating in a novel environment following diazepam injection compared to saline-injected controls. Collectively these results imply that the behavioral and physiological differences seen in postnatal handling (Caldji et al., 2000b) may be mediated through differences in the maternal-infant interaction early in life. These effects are presumed to be a non-genomic method of heritability, since partial fostering studies have shown that offspring born to "low LG-ABN" mothers, but fostered by "high LG-ABN" mothers (Low to High) exhibit increased open-field exploration compared to Low to Low offspring, and High to Low exhibit decreased open-field exploration compared to High to High (Francis et al., 1999).

In contrast to the daily manipulations of the maternal-infant interaction described above, the effects of single, 24-hr maternal separations have also been extensively studied. The outcomes of these manipulations are also critically dependent on the postnatal timing of the maternal separation. For example, the time course of the stress axis response to the mild stressor of a saline injection was examined immediately following 24 hrs of maternal separation beginning on PND3 and PND11 rats (van Oers et al., 1998a). The PND3 rats show relatively stable levels of plasma ACTH and corticosteroids initially and at 30 minute intervals following saline injection stress. Conversely, PND12 rats show immediate plasma corticosteroid elevation, and both ACTH and corticosteroid elevations at 30, 60, and 90 minutes following the saline injection stress. Interestingly, when tested at PND20, another group of PND3 MS rats show a significant increase in plasma ACTH, while the PND11 MS rats show a significant decrease in plasma ACTH (van Oers et al., 1998a). Only the PND3 MS rats show an increased c-fos mRNA expression response in the PVN, 30 minutes following saline injection stress, while the PND3 and PND11 MS rats show an increased and decreased CRF mRNA response 4 hours after saline injection stress respectively. These effects appear to be independent of the immediate corticosteroid response since injection of dexamethasone at the time of deprivation did not alter the plasma ACTH and corticosteroid levels when tested at PND20 even though this injection was shown to eliminate the initial corticosteroid response (van Oers et al., 1999). Additionally, a decreased expression of GR in the hippocampus and the PVN was observed at PND20 following MS at PND11. This phenotype was also unaffected by dexamethasone treatment at the time of deprivation, and importantly, saline injection and dexamethasone injection at the time of MS resulted in the same phenotype (van Oers et al., 1999). The latter experiment ruled out the possibility that dexamethasone was mimicking corticosteroid effects by binding its receptor.

Remarkably, intermittent feeding and stroking with a moistened paintbrush could rescue the non-MS phenotypes with respect to plasma ACTH response following stress and decreased GR mRNA expression in the hippocampus. It would be interesting to examine the maternal response upon reunion in pups that were MS for 24 hours on PND11, to see if these effects are in part mediated by maternal behavior, but this experiment has not yet been done.

A few studies have examined the longer term behavioral and physiological effects of single 24-hr MS earlier in life. Compared to nondeprived controls, PND60 rats that were MS on PND3 for 24 hours exhibit lower basal levels of CRF mRNA in the parvocellular neurons of the hypothalamic PVN and increased basal plasma ACTH and corticosteroid concentrations (Rots et al., 1996). Additionally, no differences in hippocampal GR mRNA were seen, but GR mRNA expression in the PVN and anterior pituitary were reduced. When hippocampal binding of GR was assessed at PND48 following MS on PND3 for 24 hrs, and supplemented with either 1) no treatment, 2) saline injection, or 3) $ACTH_{1-24}$ complex gender and treatment specific effects were observed (Sutanto et al., 1996). In male rats, separation alone or in conjunction with $ACTH_{1-24}$ downregulated GR receptors measured by ^{3}H RU28362 binding; separation followed by saline injection showed a nonsignificant increase in GR receptors coupled with a significantly lower affinity. In contrast the female rats showed an upregulation in GR receptors following separation and saline injection, and both an upregulation in GR receptors with a concomitant decrease in affinity. Interestingly when measured during senescence, rats that were MS on PND3 for 24 hrs show decidedly little impairments in both Morris water maze performance and reversal learning tasks (Oitzl et al., 2000).

Yet another type of maternal separation and social deprivation procedure has been examined in rats, early weaning and social isolation. These experiments create a different type

of stress by combined maternal separation with removal from littermates. Social interaction with littermates has also been observed to affect LHPA axis function (Stanton et al., 1988). In rats that were early-weaned on PND16 and socially isolated, Sanchez et al.(1998) observed reduced basal and stressed induced plasma corticosteroid levels when tested at PND75. Fewer pituitary corticotrophs were activated as evidenced by a blunted increase in ACTH-ir cell density following restraint stress compared to normally weaned and housed controls. Finally, an increase in CRF remaining in the Median Eminence following the 15-minute restraint stress period in early weaned, isolated rats also supports the hyporesponsive phenotype (less CRF has been released).

1.5. Early Life Experience in Non-human Primates

The Old World rhesus monkey is the most frequently studied experimental animal with regard to the effects of early life social experience. In the wild rhesus monkeys are indigenous to northern and central India, Afghanistan, Thailand and southern China. Rhesus monkeys prefer forest habitats, but adapt to a variety of other environments including semi-desert, dry deciduous, bamboo, and even large cities. Rhesus monkeys reside in large troops in their native environments, each containing several generations of female lineages (matrilines), and males who have joined the group after their pubertal transition. At birth, rhesus monkeys are more developed than humans; they are able to ambulate after 2 or 3 days, and in laboratory situations can learn to self-feed within two weeks. In the wild and in semi-naturalistic laboratory situations rhesus monkey infants spend large amounts of time in ventral contact with their mothers for the first few months of life, and continue to sleep in ventral contact for nearly a year, when weaning occurs (Seay and Gottfried, 1975). This close and enduring bond that develops between the

rhesus monkey mother and infant parallels the bond between a human infant and its primary caregiver. The early interaction in both has a solid grounding in attachment theory (Bowlby, 1969); both interactions are characterized by the mother acting as a sense of security and reassurance, and the infants use the mother as a secure base from which to increasingly engage the environment over development. The mother of the rhesus monkey and the primary caregiver of the human infant also train their young to understand social cues and the proper way to interact with other members of the social group. In monkeys this is characterized by the maternal regulation of early play behaviors with peers (Mears and Harlow, 1975). The strong parallels between the human caregiver-infant relationship and the rhesus monkey mother-infant relationship have made the latter an intense subject of study with regard to how disruptions in this relationship affect immediate and lifelong psychosocial behaviors, and concomitant physiology. In the remainder of this section, some of the literature on the behavioral and physiological effects of early social bond disruption in the rhesus monkey will be reviewed.

The manipulation most widely researched in the rhesus monkey is the early separation (during the 1st six months of life) of mother and infant. The landmark studies in this regard began from early research by Harlow et al. where the effects of contact comfort were assessed in monkeys that had been separated from their mothers at birth (Harlow and Zimmermann, 1959). These monkeys were reared in the presence of surrogate mothers that either contained a terry-cloth covering or were constructed of wire-mesh. These studies were instituted to determine the qualities in a surrogate that predisposed the development of a strong attachment bond, and were inspired, in part, by earlier studies in birds that suggested that early innate visual and auditory mechanisms gave rise to persistent following responses, a process known as Imprinting (Ramsay and Hess, 1954). It was observed that regardless of whether these surrogate mothers provided

nourishment for the infant, the infants preferred spending time on the terry-cloth surrogates more than the wire-mesh surrogates in times of both low and high stress. In fact, non-nursing cloth surrogates were preferred over nursing wire mesh surrogates. This finding argues that contact-comfort may be more important for bond development in rhesus monkeys compared to the previously thought "reduction of hunger drives" that was offered by other psychoanalytic theorists of the time (Dollard and Miller, 1950). The effects of a cloth surrogate were further investigated by Suomi (1973). In monkeys that spent 6 months in isolation, surrogate introduction led to decreases in self-directed behaviors, rocking, and stereotypies, and increases in locomotion and exploration. The introduction of the surrogate did not lead to increases in peer-play, suggesting that these isolated monkeys still maintained severe social effects from the initial isolation.

Other studies conducted by the Harlow group at the University of Wisconsin in the 1960's and 1970's demonstrated tremendous long and short-term behavioral deficits in monkeys that were separated from their mothers for protracted periods of time within the first year of life. The effects of social contact isolation were severe: infants that were removed from their mothers at birth and reared in cages for at least 6 months that allowed only auditory, visual, and olfactory contact with their peers developed "autistic-like" behaviors. These behaviors included stereotypical movements, compulsive nonnutritional sucking (called self-comforting behaviors in our studies), and the inability to form normal social relationships (Harlow et al., 1965; Harlow et al., 1971; Seay and Gottfried, 1975). Total social isolation exaggerated these effects; infants confined for the first year of life with no social interaction developed more self-directed behaviors, rocking, self-huddling and less social interaction and exploration when subsequently placed in social situations.

Maternal separation without subsequent enduring social isolation was also not without long-term behavioral effects. In a classic study infant rhesus monkeys were removed at birth and placed in single cages with a cloth surrogate (Young et al., 1973). These animals were then allowed two hours of peer contact each day for the first year of life, except for a 30-day complete social and physical isolation period. At one year animals were placed in a group setting (two maternally raised and two maternally separated animals). Following a variable, minimum preseparation period of four weeks, animals were subject to an experimental period where they were housed separately for 23 hrs. per day (separation condition) and in a group for one hr. per day (reunion condition). Maternally separated animals displayed increased stereotypical and self-directed behaviors, but decreased locomotion during the separation condition; they displayed increased stereotypical and self-directed behaviors, and increased locomotion during the reunion condition. Importantly, the separated animals also displayed lower levels of contact cling and proximity compared to maternally raised controls during the reunion condition.

The behavioral effects of early life maternal separation stress have also been examined in other species of monkeys, and similar outcomes were observed. An early primate maternal separation study (Kaufman and Rosenblum, 1967) described an intense depressive-like state in infant pigtailed macaques during a one-month period of isolation from their mothers beginning at 5.5 months of age. During this period observers described decreased play and increased self-directed behaviors. Upon reunion, these animals displayed hypersocial behavior characterized by significantly increased ventral-ventral contact with their mothers compared to both their preseparation levels and normal maternally raised control monkeys. The authors concluded that the hypersocial response following reunion indicated a more stressful reaction to separation than has been observed in other species of monkeys. This study also described the stages of the acute

separation as resembling those of human infants acutely separated from their mothers studied by Spitz et al. and termed "anaclitic depression" (Spitz and Wolf, 1946; Emde et al., 1965).

These distinct behavioral stages during the acute separation have been consistently observed in several different species of monkeys undergoing acute maternal separation stress (Kaufman and Rosenblum, 1967; Reite et al., 1978; Hennessy, 1986; Bayart et al., 1990). They consist of an early agitated phase characterized by increased vocalizations and locomotor activity. This period, called the protest phase, resembles the "flight-fight" response pattern proposed by Engel et al. (1962) in human infants experiencing acute stress. This is thought to be an evolutionarily conserved mechanism to increase the likelihood of reunion between the separated infant and its caregiver. The second stage as previously mentioned, usually observed following the first day of separation in monkeys, bears a striking resemblance to the syndrome of anaclitic depression (Spitz and Wolf, 1946). It is characterized by inactivity and withdrawal from the environment, a phase termed by Engel et al. (1962) as "conservation-withdrawal", and presumably functions to conserve energy and avoid injury during a prolonged separation from the caregiver. The similarity in both monkeys and children regarding the first two phases of the acute separation reaction suggests that the brain mechanisms that produce these behaviors may be similar. A third stage has been characterized in monkeys as the "recovery stage" (Kaufman and Rosenblum, 1967) and it consists of the monkey gradually increasing its level of locomotor activity in the days following the withdrawal phase. The fact that this phase is not observed in human infants has been attributed to the advanced development of the monkey's locomotor systems at the time of the acute separation that allow it to reengage the environment of its own accord.

The effects of early-life maternal infant social bond stress have also been examined in a paradigm that does not include maternal separation (Rosenblum and Paully, 1987). This paradigm used the variable availability of food to require infant bonnet macaques' mothers to spend differing amounts of time foraging, and hence, differing amounts of time in close contact with their infants. In this paradigm bonnet macaques and their 1-month to 4-month old offspring were observed during these periods of differential demand on food procurement. Maternal-Infant dyads were subject either to low foraging demand (LFD), high foraging demand (HFD), or an alternation between those two - variable foraging demand (VFD). Food consumption and weight were consistent across groups, while time spent foraging and food waste were not. Mothers of the VFD group showed the most signs of inconsistent parental care, even compared to the HFD group when the VFD mothers were in a period of LFD. Not surprisingly, the infants of the VFD mothers showed significantly fewer social behaviors, and significantly more distress behaviors compared to age matched animals in either of the other constant conditions. Anecdotal observations indicated that several of the monkeys in the VFD dyads, but none in the HFD or LFD dyads, developed a depressive-like phenotype not unlike those seen in the previously considered maternal separation models (Kaufman and Rosenblum, 1967). When mother-infant dyads were challenged with a novel environment, the VFD infants showed less play behaviors and were less willing to break contact with their mothers than the LFD infants (Andrews and Rosenblum, 1991). This finding was discussed as a potential indicator of a less-secure attachment in the VFD infants. Subsequent observations showed that the effects VFD-rearing are long lasting. Two years following the cessation of the VFD paradigm, VFD infants showed less social interaction with peers at baseline, but also less immediate and enduring arousal during the presentation of an acutely stressful event. Although this result, on the surface,

seems to conflict with earlier results showing increased levels of distress, the authors argued that this finding may indicate a behavioral downregulation to stress. This downregulation is often seen in humans suffering from Post-Traumatic Stress Disorder (PTSD), who are described as having a numbing of general responsiveness and a feeling of estrangement or detachment from others. The authors have begun intensive studies of the physiological differences between VFD-reared monkeys, and their LFD or HFD counterparts. As young adults, VFD infants were challenged with two anxiogenic agents to examine whether long-term differences in monoamine brain systems underlie any of the behavioral phenotypes. VFD-reared monkeys showed a behavioral hyperresponsivity to the noradrenergic probe yohimbine, and a behavioral hyporesponsivity to the serotinergic probe mCPP (m-chlorophenylpiperazine) when compared to LFD-reared subjects (Rosenblum et al., 1994). Furthermore differences in CRF brain systems were evident in the VFD-reared animals. When exposed to brief capture stress (approximately two minutes between capture and sedation) VFD reared monkeys display elevated levels of CSF CRF (cerebrospinal fluid cortisol releasing factor) and decreased levels of CSF cortisol (Coplan et al., 1996; Coplan et al., 2001). The seemingly disparate results were interpreted to mean that the HPA axis was suppressed during the acute stress in VFD monkeys compared to HFD or LFD monkeys, but that extrahypothalamic CRF systems are tonically overactive. Interestingly, this pattern of CSF CRF and cortisol has been found in humans with PTSD (Yehuda et al., 1991; Yehuda et al., 1995). Recent magnetic resonance imaging (MRI) studies have suggested the long lasting (10 years following rearing) anatomical/physiological brain differences in monkeys reared under VFD. In the anterior cingulate cortex, VFD-reared subjects showed an increased N-acetylaspartate (NAA) and a decreased glutamate-glutamine-γ-aminobutyric acid (Glx) resonance compared to age-matched controls (Mathew et al., 2003). These measures are thought

to reflect neuronal density/functional integrity and neurotransmitter concentrations respectively (Mathew et al., 2003). Furthermore, in the medial temporal lobe, the ratio of choline containing compounds was increased in the VFD-reared monkeys (Mathew et al., 2003). This measure is thought to reflect cell-membrane integrity (Mathew et al., 2003) and may indicate reactive fiber sprouting in response to neuronal loss as is seen when CRF is administered to the brains of immature rats (Brunson et al., 2001). One strength of the VFD paradigm lies in the fact that it more closely resembles the early life stress that a human infant may encounter compared to complete separation and isolation.

Recent experiments in rhesus monkeys have also attempted to examine the effects of less severe manipulations in early life. These experiments stem from earlier work that described deficits in social behavior and increases in self-directed behaviors following rearing with social isolations less extreme than total isolation (Suomi et al., 1971). Rhesus monkeys that are reared in the presence of peers following early maternal separation within 48 hours of birth and 45-60 days of individual housing (NR – nursery reared) also show long-term behavioral deficits compared to controls (MR – maternally reared) (Winslow et al., 2003). At 18, 24, and 36 months of age NR monkeys display lower levels of affiliative behaviors compared to MR monkeys when measured in round-robin pairings with an unfamiliar NR, unfamiliar MR, and homecage partner; these differences reach statistical significance at both the 18 and 36 month timepoints. NR monkeys also show increased aggressive behaviors in these tests that reach significance at both the 18 and 24 month timepoints. Additionally, both stereotypic behaviors and solitary behaviors are increased in NR monkeys. Studies that examined the CSF and plasma oxytocin levels in NR monkeys versus MR monkeys indicate that this neuropeptide is decreased over these same periods of time tested (Winslow et al., 2003). Oxytocin has been previously implicated in the

control of social behaviors in rodents (Insel, 1992). These findings suggest that this neuropeptide may be evolutionarily conserved to control social behaviors in mammals. Interestingly, two groups have reported a decrease in oxytocin receptor binding in the amygdala, a brain region that will be introduced later in the introduction of this thesis and examined in another model of early life social bond disruption in monkeys in chapter three, following repeated mother-infant separations or decreased maternal LG-ABN (Noonan et al., 1994; Francis et al., 2000). Finally, when exposed to a stressor, NR monkeys show increased abnormal and repetitive behaviors, decreased social contact behaviors, and are unable to buffer the subsequent plasma cortisol elevation in the presence of a companion (Winslow et al., 2003).

1.6. The Amygdala: Background

The amygdala is an almond shaped structure located deep within the medial temporal lobe. On the basis of lesion studies (Kluver and Bucy, 1937; Weiskrantz, 1956) the amygdala was found to be central in the control of emotional processing in monkeys. These studies support the amygdala's role in assigning emotional significance and producing an appropriate behavioral response to a wide array of salient environmental stimuli. To date, most of what is known about the functioning of the amygdala in the domain of emotional behaviors has been gleaned from studies of fear behavior in rats. While initial studies in the rat used avoidance conditioning and instrumental learning to further define the functions of the amygdala (Sarter and Markowitsch, 1985), more recent work has concentrated on fear conditioning as a prototype for emotional response (Davis, 1992, 1994; LeDoux, 2000). Fear conditioning is a Pavlovian conditioning response when a previously neutral stimulus such as a light or tone is paired with an aversive stimulus such as a foot shock. Following a few trials, the neutral stimulus by itself

begins to elicit behavioral responses similar to those originally evoked by the aversive stimulus alone. These fear responses are measured by freezing behavior, increased startle reflex, sweating, increased heart rate, and increased blood pressure. In humans, concomitant feelings of dread and despair accompany the autonomic responses. These responses are long-lived and robust, and as such, this model has been used extensively to investigate emotional behavior in experimental animals. Furthermore since human and animal fear share these similarities, fear conditioning has been implicated in the generation of human anxiety disorders (Rosen and Schulkin, 1998). Other studies of the amygdala suggest that a related function of this brain structure is in the modulating of social interactions (Bachevalier, 2000). The influence of the amygdala on social behavior likely also rests in its ability to coordinate many aspects of the external environment and subsequently influence behaviors accordingly. The amygdala has additionally been directly implicated in the long-term effects of social bond disruption in rats (Caldji et al., 1998; Caldji et al., 2000b; Fenoglio et al., 2004). In chapter two of this thesis, the behaviors resulting from early maternal separation of an infant monkey from its mother at two early developmental timepoints will be examined. The main effects will be in the domains of social and anxious behaviors, suggesting that there, too, the amygdala may be involved; this involvement will be probed by assays of gene expression in chapter three. Hence, in this section of the introduction, the literature relevant to the amygdala control of socioemotional behaviors will be reviewed.

1.7. The Amygdala: Anatomical Considerations

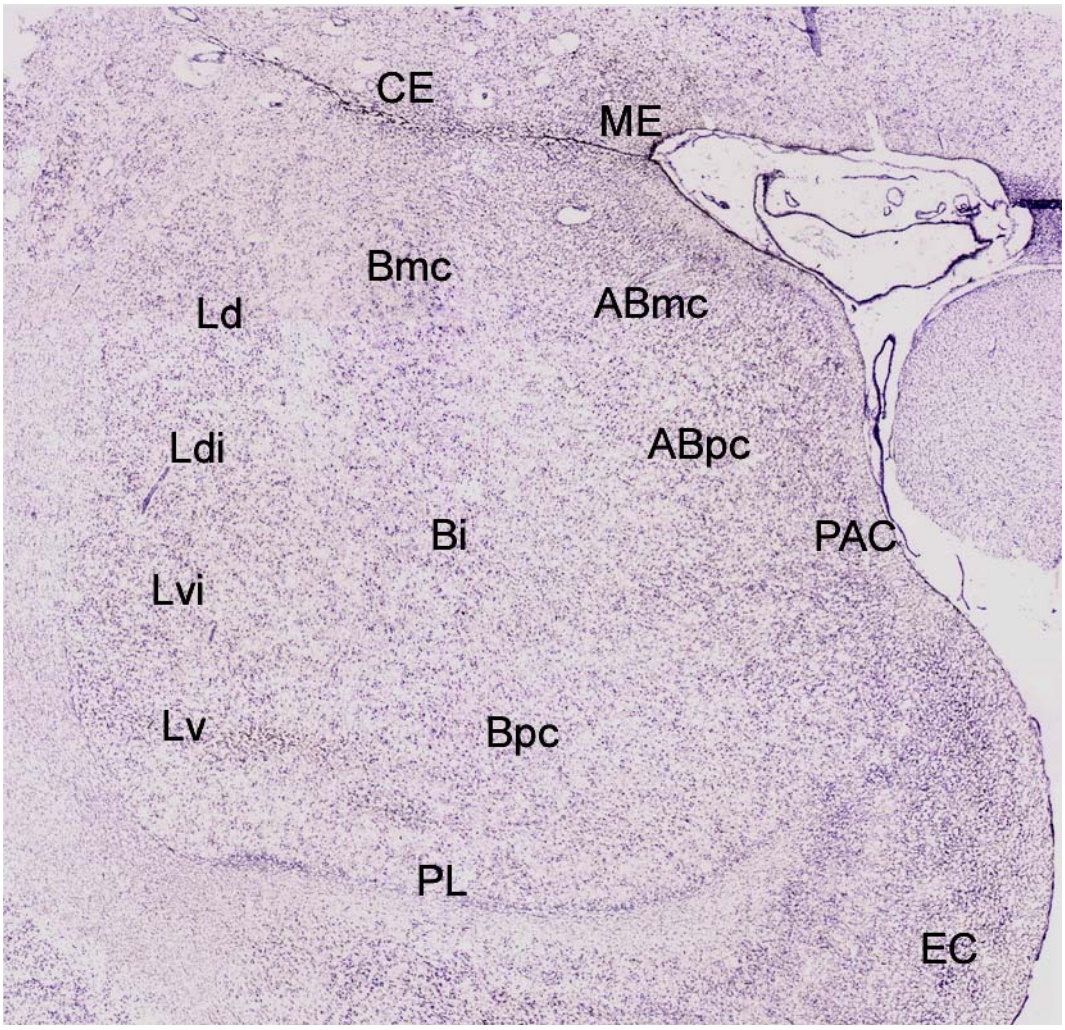
In chapter three of this thesis, the idea that the amygdala may subserve some of the behavioral responses to the early-life stress of maternal-infant social bond disruption will be

examined in the rhesus monkey. In order to appreciate the systems level functions of the amygdala, its intrinsic and extrinsic anatomy will be briefly examined in this section. The aim of this section is not to provide an exhaustive description of the cellular and connectional anatomy of this structure, but to highlight the relevant systems level connectivity in order that the reader can interpret the gene expression level changes of chapter three in a functional context. As such the nuclear structure of the amygdala will be reviewed, and its connections, both intra- and extra-amygdalar will be described. Finally, the possible functional significance of each of the major connections will be discussed.

The amygdala consists of a group of heterogeneous nuclei situated in the rostral medial temporal lobe. According to the nomenclature described by Price et al. (1987), modified slightly by Amaral et al. (Amaral and Bassett, 1989; Pitkanen and Amaral, 1998), and reviewed in Amaral et al. (1992), the amygdala can be separated into several distinct groups of subnuclei. A Nissl stained section through the amygdala that shows the anatomical locations of the individual subnuclei is shown in **Figure 1.1.** The "deep" nuclei consist of the lateral, basal (or basolateral), and accessory basal (or basomedial) nuclei. The lateral nucleus is further subdivided into the dorsal, dorsal intermediate, ventral intermediate and ventral subdivisions. The basal nucleus is further subdivided into the magnocellular, intermediate, and parvocellular subdivisions. The "cortical" nuclei consist of the anterior and posterior cortical nuclei, the periamygdaloid cortex, and the nucleus of the lateral olfactory tract. The "centromedial" nuclei are rightly composed of the central and medial nuclei. Finally the "other" nuclei consist of the anterior amygdaloid area, the intercalated nuclei, and the amygdalohippocampal area.

The intrinsic connections of the amygdala nuclei can be summarized by patterns of lateral to medial and dorsal to ventral information flow. The connections of the lateral, basal, accessory

Figure 1.1 Anatomy of the Amygdala. Nissl stained section through the amygdala at the level of the rostral most border of the lateral ventricle. CE: Central nucleus, ME: Medial nucleus, L: Lateral nucleus, B: Basal nucleus, AB: Accessory Basal nucleus, PAC: Periamygdalar cortex, PL: Paralaminar nucleus, EC: Entorhinal cortex, d: doral, di: dorsal intermediate, vi: ventral intermediate, v: ventral, mc: magnocellular, pc: parvocellular.


CE
ME
Bmc
Ld
ABmc
Ldi
ABpc
Bi
PAC
Lvi
Lv
Bpc
PL
EC

basal, and central nuclei will be discussed in detail, since these nuclei are the focus of what is known about this structure's participation in emotional behavior. The lateral nucleus projects to all subdivisions of the basal nucleus, the accessory basal nucleus, the paralaminar nucleus, the anterior cortical nucleus, the medial nucleus, and portions of the periamygdaloid cortex (Pitkanen and Amaral, 1991). Although the lateral nucleus projects to many other amygdaloid nuclei, it receives few reciprocal projections in return. Retrograde tracer injections have identified relatively weak projections to the lateral nucleus from the accessory basal, central, and basal nuclei (Aggleton, 1985). Within the lateral nucleus, lateral to medial connections are also evident. The magnocellular and intermediate portions of the basal nucleus send projections to the accessory basal nucleus, the medial portion of the central nucleus, medial nucleus, the nucleus of the lateral olfactory tract, the periamygdaloid cortex, and the amygdalohippocampal area (Aggleton, 1985). The parvocellular portion of the basal nucleus projects to the accessory basal nucleus, the central nucleus, the caudal portion of the medial nucleus the periamygdaloid cortex, and the amygdalohippocampal area (Aggleton, 1985), and also within the basal nucleus back to its magnocellular and intermediate subdivisions (Savander et al., 1995). The connection to the medial portion of the central nucleus has functional significance since this region of the central nucleus ultimately sends projections to the hypothalamus that subserve the amygdala's effects on autonomic function. Aside from the dense projection from the lateral nucleus, the basal nucleus also receives projections from the accessory basal nucleus, the central nucleus, and the periamygdaloid cortex. The accessory basal nucleus sends a dense projection to the central nucleus, and relatively weaker projections to the medial nucleus, the "cortical" nuclei, and the intercalated nuclei (Aggleton, 1985). The accessory basal nucleus receives dense input from the lateral nucleus, and less dense inputs from the basal, central, and medial nuclei (Aggleton, 1985).

The central nucleus sends intrinsic projections to the cortical nuclei, the amygdalohippocampal area, the anterior amygdaloid area and light projections to the lateral, basal, and accessory basal nuclei (Price and Amaral, 1981). In summary, there are extensive connections within and between the individual nuclei of the amygdala, which suggest that afferent information is processed substantially before efferent connections initiate the appropriate behavioral and autonomic responses.

The major inputs to the amygdala and their projection to individual subnuclei in the monkey are summarized in **Table 1.1**. Afferent projections to the amygdala can be divided, on the basis of the information content that they transmit, into two groups: those arising from the cortex and thalamus, and those arising from the hypothalamus and brainstem. The former supply the amygdala with sensory input and information from memory systems, while the latter supply the amygdala with information relevant to behavioral and autonomic systems. Most cortical inputs emanate from association areas that have integrated sensory information via extensive cortico-cortical connections; however the amygdala also receives sensory information directly from the thalamus.

The major outputs from the amygdala and their individual subnuclear origin in the monkey are summarized in **Table 1.2**. The efferent projections from the amygdala target cortical, hypothalamic, and brain stem regions. The cortical targets include the same areas from which the amygdala receives projections, but also include areas of unimodal sensory cortex. As such, one organizing feature of amygdala cortical efferent connections is that the amygdala projects back to more areas of the cortical mantle than from which it receives afferent projections. It is likely that these projections allow the amygdala to control sensory input at very early stages of processing. Interestingly, where studies have been conducted, it appears that the unimodal

Table 1.1 Summary of Afferent Projections to the Monkey Amygdala

Origin	Termination	Attributed Function	Reference(s)
Basal Forebrain	Bmc	Modulation of cholinergic signaling	(Hellendall et al., 1986)
Midline thalamic nuclei	CE, B	Modulation of higher cortical and basal ganglia functions	(Mehler, 1980; Van der Werf et al., 2002)
Posterior thalamus	LA, AB, ME, CE	Emotional response to auditory stimuli	(Mehler, 1980; LeDoux et al., 1990)
Hypothalamus	CE, ME, AB, Bpc	Stress axis feedback	(Amaral et al., 1982)
ERC	LA	Emotional modulation of memory	(Amaral et al., 1992)
Hippocampus	Bpc	Contextual Feedback	(Saunders et al., 1988)
TE	LA	Unimodal visual information	(Aggleton et al., 1980; Pandya and Yeterian, 1985)
STG	LA	Auditory input	(Turner et al., 1978)
Sensory Association Cortices	LA	Polymodal sensory input	(Turner et al., 1980)
Frontal Cortex	LA, Bpc, Bmc, AB	Cortical modulation of emotion and arousal	(Aggleton et al., 1980; Mufson et al., 1981)

Abbreviations: AB; accessory basal nucleus of the amygdala, B; basal nucleus of the amygdala (all divisions), Bmc; magnocellular division of the basal nucleus of the amygdala, Bpc; parvocellular division of the basal nucleus of the amygdala, CE; central nucleus of the amygdala, ERC; entorhinal cortex, LA; lateral nucleus of the amygdala, ME; medial nucleus of the amygdala, STG; superior temporal gyrus, TE; anterior inferior temporal gyrus.

Table 1.2 Summary of Efferent Projections from the Monkey Amygdala

Origin	Termination	Attributed Function	Reference(s)
AB	Hippocampus	Emotional memories	(Amaral and Price, 1984)
B, AB	Striatum (esp. NAcc)	Modulation of motor output	(Nauta, 1962)
B, AB, LA	Frontal Cortex	Emotional influence on cognitive function	(Barbas and De Olmos, 1990)
Bmc, AB	Visual cortex (all levels)	Modulation of visual input	(Amaral and Price, 1984)
Bpc	Basal forebrain	Modulation of cholinergic signaling via en passant synapses	(Mesulam et al., 1983)
CE, Bpc, AB	BNST	Relay of amygdala signal to hypothalamus	(Holstege et al., 1985)
CE	Lateral hypothalamus	Autonomic responses	(Price and Amaral, 1981; Swanson, 2000)
CE	Brain stem	Autonomic output	(Price and Amaral, 1981)
CE	VTA, SN	Modulation of dopamine signaling	(Price and Amaral, 1981)
CE,ME	Midline thalamic nuclei	Modulation of higher cortical and basal ganglia functions	(Price and Amaral, 1981; Van der Werf et al., 2002)
LA, AB	ERC	Emotional modulation of memory	(Insausti et al., 1987)
LA, B, AB	MD	Modulation of frontal cortex information flow	(Russchen et al., 1987)

Abbreviations: AB; accessory basal nucleus of the amygdala, B; basal nucleus of the amygdala (all divisions), Bmc; magnocellular division of the basal nucleus of the amygdala, BNST; bed nucleus of the stria terminalis, Bpc; parvocellular division of the basal nucleus of the amygdala, CE; central nucleus of the amygdala, ERC; entorhinal cortex, LA; lateral nucleus of the amygdala, MD; mediodorsal thalamus, ME; medial nucleus of the amygdala, NAcc; nucleus accumbens, STG; superior temporal gyrus, TE; anterior inferior temporal gyrus, SN; substantia nigra, VTA; ventral tegmental area.

sensory cortical efferents do not project from the same locus that received the cortical afferent projections. For example in the visual system, afferents to the amygdala terminate in the lateral nucleus, but efferents projecting back to these areas originate in the basal nucleus. This suggests a second organizing feature of amygdala cortical efferent connections: one or more levels of processing occur before the amygdalocortical loop is closed (Amaral et al., 1992). The hypothalamic efferents mainly arise from the central nucleus of the amygdala, and allow the amygdala to control aspects of ingestive, reproductive, and defensive behaviors. In addition to the direct projection to the hypothalamus, the hypothalamus also receives information from the amygdala via a projection through the bed nucleus of the stria terminalis. Furthermore both the central nucleus and the bed nucleus of the stria terminalis send dense projections to ascending monoaminergic and cholinergic nuclei. Hence the amygdala can modulate a wide range of brain systems through its contacts with serotinergic raphe nucleus and the cholinergic nucleus basalis (Amaral et al., 1992).

1.8. The Amygdala and Emotion

The notion that the amygdala was involved in emotion first was posited by Maclean (1949) when he expanded Papez' circuit to include this temporal lobe structure; however, in this report, it was considered secondary compared to the central role of the hippocampus. Later studies, however, began to emphasize its central role. The conclusive evidence that the amygdala was involved in emotional behavior, especially fear-related behavior, was offered by Weiskrantz et al. (1956) when he performed selective amygdala lesions in monkeys, and reported that the tendency to leave an enclosure which was previously associated with an electric shock extinguished much slower in lesioned animals than in controls. Lesioned animals in these

studies also displayed the unusual behavior of not avoiding, and sometimes approaching, human observers. Further, they tended to accept previously rejected items as food such as meat or feces. These data imply that selective lesions of the amygdala and periamygdalar cortex can replicate many features of the historical Kluver, Bucy syndrome (Kluver and Bucy, 1937). Among these features was a constellation of behaviors that the authors termed "psychic blindness." They summarized these behaviors as follows: "the animal in moving about behaves as if all available objects belonged to one general class, as if they were merely 'something to be approached and examined.'" (Kluver and Bucy, 1939, p. 985) Even in subsequent studies where amygdalar lesion selectivity improved and behavioral testing became more systematic, elements of "psychic blindness" persisted (Horel et al., 1975; Aggleton and Passingham, 1981). The inability to display appropriate fearful tendencies in all of these studies suggests that the amygdala is required for assigning affective value to a variety of external stimuli, and further that it functions in engaging the appropriate response systems to deal with these stimuli.

Recently, researchers have used ibotenic acid to limit lesions only to cells of the amygdala, sparing attendant fibers of passage. In one such study, behavioral tests following recovery from bilateral ibotenic acid lesions revealed a decreased latency to inspect novel objects, and a tendency to accept normally aversive objects such as rubber snakes (Emery et al., 2001). Measurement of cortisol levels following social interaction assays in these animals revealed a small, nonsignificant increase above baseline. This finding contrasted the significantly elevated levels seen in sham-operated control monkeys in the same situations. The authors concluded that these studies are in line with the hypothesis that amygdala-lesioned animals do not view novel social interactions and certain aversive objects as anxiogenic. Interestingly, a restraint stimulus could still activate the stress axis at levels comparable to

control animals, suggesting that certain nonsocial stimuli could elicit the physiological response to stressful situations even in the amygdala's absence. Nevertheless, these studies also imply that normal fear-related behaviors require an intact amygdala in nonhuman primates.

In an elegant experiment, Downer et al. (1961) worked out some aspects of the circuitry which appears to function in attaching an emotional tag to aversive visual stimuli. In this experiment the author placed a unilateral amygdala lesion together with dissection of the optic chiasm and forebrain commissures in an experimental monkey. When the monkey viewed a threatening visual stimulus through the eye that was connected to the amygdalectomized hemisphere, tameness ensued. The same stimulus, however, could elicit wild, defensive behavior when viewed through the opposite eye. This experiment suggests that cortically processed visual stimuli must reach the amygdala for the association of affective significance. This idea was solidified by later experiments that selectively lesioned the temporal cortex visual areas or their connection to the amygdala (Akert et al., 1961; Horel et al., 1975).

More recent work in rats has begun to examine in detail the circuits involved with emotional behaviors, especially fear-related behaviors. These studies use Pavlovian conditioning to elicit fear responses to stimuli that are normally viewed as nonthreatening. For example after several pairings of a neutral stimulus (e.g. tone, or light) with an aversive stimulus (e.g. foot shock), the neutral stimulus alone can elicit defensive behaviors (e.g. freezing, startle), autonomic nervous system responses (e.g. increases in blood pressure and heart rate), and neuroendocrine responses (release of pituitary and adrenal hormones) (Maren and Fanselow, 1996). These are innate responses to dangerous stimuli, and thus fear-conditioning allows previously neutral stimuli to engage innate mechanisms evolved to manage environmental threats.

In tandem with lesions, fear-conditioning has elucidated much of the intrinsic and extrinsic circuitry responsible for emotionality. The amygdala receives projections in the auditory domain from both the medial geniculate thalamus and the auditory cortex (Turner et al., 1980; Amaral et al., 1992). In a study that lesioned either the auditory portions of the thalamus that project to the amygdala, or the auditory cortex, or both; the relative ability for these areas to affect fear-conditioning was examined (Romanski and LeDoux, 1992). Only the combined lesions were able to block elevations in blood pressure and freezing behaviors elicited by the conditioned stimulus suggesting that either the thalamoamygdalar or corticoamygdalar pathway was sufficient. In this paradigm, rats will also elicit fearful responses when returned to the chamber in which the original training took place, a phenomenon known as contextual fear. Following hippocampal lesions the freezing responses to contextual stimuli are reduced, but only when the lesions are made at early timepoints following pairing (Kim and Fanselow, 1992). Interestingly, the freezing responses to the conditioned tone are not affected at any time by the same lesions. These data suggest that the association of a fearful context requires the hippocampal connections with the basal and accessory basal nuclei, but that normal freezing responses function independently of this connection. Other studies showed that the acoustic startle reflex potentiation of a light stimulus previously conditioned to a foot shock was eliminated by lesions of the central nucleus, but not by lesions of the cerebellar peduncles or the red nucleus (Hitchcock and Davis, 1986). Lesions of distinct targets of the central nucleus affected selective fear behaviors. For example damage to the lateral hypothalamus affected blood pressure but not freezing response, while damage to the periaqueductal grey interfered with freezing but not blood pressure response (LeDoux et al., 1988). Similarly damage to the bed nucleus of the stria terminalis disrupted the conditioned release of pituitary adrenal hormones but

had no effect on either blood pressure or freezing behaviors (Van de Kar et al., 1991). Although expression of the full fear response requires the central nucleus, interestingly, certain features remain following its lesion. In a study that coupled fear conditioning, learning an escape task, and lesions of either the lateral, basal, or central nucleus each lesion had a different ultimate effect (Amorapanth et al., 2000). Lesions of the lateral nucleus disrupted both the conditioned fear responses and the ability to perform the escape task, lesions of the basal nucleus only interfered with the escape task performance, and lesions of the central nucleus interfered with freezing response, but interestingly left the escape behaviors intact.

Damage to the amygdala (Bechara et al., 1995) or areas of the temporal cortex that include the amygdala (LaBar et al., 1995) also have been shown to result in deficits in fear conditioning in humans. The former study measured galvanic skin responses while subjects were exposed to a laboratory fear conditioning paradigm in three patients: one with bilateral amygdala lesions, one with bilateral hippocampus lesions, and one with bilateral lesions of both structures. The patient with bilateral amygdala lesions did not have a conditioned galvanic skin response to, however could recall the facts about, the stimulus that was paired with a sudden loud noise. Conversely, the patient with bilateral hippocampus lesions was not impaired in acquiring the galvanic skin response, but nevertheless was unable to recall the facts about which stimulus preceded the noise. The patient with lesions encompassing both structures was impaired in both galvanic skin response and factual recall. Later studies by this group examined amygdaloid area activation by fMRI in normal subjects while learning the associations between a neutral stimulus and an aversive shock stimulus (LaBar et al., 1998). An increase in signal was observed in the amygdala during the early stages of both acquisition and extinction of the stimulus pairings. Furthermore, these activations were proportional to measures of the galvanic skin response.

Thus it appears that fear-conditioning in humans shares many features with fear-conditioning in rats, namely, underlying circuitry and temporal dynamics.

In summary, the fear conditioning paradigm has been extensively studied as a model emotional circuit in the rat. Different pathways within the amygdala subserve different aspects of fear-conditioning depending on the exact nature of the conditioned stimulus and the learning task. For conditioning to an auditory stimulus, information enters the amygdala through the lateral nucleus, and the lateral nucleus to central nucleus connection is required for appropriate autonomic responses. To engage brain systems which underlie the instrumental learning of an avoidance task, the basal nucleus is required, and presumably actions are directed through its connections with the ventral striatum (Everitt and Robbins, 1992). For contextual conditioning, the lateral nucleus is not required; connections between the hippocampus, basal nucleus and central nucleus suffice. It is likely that these specific functions may apply to more general stimuli, and hence the amygdala may modulate the same types of reactions to more complex stimuli. For example, neurons in the monkey amygdala have been shown to respond to both faces and novelty (Rolls, 1992).

1.9. The Amygdala and Social Behavior

Behavioral studies in primates following bilateral amygdala lesions have provided much of the data leading to conclusions concerning its role in modulating socioemotional function. Initial studies examined the ability for lesioned monkeys to function following reintroduction into their group setting. The first study that introduced amygdala lesions for the purpose of studying their effects on social behavior (Rosvold et al., 1954) found that high-ranking rhesus monkeys fell in rank following reintroduction into a group setting. Interestingly, this study

reported differences in aggressive behavior in lesioned animals depending on whether these behaviors were measured in the group setting or in single cages. This finding may reflect the fact that aggressiveness was measured toward human observers in the single cage setting, but toward other monkeys in the group setting. In studies of free ranging rhesus monkeys, amygdala lesioned animals failed to return to their native group as opposed to their sham-operated counterparts (Dicks et al., 1968). In fact the majority of operated animals died from wounds or predation within a few weeks of release. In a similar study in free-ranging vervets, amygdalectomized animals in every case also failed to reenter social groups (Kling et al., 1970). Additionally, these animals failed to respond to social solicitations from other animals. These studies clearly demonstrated behavioral differences since unoperated, sequestered controls were able to reenter social groups upon release. However, long-term adaptation strategy could not be assessed since all animals save one were lost within a few hours of release.

These early studies were limited by the nature of the lesioning techniques available; lesions were typically accomplished by either surgical ablation or radiofrequency ablation. Both of these techniques necessarily disrupt en passant fibers coursing through the amygdala from areas of the surrounding medial temporal cortex, and additionally these techniques often directly ablate the neighboring perirhinal and entorhinal cortices. Newer lesioning methods which combine stereotaxic localization under MRI guidance with ibotenic acid are able to selectively lesion only the amygdala, and spare the fibers of passage. These experiments have refined what is known about how the amygdala controls social interactions.

The group of Amaral et al. has examined the behavior and to a limited extent, physiology, of both adult and infant rhesus monkeys receiving ibotenic acid lesions of the amygdala. In the first series of experiments, adult animals received bilateral ablations and were subsequently

tested in dyadic social interactions (Emery et al., 2001). Lesioned animals showed more prosocial behaviors as measured by an increased tendency to engage control animals, increased maintenance of spatial proximity, and decreased expression of anxious behaviors during social interaction compared to control monkeys. Interestingly, stimulus monkeys also showed increased social interaction toward lesioned animals, suggesting that something about their behaviors makes them more attractive to other conspecifics. In another series of experiments, the effects of lesions given early in infancy (two weeks) were examined (Prather et al., 2001). It was hypothesized that the amygdala may be necessary for the development of species typical interactions. Lesions at this age resulted in decreased fear of novel and frightening objects compared to control monkeys, but in opposition to the effects of adult lesions, lesions at this age resulted in increased fear behaviors during social interactions. Hence it appears that lesioning early in life is able to dissociate different types of fear systems in the monkey. Subsequent studies of the development of maternal infant interactions following neonatal amygdala lesions suggest that apart from spending more time in physical contact with their mothers, amygdala lesioned animals do not differ from controls (Bauman et al., 2004b). The increased contact time with their mothers was particularly observed when other monkeys were present in the observations, suggesting that these monkeys may again be responding to an increased level of social anxiety. Interestingly following separations from their mothers at six months of age lesioned animals fail to show a species typical separation response and a preference for their mothers upon reunion. Although it is not currently clear why age at lesioning results in different patterns of social fear, it is clear that, at all ages, amygdala lesions consistently disrupt the ability to appropriately evaluate potential social dangers.

As in studies of fear conditioning, studies in normal humans and those with amygdala damage also hinder some aspects of social function. In the first experiment that will be described, a social stimulus (angry face) was paired with an aversive stimulus (loud burst of noise) (Morris et al., 1998). After conditioning, when the angry face was presented briefly such that subjects were not able to recall seeing the face, a significant activation was observed by PET imaging in the right amygdala. When the stimulus was presented for longer periods of time a significant activation was observed in the left amygdala. This extends the earlier fear conditioning studies by using a social stimulus as the conditioned stimulus. Further, differences in right and left amygdala activations support a dissociating role dependent on whether the subjects gained conscious awareness of the angry face stimulus. Another set of experiments measured the ability of humans with bilateral amygdala damage to assess the emotional valence of face stimuli (Adolphs et al., 1998). Subjects were asked to rate on the basis of trustworthiness and approachability, the 50 faces that were most negatively rated by controls. In this task, the amygdala damaged subjects consistently rated these faces positively compared to normal and nonamygdala brain-damaged controls. Interestingly, when asked to judge a person based on a short biography, these subjects' responses were equivalent to controls suggesting that the deficit is one of gaining access to social memories based on visual stimuli versus verbal stimuli.

1.10. The Model System

In this brief introduction, we reviewed earlier studies of maternal-infant social bond disruption in a variety of mammals. Consistently behaviors were observed that indicated pathology in socioemotional behavioral functions. Among these were differences in social interaction, interactions to novelty, anxious behaviors, and abnormal stress responses to

anxiogenic stimuli. This constellation of behaviors suggests that the amygdala may be modulating some of the lasting effects of the stress of early life maternal separation. Differences in the lasting effects were seen depending on the type, duration, and timing of the maternal separation stress. We have developed a model system which examines in more detail, and in a more precise manner, how the timing of the early life stress may affect long term outcomes in rhesus monkeys. It is the purpose of this section to describe this model and highlight the relevant earlier findings.

During the past several years, as part of a program project on the "Psychopathology of Childhood Anxiety and Depression," Cameron et al. (Cameron, 2001; Bytheway, 2004; McCormick, 2005; Rockcastle, 2005) have developed a monkey model for examining the neurobiological underpinnings of how early stressful life experiences lead to changes in anxious and depressive behaviors. This model was designed in an effort to answer two questions: (1) would infant monkeys reared with maternal separation show increased anxious and depressive behaviors if they were reared in a more normative social context than in previous studies (i.e., akin to what would happen to a child if it experienced separation from its mother in early development), and (2) does the timing of experiencing an early stressful life event (maternal separation) influence the behavioral outcome?

To address these issues a study was initiated in which infant rhesus monkeys were reared in small social groups. At the time of maternal separation the mother was removed from the infant and the group, but the infant was left within the group throughout development. To determine how the timing of maternal separation influences behavioral outcome, mothers were removed at one of three times: one week of age, one month of age, or six months of age. In the colony at the University of Pittsburgh Primate Research Laboratory, as in many other breeding

programs world-wide, six months is the normal time of weaning, thus the six month group was considered the control group in this project. The behavioral effects of early maternal separation are described in the paragraphs below.

Maternal Separation at Six Months: Control infants spend the vast majority of their time in close ventral contact with their mothers during the first three months of life, and then from 3-6 months of age they progressively spend less and less time with their mothers. As they gain independence from their mothers they progressively increase a variety of categories of behavior, including social contact with other animals, locomotion and exploration, and time spent alone. Progressive increases in eating and play activities are also seen. By about six months of age infants spend relatively little time with their mother and they go on to develop species normal behaviors when she is removed then. Longer term behavioral analyses at four years of age indicate that at this age the control monkeys are spending about 20% of their time in close social contact with other monkeys (including grooming, huddling together, and sitting in physical contact), and the other 80% of their time exploring the environment, playing, eating, and sitting alone.

Maternal Separation at One Month: Infants whose mothers were removed at one month of age displayed extreme withdrawal behaviors for variable portions of the first several weeks after maternal separation. Generally, for several weeks after re-entering the pen they show little movement, generally staying curled up, and interacting very little with the environment or other monkeys. After a variable length of time they start to interact more and each formed a strong pairing with another animal. After that point they stay in close physical contact with the other monkey, and over development this level of social contact did not decrease like it does in the control monkeys. This pattern of behavior has remained apparent throughout development and is

still observed at four years of age. The increase in time spent in social contact is accompanied by a statistically significant decrease in time spent alone, however the level of other activities such as eating and locomotion are not compromised at older ages. Thus, maternal separation at one month of age had profound short-term consequences, with infants displaying dramatic behavioral withdrawal symptoms, and then showing substantial long-term increases in the amount of social contact that they seek. This pattern of behavior is very similar to the "indiscriminate" form of reactive attachment disorder found in children who have experienced neglect or abuse in early childhood (Richters and Volkmar, 1994).

Maternal Separation at One Week: Infants whose mothers were removed at one week of age also displayed marked changes in behavior, but surprisingly these changes were dissimilar to those displayed by the one-month separated monkeys. Maternal separation at one week of age resulted in no immediate behavioral withdrawal, rather the infants learned to bottle feed very rapidly and appeared fairly active and exploratory. Strikingly, however, the one-week separated monkeys did not develop social contacts with the other monkeys in the pen at any time throughout development. Other monkeys showed a moderate amount of interest in the one-week separated monkeys and routinely initiated some contact. However, when contact was initiated by penmates, the one week separated infants generally froze until the other monkey terminated contact. This pattern of behavior remained present throughout the first year of development. Long term assessment at four years of age indicates they continue to show very little social contact with penmates. In general, the freezing behavior has decreased as the animals age, such that now they appear rather calm when penmates give them attention, although they do not reciprocate or show any seeking of social contact. Interestingly, as they have started to go through puberty, some of the 1-week separated monkeys have had sexual interactions, but

without accompanying grooming or any other form of social contact. Some, but not all, of the 1-week separated monkeys have developed attachments to snuggly toys, which they carry with them much of the time throughout development. One-week separated monkeys have learned to locomote at a normal rate, and spend a similar amount of time eating and moving compared to control monkeys. However, they have spent significantly more time in isolation from the group (some of them staying by themselves in the remote nursery approximately half the time, even at 2-3 years of age) compared to control monkeys. Additionally, several of the one-week separated monkeys developed stereotypies, which include rocking (i.e., sitting in a huddled position with head down and body clasped and moving back and forth), and thumb and toe sucking behaviors. Thus, maternal separation at 1-week of age had profound developmental consequences which were different than those displayed by the 1-month separated monkeys, with a striking decrease in social contact and environmental exploration, and an increase in self-directed behaviors. This pattern of behavior is very similar to the "inhibited" form of reactive attachment disorder found in children who have experienced neglect or abuse in early childhood, with ambivalence to social interactions (Richters and Volkmar, 1994).

Thus the longitudinal development of these monkeys has been examined in detail, but so far, the acute response has not. In chapter two of this thesis, we will examine whether the acute response to maternal separation also differs with respect to the age of separation in this model. Furthermore, if a difference exists, can a monkey's acute response predict its long term outcome? As described, the long term behavioral deficits are most profound in the realm of socioemotional behaviors such as social affiliation and anxiety. Therefore, in chapter three, we will examine gene expression in the amygdala ask the questions: 1) does the stress of maternal separation

have different effects in this brain structure depending on the age at separation, and 2) do the expression of any genes in the amygdala correlate with the longer-term behavioral assessments?

2. Acute and Chronic Behavioral Reaction to Early Social Bond Disruption

2.1. Abstract

Monkeys who experience the stress of maternal –infant social bond disruption have an initial reaction characterized by increases in vocalizations, anxious behaviors, and comforting behaviors. Long-term these monkeys go on to develop differences in social interaction, self-directed behaviors, and anxious behaviors that depend on the timing of the initial stress experience. The goal of these studies was to determine if: 1) the initial reaction to maternal separation depends on the timing of the stressor, and 2) aspects of the initial reaction could predict the long-term behavioral outcome. The subjects of these studies were two groups of monkeys that experienced social bond disruption at either 1 week or 1 month of age (n=4/group). At the time of maternal removal the infants were placed in a nursery cage immediately adjacent to their social rearing group environment. Monkeys were videotaped 9-11 times (10-minute observations) over a five day period in the acute separation environment. After this initial period of learning to bottle-feed, monkeys were placed back into their social rearing groups. In their social group environment the monkeys were videotaped twice a week (15-minute observations); videotapes collected during the third month of life were analyzed for this study. For each of these observation periods behaviors were coded for duration and frequency according to a custom designed behavioral ethogram suited to each environment. Reported levels of behavioral expression are the average over all sessions for a given period (acute or 3-month). In the acute setting significant differences were observed in the time spent adjacent to the social rearing group ($p=0.001$) and the time spent in contact with a soft cotton snuggly ($p=0.03$). During the third month of life differences in separated monkeys were compared to an additional control group of maternally raised monkeys. Significant difference between groups were seen in self-comforting behaviors ($p=0.002$) and typical social behaviors ($p=0.01$). When considering all

separated monkeys together, several behaviors were correlated between the acute and 3-month periods: time spent adjacent acutely and self-comforting behavior during the third month of life ($r=-0.785$, $p=0.037$), time spent in contact with the snuggly acutely and typical social behaviors during the third month of life ($r=0.777$, $p=0.023$), and time spent in contact with the snuggly acutely and self-comforting behavior during the third month of life ($r=0.799$ $p=0.031$). Additionally measures of locomotion were significant between the two periods of observation ($r=0.846$, $p=0.016$). The relationship of these studies to studies of coping in humans will be discussed.

2.2. Introduction

Infants and children who experience adverse early life events carry a greater risk for developing future mental health problems. For example children who experience early-life stress in the form of prolonged hospitalizations, abuse and neglect, or parental loss have increased incidences of mental illness, especially anxiety and depressive disorders, in adolescence and adulthood (Harris et al., 1986; Breier et al., 1988; Kendler et al., 1992; Agid et al., 1999). Furthermore infants who have spent long periods of time in orphanages with very little social interaction display attachment problems, inattention, overactivity, autistic features, and cognitive impairment later in life (O'Connor et al., 2003), even when adopted into stable families (Rutter 2001). Similarly, monkeys who encounter early-life stress in the form of maternal separation, exhibit features of social behavioral dysfunction, anxiety and attachment disorders (Seay and Harlow, 1965; Capitanio et al., 1986; Andrews and Rosenblum, 1991; Cameron, 2001; Rockcastle, 2005). In monkeys, as in children, these stresses lead to the acute development of distress, followed by withdrawal, decreases in social interactions, increases in self-directed behavior, and increases in the display of anxious behaviors in response to stress (Harlow and

Harlow, 1965; McKinney et al., 1971; Suomi et al., 1976; Capitanio et al., 1986; Cameron, 2001; Rockcastle, 2005). These data from humans and nonhuman primates indicate that the social environment in the postnatal period is critical for the shaping of many normal and abnormal behaviors later in life, especially those related to social function and anxiety. Indeed, this has also been shown in rodents who experience differences in early maternal care (Caldji et al., 1998; Kalinichev et al., 2000; Pryce et al., 2003) The consistency of these effects suggests that the early relationship with a caregiver is of paramount importance in the developing brain.

Recent work in monkeys has shown that the timing of the early life stress is critical for determining the exact nature of the long-term behavioral expression (Cameron, 2001; McCormick, 2005). Compared to monkeys raised with their mothers for the first three months of life, monkeys experiencing maternal separation at one-week of age display decreased social interaction and increased anxious behaviors later in life, while monkeys experiencing maternal separation at one-month of age display increased social interaction and increased anxious behaviors, particularly when placed in social isolation. The importance of timing has also been elucidated by rodent work (Levine and Lewis, 1959). These studies suggest that a sensitive period of maternal care is required for the development of species-normal behaviors, and that between one week and one month of age systems underlying social behavior are particularly plastic. In these earlier studies the adult pattern of behaviors following early postnatal maternal separation begins to stabilize after an initial 1-2 month period of increased variability (Cameron, 2001; McCormick, 2005). Interestingly, at this stage some infant monkeys who experience the stress of maternal separation display relatively normal behaviors, suggesting that other environmental or heritable factors also interact to shape the behavioral response to maternal separation. Knowledge of the chronic behavior patterns at 2-3 months of age, however, may be

too late with regard to optimal therapeutic interventions. In fact, it has been shown that following maternal separation, adoption by a foster-mother can rescue species-normal behaviors, but only if this adoption occurs within one-month of maternal separation (McCormick, 2004). Since not all monkeys are affected equally, and optimal therapeutic intervention in those that are substantially affected must be initiated early, earlier indication of behavioral pathology would greatly inform treatments aimed at altering the chronic behavioral phenotype. Early indications of later psychopathology have also been important in some clinical studies of acutely stressful life events (Foa et al., 1995).

The ability to infer information about long-term behavior from the acute response to maternal deprivation in monkeys has been examined previously. These studies have correlated behavior and physiological measures of stress during a temporary, 10-day maternal separation (23-35 wks of age) with response to unfamiliar and threatening situations later in life (Snyder et al., 1981; Capitanio et al., 1986). Increased depressive-like behaviors and heart rate at separation were correlated with response to novel social and nonsocial stimuli measured 2.5-4 yrs later. It can be argued that both of these measures may reflect the animal's sensitivity to acute stress and that after the earliest time assessed (6 mos.) this response is essentially hard-wired. This interpretation may be especially relevant since the native behavior in the home environment following this brief maternal separation and reunion was not affected in the long term (Snyder et al., 1981). Although these studies elegantly showed that different measures of acute stress are related within individual monkeys over several years and through puberty, several questions remain unanswered.

In the current study we aim to examine whether the correlations between a monkey's acute reaction to maternal separation and its later behavior are maintained even when evaluated

after maternal separation much earlier in development, and furthermore whether they can be assessed by the monkeys native group behavior. Thus in this study, we will: 1) characterize the difference in the acute response to maternal separation between one-week and one-month separated monkeys, 2) confirm that the previously reported 2-3 month phenotype (Cameron et al., 2005) is evident in this cohort of monkeys, and 3) identify features of the acute behavioral reaction to maternal separation that may predict the 2-3 month behavioral phenotype, the earliest time point when the chronic behavioral reaction manifests.

2.3. Methods

Animal Subjects

A total of twelve female rhesus monkeys born in the breeding colony at the University of Pittsburgh Primate Research Laboratory were used for these institutional IACUC approved studies. At birth each monkey was arbitrarily selected to enter the "one-week separated", "one-month separated", or "control" experimental groups (n=4 per group). All monkeys in this study spent the first week of life in a single cage with their mother. During this initial period mothers and infants were fed Purina Monkey Chow once daily (no. 5045, Ralston Purina Co, St. Louis, MO), and offered water ad libitum, although at this age the infants receive their nutrition primarily from breast milk. They were housed in 4-bank cages in temperature controlled (24 +/- 2°C) communal rooms with artificial lighting from 0700 to 1900h. After the first week of life the animals were handled differently depending on experimental group assignment. At one-week of age monkeys designated as "one-week separated" were removed from their mothers and placed in a single nursery cage juxtaposed to the group rearing pen that they would ultimately join. After a 5-7 day period of learning to bottle-feed, the monkeys were introduced into the group rearing environment. One-week separated monkeys spent weeks 3-12 in the group rearing

environment. Conversely, at one-week of age, monkeys designated as "one-month separated" were introduced into the group rearing pen with their mothers. One-month separated monkeys spent weeks 2-4 in the group rearing environment. At 4 weeks of age, one-month separated monkeys' mothers were removed from the group rearing environment and the infants were placed in the single nursery cage positioned identically as described for the one-week separated group. After a 5-7 day period of learning to bottle-feed, these monkeys were reintroduced into the group rearing environment. One-month separated monkeys then spent weeks 6-12 in the group rearing environment. "Control" monkeys were similarly introduced into the group rearing pen at 1 week of age with their mother, and they remained there for the study's entirety, weeks 2-12. For complete descriptions of the nursery cage and group rearing environment see below.

Nursery Cage Environment. The single nursery cage was placed approximately 1-2 feet immediately in front of the group rearing pen. This position of the single nursery cage allowed the infant to maintain visual and auditory interaction with the monkeys in the group rearing pen. The single nursery cage contained two hanging bottles of infant formula, towels for bedding, and a snuggly toy. Artificial lighting was on between 0700 and 1900h, and some natural light entered the area via skylights above each pen. The infant monkeys were fed initially 4 times per day for the first 2-3 days following maternal removal, and then 2-3 times per day thereafter, depending on willingness to self-feed from the hanging bottles. For each feeding, the formula was refreshed and hung on the cage wall adjacent to the group rearing pen. When necessary the infants were held up to the hanging bottles to stimulate feeding. This arrangement offered the best compromise between ensuring adequate nutrition while minimizing human contact. Weight was monitored daily to guarantee that the monkeys were ingesting appropriate amounts of

formula. Both groups of monkeys required about 1 week in the nursery cage to learn bottle-feeding; after that period monkeys were moved into their adjacent group rearing pen.

Group Rearing Environment. The group rearing pen consisted of 4-5 monkeys of varying ages with no monkey more dominant than the adult female mother (when present). This age / sex distribution minimized any disturbances that may be caused by competition for dominance. The group rearing pen, measuring 14 ft. by 11 ft. by 12 ft., was cement block construction with a several inch bed of wood shavings on the floor, 4 perches at various heights, and a chain-link penfront. A "nursery chamber", measuring 2 ft x 2 ft x 3 ft, was placed in each pen and locked to the chain-link penfront so that it could not be moved. The nursery had a small doorway with dimensions such that infants could enter but larger animals in the pen could not enter. Bottles of formula were provided in the nursery for infants whose mothers had been removed in the first month of life, so that the formula was available to the infants but not to all monkeys in the pen. Toys were often available in the pens, including tire swings, swinging balls, chew toys, and snuggly toys. Monkeys were fed one meal a day between 0900 and 1000 hours of Purina high protein monkey chow supplemented several times a week with fresh fruit and seeds strewn in the bedding to encourage foraging. Water was available *ad libitum*. Artificial lighting in the hallways was on from 0700 to 1900 hours each day, and sky lights above each pen admitted natural light. Temperature was maintained at 24±2 C.

Experimental Paradigm

All experiments were reviewed and approved by the Animal Care and Use Committee of the University of Pittsburgh.

Acute Separation Behavior. Each maternally separated monkey (4 -one week separated and 4 – one month separated) was videotaped in the single nursery cage setting for 10 minutes per focal

session. This occurred 2-4 times per day for the first 3 days following maternal separation, and every other day thereafter, until the infant was transferred to the group housing pen. An acute behavior ethogram was developed that consisted of 15 behaviors distributed in 3 channels (Table 2.1). Multiple channels allowed the simultaneous scoring of several exclusive behaviors. For example, a monkey could be scored as "adjacent" on the location channel, while simultaneously being scored as "climbing" on the behavior channel. For each focal session, the total time scored for all elements in a given channel must sum to 10 minutes. Scoring was accomplished by a single observer via videotape analysis with the aid of behavioral observation software (Observer Video Pro, version 5.0, Noldus Information Technology, The Netherlands). Although blinding was impossible because the group differences were distinct, it was unlikely since: 1) the tapes are observed in a frame-by-frame analysis in slow motion, and 2) behaviors are rigorously defined, leaving no room for observer interpretation. For each behavior session, behaviors were systematically coded in a frame-by-frame manner with the aid of a VCR equipped with freeze-frame and slow motion capabilities. Each behavior was linked with a unique series of keystrokes, and the software tracked the duration and frequency of each behavior via its linkage to the VCR time-clock. For each session the total time spent engaging in each behavior was compiled. For most behaviors, the total time for each focal session was adjusted for the time spent in passive sit, a behavior that includes, and is not distinguishable from sleeping. Hence, passive sit, when reported is a percentage of total time, while all other behaviors are reported as a percentage of time not spent in passive sit. For each monkey, the duration data were averaged over all of the focal sessions (9-11 per animal) to obtain a single measure for each behavior. These values were then used to compute the mean and standard deviation for the duration of each behavior in each group of experimental monkeys. In some cases, aggregate behaviors were

Table 2.1 Acute separation ethogram

Behavior	*Description*
Adjacent	Touching the wall adjacent to and facing the group-rearing pen
Snuggly	Contact with the snuggly toy
Climbing	Moving up or down on cage wall
Stationary	Not moving in a standing position or hanging on a cage wall
Locomote	Walking, running, or pacing on the cage floor
Jumping	Jumping on cage floor or from wall to cage floor
Cage Bite	Biting the cage
Self-comforting	Thumb-sucking or toe-sucking
Self Scratch	Scratching oneself or picking at hair or skin
Active Sit	Sitting position with head raised and alert
Passive Sit	Sitting position with head down or sleeping
Drink	Drinking infant formula
Agitated	Rapid head movements
Groom	Picking or brushing ones hair or skin
Explore	Manipulation of cage or cage objects

defined that were the sum of several exclusive behaviors. For example, the aggregate behavior "total locomotion" consisted of the sum of the individual behaviors "locomote", "climb", and "jump".

3-month Social Group Behavior. Each focal monkey (4 per group in one-week separated, one-month separated, and control groups) was videotaped in its group rearing environment twice per week for 15 minutes. One focal session per week was obtained in the morning prior to the 0900 feeding, and one session was obtained in the afternoon. A social group housing ethogram was adapted from previous studies in our lab (Cameron, 2001) that consisted of 18 behaviors distributed in 4 channels (Table 2.2). Multiple channels allowed the simultaneous scoring of several unrelated behaviors. For each focal session, the total time scored for all elements in a given channel must sum to 15 minutes. Scoring was accomplished by a single observer via videotape analysis with the aid of behavioral observation software (Observer Video Pro) as described in the acute behavior section previously. Each 15-minute, individual focal session was completely analyzed for duration and frequency of all behaviors. For most behaviors, the total time for each focal session was adjusted for the time spent in the nursery chamber on the location channel. This chamber did not allow an unobstructed view of the experimental monkeys, and therefore the time spent in the chamber could not be reliably scored. Hence "Nursery", when reported, is a percentage of the total time, while all other behaviors (unless noted) are reported as a percentage of the time not spent in the nursery chamber. However, the behavioral aggregate termed "Total Nonsocial" includes all behaviors deemed "nonsocial" including "Nursery" (Table 2.2). For this particular aggregate, raw measures of the other behaviors not corrected for the time spent in the nursery were used. For each monkey, the 8 focal sessions (2 per week) during

Table 2.2 Native Social Group Housing Ethogram

Behavior	*Description*
Ventral Contact	Initiate or receive a face-to-face hold with a pen mate
Huddle	Sitting with torso in contact with a pen mate (but not a face-to-face hold)
Touch	Receive or initiate contact with pen mate, not defined as ventral, huddle, or groom
Groom	Picking or brushing a penmates hair or skin
Proximity	Sitting within an arms length of a pen mate, but not touching
Social Play	Initiate or receive play with a pen mate
Atypical Social	Thumb-sucking or toe-sucking while in contact with or proximity to a penmate
Self-comforting	Thumb-sucking or toe-sucking
Agitation	Rapid head movements
Object Play	Manipulating cage or cage objects
Aggression	Receive or initiate threatening gesture or aggressive contact
Stationary	Stationary
Locomote	Walking, running, and pacing in the pen or on a perch
Eat	Foraging for food, putting food in mouth, drinking
Nursery	Inside nursery chamber
Snuggly	Contact with snuggly toy
Fence Biting	Biting or chewing cage at pen front
Scratch	Scratch oneself

weeks 9-12 were used for analysis. Previous studies in our lab suggest that this number of sessions produces a reliable measure of stable behavior at this age (Cameron, 2001).

Statistical Analysis

Acute Separation Behavior. Our first goal was to examine the differences in the acute reaction to maternal separation stress between the one-week and one-month separated monkeys. As described, the behavioral measures in each of the acute focal sessions were averaged for each monkey; these averages were then used to calculate group means and standard deviations for both the one-week separated and one-month separated groups. The distribution of each behavior was tested for normality using the Kolmogorov-Smirnoff statistic. For normally distributed behaviors, we performed a Levene's test for equality of variances and a Student's t-test to compare means. If Levene's test was not significant then the t-test p-value was calculated assuming equal variances, however if Levene's test was significant then the p-value was calculated not assuming equal variances. Each p-value was two-tailed and considered statistically significant at </= 0.05. For behavioral measures that were not distributed normally, an attempt was made to normalize the data using a square root or natural log transformation. If the data failed to normalize then the nonparametric Mann-Whitney test was performed.

3-month Social Group Behavior. Next we wanted to examine group differences in the 3 month stable behavior in this cohort of monkeys. As described, behavioral measures in each of the 8 focal sessions during weeks 9-12 were averaged for each monkey; these averages were then used to calculate group means and standard deviations for the one-week separated, one-month separated groups, and control groups. The distribution of each behavioral measure was tested for normality using the Kolmogorov-Smirnoff statistic. For normally distributed behaviors, we performed a Levene's test for equality of variances and a one-way ANOVA to compare means.

Each ANOVA p-value was two-tailed and considered statistically significant at </= 0.05. When significant, group differences were compared using either *post-hoc* Least Significant Difference contrasts when homogeneity of variance could be assumed or the Games-Howell pairwise comparison test when homogeneity of variance could not be assumed (Atypical Social and Total Self-comforting behaviors). For behavioral measures that were not distributed normally, an attempt was made to normalize the data using a square root or natural log transformation. If the data failed to normalize then the nonparametric Kruskal-Wallis test was performed. Group differences in behavioral measures with a significant Kruskal-Wallis test were compared using the Mann Whitney U-test. Each p-value was two-tailed and considered significant at </= 0.05.

Acute to 3-month Behavior Correlations. Finally, we wanted to examine whether any behavioral aspects of the acute separation period were reflected in the 3-month stable behavior. Selected behavioral measures from both groups were plotted against each other in a scatter plot to determine possible linear relationships. For comparisons which appeared to be linearly related we ran Pearson correlations to determine r values and used a t distribution table with n-2 degrees of freedom to determine the associated two-tailed p-values. R values were considered significant when the associated p-value was </= 0.05. We report both the r value and its associated p value in the results.

2.4. Results

2.4.1. Acute response to maternal separation

For one-week and one-month separated monkeys, each monkey was videotaped for 9-11, 10-minute focal sessions during the immediate post-separation period. In this way we were able to examine the acute response to maternal separation by measuring behaviors relevant to this

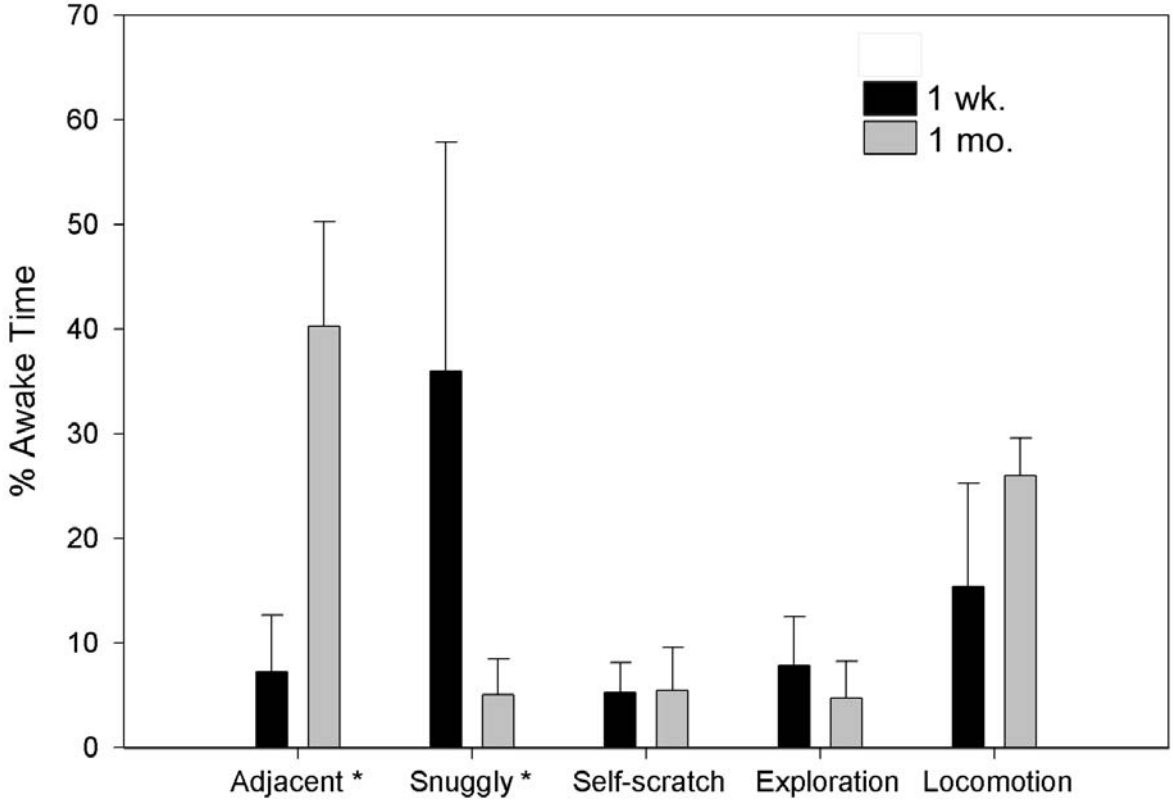
setting (for a complete explanation of the behavioral ethogram see sec. 2.2 "Experimental Paradigm: Acute Behavior" and Table 2.1).

Location. We defined a location channel that indicated what portion of the single cage the acutely separated monkeys preferred. As indicated in Table 2.1, monkeys were scored as *adjacent* if they were in contact with and facing the portion of the single cage wire mesh wall juxtaposed to the group pen environment. When a monkey's behavior did not meet these criteria, they were scored as *not adjacent* on the location channel. These particular behaviors were scored independent of any other behaviors and together were mutually exclusive and collectively exhaustive. One-week separated monkeys spent 7.27 +/- 5.41% of their time adjacent, while one-month separated monkeys spent 40.3 +/- 10.0% of their time adjacent **(**Fig. 2.1). This difference was statistically significant (p=0.001), and suggested that one-month separated monkeys preferred spending more time in social contact with other monkeys during the acute stress of maternal separation.

Snuggly. The behavior "snuggly" is defined as the amount of time a monkey spends in contact with a stuffed animal snuggly toy provided in the acute separation environment. Previous studies suggest that the texture of this toy is comforting to monkeys, and that contact with the snuggly may help relieve stress or reduce its onset. One-week separated monkeys spent 36.0 +/- 21.9% of their time in contact with the snuggly toy, while one-month separated monkeys spent 5.1 +/- 3.4% of their time in contact with the snuggly (Fig. 2.1). A student's t-test revealed that this behavioral measure was significantly different between separation groups (p=0.03).

Self-Scratch. The behavior "self-scratch" is defined as scratching oneself or picking at hair or skin. Previous studies have used self-scratch as a measure of anxiety. One-week separated monkeys spent 5.28 +/- 2.85% of their time engaging in self-scratching behaviors, while one-

Figure 2.1. Acute response to maternal separation. Each separated monkey was videotaped in its nursery cage environment for 9-11 sessions over the 5-7 days following the acute maternal separation. Behaviors were corrected for the time spent in passive sit, a behavior indistuingshable from sleeping, and averaged across all sessions to establish the % awake time each monkey spent in a given behavior. Each bar represents the mean percent time engaged in a given behavior for each of the separation groups. Black bars represent the one-week separated group; grey bars represent the one-month separated group. *Adjacent and Snuggly behaviors showed a significant difference between separation groups ($p < 0.05$). Error bars are one st. dev.


70
60
50
40
30
20
10
0
% Awake Time
1 wk.
1 mo.
Adjacent *
Snuggly *
Self-scratch
Exploration
Locomotion

month separated monkeys spent 5.45 +/- 4.15% of their time engaging in self-scratching behaviors. These data were not significant between separated monkey groups (p=0.949) (Fig. 2.1).

Aggregate Acute Behaviors. We also defined *exploration* in the acute separation environment as digital manipulation and/or close inspection of objects such as bedding towels, formula bottles, or wire-mesh cage. One-week separated monkeys explored their acute separation environment 7.0 +/- 4.7% of their time while one-month separated monkeys explored 4.7 +/- 3.5%. These data were not significant between separated monkey groups (p=0.32) (Fig. 2.1). Another behavioral aggregate, *locomotion,* included the individual behaviors locomotion, climbing, and jumping. One-week separated monkeys spent 15.4 +/- 9.9% of their time engaging in locomotive behaviors, while one-month separated monkeys spent 26.0 +/- 3.6%. These data were also not statistically significant (p=0.089) (Fig. 2.1).

2.4.2. 3-month behavioral response to maternal separation

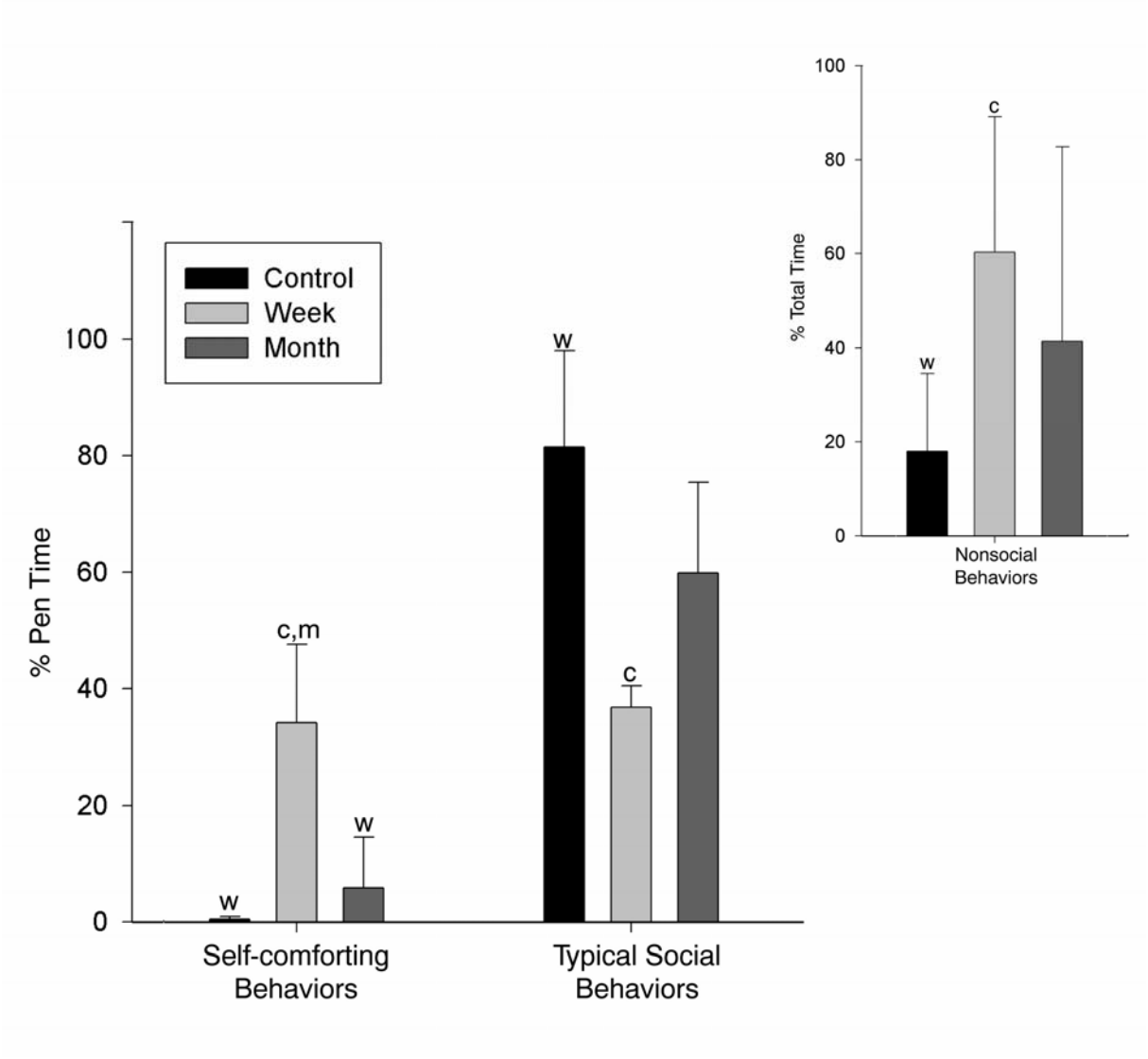
Self-comforting Behavior. In our ethogram, self-comforting behavior is defined as thumb-sucking, toe-sucking, or rocking (repeated back and forth motions usually while grasping the front of the pen) displayed while an animal is not in a social setting. In this cohort during the third month of life, rocking behaviors were not observed, and therefore did not contribute to "self-comforting behaviors". Maternally raised infants engage in scant self-comforting behavior, comprising only 0.12 +/- 0.15% of their group rearing pen time. One-week separated infants spend 14.6 +/- 1.9% and one-month separated infants spend 2.43 +/- 4.18% of their group rearing pen time engaging in self-comforting behavior. One-way analysis of variance suggests that a significance difference exists between groups (p=0.002). Post hoc tests revealed that

significant differences exist between the CO and WK groups (p=0.001) and the WK and MO groups (p=0.003), but no significant difference exists between the CO and MO groups (p=0.257).

In this cohort of monkeys we defined a behavior, "atypical social", which was not previously defined in our past ethograms. We defined this behavior as self-comforting behavior (thumb-sucking or toe-sucking) that occurred in a social setting. The addition of this behavior allowed us to differentiate between typical and atypical social interactions, and also allowed us to track both behaviors when they occurred together. During the third month of life, maternally raised monkeys display negligible atypical social behavior at 0.38 +/- 0.41% of their pen time. One-week separated infants spend 19.5 +/- 12.3% of their pen time engaging in atypical social behavior, while one-month separated infants spend 3.3 +/- 4.7% of their pen time. One-way analysis of variance suggested that there was a statistical difference between groups (p=0.049). Although these data did not statistically meet the criterion of homoscedasity, post hoc tests that did not assume equal variance suggested that a statistical difference existed between the CO and WK groups (p=0.023), but not between the CO and MO groups (p=0.938) or the WK an MO groups (p=0.146). It is worth noting that post hoc test assuming equal variances did suggest a significant difference between the WK and MO groups (p=0.039).

We also evaluated "total self-comforting behaviors" by summing both the self-comforting and atypical social behaviors. Maternally raised infants spent .50 +/- .42%, one-week separated infants spent 34.18 +/- 13.34%, and one-month separated infants spent 5.78% +/- 8.88% of their pen time displaying atypical behavior regardless of social context (Fig. 2.2). One way analysis of variance suggested a group difference in total atypical behavior (p=0.004). Post hoc tests revealed that significant differences existed in both the CO-WK comparison (p=0.024) and WK-MO comparison (p=0.049), but not in the CO-MO comparison (p=0.647) (Fig 2.2).

Figure 2.2. 3-month chronic behavioral response to maternal separation. Each monkey was videotaped in its group rearing environment two times per week, during weeks 9-12. Self-comforting and typical social behaviors were corrected for the time spent in the nursery chamber and averaged across all sessions to establish the % pen-time each monkey spent in a given behavior. Each bar represents the mean percent time engaged in a given behavior for each of the two separation groups and the maternally raised controls. Inset: Nonsocial behaviors included the time spent in the nursery chamber; hence for this measurement behaviors were not corrected. Nonsocial behaviors are measured as a % of total time. c: significantly different from control $p<0.05$, w: significantly different from one-week separated $p<0.05$, m: significantly different from one-month separated $p<0.05$. Error bars are one st. dev.


Control
Week
Month
% Pen Time
100
80
60
40
20
0
w
c,m
w
w
c
Self-comforting Behaviors
Typical Social Behaviors
% Total Time
100
80
60
40
20
0
w
c
Nonsocial Behaviors

Atypical social behaviors accounted for 76%, 57%, and 57% of total atypical behavior for the CO, WK, and MO groups respectively.

Social Behavior. Previous mixed sex cohorts of monkeys in this experimental design displayed large differences in measures of close social behavior at three months of age; maternally raised, one-month separated, and one-week separated spent 75%, 40%, and 10% of their time respectively in close social contact (Cameron, 2001). In this cohort of all female monkeys, we measured the related variable "typical social behaviors." Typical social behaviors consist of ventral contact, touch, huddle, groom, proximity, and social play as defined in Table 2.1. This group of behaviors also requires that the monkey is not engaging in concurrent self-comforting behavior. Considered separately, none of these individual behaviors reach significance at the p=0.05 level because distinct monkeys express their social behaviors in different ways, and this sample size does not allow us to uncover the smaller differences seen in the individual behaviors that were significant in previous studies. However, considered together, in this behavioral aggregate during the third month of life, significant group differences were evident. Maternally raised monkeys spent 81.5 +/- 16.5% of their time engaging in typical social behavior. One-week separated and one-month separated infants spent less time engaging in typical social behavior, 36.8 +/- 3.6% and 59.9 +/- 15.5% respectively (Fig 2.2). One-way analysis of variance suggested that a significant difference exists between groups (p=0.01). Post-hoc tests revealed a significant difference in the CO to WK comparison (p=0.003), and nonsignificant trends in both the CO to MO comparison (p=0.061) and WK to MO comparison (p=0.063). This all-female cohort, then, resembles the previously described results for a mixed-sex cohort at this developmental stage. This aggregate behavior best stratifies the three experimental groups.

We also considered the behavioral group "total social behavior" which includes the typical social behaviors considered above together with atypical social behavior. This measure, then, considered the total time spent in social contact whether that contact was typical or atypical. Maternally raised infants spent 81.85 +/- 16.51% of their home pen time in social contact with other monkeys. One-week separated infants spent 56.36 +/- 15.86% and one-month separated infants spent 63.24 +/- 11.36% of their home pen time in social contact with other monkeys. One-way analysis of variance indicates that this behavioral grouping is not significant at the p=0.05 level between the three experimental groups (p=0.109). However, a student's t-test between control and separated animals (regardless of separation time) revealed a significant difference between groups (p=0.037).

Anxious Behaviors. Studies from our and other laboratories suggest that scratching, cage-biting, and agitation may be related to anxiety in some situations in monkeys. In this cohort, during the third month of life these behaviors were exhibited at modest levels when considered together in a behavioral aggregate labeled "total anxious behaviors". Maternally raised infants displayed anxious behaviors 3.29 +/- 2.03% of their pen time, one-week separated infants spent 3.20 +/- .20%, and one-month separated infants spent 3.78 +/- 2.08%. These behaviors were not significantly changed between these groups of monkeys (p=0.893).

Locomotor Behaviors. Our behavioral ethogram included two behaviors that measure the general locomotor activity of these monkeys, stationary and locomote. These behaviors are scored only when monkeys are not in social contact. Maternally raised monkeys spent 10.01 +/- 9.70%, one-week separated monkeys spent 11.53 +/- 5.24%, and one-month separated monkeys spent 18.62 +/- 5.29% of their native group housing time engaging in stationary behavior. One-way analysis of variance did not support a significant difference in this behavior between groups (p=0.264).

Conversely, maternally raised monkeys spent 6.69 +/- 5.84%, one-week separated monkeys spent 10.01 +/- 6.85%, and one-month separated monkeys spent 12.07 +/- 1.09% of their time engaging in locomotor behaviors. One-way analysis of variance also did not support a significant difference in this behavior between experimental groups (p=0.368). Taken together, these data suggested that general locomotor activity was not affected by experimental group assignment.

Nonsocial Behaviors. We measured two aggregate behaviors reflecting the amount of time that the monkeys spent not engaging in social behaviors, time alone and total nonsocial. Time alone is designated as the amount of pen time that the monkeys spent not engaging in behaviors designated as social; these behaviors include stationary, locomote, eat, agitation, atypical, and object play. Maternally reared monkeys spent 18.15 +/- 17.20% of their pen time in time alone, one-week separated monkeys spent 43.26 +/- 20.41%, and one-month separated monkeys spent 36.72 +/- 13.59%. One-way analysis of variance did not suggest a significant difference between experimental groups (p=0.109). However, a student's t-test between control and separated animals (regardless of separation time) revealed a significant difference between groups (p=0.037).

Total nonsocial behavior was defined as the total time, regardless of location, that a monkey spent in behaviors that were not considered social behaviors. For this measure the time spent in voluntary isolation in the nursery area was considered a nonsocial behavior. Since we included this behavior, the pen behaviors were not corrected for time spent in the nursery, as they were in all previous measures (see methods sec. 2.2). Maternally-raised control monkeys spent 18.0 +/- 16.6% of their time engaging in behaviors contributing to total nonsocial behavior, while one-week separated monkeys spent 60.3 +/- 28.8% and one-month monkeys spent 41.4 +/-

12.9% (Fig. 2.2, inset). One-way analysis of variance suggested that a significant difference existed between experimental groups (p=0.05). Post hoc tests revealed that a significant difference existed between the CO and WK groups (p=0.017), but not between the CO and MO groups (p=0.143) or the WK and MO separated groups (p=0.227).

2.4.3. Acute Behavior Predicts 3-month Behavior

We investigated potential bivariate linear relationships between acute separation environment behaviors and 3-month group-rearing pen environment behaviors using Pearson correlations. Two measures of the acute behavioral response correlate well with the 3-month behavior "self-comforting". The acute behavioral measure "Adjacent" was significantly correlated with the 3-month behavior "self-comforting" (r=-0.785 p=0.037) (Fig 2.3a), while the acute behavioral measure "Snuggly" was significantly correlated with the 3-month group behaviors "self-comforting" (r=0.799 p=0.031) (Fig 2.3b) and "Total Nonsocial" (r=0.777 (p=0.023) (Fig 2.3c). Finally, we examined the relationship between locomotor behaviors in the acute separation period setting and during the third month of life. The two measures were significantly correlated (r=0.846, p=0.016) (Fig 2.4).

2.5. Discussion

In this study we have shown that the types of behaviors displayed acutely following the stress of maternal separation depend on the timing of the maternal-infant bond disruption. Monkeys who experience maternal separation at one-week of age responded acutely by increasing their self-comforting behaviors as measured by their contact preference for a soft cotton snuggly toy compared to monkeys who experience maternal separation just three weeks later, at one-month of age. Compared to the one-week separated monkeys, the one-month separated monkeys responded acutely by increasing their seeking of social contact as measured

Figure 2.3. Acute behavior predicts 3-month social group behavior. Acute behaviors are on the x-axis, and 3-month social group behaviors are on the y-axis. Black circles are one-week separated animals, and grey circles are one-month separated animals. A) the acute % time in adjacent behavior correlates with the % time spent in self-comforting behaviors during the 3rd month ($r=-0.785$, $p=0.037$). B) the acute % time spent in contact with the snuggly correlates with the time spent in self-comforting behaviors during the 3rd month ($r=-0.799$, $p=0.031$). C) the acute % time spent in contact with the snuggly correlates with the time spent engaging in nonsocial behaviors during the 3rd month ($r=0.777$, $p=0.023$).

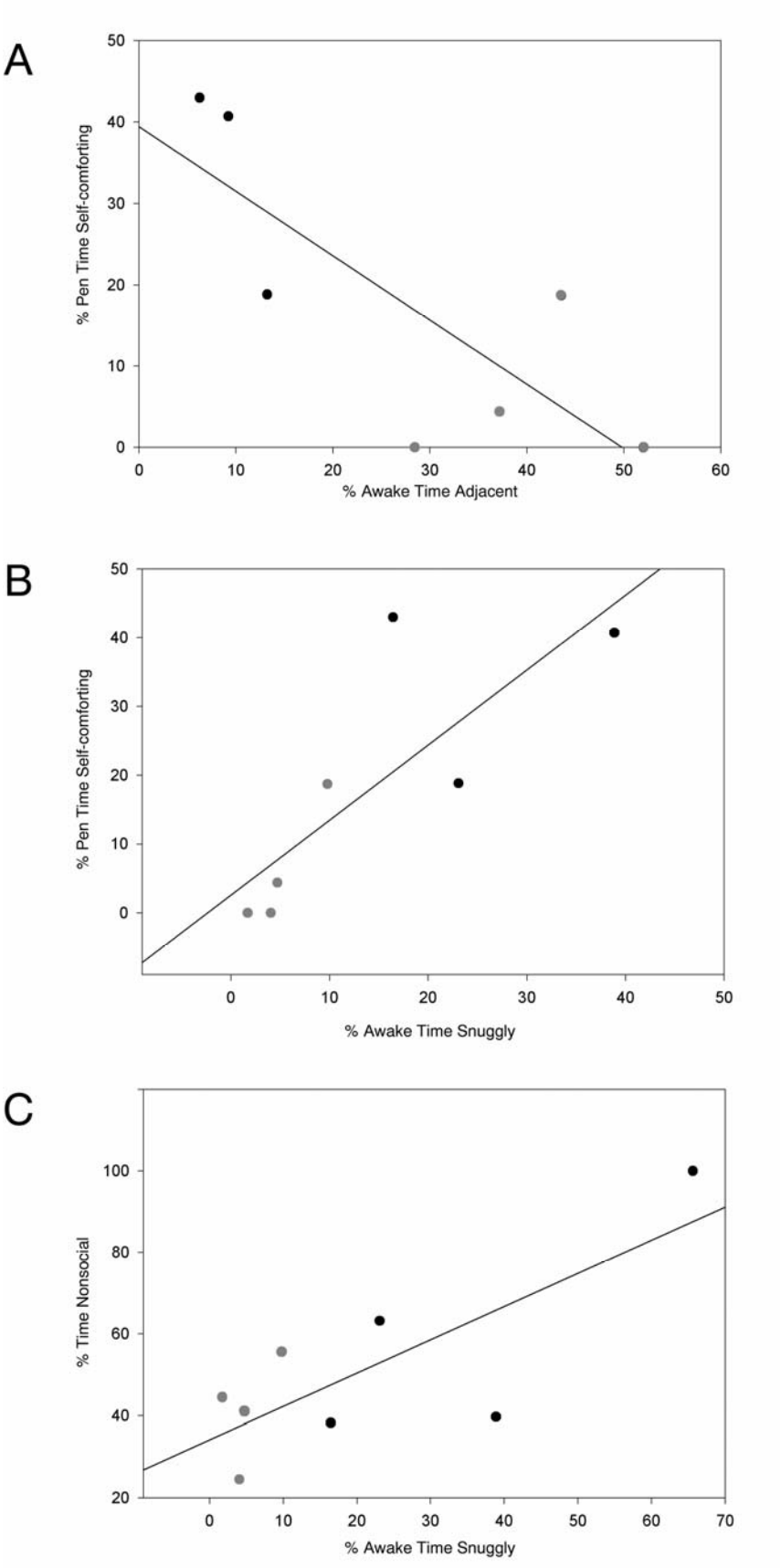
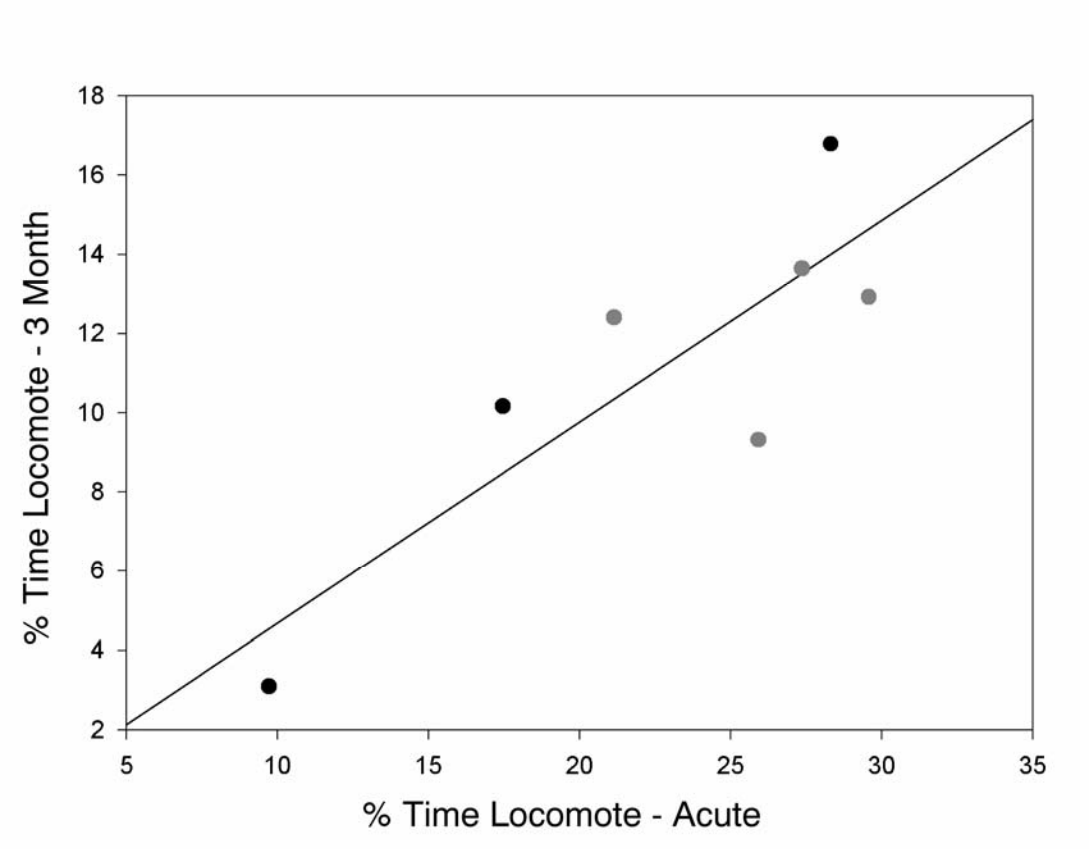

A
% Pen Time Self-comforting
% Awake Time Adjacent
B
% Pen Time Self-comforting
% Awake Time Snuggly
C
% Time Nonsocial
% Awake Time Snuggly

Figure 2.4 Locomotor behaviors. Locomotor behaviors are significantly correlated between the acute separation environment following maternal separation, and the group rearing environment during the 3rd month of life ($r=0.846$, $p=0.016$). Black circles are one-week separated animals; grey circles are one-month separated animals.


% Time Locomote - 3 Month
% Time Locomote - Acute
18
16
14
12
10
8
6
4
2
5
10
15
20
25
30
35

by their increased tendency to locate themselves adjacent to their group-rearing environment. These behaviors have parallels with different coping behaviors exhibited by children responding to stress, namely active coping and avoidant coping (Connor-Smith et al., 2000). Further, these two behaviors are specific since other measures in the acute separation environment between these groups of monkeys do not show significant group differences; exploratory behaviors, locomotive behaviors, anxious behaviors, and stationary behaviors are similar in one-week separated and one-month separated monkeys. Interestingly, these same two behaviors are significantly correlated with aspects of the native behavior assayed in the social group during the third month of life that are indicators of social adjustment and fitness: self-comforting behaviors and social behaviors. It has been previously established that, in this maternal separation experimental design, native social group behavior during the third month of life is the earliest reliable indicator of the chronic behavioral response to maternal separation (Cameron et al., 2005). Taken together, these data provide evidence that facets of the chronic behavioral reaction to the early-life stress of maternal-infant bond disruption in monkeys can be predicted by certain aspects of the monkey's acute response. The parallels to children experiencing stress suggest that this manipulation may be useful as a nonhuman primate model of coping style.

One methodological issue should be addressed before the scientific discussion of the data begins. In order to determine whether aspects of the chronic behavioral response to the early life stress of maternal separation could be predicted by the acute separation response, the chronic behaviors needed to be assessed at a time-point after they mature. Previous work has shown that in this separation paradigm, traces of the acute disturbance to maternal removal persist until 2 months of age for the one-month separated monkeys (Cameron, 2001) After this transition period, the behaviors quickly stabilize and resemble their adolescent and adult patterns. Using

our study methods, the reliable measurement of behaviors requires at least 2 hours of focal sessions for each subject (i.e. 8, 15-minute focal observations). As such, the third month of life is the first time that the chronic response to early maternal separation can be reliably inferred. This time point was chosen since at this time the greatest difference was seen between social behaviors in the one-week and one-month separated infants.

The ability to predict the long-term outcome in monkeys experiencing the substantial early life stress of maternal separation by the individual monkey's acute stress reaction has received little attention in past studies. In the studies that do exist, the maternal separation stress occurred at a much later time in development compared to the current study. For example, in a study of monkeys experiencing the stress of a ten-day maternal separation between four and eight months of age, the response to novel objects in a novel testing environment was related to the level of disturbance displayed during the acute separation phase (Snyder et al., 1981). The predictive power of this study was limited, though, since the age at separation and the age at subsequent testing varied considerably in this sample. Furthermore, the elicited behaviors were negatively correlated with the age of the monkey at testing and positively correlated with the age of the monkey at separation. These correlations suggest that many factors may have contributed to the individual responses. A subsequent study by the same group attempted to partly eliminate the effects of age by examining a subset of these monkeys at a much older age (3.2 - 5.4 years) (Capitanio et al., 1986). In this pool of monkeys there was no correlation between age of initial separation and age at the time of study. The degree of "slouching" during maternal separation, thought to be an indicator of a depressive response, was significantly positively correlated with disturbance behaviors (e.g. stereotypies, crouching, bizarre posturing) during novel cage and object presentations. Interestingly, in contrast with the current study, no correlations with self-

comforting behaviors reached significance. Nevertheless, these studies suggest that reactivity to an acutely stressful situation can predict later adult behavioral responses to anxiogenic stimuli. The present study adds several features to this model. We have shown that the predictive power of the acute stress reaction is evident even when the stressor occurs at very early stages of development in the monkey, and further that it can predict certain features evident in the monkey's native social group environment behavior. Presumably, the monkey's behavior in the social rearing group is not driven by acute stress axis activation, so the fact that the correlation is significant implies that even elements of basal behaviors are modulated by factors which may also influence the acute reaction to maternal separation. It is also possible that there is a tonic elevation of the diurnal cortisol rhythm similar to what is observed in orphanage reared children and children with PTSD and a history of sexual abuse (Carlson and Earls, 1997; De Bellis et al., 1999). It is important to note that although the acute measurements between these studies were very similar (behavior in the immediate post-separation period), the ultimate effects may be quite different since in the previous studies (Snyder et al., 1981; Capitanio et al., 1986) the monkeys were reunited with their mothers after the ten-day separation, but in the current study the monkeys were permanently separated and reared in social groups. The data from this and earlier studies suggest aspects of an individual monkey's temperament already in place early in life contribute to both the acute response to the stress of maternal separation, and the later behavioral responses measured in several different paradigms.

Both the current study and the previous studies (Snyder et al., 1981; Capitanio et al., 1986) that measure the relationship of the acute behavioral response to the stress of maternal separation and its relationship to later behaviors are limited by an inability to determine whether the differences in behavior were mediated by the environment, by genetic tendencies, or more

likely a combination of both. In part, this limitation is due to the fact that all of the studies described utilized the stress of maternal separation at different times in development to produce greater variability in the individual measurements of behavior. This difference in developmental age represents a large environmental factor, and hence, it is not clear whether the heritable factors alone can drive a similar correlation. It should be noted, though, that in the present study, the correlation between the time spent in contact with the snuggly acutely and the time spent engaging in self-comforting behaviors during the third month of life is significant even when limited only to one-month separated monkeys. In order to better tease apart the genetic liability from the environmental influence, a study using many more animals from within one group (either one-week or one-month separated) of monkeys would be required. The conclusions of even a study of this kind would be subject to differences in environment as well, since both the pre-separation period of maternal rearing and the post-separation social environment would be different among individuals. Differences in maternal rearing (Andrews and Rosenblum, 1991) and social environment following maternal separation (Hinde and Davies, 1972; Chappell and Meier, 1975; Hinde et al., 1978) have both been shown to mediate the ultimate behavioral effects in monkeys exposed to early life stress. For these reasons the behavior of other monkeys toward the separated monkey during every phase of development would need to be assessed to positively exclude them from a causative role.

The current study also differed from the others on the basis of the measurement of the long-term effects of the maternal separation stress. In the other studies, the long-term outcome was assayed in acutely anxiogenic testing situations; however in this study, the long-term outcome was assayed by normative social behavior in the stable social group environment. Convergent evidence from other studies of one-month and one-week separated monkeys reared

in social groups suggests that the decreased social and increased self-comforting behaviors are a feature of the one-week separated monkeys; and increased social behavior is a feature of the one-month separated monkeys, in many different assays of the long-term outcome of maternal separation (Cameron, 2001; Bytheway, 2004; McCormick, 2005). These assays include longitudinal assessment over the first year of life in the native social group environment, response to anxiogenic stimuli measured at one-year, and now, the acute behavioral response to the maternal separation stress. The fact that a similar phenotype exists across many different assays of behavior and that they are evident immediately may be suggestive of several features. First, it can be argued that the behavioral deficits observed in the one-week separated monkeys are more likely the result of a failure to develop rather than a separation induced decline. Second, similar brain mechanisms may be responsible for the generation of these behaviors in the different contexts. The amygdala has been shown to participate in acute fear responses across many studied mammals, including rats (Davis, 1992), monkeys (Mason, 1959), and humans (Chapman et al., 1954; Bechara et al., 1995). Furthermore it has been associated with both anxious (Davis, 2000) and social behaviors (Amaral, 2003). Since, depending on the timing of the initial separation, the animals in the current study demonstrate specific acute responses, social group behaviors, and responses to anxiogenic tests measured at one-year (Cameron, 2001; McCormick, 2005; Rockcastle, 2005) that are all characterized by differences in anxious and social behaviors, the amygdala is a possible site for the brain substrate underlying these features. In chapter three of this thesis, we will examine the amygdala as a potential contributor to these behavioral phenotypes.

As alluded to earlier, with respect to age-related behavioral outcomes, the most similar human analog to the experiments performed in this chapter is the coping responses of children

who were previously victims of child abuse, and their subsequent long term psychiatric risks. It has been well documented that adults who were victims of child abuse are at increased risk for myriad psychiatric disturbances later in life including anxiety (Yama et al., 1993), depression (Bifulco et al., 1991), posttraumatic stress disorder (Kiser et al., 1991; Famularo et al., 1994), self-destructive behaviors (Boudewyn and Liem, 1995), and poor self-regulation and self-integrity (Cole and Putnam, 1992). Many victims, however, emerge from these experiences without signs of mental health problems (Mullen et al., 1993). As a result considerable research has been conducted to determine what factors are responsible for this relative protection. Consistently, the coping style of "seeking of social support" is associated with more adaptive outcomes (Himelein and McElrath, 1996), while the coping styles described as "avoidant" and "emotion suppressing" are associated with poorer outcomes (Leitenberg et al., 1992). In principle, during the acute separation response, the one-week separated infants of this study can be described as engaging in avoidant coping, and the one-month separated infants can be described as engaging in "seeking of social support" coping. We can also draw parallels between humans and the animal subjects of this study with respect to the long-term outcomes. The one-week separated animals engage in greatly increased species atypical behaviors compared to the one-month separated animals, both during the 3rd month (this study), and throughout the first years of life (Cameron, 2001; Rockcastle, 2005). Hence it is reasonable to conclude that the model system that is the subject of this report may also be useful as a model of successful versus detrimental coping. The first and most obvious conclusion of this model would be that the development of successful coping strategies in monkeys requires a longer period of maternal infant contact earlier in life. The utilities of an animal model of successful coping would include the fact that the biological basis of coping style may be probed, and that

environmental factors which contribute to coping could be better controlled. A group has recently identified two strains of wild-house mice, short-attack-latency and long-attack-latency, thought to exhibit differences in coping behaviors (Veenema et al., 2003a; Veenema et al., 2003b). The mice considered to exhibit "active" coping released decreased amounts of corticosterone and gained rather than lost weight over a period of chronic exposure to psychosocial stress compared to those exhibiting "avoidant" coping. More studies in these models would greatly help in validating their usefulness as representing coping styles. As an example, coping style and cortisol response have been assayed in persons tested under stressful conditions that were out of their control (Bohnen et al., 1991). In this test, the coping style of "comforting cognition" was thought to be adaptive, and it was negatively correlated with cortisol levels. It would be interesting to examine whether the levels of social seeking versus avoidant coping styles in the acute stress responses exhibited by the separated monkeys are, too, correlated with cortisol responses.

The relationship between coping strategy and certain measures of psychosocial health have been identified in several previous studies in other populations that were not abused as children. For example, Vitiliano et al. (1987) assessed coping using five general categories: problem focused, seeking of social support, blaming self, wishful thinking, and avoidance. In the three samples of this study (psychiatric inpatients of mean age 32.5, spouses of Alzheimer's disease patients of mean age 65.8, and medical students of mean age 25.8), the strategy termed problem focused coping (e.g. acceptance and growth from a stressor) was negatively correlated with a score representing the presence of depressive symptoms. Conversely, the strategy termed wishful thinking coping (e.g. fantasizing the stressor would just go away) was positively correlated with the presence of depressive symptoms. In the psychiatric inpatient and medical

school student groups, the style of coping characterized by the seeking of social support was significantly negatively correlated with depressive symptoms. Finally only in the medical student group avoidant coping (e.g. avoiding people or internalizing feelings) and self-blaming were significantly positively correlated with depressive symptoms. Retrospective studies, however, always contain the potential confound of recall bias. In cases like these, it is reasonable to question whether the historical description of coping is jaded by the current depressive-like state. Longitudinal studies, although not devoid of reporter bias, provide some protection against recall bias. In another study inner-city adolescents were assessed longitudinally to examine whether coping style predicted symptoms of psychopathology (Tolan et al., 2002). It was found that emotion-focused coping (e.g. venting of negative emotions and distraction strategies) was significantly associated with increased symptoms of psychopathology while support and guidance seeking coping was associated with fewer symptoms. Thus, the relationship of the monkey model presented here to human studies of coping has face validity in that: 1) the acute measures are similar: avoidant coping resembles contact and self-comforting behaviors, and seeking of social comfort resembles active coping, and 2) the ultimate outcomes are similar: avoidant coping and self-comforting behaviors are associated with increased likelihood of later psychopathology, and seeking of social comfort and active coping are associated with decreased likelihood of later psychopathology.

Additionally a monkey model of coping styles may be able to better address issues with causality that are prevalent in cross-sectional clinical studies. For example, the questions of whether maladaptive coping styles lead to psychopathology, whether early expression of psychopathology includes maladaptive coping styles, or whether other causative agents result in both are often ambiguous in human studies. The data presented in this study suggest either of the

latter two possibilities in monkeys, since the environmental manipulation of maternal separation at different early times in development results in both maladaptive coping styles and increased symptoms of psychopathology in a variety of long-term tests of behavior. A potential causative mediator of both coping style and risk for developing psychopathology that has been offered in the clinical literature is temperament. Individual differences in reactivity and temperament are expected to be related to coping since they modulate an individual's avolitional response to stress and may facilitate or limit certain coping responses (Compas, 1987). In a longitudinal study of children, those who expressed an inhibited temperament between ages two and three, continued to show an inhibited temperament when assessed at age six (Kagan et al., 1987). Additionally, when confronted with novelty, children with an inhibited temperament have distinctive physiological differences in measurements such as heart rate, heart rate variability, vocal cord tension when speaking under stress, and salivary cortisol levels (Kagan et al., 1987, 1988). Inhibited children were more likely to develop social phobia later in life (Schwartz et al., 1999) and uninhibited children were more likely to develop externalizing behaviors such as aggression (Schwartz et al., 1996). Further, a fMRI study measuring activation in the amygdala following the presentation of novel faces in a group of adults (mean age 21.8 years) who had been characterized as inhibited at two years of age revealed increased activity in both the left and the right amygdala compared to adults characterized as uninhibited two year olds (Schwartz et al., 2003). The present study suggests that the inhibited - uninhibited temperament styles may be an immediately evident feature following maternal separation at one-week and one-month respectively. If coping style is, indeed, an indicator of temperament that appears immediately after the removal of the mother, then these findings are highly suggestive that there is a normal developmental period of temperament that requires the presence of a secure attachment. In

clinical samples of monozygotic twins and dizygotic twins, heritable factors were reported as more important for the development of temperament than environmental factors (Robinson et al., 2002). A possible rectification of both of these studies may be that the developmental potential of temperament is mediated by genetics, but the actual realization is affected by minimal early-life experience. As an example, the interaction of heritable factors and environmental factors has been offered as an explanation for why certain children adopted from orphanages form a pattern of inhibited reactive attachment disorder and others develop disinhibited reactive attachment disorder (Zeanah and Fox, 2004).

The early ability to discern the most affected (or most likely to become affected) individuals after periods of toxic stress may have clinical implications. This fact has already been realized for victims acutely following rape. For example, Foa et al. has found that a short 4-session preventative treatment program, if initiated within 14 days of the assault, decreases the chances that a victim will go on to develop PTSD (Foa et al., 1995). The effect is very dramatic in the short term, a 70% likelihood of developing PTSD at 3 months drops to a 10% likelihood, (although at 5 months the benefit is marginal since the symptoms decrease rapidly over time in this population). As such indicators of a predisposition to develop PTSD could be of clinical benefit. In fact efforts have been underway to identify early symptoms that may signal the forthcoming illness. Feelings of fear, anxiety, and especially intrusive feelings have all been implicated as early predictors of pathology (Resnick, 1997). Indeed, the value of positive coping has been also identified in this population (Valentiner et al., 1996). In another clinical population, the effect of negative coping strategies after mild brain trauma (Harvey and Bryant, 1999) and more severe brain trauma (Bryant and Harvey, 1995; Harvey and Bryant, 1999) has been investigated. Avoidant coping strategies are again consistently linked with the subsequent

development of PTSD. As in other studies it is not clear whether the maladaptive coping strategies are a symptom or cause of PTSD, but nevertheless, this population may also benefit from education about healthy ways to cope. It appears that in the case of the rape victims, treatment within 2 wks is essential for clinical benefit. Interestingly, in the monkey model of this study, adoption therapy could rescue species-typical interactions, but only when it was implemented within one month of separation (McCormick, 2004).

Lastly, one more finding of note should be discussed from this study. Locomotor behaviors in the acute separation setting were found to be correlated with locomotor behaviors during the third month of life in the native social environment. Unlike the other correlations presented in this report this correlation did not segregate the data based on age at separation. This suggests that the tendency to locomote is in place early in development and is not subject to modulation by early maternal separation. It will be of interest to determine whether these correlations hold up as the monkeys mature, or whether they represent a developmental delay in some monkeys at this early stage in life. In free ranging monkeys much refinement of motor control occurs after 3 months of age (Dunbar and Badam, 1998).

3. Early Social Bond Disruption and Amygdala Gene Expression

3.1. Abstract

Monkeys exposed to social bond disruption early in life develop long-term alterations in socioemotional behavior that vary depending on the timing of the disruption, even when they are raised in social groups. Monkeys experiencing maternal separation at 1 mo of age show more social behavior, whereas monkeys experiencing maternal separation at 1 wk of age show less social behavior and an increase in self-comforting behaviors (e.g., thumb-sucking). Knowledge of the neural substrates that mediate these behavioral differences may be relevant to psychiatric illnesses which share similar phenotypic features such as anxiety, depression or autism. We sought to identify neural systems that underlie these stress-induced behavioral changes by examining changes in mRNA content in amygdala tissue collected from 1 wk-/1 mo-separated, and maternally-reared infants at 3 mos. of age (n=4/group). TotRNA was isolated from the right medial temporal lobe region, predominantly the amygdala, and analyzed using Affymetrix® U133A 2.0 arrays. ^{35}S-CTP In situ hybridization was performed on adjacent thaw-mounted sections. 8,405 out of >18,000 unique transcripts were expressed at levels high enough for analysis. According to stringent expression difference criteria, 12 genes differed between the 1 wk and control groups, 24 genes differed between the 1 mo and control groups, and 1 gene (Guanylate Cyclase 1 α3) differed between the 1 wk and 1 mo groups. Genes involved in development and cell signaling were among the changed genes. The differential expression of Guanylate Cyclase 1 α3 was verified by in situ hybridization. The expression of this gene was significantly correlated with two behaviors that were previously measured during the third month of life: close social behavior ($r=0.708$, $p=0.015$), and self-comforting behaviors ($r= -0.88$, $p<0.001$). Therefore we conclude that Guanylate Cyclase 1 α 3 may contribute to the altered

behavioral phenotypes observed following differentially timed maternal separation in rhesus macaques.

3.2. Introduction

The early social environment in infants and children is important for long-term behavioral and cognitive functioning later in life. Epidemiological studies suggest that early social stress leads to many negative outcomes including increased risk for developing psychiatric illnesses (Agid et al., 1999), substance abuse (Furukawa et al., 1998), and behavioral problems later in life. The social stressors specifically shown to impact long-term mental health include early parental loss from death or separation (Mireault and Bond, 1992), parental abuse or neglect (Pianta et al., 1989; Sedlak and Broadhurst, 1996), and orphanage-rearing (O'Connor, 1999). In animal models, manipulations of the early maternal-infant bond have been used to investigate the effects of early-life stress.

Many different paradigms in experimental animals have modeled early social stress including maternal deprivation in the rat (Francis and Meaney, 1999; Caldji et al., 2000a), maternal separation in the monkey (Ruppenthal et al., 1976; Capitanio et al., 1986; Cameron, 2001; Rockcastle, 2005), or variable maternal care in the monkey (Coplan et al., 1996). Although these manipulations vary with respect to the nature and timing of the maternal-infant bond disruption, they consistently lead to increases in anxious and depressive behaviors, differences in social behaviors, and a dysregulation of the HPA axis. Abnormal responses to anxiogenic stimuli are also observed as increased startle reflexes in rodents (Ellenbroek et al., 2004) and in monkeys (Parr et al., 2002; Sanchez et al., 2005) later in life following earlier maternal deprivation. These studies suggest that the postnatal social environment has profound effects on later socioemotional behavior, but the mechanisms underlying this association are not

completely understood. It is likely that they involve activation of the stress axis since these manipulations lead to increases in circulating corticosteroids (Lyons et al., 2000), and changes in the patterns of glucocorticoid receptor distribution and activation in the brain (Liu et al., 1997; Fenoglio et al., 2004). Other effects independent of HPA axis activation may also be involved since suppression of adrenal responses by adrenalectomy or dexamethasone do not eliminate the behavioral and neurochemical changes observed in some cases (van Oers et al., 1998b; van Oers et al., 1999). Further investigation of the neural substrate associated with the behavioral changes following early life stress may lead to both an increased understanding of the biological underpinnings of certain neurodevelopmental psychiatric illnesses and potential therapeutic targets for their ameliorization.

A growing body of preclinical and clinical evidence suggests that the amygdala participates in the behavioral and neurochemical responses to stressful situations, and mediates anxious and social behaviors. Monkeys who undergo selective bilateral amygdalectomy as adults display extroverted behavior when tested in dyadic interactions in the laboratory environment and decreased responding to inanimate fearful stimuli (Prather et al., 2001). Similar tests in monkeys following lesions made at 2 weeks of age show similar responses to inanimate fearful stimuli as adult lesioned animals, but interestingly, more fear in social dyadic interactions (Emery et al., 2001). The role of the amygdala in mediating anxious behaviors is suggested by the high density of benzodiazepine receptors in monkeys (Onoe et al., 1996), rodents (Niehoff and Kuhar, 1983) and humans (Niehoff and Whitehouse, 1983). In mice, the amygdalar expression of the benzodiazepine receptor is positively modulated by early rearing environment, and increases in this receptor are associated with decreased fearfulness in the presence of novelty (Caldji et al., 2000b; Fries et al., 2004). In humans, bilateral amygdala damage due to surgery or

disease is associated with a decreased ability to interpret fearful facial expressions (Adolphs et al., 1994; Adolphs et al., 1998). Furthermore, functional MRI studies in humans have implicated the amygdala in autism (Grelotti et al., 2005), anxiety and anxiolytic action (Etkin et al., 2004; Lorberbaum et al., 2004), fear-conditioning (Knight et al., 2004; Knight et al., 2005), and major depression (Drevets, 1999; Drevets et al., 2002).

In the present study, we identified socioemotional behavioral differences and amygdala gene expression changes at three months of age in three groups of female monkeys that differ with respect to early maternal care and rearing. Studies in this early-life stress paradigm have previously shown that the exact behavioral phenotype exhibited following maternal separation critically depends on the age at separation, and is highly suggestive that a sensitive period of maternal-infant interaction between one-week and one-month of age is required for the developmental of species-typical social interactions (Cameron, 2001; Rockcastle, 2005). An initial screen of expression differences was performed in the amygdala and surrounding periamygdalar cortex using microarrays. The most robustly regulated gene between one-week separated monkeys and maternally raised monkeys was soluble guanylate cyclase 1 α 3 (GUCY1A3). The product of this gene has been previously shown to be an element of the NO signaling cascade (Russwurm and Koesling, 2004), and to be expressed in limbic structures of the prenatal and postnatal rodent brain (Furuyama et al., 1993; Smigrodzki and Levitt, 1996). The expression of GUCY1A3 was verified by in situ hybridization and found to be significantly correlated with two behaviors measured in the social group during the third month of life: typical social behaviors and self-comforting behaviors. This study describes the first gene shown to be regulated in the monkey brain by maternal separation, and raises the possibility that GUCY1A3 may represent a novel therapeutic target for psychiatric illnesses such as anxiety and depression.

3.3. Methods

1. Maternal Separation Paradigm.

A total of twelve female rhesus monkeys born in the breeding colony at the University of Pittsburgh Primate Research Laboratory were used for these studies. At birth each monkey was arbitrarily selected to enter the "one-week separated", "one-month separated", or "control" experimental groups (n=4 per group). All monkeys in this study spent the first week of life in a single cage with their mother. During this initial period mothers and infants were fed Purina Monkey Chow once daily (no. 5045, Ralston Purina Co, St. Louis, MO), and offered water ad libitum, although at this age the infants receive their nutrition primarily from breast milk. They were housed in 4-bank cages in temperature controlled (24 +/- 2°C) communal rooms with artificial lighting from 0700 to 1900h. After the first week of life the animals were handled differently depending on experimental group assignment. At one-week of age monkeys designated as "one-week separated" were removed from their mothers and placed in a single nursery cage juxtaposed to the group rearing pen that they would ultimately join. After a 5-7 day period of learning to bottle-feed, the monkeys were introduced into the group rearing environment. One-week separated monkeys spent weeks 3-12 in the group rearing environment. Conversely, at one-week of age, monkeys designated as "one-month separated" were introduced into the group rearing pen with their mothers. One-month separated monkeys spent weeks 2-4 in the group rearing environment with their mothers. At 4 weeks of age, one-month separated monkeys' mothers were removed from the group rearing environment and the infants were placed in the single nursery cage positioned identically as described for the one-week separated group. After a 5-7 day period of learning to bottle-feed, these monkeys were reintroduced into

the group rearing environment. One-month separated infants then spent weeks 6-12 in the group rearing environment. "Control" monkeys were similarly introduced into the group rearing pen at 1 week of age with their mother, and they remained there for the study's entirety, weeks 2-12. The group rearing pen consisted of 4-5 monkeys of varying ages with no monkey more dominant than the adult female mother (when present). An adolescent female was always present in the social rearing groups as they have been shown to be particularly attentive to infants. This age / sex distribution minimized any disturbances that may be caused by competition for dominance. The group rearing pen, measuring 14 ft. by 11 ft. by 12 ft., was cement block construction with a several inch bed of wood shavings on the floor, 4 perches at various heights, and a chain-link penfront. A "nursery chamber", measuring 2 ft x 2 ft x 3 ft, was placed in each pen and locked to the chain-link penfront so that it could not be moved. The nursery had a small doorway with dimensions such that infants could enter but larger animals in the pen could not enter. Bottles of formula were provided in the nursery for infants whose mothers had been removed in the first month of life, so that the formula was available to the infants but not to all monkeys in the pen. Toys were often available in the pens, including tire swings, swinging balls, chew toys, and snuggly toys. Monkeys were fed one meal a day between 0900 and 1000 hours of Purina high protein monkey chow supplemented several times a week with fresh fruit and seeds strewn in the bedding to encourage foraging. Water was available *ad libitum*. Artificial lighting in the hallways was on from 0700 to 1900 hours each day, and sky lights above each pen admitted natural light. Temperature was maintained at 24±2 C.

2. Medial Temporal Lobe Tissue Collection

Following the behavioral analyses at 12 weeks of age, the medial temporal lobe brain tissue was collected from each experimental monkey for molecular studies. Monkeys were

anesthetized with ketamine hydrochloride and sodium pentobarbitol, quickly decapitated, and the entire brain was removed from the skull. The brain was then hemisected by a midline saggital cut. The right hemisphere was cut into ~5 mm-thick coronal blocks, flash-frozen in isopentane over dry ice, and stored at -80°C until cryostat sectioning as described previously (Volk et al., 2000). Immediately prior to cryostat sectioning, the amygdala and periamygdalar cortex was dissected from the coronal blocks using a cut parallel to the midline, between the amygdala lateral nucleus boundary and the invagination of the superior temporal sulcus, and a perpendicular cut just superior to the optic tract. The dissected blocks were then sectioned using a cryostat at 20 μm throughout the region containing the amygdala. From each set of 20 sections (400 μm), 11 sections were thaw-mounted onto Superfrost Plus micro slides (VWR Scientific, U.S.A) and 9 sections were collected into TRIzol® reagent for totRNA isolation, vortexed for 1-minute, and frozen at -80°C. One thaw-mounted section in each set of 20 was stained for Nissl substance using Thionin. The identification of two distinct landmarks, the most rostral aspect of the lateral ventricle and the glomerulus of the paralaminar nucleus allowed the mapping of each medial temporal lobe onto one another, ensuring that the same region of the medial temporal lobe was used for each subject. The set of 20 sections nearest the most rostral boundary of the lateral ventricle was used for both microarray and in situ hybridization experiments.

3. Microarray Experiments

Nine cryostat sections from the medial temporal lobe adjacent to the most rostral portion of the lateral ventricle, predominantly the amygdala, were collected into TRIzol® reagent (Invitrogen™, Carlsbad, CA) for each of the 12 experimental subjects. TotRNA was isolated according to the manufacturer's instructions including all optional steps. The isolations yielded between 10-15 ug of totRNA from each subject. Each totRNA sample was assessed for purity

using the absorption ratio between 260 nm and 280 nm in an ultraviolet spectrophotometer, and quality using the 28s to 18s ribosomal subunit ratio from the electropherogram of the BioAnalyzer (Agilent, Palo Alto, CA). Samples were considered appropriate for microarrays with an A260/A280 $\geq$ 1.8, and a 28s/18s $\geq$ 1.4. Reverse transcription, in vitro transcription amplification, and fragmentation were performed according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Using the Affymetrix hybridization station, each sample was hybridized onto an Affymetrix Human Genome U133A 2.0 Array which contained roughly 22,000 probesets that characterize greater than 18,500 unique transcripts. To avoid microarray batch variation only microarrays from a single lot were used. Segmentation of scanned microarray images was performed by Microarray Analysis Suite 5.0 (MAS5). Determination of expression levels and scaling were performed using Robust Multi-array Average (RMA) (Irizarry et al., 2003). Microarrays were considered for use only if the average 3':5' ratio for GAPDH and actin did not exceed 1:1.2.

4. GUCY1A3 In Situ Hybridization

In situ hybridization studies were performed according to methods described previously (Mirnics et al., 2001). Briefly, gene-specific 20-mer primers were designed using the human soluble guanylate cyclase 1, α 3 subunit (GUCY1A3) sequence, GenBank accession # NM_000856. Forward primer was TGTACACTCGCTTCGACCAG, reverse primer was TCCAGAAAATGGCAGATTCC. Using these primers a 448 base-pair amplicon was generated by PCR amplification using monkey medial temporal lobe cDNA as template. The resulting fragment was T/A cloned into the Acceptor™ pstBlue-1 vector (Novagen®, San Diego). The GUCY1A3 clone was confirmed by sequencing. Dual opposed promoters allowed efficient in vitro transcription of both the forward and reverse products. Labeled anti-sense RNA probes

were generated using in vitro transcription in the presence of ^{35}S-labeled CTP (Amersham Biosciences, Piscataway, NJ). An amount of probe yielding 2 million dpm was hybridized overnight onto 20 μm-thick, thaw-mounted tissue sections that were lightly fixed in 4% paraformaldehyde, dehydrated in graded alcohols, and delipidated in chloroform. Following a series of stringent washes and RNase treatment to limit nonspecific binding, slides were dried and exposed to x-ray film (Kodak MR Gold) for 2 days. Known 14C standard slides were included for quantitative analysis. A sense RNA probe was treated in parallel as a specificity control, and no signal was present after 2 days exposure. Radiographic films were scanned at 800 dpi and imported into Scion Image (Scion Corporation, U.S.A) for optical density measurements. The slides were subsequently dipped in photo emulsion. Dipped slides were exposed for 7 days, developed with Kodak D-19 developer, fixed with 30% sodium thiosulfate, and visualized under light and dark-field microscopy.

5. GUCY1A3 Postnatal Developmental In situ hybridization

For the developmental studies we chose five postnatal timepoints: 1 week, 4 weeks, 12 weeks, 63 weeks, and 176 weeks. Four monkeys each were analyzed at 1 and 4 weeks, 6 monkeys at 12 weeks, and 3 monkeys were analyzed at 63 and 176 weeks. None of the monkeys used for these experiments were subjected to early-life social stress. Some monkeys (n=6) had undergone previous brain biopsy of the prefrontal cortex. All monkeys from the 1 week and 1 month groups, and 4/6 monkeys from the 12 week group were raised in social groups of 3-5 monkeys of varying ages. Monkeys from the 63 week and 176 week groups, and 2/6 monkeys from the 12 week group were reared with their mothers in single cages. In the single-cage reared monkeys weaning occurred at 6 months of age when applicable. Amygdala collection and sectioning was

identical to the monkeys in the maternal separation paradigm. In situ hybridization was accomplished by the same method and used the same template for probe production.

6. Data Analysis

Microarray. RMA analysis yielded normalized, Log2 transformed expression levels for each of the > 18,500 transcripts probed. We considered a gene present when its RMA – scaled expression level was > 6.49. Two-tailed Student's t-tests were performed between experimental groups, and a transcript was considered differentially regulated between groups when: 1) the difference between the group averages was at least 1.25-fold, and 2) the t-test p-value was </= 0.02. This present-absent cutoff has been set based on previous success of verifying expression levels of genes at or above this level by either real-time PCR or in situ hybridization.

In Situ Hybridization. The emission of beta particles from specifically bound probe was determined by measuring the mean gray level around the extent of the amygdala on the radiographic film images using the polygonal selection tool in Scion Image. The observer was blinded to experimental group. Known carbon-14 standards were included in each exposure to ensure that the maximum gray level measured within the amygdala was within the linear range of the radiographic film and to calibrate the gene expression data. The background gray level for the film surrounding the section was subtracted from each measurement. The background-subtracted gray level measurements were transformed into uncalibrated optical densities (OD) using the following transformation: Uncalibrated OD = log10 (255 / (255 - Gray Value)). The uncalibrated O.D. measurements were then calibrated to known quantities of ^{14}C. The expression levels from 3 independent sections from each experimental animal were averaged together. Expression levels are reported as μCi/g of ^{14}C. The Kolmogorov-Smirnoff test was used to test for normality. A one-way ANOVA was performed, and following a Levene's test to

ensure homoscedasity, Least Significant Difference post-hoc tests were performed. Significance was defined at p </= 0.05.

Gene-Behavior Correlations. Inspection of the mean data for self-comforting behaviors and typical social behaviors suggested a possible relationship with the GUCY1A3 expression data. To examine this, we ran Pearson correlations to determine r values and used a t distribution table with n-2 degrees of freedom to determine the associated two-tailed p-values. R values were considered significant when the associated p-value was </= 0.05. We report both the r value and its associated p value in the results.

3.4. Results

3.4.1. Microarray Experiments

Microarray studies were used to gain an unbiased survey of gene expression differences in the medial temporal lobe, predominantly the amygdala, between monkeys whose mothers were removed from their social group at different developmental time points. Based on previous work in our lab, and empirically on this data set, we defined a gene as present if its RMA normalized gene expression value was at least 6.49 when averaged across all subjects. Of the greater than 18,500 unique transcripts measured, 8,405 were called present according to this criterion. From these genes we designated a gene as differentially regulated between groups if in at least one of the three comparisons (Control – Week, Control – Month, and Week – Month): 1) the difference of the group averaged RMA expression levels was at least 0.322 (1.25 fold difference), and 2) the Student's t-test p-value was less than 0.02. Using these criteria, 12 genes were differentially regulated in the control-week comparison, 24 genes were differentially regulated in the control-month comparison, and 1 gene was differentially regulated in the week-month comparison

(Table 3.1). Four genes (GUCY1A3, FABP5, CUGBP1, and TSC22D2) were present in more than one comparison. Given the heterogeneous nature of the tissue samples, and the nature of the arrays (monkey hybridized to human) each gene should be validated by an alternate method; in situ hybridization studies are well-suited for this task since they additionally provide anatomical detail. In this chapter we chose to examine GUCY1A3 in more detail since its expression profile resembles the behavioral data for these monkeys (see Fig 2.2). The expression of the GUCY1A3 gene for each individual monkey as assayed by microarray reveals that maternally-raised monkeys display a level of 381 +/- 47 relative units, one-week separated monkeys display a level of 270 +/- 23, and one-month separated monkeys display a level of 346 +/- 33 in the rostral medial temporal lobe at 12 weeks of age (Fig 3.2a).

3.4.2. GUCY1A3 is decreased in the amygdala following early social bond disruption

In situ hybridization on tissue sections adjacent to those used for microarray was performed for the gene that is most regulated between the control and one-week separated groups, GUCY1A3. One representative field from the Ldi (Lateral dorsal intermediate) and Bmc (Basal magnocellular) nuclei from one subject of each group appears in Figure 3.1. Hybridization appears as developed silver grains over cell bodies counterstained with cresyl-violet (see arrows for typical appearance of an expressing cell). Silver grains were observed in large, lightly counterstained cells, presumably projection neurons, and absent from smaller more darkly counterstained cell bodies. Inspection of the other subnuclei in the amygdala revealed a similar pattern of expression (data not shown).

The gene expression of GUCY1A3 was quantified by exposing radiographic film to the tissue sections that were labeled with ^{35}S-CTP riboprobe. Amygdala specific expression is reported as μCi/g of a known quantity of ^{14}C standard (Fig 3.2b). Maternally-raised monkeys

Table 3.1 Microarray Gene Expression Changes. Summary of gene expression changes in each of the three possible comparison groups. ALR: Difference of the Log_2 RMA normalized expression level. Genes are color-coded based on functional pathway. For reference: ALR of 0.322 is equivalent to 1.25-fold change, ALR of 0.585 is equivalent to 1.5-fold change.

			Comparison					
			Control - Week		Control - Month		Week - Month	
Control-Week Regulated Genes	Abbreviation	Accession#	ALR	p-value	ALR	p-value	ALR	p-value
guanylate cyclase 1, soluble, alpha 3	GUCY1A3	AI719730	-0.490	0.005	-0.135	0.268	-0.355	0.009
calreticulin	CALR	AI348935	-0.465	0.004	-0.204	0.113	-0.261	0.028
LIM homeobox protein 2	SPN	NM_004789.1	-0.444	0.010	-0.412	0.023	-0.032	0.821
FK506 binding protein 1A, 12kDa	FKBP1A	NM_000801.1	-0.426	0.013	-0.350	0.030	-0.076	0.610
ubiquitin specific protease 7	USP7	AI160440	-0.407	0.013	-0.308	0.152	-0.099	0.515
fatty acid binding protein 5	FABP5	NM_001444.1	-0.394	0.009	-0.461	0.007	0.067	0.692
splicing factor, arginine/serine-rich 11	SFRS11	AW241752	-0.383	0.016	-0.294	0.057	-0.089	0.366
CUG triplet repeat, RNA binding protein 1	CUGBP1	BF037823	-0.372	0.009	-0.361	0.001	-0.010	0.981
sialophorin (gpL115, leukosialin, CD43)	SPN	NM_003123.1	0.352	0.009	0.311	0.086	0.041	0.848
AT rich interactive domain 1A (SWI- like)	ARID1A	AF231056.1	-0.338	0.009	-0.302	0.068	-0.036	0.695
hypothetical gene CG018	CG018	AL049785.1	0.325	0.007	0.311	0.012	0.015	0.888
origin recognition complex, subunit 5-like	ORC5L	NM_002553.1	-0.323	0.006	-0.207	0.020	-0.116	0.244

Control-Month Regulated Genes	Abbreviation	Accession#	ALR	p-value	ALR	p-value	ALR	p-value
TSC22 domain family 2	TSC22D2	NM_014779.1	-0.513	0.014	-0.682	0.020	0.170	0.565
fatty acid binding protein 5	FABP5	NM_001444.1	-0.394	0.009	-0.461	0.007	0.067	0.692
protein kinase C binding protein 1	PRKCBP1	BC001004.1	-0.405	0.023	-0.460	0.016	0.055	0.630
regulator of G-protein signalling 5	RGS5	AI183997	0.253	0.070	0.450	0.017	-0.197	0.270
vacuolar proton-ATPase subunit	C7orf32	AI884867	-0.239	0.119	-0.418	0.019	0.179	0.067
calmodulin 3 (phosphorylase kinase, delta)	CALM3	AV685208	0.180	0.296	0.395	0.016	-0.215	0.072
transforming acidic coiled-coil protein 2	TACC2	NM_006997.1	-0.287	0.013	-0.389	0.006	0.102	0.250
polo-like kinase 2	PLK2	NM_006622.1	-0.206	0.024	-0.383	0.010	0.177	0.074
ectodermal-neural cortex	ENC1	AF010314.1	-0.363	0.027	-0.376	0.007	0.013	0.885
5,10-methenyltetrahydrofolate synthetase	MTHFS	NM_006441.1	0.333	0.071	0.373	0.018	-0.040	0.835
peroxiredoxin 1	PRDX1	L19184.1	0.276	0.059	0.367	0.013	-0.091	0.524
high mobility group AT-hook 1	HMGA1	AF176039.1	0.152	0.135	0.366	0.001	-0.213	0.024
HMP19 protein	HMP19	NM_015980.1	-0.171	0.031	-0.363	0.005	0.192	0.134
CUG triplet repeat, RNA binding protein 1	CUGBP1	BF037823	-0.372	0.009	-0.361	0.001	-0.010	0.981
ras homolog gene family, member Q	RHOQ	BF348067	-0.247	0.171	-0.356	0.005	0.109	0.473
Major histocompatibility complex, class I, C	HLA-C	U62824.1	0.185	0.217	0.347	0.015	-0.163	0.105
PI-4-phosphate 5-kinase, type II, gamma	PIP5K2C	NM_024779.1	-0.282	0.008	-0.345	0.008	0.063	0.564
discs, large (Drosophila) homolog 4	DLG4	AF028825.1	0.258	0.219	0.343	0.006	-0.085	0.758
ras homolog gene family, member C	RHOC	NM_005167.1	0.103	0.166	0.330	0.014	-0.227	0.060
general transcription factor IIH, polypeptide 1	GTF2H1	BC000365.1	-0.279	0.050	-0.329	0.014	0.050	0.604
phosphatase and actin regulator 2	PHACTR2	NM_014721.1	-0.195	0.315	-0.328	0.015	0.133	0.436
hypothetical protein DKFZp762N1910	DKFZp762N19	BG167570	0.203	0.041	0.327	0.010	-0.124	0.264
platelet-activating factor receptor	PTAFR	D10202.1	0.276	0.174	0.325	0.010	-0.049	0.886
KH domain, RNA binding, sig. trans. assoc. 3	KHDRBS3	AF069681.1	-0.190	0.009	-0.324	0.011	0.134	0.236

Week-Month Regulated Genes	Abbreviation	Accession#	ALR	p-value	ALR	p-value	ALR	p-value
guanylate cyclase 1, soluble, alpha 3	GUCY1A3	AI719730	-0.490	0.005	-0.135	0.268	-0.355	0.009

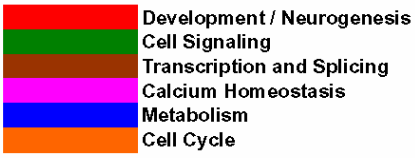


Figure 3.1 Cellular and Regional Distribution of GUCY1A3. Representative photomicrographs from the Ldi (A,C,E) and Bmc (B,D,F) amygdala from one monkey per group. ^{35}S-labeled riboprobes for GUCY1A3 appear as silver grains clustered around cell bodies counterstained with cresyl-violet. Arrows depict typically labeled cells. Note that the silver grains label lightly counterstained large cells in both regions. A,B: Maternally-reared control. C,D: One-month separated. E,F: One-week separated. Scale bar, 40 μm.

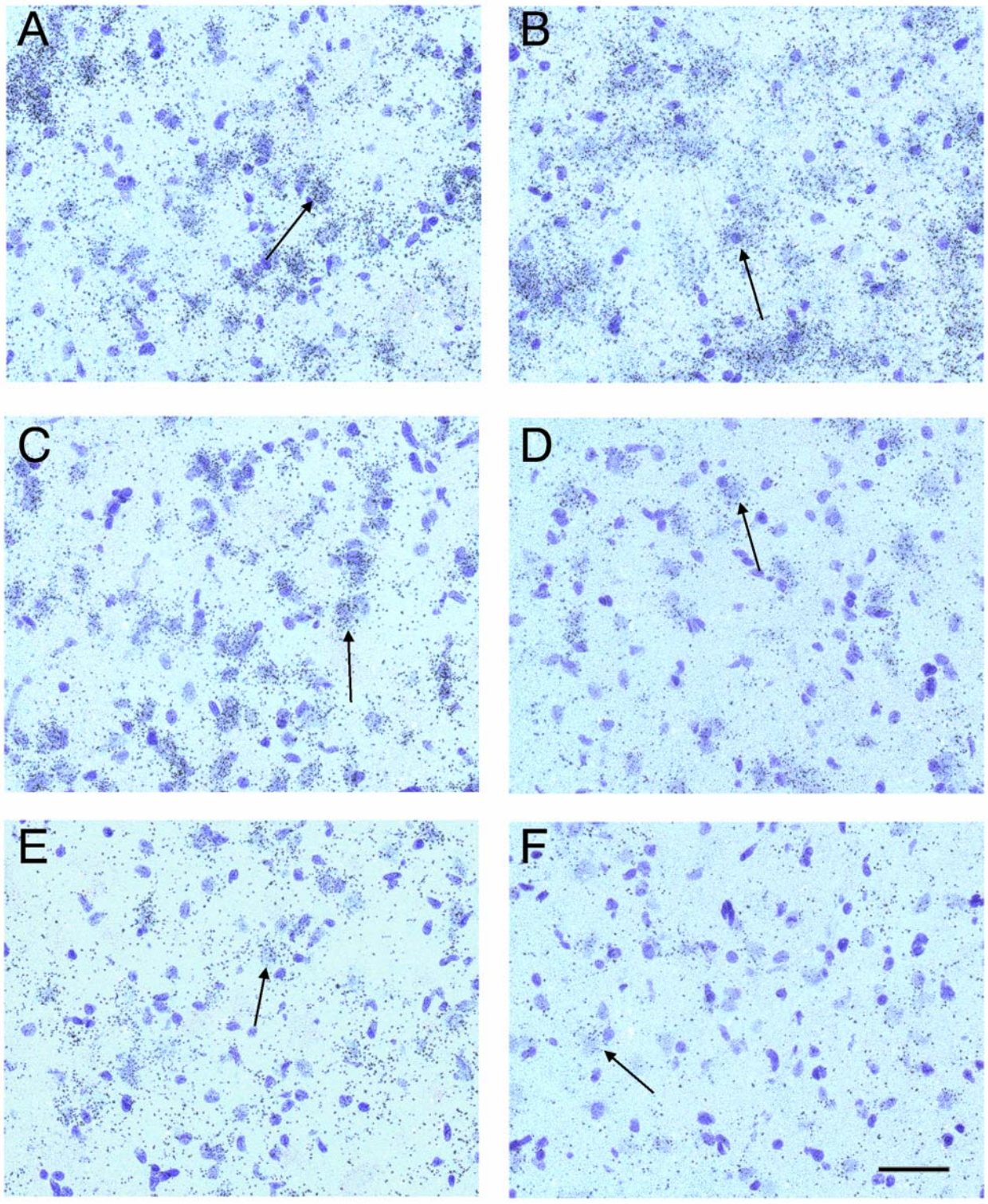
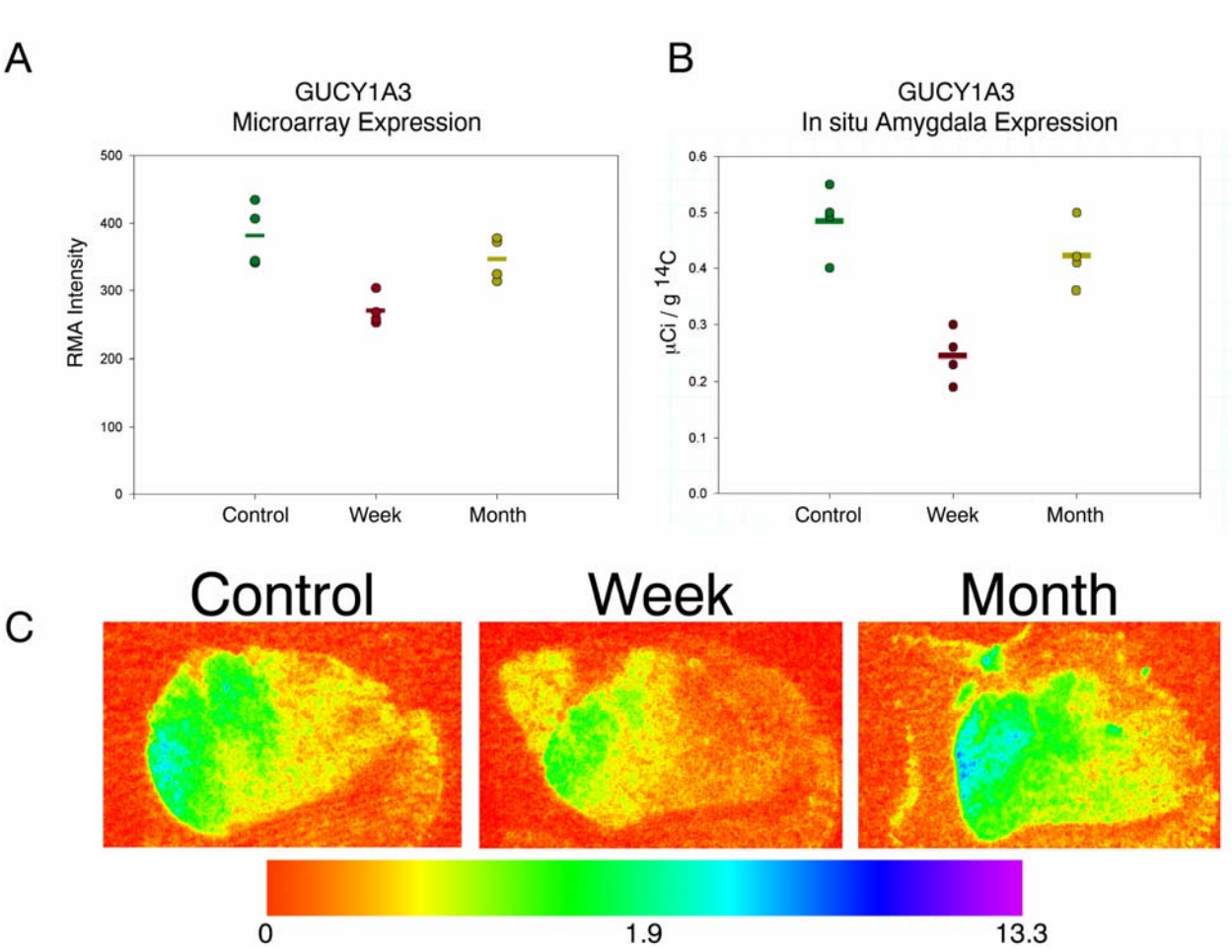

A
B
C
D
E
F

Figure 3.2 GUCY1A3 mRNA Expression. A) GUCY1A3 microarray gene expression level in the rostral medial temporal lobe. Circles represent the RMA normalized expression levels from each individual monkey, bars represent group average. B) Quantification of radiograph of GUCY1A3 ^{35}S-labeled ribroprobe around the extent of the amygdala. Optical density levels were converted to known activities of ^{14}C standards and expressed in μCi / g. Circles represent the expression levels from each individual monkey, bars represent group average. C) Representative pseudocolored image depicting regional amygdalar expression in one animal per group expressed in a Log scale.


A
GUCY1A3
Microarray Expression
RMA Intensity
500
400
300
200
100
0
Control
Week
Month
B
GUCY1A3
In situ Amygdala Expression
µCi / g 14C
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control
Week
Month
C
Control
Week
Month
0
1.9
13.3

display an expression level of 0.486 +/- 0.063 μCi/g, one-week separated monkeys display an expression level of 0.245 +/- 0.048 μCi/g, and one-month separated monkeys display an expression level of 0.422 +/- 0.060 μCi/g. An ANOVA revealed that this difference is significant between groups, ($F(2,9)=21.181$, $p<.001$). Post-hoc tests reveal that a significant difference exists in the Control to Week comparison ($p<0.001$) and in the Week to Month comparison ($p=0.001$), but not in the Control to Month comparison ($p=0.194$). Data from three representative sections reveal that the decrease in the one-week separated monkeys is distributed throughout the amygdala (Fig 3.2c), but is especially prominent in the lateral and basal nuclei. These are the areas of the amygdala that are the highest expressors in control monkeys.

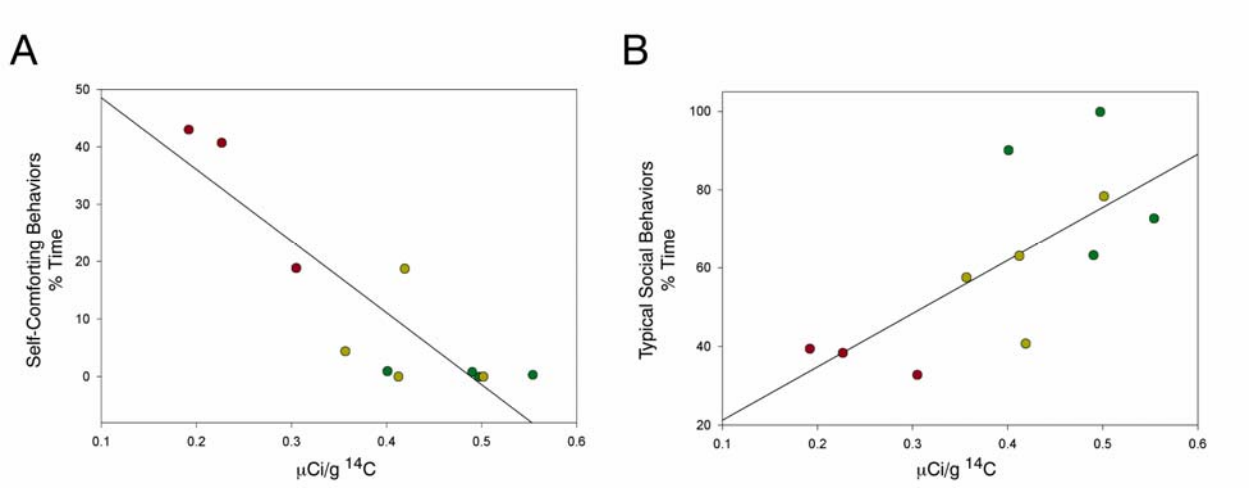
3.4.3. GUCY1A3 is correlated with behavior

The expression data of GUCY1A3 appeared to resemble the behavioral phenotypes observed during the 3rd month of life: one-week separated monkeys were significantly different from controls, and one-month separated animals were intermediate between the two, but closer to the control expression in both the behavior and gene expression measurements. We examined this relationship via Pearson correlations. Self-comforting behavior was significantly negatively correlated with GUCY1A3 amygdala expression ($r= -0.878$, $p<0.001$, Fig 3.3a), and typical social behavior was significantly positively correlated with GUCY1A3 amygdala expression ($r=0.708$, $p=0.015$, Fig 3.3b). These data raise the possibility that this gene plays a causative role in these behaviors.

3.4.4. GUCY1A3 is expressed at adult levels by one-week of age

We next investigated whether the observed lower expression in one-week separated animals was due to a decreased expression following maternal separation, or a failure to increase over development. Five time points between 1 week postnatal and 176 weeks postnatal were

Figure 3.3 Gene-Behavior Correlations. Red circles represent individual one-week separated monkeys, yellow circles represent individual one-month separated monkeys, and green circles represent individual maternally-reared monkeys. A) Level of Self-comforting behavior during the third month of life is significantly correlated with GUCY1A3 mRNA levels in the amygdala ($r=-0.878$, $p<0.001$). B) Level of typical social behavior during the third month of life is significantly correlated with GUCY1A3 mRNA levels in the amygdala ($r=0.708$, $p=0.015$).


A
Self-Comforting Behaviors
% Time
50
40
30
20
10
0
0.1
0.2
0.3
0.4
0.5
0.6
μCi/g 14C
B
Typical Social Behaviors
% Time
100
80
60
40
20
0.1
0.2
0.3
0.4
0.5
0.6
μCi/g 14C

examined by in situ hybridization in monkeys that had not been subjected to any early life stress (Fig 3.4). An ANOVA did not support a significant difference between ages for the expression of this gene ($F(4,15)=0.937$, $p=0.469$) at any developmental age. Inspection of the anatomical distribution of GUCY1A3 expression in the medial temporal lobe over development revealed that similar patterns exist in the amygdala at all ages examined, while the cortical expression seems to vary with age (Fig 3.5). Additionally, given the relative size of the amygdala at one-week, it is unlikely that a developmental increase in expression is being masked by a decreased cell-packing density. These data suggest that the GUCY1A3 is expressed at or near adult levels by one-week of age.

3.5. Discussion

In this study we have examined gene expression changes in the amygdala at three months of age in three groups of monkeys (one-week separated, one-month separated, maternally reared) that differed with respect to period of early maternal rearing. Microarray analysis revealed 37 gene products that were differentially regulated between at least one of the three possible comparisons. In this report we chose to concentrate on Guanylate Cyclase 1 α3 (GUCY1A3) since: 1) it was the most changed gene between the one-week separated and maternally reared groups, 2) it was the only gene product that was differentially regulated between the one-week separated and one-month separated groups, and 3) and it was one of the highest expressors of the genes identified. The subsequent quantitative in situ hybridization studies confirmed a robust downregulation of GUCY1A3 mRNA throughout the amygdala in the one-week separated group compared to both the maternally reared and one-month separated groups. This downregulation was most apparent in the lateral and basal nuclei where this gene is heavily expressed in maternally reared controls. In chapter 2, data were presented that described the chronic

Figure 3.4 Quantitative Analysis of the Development of GUCY1A3 mRNA expression. Expression level in the amygdala was quantified from the radiographic O.D. and converted to known a known quantity of ^{14}C standard. Each bar represents the average quantified GUCY1A3 mRNA level from 3-6 animals per age. Error bars represent 1 S.D. There was no significant difference between any of the ages examined $F(4,15)=0.937$, $p=0.469$).

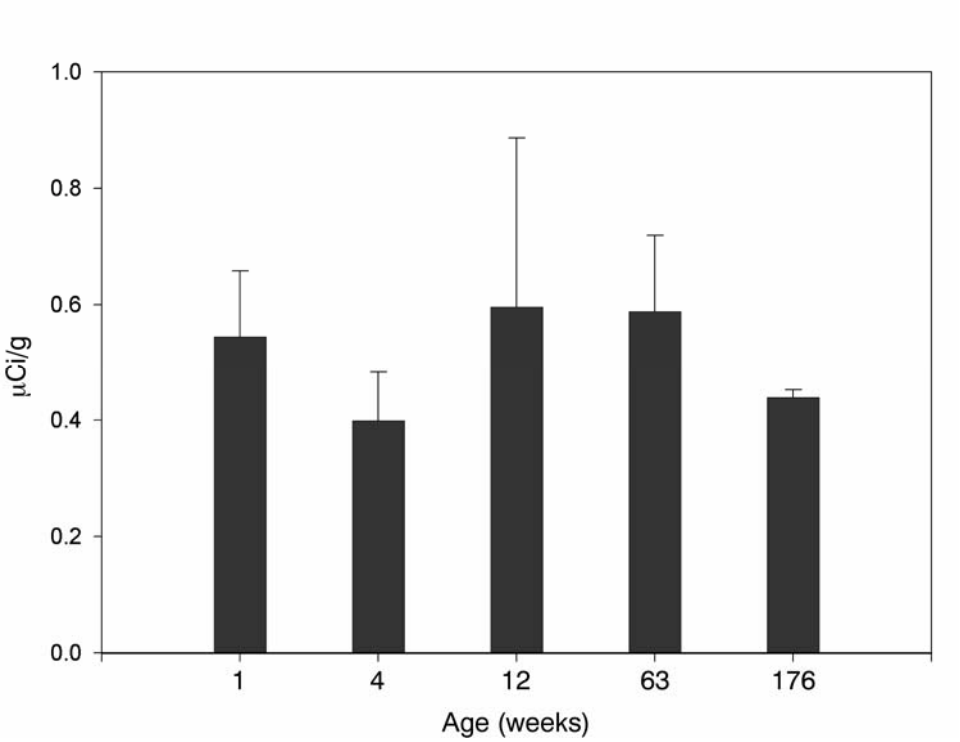
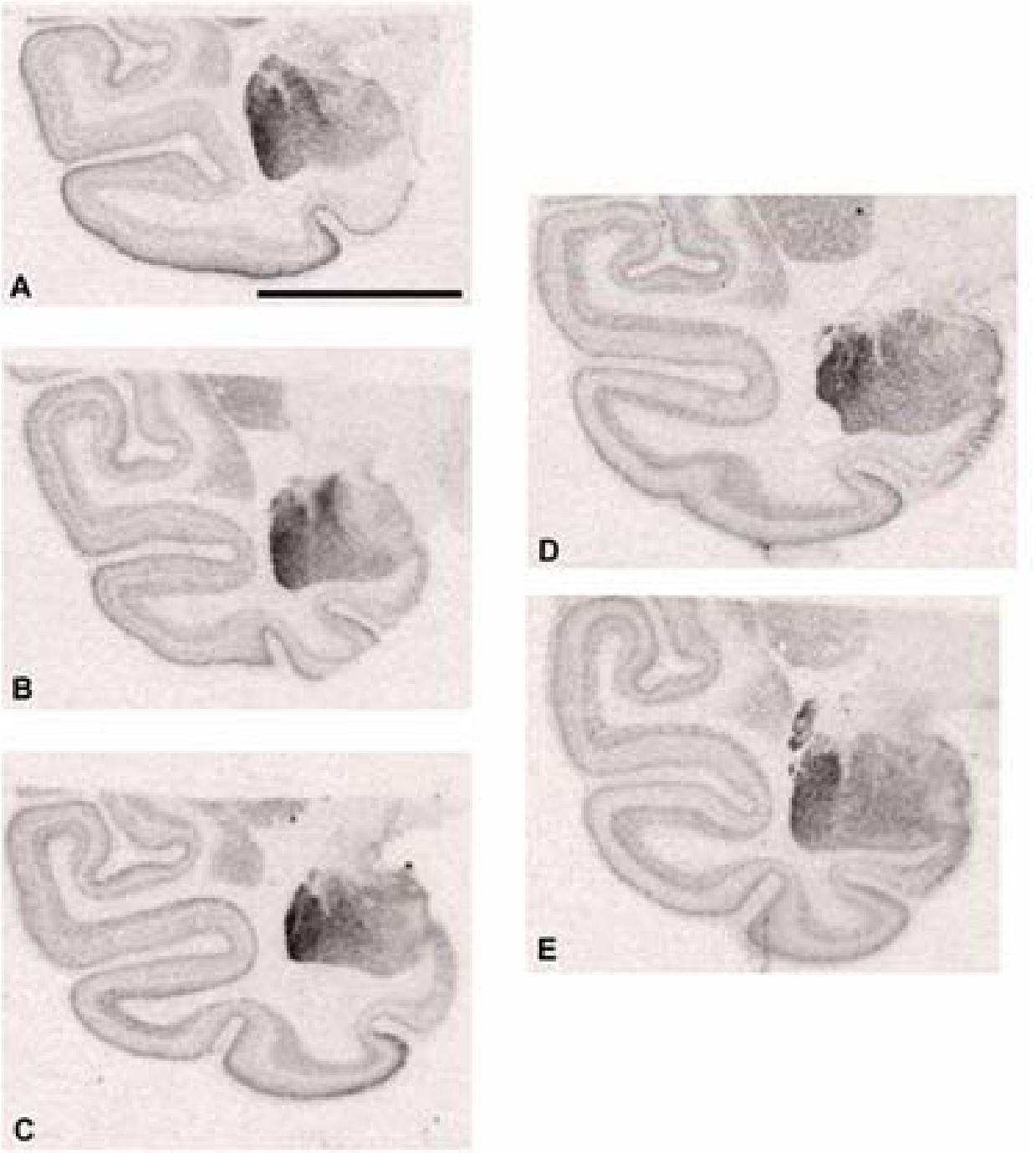

1.0
0.8
0.6
0.4
0.2
0.0
µCi/g
1
4
12
63
176
Age (weeks)

Figure 3.5 Anatomical Expression of the Development of GUCY1A3 mRNA expression. Images of representative scans of the radiographic films from one animal per age: A) 1-week, B) 4-weeks, C) 12-weeks, D) 63-weeks, and E) 176-weeks. Each image depicts the amygdala and surrounding cortical areas. Darker shades of grey represent high levels of GUCY1A3 mRNA expression while lighter shades of grey represent low levels of GUCY1A3 mRNA expression. Intense labeling is present in the amygdala at all ages examined. Scale bar, 10 mm.


A
B
C
D
E

behavioral effects in this same model of early social bond disruption. In terms of those behavioral studies, the biggest difference between groups existed between the maternally reared controls and the one-week separated monkeys, and the behavioral phenotype of the one-month separated monkeys fell in between. Interestingly, the GUCY1A3 gene expression data resemble the behavioral data in that the largest difference exists between the maternally reared controls and the one-week separated monkeys, while the expression level in the one-month separated monkeys is intermediate between the two. These similarities raised the possibility that not only is this gene regulated by early maternal care, but also that it may be functionally responsible for the differences in long term behavior. To further examine this relationship we performed a correlational analysis between aspects of the behavior that differentiate the three groups of monkeys during the third month of life and the expression level of GUCY1A3 mRNA specifically in the amygdala. The expression of this gene was significantly correlated with two behaviors that were measured in the native social group environment during the month preceding the study: self-comforting behaviors (thumb-sucking and toe-sucking), and typical social behaviors. We hypothesize that this relationship is more than a correlation, but also may be causal in nature. This hypothesis is supported by the fact that intraamygdaline injection of an inhibitor of NO synthase results in increased anxiety in rats (Monzon et al., 2001). To assess whether this downregulation was the result of decreased expression or a failure to develop a normally increasing expression, we conducted another series of in situ hybridization studies on unstressed monkeys at five ages spanning one-week to 176 weeks. There were no significant differences in GUCY1A3 expression levels with respect to age at any of the timepoints examined. We interpret these data as indicating that the adult level of GUCY1A3 gene expression is already in place by one week of age. Given the level of the reduction in

GUCY1A3 expression, we favor the explanation that normally expressing cells decrease their expression over the alternate possibility that subsets of cells that express GUCY1A3 mRNA are eliminated. The fact that the GUCY1A3 is well correlated with salient behaviors in the social group may signify that it deserves further attention as a possible direct modifier of socioemotional behaviors.

The definitive role of GUCY1A3 in the amygdala is unknown, but we can glean some clues as to the potential functional result of the downregulation of GUCY1A3 mRNA observed in this study in the context of what is known about the protein and its interactions. GUCY1A3 acts as the soluble receptor for nitric oxide (NO) in a variety of brain tissues, where it responds to NO binding by stimulating the production of cGMP (Ahern et al., 2002). It is thought to mediate its actions via downstream protein kinase G, cyclic-nucleotide-gated channels, and cGMP dependent phosphodiesterases. In our study, specific labeling by antisense riboprobes was located around and over lightly counterstained, large cells in all subnuclei examined. By virtue of their morphology, these neurons are likely the pyramidal-like neurons described by others (Pare et al., 1995; Faber et al., 2001), although it is possible that a subset may be interneurons since they comprise about 25% of the neurons in the monkey basolateral amygdala and there is evidence that some nitric oxide producing neurons also express neuropeptide Y and somatostatin in the basal nucleus (McDonald and Augustine, 1993; McDonald et al., 1993). Even though this gene is most likely expressed in excitatory cells, its role in the cell's activity remains uncertain. Indeed NO has been shown to have excitatory, inhibitory, and modulatory effects depending on where and how it is studied (Dawson and Snyder, 1994; Mothet et al., 1996).

One definitively reported function of guanylate cyclase is the facilitation of long term potentiation in the amygdala (Chien et al., 2003). In rat amygdala slice recordings, stimulation

of guanylate cyclase by the drug YC-1 leads to enhanced and enduring potentiation of the cortico-amygdala pathway; this potentiation could be eliminated by concomitant inhibition of NO synthase by L-NAME (Chien et al., 2005). Other studies have shown in vivo that inhibition of NO synthase impairs memory in rats as assessed by the Morris water maze (Holscher et al., 1996), and recently, Chien et al. (2005) have tested YC-1 in vivo and found improvements in Morris water maze performance. Thus if decreased long term potentiation in the amygdala is responsible for the behavioral deficits in early separated monkeys, drug treatment with YC-1 may be a promising avenue for future studies of therapeutic intervention. More often, though, increased amygdala LTP is associated with negative aspects of emotion. LTP in the amygdala has been postulated as the mediating factor of fear consolidation (Schafe et al., 2001). It has been suggested that NO generated postsynaptically by Ca2+-calmodulin dependent NO synthase acts as a retrograde messenger and binds to presynaptic guanylate cyclase producing cGMP dependent downstream effects, which strengthen the synaptic connection between these coordinately firing cells (Hawkins et al., 1998; Son et al., 1998). At first glance, it would seem surprising if decreased signaling through GUCY1A3, causing deficits in LTP, was contributing to the behavioral phenotypes observed in this study. Since this pathway modulates fear consolidation memory, you would expect that decreased signaling through GUCY1A3 would lead to decreased anxiety. However, since we are observing GUCY1A3 mRNA, in amygdala cells, then this necessarily means, if they are involved in LTP, that these cells are the presynaptic cells and not the postsynaptic cells. Any LTP occurring through GUCY1A3 in these cells would be in efferent pathways and not the afferent pathways that provide information about the unconditioned stimulus in fear consolidation. It is likely that the dense labeling in the lateral nucleus is in cells that project to the accessory basal, basal, and central nuclei. Other potential

projections would be projections to self, some cortical areas, most of the remaining subnuclei, and the medial temporal lobe memory system. In this study, monkeys exhibit decreased GUCY1A3 and increased anxiety-like behavior. Both of these facts, however, could be rectified if the main effect of the decreased LTP as a result of lower levels of GUCY1A3 resulted in the decreased ability for the amygdala to form stimulus reward relationships (Baxter and Murray, 2002). This deficit would hamper a monkey's ability to make stimulus reinforcement evaluations.

NO signaling has also been implicated in neuronal development in a variety of systems studied. In vertebrates, NO signaling contributes to the development of retinotectal projections (Wu et al., 1994; Angelucci et al., 1997), and the activity dependent synaptic suppression at the developing neuromuscular junction (Wang et al., 1995). Additionally it is critical for normal development in drosophila, as a mutation in the NO synthase gene is lethal late in embryogenesis (Regulski et al., 2004). Its functions in neuronal development may be related to its ability to modulate neurite outgrowth. NO-releasing compounds have been shown to either induce growth cone collapse (Hess et al., 1993; He et al., 2002) or increase neurite extension (Hindley et al., 1997; Rialas et al., 2000) in different experimental systems. It may be relevant to the current study since it is known that the amygdala continues to develop in postnatal life in many vertebrates. For example, rabbits continue to add neurons to the lateral nucleus of the amygdala through PND60, and in the accessory basal, and basal nuclei until PND90 (Jagalska-Majewska et al., 2003). In the cat, increases in dendritic length, branching, dendritic field, and number of cell types in the basolateral group of nuclei occur through PND18 (Wakefield and Levine, 1985). In rats, addition of neurons to the basal nucleus extends postnatally to PND7 and in the lateral nucleus postnatally to PND14 (Berdel et al., 1997). Also in rats, injections of anterograde and

retrograde tracers suggest that a period of refinement in amygdalocortical connections occurs between P9 and PND11 where a diffuse pattern matures into the adult restricted pattern of innervation (Bouwmeester et al., 2002b; Bouwmeester et al., 2002a). There is little data with regard to the development of the connectional anatomy of the amygdala in the monkey, but one study reported that there were connections with the temporal cortex (area TEO) at 1 wk of age that did not exist in adult monkeys (Webster et al., 1991). Together with the fact that it is known that area TE does not become fully functional until three months of age (Rodman and Consuelos, 1994), it is possible that at that time, the amygdala temporal lobe connection transforms into its adult-like form. If the guanylate cyclase reduction results in the aberrant development of amygdala efferent target sites, then the effects of this reduction may be realized in parts of the brain throughout the amygdala's projection field as well as within the heavily interconnected subnuclei. If distal amygdala connections are more important for the impairments of early separated monkeys, then the global delivery of a drug such as YC-1 may be superior to local infusion into the amygdala.

One obvious conclusion of the current study is that the ultimate effect of maternal separation on amygdala pathology is intimately dependent on the timing of the separation. This suggests that there are developmental events involving the amygdala between one-week and one-month of age in the monkey that are sensitive to maternal care. One possibility is that a normally functioning amygdala is required for the normal development of other brain regions that it targets. This notion is supported by amygdala lesion studies in several vertebrates; lesions that are given at different developmental time-points result in different chronic effects. For example, monkeys who receive bilateral amygdala lesions as adults show decreased social fear and decreased fear of novel objects (Emery et al., 2001), while monkeys who receive the same

bilateral amygdala lesions at two weeks of age show decreased fear of novel objects, but increased social fear (Prather et al., 2001) even when tested later in development (Bauman et al., 2004a). Although the latest that the neonatally lesioned animals were tested in social interaction (Bauman et al., 2004a) was still earlier than when the adult lesioned animals were tested (Emery et al., 2001), it is believed that increased fear in social interactions will be a stable life-long trait in the neonatally lesioned monkeys. If so, then it must signify that the amygdala is responsible for the normal development of other brain regions. Relating this to the current study, it is possible that, given guanylate cyclase's known functions in development, maternal separation creates a functional amygdala lesion in infant monkeys. The earlier this functional lesion occurs, the greater the effect on target sites that require amygdala input for proper development. If one of these sites was the aforementioned TE, TEO areas, for example, then early maternal separation may chronically and fundamentally affect how the infant monkey perceives its environment.

Evidence from other animals and humans support the dissociating effects of differentially timed amygdala lesions. For example the group of van Ree et al. has extensively examined the long term behavioral and anatomical consequences of amygdala lesions in the rat that were initiated at either PND7 or PND21. They have observed differences in social behavior, exploratory activity, sensorimotor gating, and stress reactivity (Daenen et al., 2001; Wolterink et al., 2001; Daenen et al., 2003) when measured at the same time in adulthood in both lesioned groups. As a general organizing principle, deficits in these behaviors follow lesion at PND7, but PND21 lesioned rats are spared. Another study from this group tested the hypothesis that differentially timed lesions would affect tasks dependent on mPFC functioning. The impetus for these tests stemmed from the fact that the basal nucleus - mPFC connection is refined after PND7 in nonlesioned animals (Bouwmeester et al., 2002b; Bouwmeester et al., 2002a). As they

expected, the authors of this study observed deficits in food hoarding and spontaneous alternation with a sparing in water maze performance in PND7 lesioned animals compared to PND21 lesioned animals (Diergaarde et al., 2005). The former tasks have been previously shown to be dependent on mPFC viability (de Brabander et al., 1991). Additionally time dependent lesion effects have been observed in humans with amygdala damage (Shaw et al., 2004). In complex tasks designed to establish whether an individual can attribute mental states to others (called "theory of mind"), adults who sustained early damage to the amygdala performed poorer compared to those who had sustained late damage to the amygdala. In fact, the persons with late-damage were relatively unimpaired. These findings raise the possibility that the amygdala supports the development of "theory of mind" but is not necessary for its maintenance. All of these data converge on the fact that a normally functioning amygdala is required for the development of other areas of the brain. As such, the types of deficits that are observed in the one-week versus one-month separated animals are possibly due to amygdala-driven development of the cortex, or one of its other connecting sites. In future studies it will be important to consider the orbital frontal cortex as a potential site mediated by abnormal amygdala function. This cortical area is heavily connected with the amygdala, and it, too has been implicated in the generation of reward related behavior (Holland and Gallagher, 2004)

The effects of the NO – Guanylate Cyclase – cGMP signaling in emotional behaviors have been more directly examined in rodents. Interruption of this pathway has led to mixed findings with regard to anxiety-like behaviors. In one study, knockout of the major downstream target of cGMP, cGKII, led to increased anxiety-like behavior in light/dark test and elevated maze test (Werner et al., 2004). Although cGKII is highly expressed mainly in limbic brain areas, the anxiogenic features evident after its knockout may possibly have resulted from

extraamygdalar effects. Another study, however, offered evidence directly implicating the amygdala. In this study, intraamygdalar injection of the inhibitor of NO synthase, L-NOARG, resulted in decreased time spent on open-arms in the elevated plus maze (Monzon et al., 2001). We think that this is the clearest evidence arguing for an anxiolytic property of this pathway in the amygdala, however some other studies report opposite effects for systemic treatments. Volke et al. reported that systemic administration of L-NAME or 7-nitroindazole, both NO synthase inhibitors, reduced anxiety in the elevated plus maze (Volke et al., 1995; Volke et al., 1997). These studies highlight the possibility that inhibition of NO synthase specifically in the amygdala may have different consequences compared to global inhibition.

The finding that the decrease in GUCY1A3 mRNA was throughout the amygdala, but especially prevalent in the lateral and basal nuclei, merits some discussion. It is possible that the mediator of this effect acts through the bloodstream to gain access across the entire structure. One potential candidate would be adrenal corticosteroid binding to glucocorticoid receptors (GR) or mineralocorticoid receptors. Glucocorticoid receptors are expressed in the amygdala of the monkey brain especially in the lateral nucleus (Patel et al., 2000). Altered HPA axis function is a common feature observed in animal models of animal models of early maternal separation (Meyer and Bowman, 1972; Clarke et al., 1998).

Another potential mediator of a global change in the amygdala would be from ascending serotinergic fibers from the dorsal raphe nucleus (Mehler, 1980). Antibodies directed against serotonin reveal dense fiber plexuses in the lateral, basal, and central nuclei (Sadikot and Parent, 1990), with relatively poor labeling of the accessory basal. Interestingly, this pattern of labeling closely resembles the differences in GUCY1A3 expression in control (and separated) monkeys. Importantly, the density of this marker was reported as relatively high compared to both

dopaminergic and noradrenergic markers. Serotonin 1a receptors are abundant in the lateral nucleus, basal nucleus, and periamygdaloid cortex (Stuart et al., 1986). Recently, the ability of serotonin to modulate the early expression of markers of nitrergic cells in the amygdala of the rat was investigated (Tagliaferro et al., 2003). Depletion of serotonin by two parachloroamphetamine injections given to rats on PND3 and PND4 caused an increase in neuronal NO synthase and NADPH diaphorase (a marker of NO synthase cells) over early development. This experiment might suggest that overproduction of serotonin might be leading to the reduction in GUCY1A3. There is some indication in rats that impaired feedback through autoreceptors in the dorsal raphe may lead to a hyper-release of serotonin following early maternal separation (Gartside et al., 2003). However since there is one more step in the sequence of events (NO must bind to GUCY1A3) it is entirely possible that it would predict the opposite. Namely, that underproduction of serotonin causes an overproduction of NO which causes a compensatory decrease in GUCY1A3. Indeed, other studies show that treatment with an SSRI eliminates some of the behavioral and neuroendocrine consequences of early maternal separation in rats (Huot et al., 2001). The only study to the author's knowledge that examined serotonin in response to maternal separation in monkeys did not find it to be regulated over development (Kraemer et al., 1989), however the measurements were taken once a month from the CSF over the first two years, so only a very broad indication of whole brain serotonin output was assayed. More work needs clearly needs to be done to examine how early stress affects serotonin function.

A limitation of this study is that the gene expression measurements were only measured in the right amygdala. Differences in right and left activation in the amygdala have been a consistent feature in many studies of amygdala function. Several explanations have been offered to explain this consistent finding: 1) the left amygdala is more involved with detailed feature

extraction and longer processing times, while the right is more involved with pictorial or image-related processing and shorter processing times (Markowitsch, 1998; Phelps et al., 2001), 2) explicit versus implicit task instructions leads to greater left activation (Lange et al., 2003), and 3) right activation habituates faster than left activation and therefore is less likely to be significant in longer imaging times (Wright et al., 2001). A recent meta-analysis of functional imaging studies suggests that although indeed left amygdala activation is more commonly reported than right amygdala activation (Baas et al., 2004), this activation does not seem to be affected by either task instruction or level of processing. Another possible interaction is with the sex of the individuals, Cahill et al. (Cahill et al., 2001) found that with respect to memory for emotionally arousing films, males had greater activation in the right amygdala and females had greater activation in the left amygdala. It should be noted that the preponderance of studies that report lateralization have been conducted in humans, and laterality in humans may not translate to laterality in monkeys. For example, when measured early in development. Rhesus macaques were noted to have a preference for using the left hand and not the right hand in various coordination tasks (Westergaard et al., 1997). Hence although laterality may be a feature of amygdala activation, and it would be important to examine whether the GUCY1A3 downregulation is also evident in the left amygdala, it does not minimize the importance of the current finding.

4. Conclusions and Future Directions

4.1. Summary of Findings

Over the last several years, our lab has developed a compelling model of the effects of early life social stress on the developing brain and behavior (Cameron, 2001; Bytheway, 2004; McCormick, 2005; Rockcastle, 2005). In this model, rhesus monkeys are exposed to the stress of early life maternal separation at either one-week or one-month of age. Monkeys who experience maternal separation at one-week of age develop long-term deficits in social behavior and also exhibit many anxious behaviors both in their native social group interactions and in laboratory tests of temperament. Monkeys who experience maternal separation at one-month of age develop long-term increases in social behavior and also display anxious behaviors in test of temperament, but the types of anxious behaviors they display are distinct from those that are displayed by the one-week separated monkeys (Rockcastle, 2005). Interestingly, the decreased and increased tendencies to socially interact for the one-week and one-month separated monkeys, respectively, remain evident through puberty and into adulthood. Thus, the influence of only three extra weeks of maternal rearing, when occurring early in life, produces profound long-term consequences. The work in this dissertation has focused on answering two main questions in this model of early life social stress in rhesus monkeys. These questions are: 1) can the long-term reaction to the stress of maternal separation be predicted from aspects of acute separation response? and 2) are there gene expression changes in the amygdala that may subserve these very different long-term behavioral outcomes? The answers to these questions may have clinical significance for children who are exposed to early life stress for example those who are reared in orphanages.

Acute Behavioral Response. With respect to question one, we examined the acute behavioral response over the ensuing five day period following early maternal separation in both one-week separated and one-month separated female rhesus monkeys. Distinct patterns of behavior were evident that depended on the timing of the separation and were strikingly similar to the way people cope with stressful life events. One-week separated animals responded by increasing their levels of contact comfort with a soft cotton snuggly toy compared to one-month separated monkeys. Children responding to stress will often grab an attachment object such as a blanket to cope. The preference for similar objects has been a consistent finding in studies of maternally separated rhesus monkeys (Harlow and Zimmermann, 1959; Suomi, 1973). On the other hand, one-month separated monkeys responded by increasing their seeking of social comfort as measured by their tendency to locate themselves adjacent to their group rearing environment. This tendency may share some similarities with a style of coping known as "engagement coping" or "seeking of social support". In people who are dealing with acutely stressful life situations, this method of coping is associated with better long-term outcomes.

Longer-term Behavioral Response. Next we measured long-term behaviors in these monkeys in their social rearing group. For these comparisons we added a group of maternally reared monkeys to serve as undisturbed controls. In their social rearing group, one-week separated monkeys spent significantly less amounts of time engaging in species typical social behavior and significantly more time engaging in self-comforting behaviors compared to both one-month separated monkeys and maternally reared controls. One-month separated monkeys displayed an intermediate phenotype between the other two groups of monkeys with respect to these behaviors. These data are in agreement with previous studies of our laboratory conducted in a mixed-sex cohort at this age (Cameron, 2001).

Predictors of Long-term Behavior. In an effort to decode which behaviors in the acute separation period might be predictors of behavior in the chronic setting of the native social group, we ran a correlational analysis. In this analysis we determined that in one-week and one-month separated monkeys considered together, the level of contact-comforting behaviors in the acute setting predicts both the level of time spent engaging in self-comforting behaviors and the amount of time spent engaging in nonsocial behaviors in the social rearing group later in life. Both of these relationships varied directly, as one would expect. Additionally, the amount of time seeking social comforting, as assayed by preference for locating oneself in the acute separation cage on the partition that abuts the native social group pen, predicts the amount of time spent engaging in nonsocial behaviors in an inverse relationship, again, as one would expect. Another behavior, locomotion, that was not significantly different between groups in either setting, nevertheless showed a significant correlation in the acute setting compared to the native social group setting. One may contend, then, that the tendency to locomote is in place early, and is not stratified by the age at which an individual monkey encounters the environmental stress of early life separation.

Gene Expression Correlates of Behavior. With respect to question two posed earlier, we examined gene expression in the amygdala at three months of age in monkeys that had been maternally raised, maternally separated at one-week, or maternally-separated at one-month. Microarray analysis suggested many potential candidates for downstream analysis; we chose to follow up on the gene that was most differentially expressed between the one week separated and maternally raised controls, GUCY1A3. We confirmed the robust downregulation by quantitative in situ hybridization and localized the anatomy of the expression change to large, lightly counterstained neurons, which likely correspond to projection neurons (Pare et al., 1995; Faber et

al., 2001). The downregulation was most evident in the lateral and basal nuclei, the location where this gene is most highly expressed. Since the gene expression change resembled, in principle, aspects of the behavioral phenotype during the third month of life, we ran a correlational analysis. GUCY1A3 was found to be negatively correlated with the amount of time that an animal spends engaging in self-comforting behaviors, and positively correlated with the amount of time an animal engages in nonsocial behaviors. We propose that this relationship is more than a correlation, but also may be causation. This hypothesis is supported by the fact that intraamygdaline injection of an inhibitor of NO synthase results in increased anxiety in rats (Monzon et al., 2001). To assess whether this downregulation was the result of decreased expression or a failure to develop a normally increasing expression, we conducted another series of in situ hybridization studies on unstressed monkeys at five ages spanning one-week to 176 weeks. There were no significant differences in GUCY1A3 expression levels with respect to age at any of the time points examined. We interpret these data as indicating that the adult level of GUCY1A3 gene expression is already in place by one week of age. Given the level of the reduction in GUCY1A3 expression, we favor the explanation that normally expressing cells decrease their expression over the alternate possibility that a subset of cells that express GUCY1A3 mRNA are eliminated.

We feel that this gene product is an excellent candidate for possible pharmacotherapeutic interventions. It is directly modulated by the activator YC-1, and can be indirectly deactivated by many inhibitors of NO synthase, such as 7-nitro indazole. The efficacy of these drugs in affecting NO dependent tasks has been previously established. Further the fact that these drugs have opposite effects on Morris water maze behavior in rats suggests that their gene target may be involved with learning and memory (Holscher et al., 1996; Chien et al., 2005).

4.2. Clinical Relevance

As described in more detail in chapter two, the findings of the present study suggest that the early acute response to maternal separation in rhesus monkeys may serve as a useful model for coping style in humans. In humans coping style has been consistently associated with outcomes such as level of depressive symptoms (Vitaliano et al., 1987; Tolan et al., 2002) and PTSD (Foa et al., 1995; Harvey and Bryant, 1999), and in monkeys, the current study has implicated coping style in predicting differences in social interaction and self-directed behaviors, but how differences in coping styles are functionally translated to psychiatric illnesses is not well understood. In both cases, the early coping styles of seeking of social contact are associated with more beneficial outcomes, and the early coping styles of avoidance and internalizing are associated with negative outcomes (Leitenberg et al., 1992; Himelein and McElrath, 1996). The utility of a nonhuman primate model of coping style lies in the ability to: 1) experimentally manipulate coping style through the environmental manipulation of maternal separation, 2) longitudinally follow the outcomes of different coping styles in a more controlled manner, one that is not affected by certain biases that plague retrospective and longitudinal studies in humans, and 3) the ability to associate different styles of coping with more invasive studies of the brain and physiology. Although the current study is not able to explicitly differentiate whether coping style and long term well being are more determined by heritable factors, or by environment, future studies that rigorously control for environmental differences in monkeys will be able to answer these questions. Human studies of temperament, a factor that partly explains coping style (Compas, 1987) have suggested that there is a strong heritable component of how individuals react to stressful stimuli (Robinson et al., 2002). The current study is highly suggestive that

early interactions with a securely attached caregiver are also critical for determining temperament and coping style. In chapter two it was posited that both of these findings may be rectified by the assertion that the mature expression of temperament is largely determined by hereditary factors, but to realize this level of mature expression an early secure attachment with a caregiver is mandatory.

In children who are exposed to orphanages early in life, the later development of attachment disorders is a common consequence (O'Connor, 1999). Most children fare quite well after adoption, a testament to the resilience and plasticity of the human brain, but others go on to develop reactive attachment disorders. It has been suggested that the length of time spent in the orphanage is a predictor of later attachment tendencies (O'Connor et al., 2003), but exactly what factors lead to the development of attachment disorders are unknown. Based on the results of chapter two, one prediction may be that differences in the coping styles of the child early in life affect the later expression of psychopathology. In fact, in other clinical populations, early behavioral therapy aimed at altering coping styles has lead to better long-term outcomes (Foa et al., 1995). Hence, teaching children better coping skills may also lead to better long term mental health.

In the study of the long-term effects of early social bond disruption, a consistently emerging feature is that monkeys who experience the stress of maternal separation go on to express differences in social behaviors and anxious behaviors (Cameron, 2001; Rockcastle, 2005, and chapter 2). These features may not be completely separable, since differences in social behaviors may be explained, in part, by social anxiety. In chapter three of this thesis we have uncovered a gene product in the amygdala that is expressed in an inverse relationship to the level of anxious behaviors that are observed in monkeys that have experienced maternal

separation stress early in life. Based on experimental tests of anxiety in rodents (Monzon et al., 2001), it is possible that this gene directly contributes to the anxious to the anxious phenotype. Furthermore, a specific potentiator of this gene product's actions, the drug YC-1, can enhance memory in amygdala directed tasks in rodents (Chien et al., 2005). We suggest based on the experiments presented, that this drug may also be effective in moderating anxiety in monkeys. This work may also support later clinical studies of YC-1 in human anxiety disorders.

4.3. A Proposed Model

One obvious conclusion of the current study is that the ultimate effect of maternal separation on amygdala pathology is intimately dependent on the timing of the separation. This suggests that there are developmental events that involve the amygdala between one-week and one-month of age in the monkey that are sensitive to maternal care. Thus when the stress is experienced at one-month, the extra three weeks of maternal care offer a relative resistance to amygdala pathology. In the discussion of chapter three, I offered the possibility that three different early-versus-late amygdala lesion studies (rat, human, and monkey) led to the conclusion that there are postnatal developmental events occurring in all of these vertebrates that depend on the amygdala. In these three studies, after the amygdala dependent developmental phase, the amygdala lesions do not have an adverse effect on tasks which require only these other brain regions. Specifically, in the rat, the development of the mPFC task of food-hoarding requires the amygdala, but once the mPFC is developed, the amygdala is no longer required for this behavior. Experimentally this was borne out by the observation that amygdala lesions occurring on PND7 but not on PND21 affected the adult expression of this food-hoarding task (Diergaarde et al., 2005). In monkeys, early amygdala lesions lead to increased anxiety in social

interaction later in life, but adult lesions lead to decreased anxiety in social interactions (Emery et al., 2001; Bauman et al., 2004a). Finally in humans, early amygdala damage leads to adult deficits in theory of mind tasks, while late amygdala damage does not affect these same tasks (Shaw et al., 2004). Based on the arguments offered previously, the substrate for this dependence may, in fact, be related to the development of efferent amygdala connections and their reciprocal developmental effect on their targets. Relating this to the current study, it is possible that, given 1) guanylate cyclase's known functions in neuronal development (Hess et al., 1993; Wu et al., 1994; Angelucci et al., 1997; Hindley et al., 1997; He et al., 2002; Regulski et al., 2004) and 2) the finding that GUCY1A3 is substantially reduced following maternal separation, this early stress creates a functional amygdala lesion in infant monkeys. The earlier this functional lesion occurs, the greater the effect on target sites that require amygdala input for proper development. If one of these sites was TEO (Webster et al., 1991), for example, then early maternal separation may fundamentally affect how the infant monkey perceives its environment. This model would predict, then, that rescuing the GUCY1A3 expression late in development may not reverse some of the changes that have ensued, while rescuing GUCY1A3 expression early would have more beneficial effects on later behavior.

4.4. Future Directions

Based on the experiments presented in this thesis, and partly on the proposed model of the last section, we can suggest several important future studies. These suggestions will be in two broad categories: A) Behavior and gross amygdala function, and B) The GUCY1A3 pathway.

A. Behavior and Gross Amygdala Function. The two main outstanding questions left from the studies presented in chapter two is whether heritable factors or environmental factors are more

important for predicting the long-term behavioral response to maternal separation, and whether the correlation will continue to be significant at time points downstream of three months. In our studies, a small sample size for each separation group precluded a definitive answer to this question, although as noted, one of the predictive relationships remained significant even among one of the separated groups. The ability for the level of contact comforting in the acute reaction to predict levels of chronic self-comforting behaviors remained significant even when limited to monkeys who received same environmental manipulation of one-month maternal separation. Nevertheless another study to solidify this finding and extend it also to the one-week separated monkeys would be of enormous value in definitively answering this question. One of the reasons we were able to demonstrate significant correlations with a relatively small sample size was the fact that the environmental manipulation led to an augmentation of variability in both the acute and chronic responses. In the absence of this environmental augmentation, many more animals will need to be examined in each group to achieve enough variability to support a correlational analysis at the level of the individual monkeys. As such future work should examine at least 20 animals per group in each of the one-week separated and one-month separated groups. The chronic behavioral assessment should also include data from later developmental time points, for instance before and after puberty. These later time points would provide information about whether the later environmental experiences can significantly alter the course of an individual's behavioral development, or whether this trajectory is mainly determined by heritable factors. To definitively rule out differences in environment in the early correlation (acute – three month response), behavioral assessments of maternal-infant interaction prior to separation should also be measured. Finally, as much as possible, infants within each group should be genetically

unrelated. This fact would aid in ensuring variability in the acute response to maternal separation.

Even though we have discovered a potential target brain area that is affected by maternal separation, the amygdala, it is unknown exactly how the differences in amygdala activity might affect behavior. Previous experiments regarding the functions of the amygdala may lead to competing hypotheses. For example, the amygdala is known to be intimately involved in fear conditioning and the assessment of the environment for danger (Davis, 1992). Based on this function one might expect that individuals that show more anxious behaviors would have a hyperactive amygdala. However, ablation of the amygdala completely in infant monkeys at two weeks of age can lead to increased anxiety in social situations (Prather et al., 2001). From this work, one might expect that decreased amygdala activity early in life leads to increased anxiety in social situations. Furthermore, one aspect of attachment theory predicts a dissociating of fear responses from attachment responses (Bowlby, 1982). Therefore on the basis of these studies and predictions, the exact function of the amygdala following maternal separation may be unclear. To examine how the amygdala is functioning at the gross level in monkeys who experience maternal separation, the following experiment is proposed. At three months of age, monkeys of both separation groups and maternally raised controls would receive an injection of fluorodeoxyglucose. They would then be returned to their social group with as little disturbance as possible. After a 30-minute period of observation while the monkeys are assessed for stress response and social functions, they would be anesthetized and scanned using PET. Differences in amygdala oxygen utilization could then be interpreted with respect to functioning of the amygdala. Recent experiments using this method have suggested the ability to highlight amygdala – prefrontal circuits involved in anxiety (Kalin and Shelton, 2003).

B. The GUCY1A3 pathway. We have identified a key element - GUCY1A3 - that is distinctly regulated by maternal separation at different early time points and correlated with behavioral responses to these separations, but exactly how differences in this gene may result in differences in behaviors requires more work. GUCY1A3 is a component of the nitric oxide signaling pathway (Ahern et al., 2002). Upstream of GUCY1A3 signaling events activate NO synthase, NO then binds to GUCY1A3 resulting in the formation of cGMP. Downstream, cGMP can act through several effectors to influence neuronal activity. Directly increasing the activity of the guanylate cyclase in rodents by injection of YC-1 has been shown to enhance memory in certain amygdala related tasks (Chien et al., 2005), suggesting that a major site of action is the amygdala. Future work could directly examine the effects of YC-1 in both one-week and one-month maternally separated monkeys. After YC-1 treatment monkeys could be assessed either in their homepen for differences in social behavior, or in tests specifically designed to examine temperament (Cameron, 2001; Rockcastle, 2005). We expect that if GUCY1A3 is directly related to the behavioral deficits, then treatment with YC-1 may lead to increased social interaction in one-week separated monkeys, and decreased anxiety in both one-week and one-month separated monkeys.

Previous work in our group suggests that monkeys who experience maternal separation at one-week of age can benefit from adoption by a foster mother, as long as this adoption occurs within a suitable time period (McCormick, 2004). In the context of this thesis, these studies beg the question: does fostering rescue GUCY1A3 expression? To examine this, one week separated monkeys could be assigned either to fostering or no fostering during weeks two through four, and at three months their amygdala GUCY1A3 expression could be assessed. If the expression

is rescued, then more detailed developmental studies may be worthwhile to parse out the exact timeframe of how GUCY1A3 is modulated.

Other studies may examine whether GUCY1A3 amygdala function is a critical component for social and anxious behaviors in rodents. For example, whether GUCY1A3 knockout limited to the amygdala would result in differences in anxiety as measured on the elevated plus maze could be examined. Furthermore in a time and spatially limited conditional knockout, one could look at the time dependency issues that were introduced in the proposed model above. It would be worthwhile to examine how KO of GUCY1A3 around PND4 differs with respect to KO at PND21. At these ages, many studies have seen differences in how gross amygdala lesions affect adult behavior in rodents (Daenen et al., 2001; Wolterink et al., 2001; Daenen et al., 2003).

Finally, other components of the NO signaling pathway should be examined in the amygdala of maternally separated monkeys. Knowledge of how this pathway is coregulated may be useful for many reasons. First, if downstream effectors of GUCY1A3 are also downregulated, then treatments aimed at increasing the action of GUCY1A3 might have no effect. Furthermore, this knowledge would be useful to determine whether the decrease in GUCY1A3 is a specific effect, or if other factors are acting to decrease the output expression of the whole pathway. As such, it would be important to look upstream at NO synthase, and downstream at cGKII, protein kinase G, cyclic nucleotide phosphodiesterases, and levels of cyclic nucleotide gated channels. Additionally, one further upstream marker of considerable interest may be serotonin. Recently, serotonin was shown to play a critical role in the early development of the NO phenotype in three brain regions (Tagliaferro et al., 2003). In the context of this study, future research should

also critically examine serotonin receptors in, and serotonin innervation of, the amygdala as a potential mediator of disturbed the disturbed NO signaling pathway.

4.5. Conclusion

In summary, the experiments in this thesis have demonstrated two correlates of the long-term behaviors that result from maternal separation in monkeys. These long-term behaviors are related to social interaction and anxiety. In these studies they have been correlated with the initial response to maternal separation and with GUCY1A3 mRNA in the amygdala. The former correlation raises the possibility that individuals who are at most risk for developing adverse outcomes can be identified early in the course of pathology. This may have clinical application for those who suffer early stress in childhood. The latter correlation suggests that some of the downstream effects of early stress may be directed by the amygdala, and that this gene may drive the associated behavioral responses. Further, many drugs target the signaling system that contains GUCY1A3, so perhaps these drugs would be beneficial for mediating similar behavioral pathologies in humans.

APPENDIX A

Summary of Unconfirmed Gene Expression Data

As a consequence of our continued effort to uncover the gene expression changes in the rostral medial temporal lobe that were ultimately described in chapter three, we attempted three distinct microarray platforms (Codelink, Amersham Biosciences; ABI 1700, Applied Biosystems; and GeneChips, Affymetrix), using both individual and pooled samples for one of these platforms (Codelink, Amersham Biosciences). Many genes that were found to be differentially expressed between one or more of the separation group comparisons on one or more of the microarray platforms were pursued with either quantitative RT- PCR or in situ hybridization validation. Table A.1 summarizes the differential expression data across the multiple methods on all genes that ultimately proceeded to in situ hybridization analysis. For each platform we imposed an expression difference cutoff criterion based on previous assessment of false discovery using custom bootstrap simulations and on previous data from our laboratory (for an example of FDR assessment, see Mirnics et al., 2005). Several genes reached our imposed criteria for differential expression between at least one of the groups of monkeys compared (see section 3.2 for comparisons) on more than one microarray platform. However, only one gene, GUCY1A3, was reported as differentially regulated by more than one platform and also by in situ hybridization studies. One other gene, LIM2 (Lim Homeobox Domain 2), was reported as differentially regulated by more than one microarray platform and also trended in the same direction according to RT-PCR. It should be emphasized here that LIM2 was not found to be significantly changed by in situ hybridization studies.

Table A.1 Summary of Expression Data for Genes which Proceeded to In Situ Hybridization

			Codelink			ABI 1700 (Pooled Sample)		Affymetrix			RT-PCR			In Situ		
Gene Name	Accession	Comparison	Direction	Fold	p-value	Direction	Fold	Direction	Fold	p-value	Direction	Fold	p-value	Direction	Fold	p-value
5 HT1aR	NM_000524	Con-Mon									▼	1.26	0.098			
Protein Phosphatase 1 β	NM_002709	Con-Mon	▲	3.05	0.070											
GABA A β 1	NM_000812	Con-Sep	▼	1.65	0.024											
CAM Kinase II β	NM_001220	Con-Wk	▼	5.00	0.064	▼	1.48									
LIM2	NM_004789	Con-Wk				▼	1.25	▼	1.36	0.010	▼	1.27	0.092			
Ectodermal Neural Cortex	NM_003633	Con-Wk						▼	1.29	0.030						
		Con-Mon						▼	1.30	0.008						
TNFα Rec. Assoc. Factor 4	NM_004295	Con-Wk	▲	1.28	0.007											
		Mon-Wk	▲	1.25	0.044											
NMDAR 2B	NM_000834	Con-Wk				▼	1.69									
BDNF	NM_001709	Con-Wk									▲	1.32	0.081			
Guanylate Cyclase 1 α3	NM_000856	Con-Wk				▼	1.83	▼	1.40	0.004	▼	1.29	0.088	▼	1.98	0.001
		Mon-Wk						▼	1.28	0.008				▼	1.72	0.003
FK506 Binding Protein 1a	NM_000801	Con-Wk						▼	1.34	0.014						
Glycogen Synthase Kinase 3 α	NM_019884	Con-Wk				▼	5.24									
NGAP-like Protein	NM_032552	Con-Wk	▲	1.47	0.001											
		Con-Mon	▲	1.27	0.011											
DAB1	NM_021080	Con-Wk	▼	1.41	0.015											

For comparisons, the first group listed is considered the control, and the second group listed is the comparison group. The direction of the expression change corresponds to the expression in the comparison group versus the control group. Fold changes represent the de-logged absolute value of the difference of the Log2 expression levels for each comparison. For all methods, genes were not considered differentially expressed unless there was at least a 1.25-fold change between control and comparison groups. Text in green represents mRNA expression changes between groups with an associated p-value between 0.00 and 0.05. Text in blue represents mRNA expression changes between groups with an associated p-value between 0.05 and 0.10. White squares represent assays that were completed, but no significant differences or trends were observed; grey squares represent assays which were ***not*** completed because either 1) Con – Month comparison was not done on the ABI 1700 platform, or 2) gene was not present on the given platform. For the ABI 1700 platform, pooled samples of Control and 1-Week separated animals were used and therefore there is no associated p-value computed.

The lack of successful validation for most of the genes tested has many potential explanations; the most likely explanations in these studies are cross-species hybridization, small sample size, and heterogeneity in the tissue assayed. It should also be noted that although for this discussion in situ hybridization (ISH) is considered as the gold standard, but it, too, is not without its experimental limitations. We expect that ISH measurements are very specific, but the sensitivity of the assay is limited. It has been suggested by others that gene expression changes must be on the order of 30% to be reliably detected by ISH; however this percentage likely depends on factors such as sample size and anatomical distribution. The former would be expected to vary directly with sensitivity while the latter's effects are less well delineated.

The ability to measure gene expression differences in monkey brain material on a global scale was, at the time of these studies, limited by the availability of only human microarrays. Hence, the cross-species hybridization of monkey mRNA to human-derived sequence added another source of variance to the data. Although reports of sequence similarity between humans and rhesus monkeys indicate approximately 94% concordance at the mRNA level and 92% at the amino acid level (Jaeger et al., 1993; Bals et al., 2001), these differences are not uniformly distributed throughout the genetic code (Frazer et al., 2003). Hence, certain regions of a particular genes sequence may show greater concordance than others. In fact, the comparison of 27 mb (megabases) out of the roughly 3000 mb in human and chimpanzee (thought to be our closest evolutionary relative) revealed 57 significant rearrangements (Frazer et al., 2003). It appears that there is no a priori way of determining exactly which genes will have significant rearrangements, and if they do, where these rearrangements will occur. Hence, on the basis of these arguments, and Table A.1, there seems to be enough variability between the human and rhesus monkey genome to affect the levels of hybridization in microarray studies.

However, even given that sequence differences occur, this does not necessarily completely explain why microarrays coded with human sequence underperformed in our studies. Even if differences in sequence between human and monkey were relevant to expression levels, each monkey sample presumably carries the same genetic difference, and therefore the levels of binding should be comparably reduced. Another likely explanation is that the normal experimental variation in the processes leading up to and including microarray analysis carries sufficient noise such that the small sample size of the present studies led to inconsistent results. On the basis of sheer numbers of genes assayed, many false leads may be expected.

Finally, the most likely explanation is in variability in the distribution of cortex and amygdalar elements in these samples. The ratio of cortex to amygdala was assessed from Nissl stained sections and they spanned the range of 0.8 to 1.7. Nevertheless, the difference between groups of monkeys was not significant. In any event, genes which are highly expressed in the cortex may lead to inconsistent results on our array samples. It should be noted that the major finding of chapter 3, GUCY1A3 was expressed at much higher levels in the amygdala than in the surrounding cortex. I would offer that as one reason why downstream experiments continued to support differential expression between groups.

Cross-species hybridization should not be problematic in future monkey work since monkey specific chip are now being marketed by several sources. In any event, the potential problems of totRNA isolation variability and tissue content variability will still exist. For future array experiments it would be beneficial to use laser-capture microdissection techniques to limit the latter of these two problems.

APPENDIX B

Abbreviation	*Description*
AB	Accessory Basal Nucleus of the Amygdala
Abmc	Accessory Basal Nucleus of the Amygdala, magnocellular division
Abpc	Accessory Basal Nucleus of the Amygdala, parvocellular division
ACTH	Adrenocorticotropic Hormone
ALR	Average Log Ratio
B	Basal Nucleus of the Amygdala
Bi	Basal Nucleus of the Amygdala, intermediate division
Bmc	Basal Nucleus of the Amygdala, magnocellular division
BNST	Bed Nucleus of the Stria Terminalis
Bpc	Basal Nucleus of the Amygdala, parvocellular division
BPD	Bipolar Disorder
CA	Central Nucleus of the Amygdala
CBZ	Central Benzodiazepine Receptor
cGKII	cGMP Dependent Protein Kinase II
cGMP	Cyclic Guanosine Monophosphate
CNS	Central Nervous System
CRF	Cortisol Releasing Factor
CSF	Cerebrospinal Fluid
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders IV
EH	Early Handled
EPL	Early Parental Loss
ERC	Entorhinal Cortex
GABA	Gamma Amino Butyric Acid
GR	Glucocorticoid Receptor
GUCY1A3	Guanylate Cyclase 1 α3 subunit
HFD	High Foraging Demand
HPA	Hypothalamic Pituitary Adrenal
ISH	In Situ Hybridization
KO	Knockout
LA	Lateral Nucleus of the Amygdala
LC	Locus Coeruleus
Ld	Lateral Nucleus of the Amygdala, dorsal division
Ldi	Lateral Nucleus of the Amygdala, dorsal intermediate division
LFD	Low Foraging Demand
LG-ABN	Licking and Grooming - Arched Back Nursing
LHPA	Limbic Hypothalamic Pituitary Adrenal

LIM2	Lim Homeobox 2
Lv	Lateral Nucleus of the Amygdala, ventral division
Lvi	Lateral Nucleus of the Amygdala, ventral intermediate division
MD	Mediodorsal Thalamus
MDD	Major Depressive Disorder
ME	Medial Nucleus of the Amygdala
mPFC	Medial Prefrontal Cortex
MR	Mother Reared
mRNA	Messenger Ribonucleic Acid
MS	Maternally Separated
NAcc	Nucleus Accumbens
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
NH	Nonhandled
NO	Nitric Oxide
NR	Nursery Reared
PET	Positron Emission Tomography
PND	Postnatal Day
PR	Peer Reared
PTSD	Posttraumatic Stress Disorder
PVN	Paraventricular Nucleus
RMA	Robust Multiarray Average
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SCZ	Schizophrenia
SN	Substantia Nigra
SSRI	Selective Serotonin Reuptake Inhibitor
STG	Superior Temporal Gyrus
totRNA	Total Ribonucleic Acid
UV	Ultrasonic Vocalizations
VFD	Variable Foraging Demand
VTA	Ventral Tegmental Area

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