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A Longitudinal Study of the Effect of at Birth Adoptions on Anxiety, Stress Hormones and Adolescent Alcohol Intake: A Nonhuman Primate Model

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ABSTRACT

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Adopted individuals have an increased risk for a variety of psychopathological disorders. Studies of the effects adoption in humans are difficult to perform because of the difficulty separating genetic risk and treatment effects. This is a developmental study investigating the effects of at birth adoption using a nonhuman primate model. Three experimental paradigms were used to assess maternal treatment, stress-related behavior, and physiology late in infancy and again later in life. Rhesus monkeys were reared for their first six months of life by either their biological mother or an unrelated, lactating adult female. Adoptions occurred immediately following birth. At six months of age, both groups were exposed to four, 4-day mother-infant separations. Behavioral observations and plasma stress hormones were used to compare the two group's responses to the separation stressor. Maternal treatments were also compared. In a second experiment performed about three years later when subjects were adolescents or young adults, an unfamiliar intruder was placed outside their home pen and stress-related behavioral responses were again measured. In the third experiment, adolescent subjects were allowed free access to a sweetened alcohol solution and daily alcohol consumption was measured across 8-10 weeks. Analyses showed that adopted subjects exhibited more behavior withdrawal and higher ACTH during the Acute and Chronic phases of the separation than infants reared by their biological mothers. This persisted when subjects were again tested with an intruder stressor 1-3 years later, with adopted subjects still showing more behavioral withdrawal during the Intruder Challenge stressor. Adopted subjects also differ in their relationship with their mother, showing more independence at an early age in non-stressful environments. Paradoxically, alcohol intake was lower in adolescents raised by an adoptive mother. Differences in maternal treatment and mismatches in temperament between the adopted mother and her infant are potential mechanisms that lead to the increased stress and anxiety in subjects raised by an adopted mother.

Keywords: adoption, alcohol drinking, adrenocorticotrophic hormone, early experience, stress, physiological, *Macaca mulatta*, mother-infant, separation stress, rhesus monkey

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A Longitudinal Study of the Effect of at Birth Adoptions on Anxiety, Stress Hormones and Adolescent Alcohol Intake: A Nonhuman Primate Model

In the United States, about 120,000 children (around 1-2% of all children in the US) are adopted annually by parents genetically unrelated to their adopted offspring (Nickman et al., 2005). The majority of these adopted children are raised in homes with healthy family relations and normal mental health, and most show little or no adverse outcomes. However, when compared to children reared by their biological mother, even when adopted within a year of birth, a disproportionate number of adopted children develop mental health disorders and maladjustment problems (Brodzinsky, 1993; Wierzbicki, 1993). For example, while adopted children make up only 1-2% of the population, Brodzinsky (1993) noted that children adopted from birth to 3.6 years make up 5% of child outpatient mental health referrals and as much as 10-15% of children in inpatient residential treatment or psychiatric settings, a figure much higher than the 1-2% that would be expected. Adopted children also show higher rates of antisocial personality disorder, ADHD, eating disorders and learning disabilities (Brodzinsky, 1993). Parental ratings indicate that adopted female children exhibit a higher number of internalizing (depression) and externalizing problems (hyperactivity and aggression) (Glover, Mullineaux, Deater-Deckard, & Petrill, 2010) when compared to girls reared by their biological parents (Brodzinsky, 1993). Similarly, studies by Brodzinsky found that adopted male children are seen as having more externalizing problems, specifically hyperactivity, aggression, delinquency, and uncommunicative behavior. In both private and public schools looking at 84 children, adopted children account for 6.7% of neurologically impaired children, 5.4% of perceptually impaired, and 7.2% of emotionally disturbed children (Brodzinsky & Steiger, 1991). Adopted children have lower ratings for social competence, self-concept, and more behavioral problems when compared to a nonadopted control group, although generally the risk is still within the normal range of behaviors on the Child Behavior Profile (Brodzinsky, Radice, Huffman, & Merkler, 1987; Brodzinsky, Schechter, Braff, & Singer, 1984). Nevertheless, these outcomes may also be moderated by age at adoption, pre-adoption conditions, family satisfaction and conditions, and other social factors related to adoption (Fergusson, Lynskey, & Horwood, 1995; Levy-shiff, 2001).

Genetic and Other Effects Resulting from Adoption

One explanation for the increased risk for psychopathology in adopted children is that adopted children carry an increased genetic loading for various forms of psychopathology. Under this explanation, the high rates of emotional and mental health problems found in adoptees are often attributed to genetic influences inherited from their biological parents and there are several studies that show the biological parents of adoptees are more likely to suffer from genetically-mediated psychological disorders such as depression, addiction, delinquent behaviors or other mental disorders (Beaver, 2011). This genetic risk is, in turn, passed on to the children that are put up for adoption (Beaver, 2011; Cadoret, Troughton, Bagford, & Woodworth, 1990). In an adoption outcome study by Brodzinsky (1993), adopted children are more likely than nonadopted children to have experienced aversive prenatal experiences that are thought to influence both post natal development, as well as their relationship with adoptive parents. Many children put up for adoption come from young unwed mothers with greater risk of inadequate medical care, fetal exposure to alcohol, poor nutrition, prenatal maternal stress, or other teratogenic agents (Brodzinsky, 1993; Everett & Schechter, 1971). These untoward conditions of the biological mother may in turn affect the stress reaction or the mental health of the infant adopted postnatally (Entringer, Kumsta, Hellhammer, Wadhwa, & Wust, 2009;

Pesonen, Raikkonen, Strandberg, & Jarvenpaa, 2006). Untangling the environmental and genetic effects is, at best, difficult in human studies.

Environmental Effects and Mother-Infant Relationships on Adoption

Another strategy in adoption research is to assess the conflict between child and parent and to link such conflict to mental and physical developmental problems in adoptive children. However, the results of such studies that have compared adoptee and biological parent-child relationships are mixed, with some failing to find differences in treatment between the two groups and others indicating differences in the mother-infant relationship. For example, adopted infants show similar rates of secure attachment with their adoptive mother when compared to infants who are reared by their biological parents (Juffer & Rosenboom, 1997; Van Londen, Juffer, & Van Ijzendoorn, 2007). At least one rodent study showed that mice cross-fostered to an unrelated mother received similar maternal care and treatment when compared to mice reared by their biological mother (Bartolomucci et al., 2004). On the other hand, in a study of 125 infants adopted before 6 months of age, 49 showed secure attachments while 76 showed nonsecure attachment styles at 12 months of age, a much higher rate of nonsecure attachment than is found in the general population (Beijersbergen, Juffer, Bakermans-Kranenburg, & Van Ijzendoorn, 2012). In a retrospective study, adults adopted as infants were measured on attachment and results showed that nonsecure attachment was more widespread among adopted individuals, in particular adoptee's scored high on avoidance and anxiety measures (Feeney, Paaamore, & Peterson, 2007).

Parent-Infant Relationship as a Function of Shared or Different Temperaments

One possible alternative explanation that may account for both treatment and genetic effects that increase the risk for pathological outcomes in adopted children is what Thomas and

Chess described as parent-child "goodness of fit". Conflict is evident in virtually all parent-child relationships (Trivers, 1974), but more so in some than in others. A child's temperament can fit in a "glove-like fashion" with the caregiver's personality but in other cases, the care-giver-infant temperaments can be quite different, resulting in increased probability for conflict. When the parent and child's temperament match, Chess and Thomas called this a goodness of fit between parent and child temperaments and for these dyads, relationships are less likely to show high levels of conflict (Chess & Thomas, 1977a, 1977b; Thomas, Chess, & Birch, 1970). Research on parent-offspring relationships that have investigated adoptive families has predominately assessed the level of conflict between parents and adoptive children, or in essence the lack of goodness of fit (Blum, 1983; Klahr, McGue, Iacono, & Burt, 2011; Rueter, Keyes, Iacono, & McGue, 2009). Although Thomas and Chess did not discuss adoption, their research may explain the developmental difficulties and psychopathological outcomes seen in adopted children. Goodness of fit between a child and their biological parent could be postulated to occur more often because, on average, there are more genetic similarities between biological parents and their offspring than between adopted parents and their offspring. This is likely to result in more temperament similarities between biological parents and their offspring than in adopted parents and offspring, and may result in increased parental insight concerning how to deal with the child's disposition. In an adopted child, parent-child interactions might be rockier because on average, adopted children share fewer genes with their adopted parents than children with their biological parents share. Consequently, parent-offspring temperaments are less likely to match, resulting in more conflict and more difficulties for the parent to adapt to their infant's behavior. Nevertheless, even when parent and offspring temperaments are disparate or a child has a difficult temperament, goodness of fit can be created when a parent is able to adjust their parenting techniques to match the needs of the child's temperament (Thomas et al., 1970).

One difficulty in studying the underlying causes of adverse outcomes in adoptees is that in adoptions, children are not randomly assigned to their adopted parents. Parents who adopt are often higher in SES, better educated and strongly desire children (Hansen, 2006; Welsh, Viana, Petrill, & Mathias, 2008). Adoption agencies are highly selective in placement and, at times, the biological mother is allowed to choose parents from many potential candidates. In a similar fashion, infants and children who are adopted are not random in their backgrounds either, with rates of biological parental psychopathology typically being higher than the rest of the population (see earlier discussion). Given that adopted children are more likely to have biological parents with genetically mediated mental health disorders, the risk of these mental health disorders in the child increases due to genetic heritage. Moreover, genetically mediated mental health disorders such as hyperactivity, depression, and aggression can lead to difficult interactions, producing higher conflict and a diminished probability of goodness of fit between parent and child; therefore, making the genetic influence hard to distinguish from environmental effects. Given such difficulties in untangling the potential etiological mechanisms that underlie the untoward outcomes seen in some adoptees, we proposed using a nonhuman primate model to investigate the effects of adoption.

A Nonhuman Primate Model

Nonhuman primates offer numerous advantages in modeling human psychopathology. They are our closest phylogenetic relatives and like humans, have complex social lives as well as an extended period of maturation and parental investment. As in humans, mother-infant interactions are of paramount importance in developmental outcomes with wide individual differences in treatment and temperaments. A major advantage to using nonhuman primates is that one can control rearing history by assigning infants randomly to mothers in order to test hypotheses concerning the role of early maternal treatment. Like human infants, during the first few months of an infant's life, its mother is involved in nearly all of the infant's activities and infants spend almost all of their time being held and protected by their mother (Hinde & Spencer-Booth, 1967; Suomi, 2005). During this period mothers tend to be protective, frequently restraining their infant's attempts to explore his or her environment, and maintain close proximity to the infant (Hinde & Spencer-Booth, 1967). While early in life most mothers maintain a more or less constant close proximity and hardly ever reject their infants, by six months of age, rhesus mothers regularly contribute to mother-infant independence by rejecting the infant's attempts to nurse. Nevertheless, as in humans, there are substantial individual differences both in maternal treatment, as well as infant temperament and response to mother (Chess & Thomas, 1977b; Suomi, 2005).

Nonhuman primates have also been used to model the effects of early experience on the stress response, and they are frequently used to model developmental outcomes in stress-related behaviors and psychopathology, hence they are ideally suited to model the effects of adoption on stress-responsiveness. Moreover, unlike New World species of nonhuman primates, rhesus macaques have a similar if not homologous stress-mediated hypothalamic pituitary adrenal (HPA) axis response with that of humans (see for example (Higley, Suomi, & Linnoila, 1992)), and consequently, they are frequently used to model stress-related HPA axis responses. They are also used to model other forms of stress-related psychopathology, such as anxiety-mediated alcohol abuse. When offered a palatable solution with an alcohol concentration less than 15%, most monkeys will voluntarily consume sufficient alcohol to experience its pharmacological effects (Higley & Bennett, 1999; Higley, Suomi, & Linnoila, 1996a, 1996b; Kraemer & McKinney, 1985), with the majority drinking the equivalent of 1-2 drinks in humans. But paralleling rates in humans, 10-20% of normally reared monkeys consume alcohol at rates that produce intoxication. Cloninger's typology model of alcoholism and abuse suggested that one form of alcohol-related abuse (Type 1) is mediated by stress and anxiety, and nonhuman primates also show similar Type-1, stress-induced increases in alcohol intake (Cloninger, 1988; Higley, Hasert, Suomi, & Linnoila, 1991).

In a decade of studying adoption in nonhuman primates, we have never seen an adoptee's mother abuse her adopted infant; nor do mothers of adoptees show any evidence that they recognize that their adopted infant is not their own biological offspring when the rearing procedure is performed. Nevertheless, there is evidence that the relationship between an adopted infant and mother is different from that of a biological infant and mother. In one recent study of the subjects used in this study, we investigated adopted mother's treatment of their infants and the effect of adoption on early mother-infant interactions. Bogh's (2010) investigation of maternal treatment and early life infant behavior in these subjects, found that even early in life, adopted rhesus macaque infants were primarily responsible for maintaining the mother-infant relationship (as measured by who approaches whom). This is a relatively atypical pattern of behavior because in early infancy, the typical mother engages in high rates of retrievals, and often restrains her infant to keep it safe. Bogh's (2010) research showed that when compared to infants reared by their biological mother, at an age when most infants remain close to their mothers, adopted infants exhibit increased time away from their mother, accompanied by more environmental exploration and activity, but also more anxiety-like self-directed behaviors, and they received more aggression from other group members. Bogh suggested that this is evidence of premature independence, leading to increased anxiety and negative emotionality. Moreover, at an age when infants seldom are rejected from nursing or being held to breast, the adopted infants were rejected more frequently than infants reared by their biological mothers, and adopted infants initiated and received more aggression from other group members (Bogh, 2010). Similarly, Bassett (2010) found that such early or premature rejections, even in infants reared by their biological mothers, results in a variety of anxious and aggressive behaviors (see also (McCormack, Newman, Higley, Maestripieri, & Sanchez, 2009)).

Separation Stress and Alcoholism

Nonhuman primate social separation is commonly used to model the stress response and to model human depression (Suomi, Chaffin, & Higley, 2011). Initially, social separation typically produces a series of behaviors designed to elicit maternal retrieval, but as maternal separation is prolonged, behaviors of retrieval are replaced by behavioral withdrawal, and HPA axis activation. Prolonged separation results in chronically elevated plasma adrenocorticotropic hormone (ACTH) and cortisol, which when it is prolonged can result in pathological changes in the brain and elsewhere (Cacioppo & Berntson, 2007). Early problematic rearing and maternal absence appear to produce long-term HPA changes leading to high or blunted ACTH and cortisol levels (Fahlke et al., 2000; Higley et al., 1992).

High levels of cortisol in an infant during mother-infant social separation is strongly predictive of high alcohol intake later in life, when the infants reach adolescence (Fahlke et al., 2000), suggesting that trait-like anxiety is a risk factor for high alcohol intake, a finding consistent with predictions of Cloninger's Type 1 alcoholism typology (Cloninger, 1988). Many studies have shown that stress can increase alcohol intake, and studies measuring the social separation stress response show increased alcohol intake in adolescent monkeys (Higley et al., 1991; Kraemer, Lin, Moran, & McKinney, 1981; Kraemer & McKinney, 1985). These studies show, for example, that alcohol consumption during separation results in increased social play, sexual activity, and environmental exploration, suggesting that in nonhuman primates alcohol is also used for its anxiolytic effects.

Hypothesis

Based on studies showing some deleterious outcomes in early life human adoptees, and the study of at birth adoption in rhesus by Bogh (2010), we designed a study to model the effects of adoption and to test for long-term deficits. To perform this study, infants were reared either by their biological mothers or randomly fostered to unrelated lactating females (adopted mothers). A nonhuman primate model has a number of advantages for understanding etiological mechanisms because the infants are more-or-less randomly assigned to the adopted mother (more or less because the adopted mother must have recently given birth). Therefore, any differences between the two groups of infants are unlikely to be a result of genetic loading in adopted infants but instead are likely a result of maternal treatment. Given the increased probability of deleterious outcomes for human adoptees, we hypothesized that under conditions of stress, (1. Adopted infants reared by an unrelated mother would show increased anxiety, as measured by behavioral withdrawal; (2. Increased HPA output, and (3. High alcohol intake during adolescence.

Methods and Materials

Experiment 1—Separations

Subjects. Subjects were 136 socially-housed rhesus macaque infants (73 females and 72 males) living with their mother and housed in indoor-outdoor enclosures for at least the first six months of life (indoor 2.44x3.05x2.21 m, outdoor 2.44x3.0x2.44 m). Of those subjects, 105 infants were raised by their biological mother (53 female and 52 male), while the remaining 32 (16 female and 16 male) were fostered to an unrelated, lactating, adoptive mother shortly after birth. Other than the early fostering, all subjects received identical treatment. The infants lived in mixed-sex social groups containing two adult males and eight to twelve adult females with their infant offspring, a condition that approximates the rhesus natural condition. There were typically two to five additional same age infants inside these social groups, allowing for species-typical socialization and peer-socialization. Within the enclosures, a light cycle of 12:12 was maintained (7:00 a.m. to 7:00 p.m.), although in the outdoor area of the pen, the monkeys were subject to natural seasonal cycles. Monkeys in the social groups were fed with high-protein monkey chow and had access to water ad libitum. Supplementary fruit and other food-based enrichment were also provided daily. The sample included monkeys from thirteen birth-year cohorts, born between the years 1991-2005. All cohorts received identical treatment.

Rearing. All mothers in the study had given birth previously. Infants fostered to an adoptive mother were pseudo randomly assigned (i.e., random except that to qualify as an adoptive mother, females had to have given birth within a week of the fostering procedure, had to be unrelated, living in a social group that did not also house the biological mother), and were medically evaluated to assure lactation. Infants fostered to adoptive mothers were removed from their biological mother within 48 hours of giving birth during a routine medical check that all infants and mothers undergo. The adoption process for this study utilized a common fostering procedure that is used in many nonhuman primate laboratories. The infants were removed from their mothers shortly after birth while the mother was anesthetized for her medical evaluation, and placed in a box-like, monkey transport cage with see-through sides. The transport cage was then placed outside the adoptive mother's holding cage, and the transport cage door was opened so that the adoptive mother could retrieve the infant. If the mother retrieved the infant, nursed and cared for it, the adoption was considered successful. The mother-infant pair was then monitored closely for 24 hours for any signs of rejection, as well as proper nursing behaviors. If the infant was not nursed in this 24-hour period, they were separated and the infant was returned to its biological mother or placed in a neonatal nursery for upbringing (less than 10% of the total attempts). Fostering to a new mother was based on similar dates of giving birth for the biological and adoptive mother and was performed within three days of the birth of the infant to be adopted in order to facilitate acceptance by the adoptive mother. All of the adoptee's mothers rapidly accepted their adopted infant, treating it as if it was their own biological infant. Our observations indicated that none of the adopted mothers showed evidence that they could tell the difference between the adopted infant and their biological infant (i.e., the mothers did not show evidence that they recognized the infant as different from their biological infant). None of the infants were observed to receive any form of mistreatment or abuse. To avoid future contact with the biological mother, adopted infants were assigned to a mother in a social group that did not house the biological mother. Thus, none of the infants had any further contact with their biological mother.

Separation stress and maternal treatment. When all infants reached six months of age, they underwent four sequential, 4-day long separations, with each followed by three days of mother-infant reunion. To perform these separation-reunions, on Monday afternoon during each of the separations, the biological mother or foster mother was removed from the home pen and the infant was left with the rest of the social group. After each of the four-day separations, the mother was returned to the pen and a three-day mother-infant reunion period ensued. For each separation, behavioral data and blood samples were collected. As in other studies from our laboratory, day-one (D1) of each separation period was considered the Acute phase, and days two through four the Chronic phase (Higley et al., 1992; Spinelli et al., 2007). Maternal treatment of her infant was assessed during the three-day, mother-infant reunion period by collecting behavior from the mother. As this is the approximate age in the natural setting for weaning, following the separations, all subjects from the same cohort were removed from their mothers and placed into a large pen with 25-40 other infants. They remained in this larger group throughout the rest of the study with all subjects receiving the same treatment thereafter.

Infant behavioral data collection. For the infants, behavioral observations were made each day during a two-week baseline, and each day during the four, 4-day separations. Trained observers collected two 5-minute scores from the infant each day, with the exception of D1 (the Acute phase), when three 5-minute scoring sessions were performed during the first two hours of the separation. Inter-rater reliability for behavioral scoring was consistently at $r = 0.85$ or above. For the separations, infant behavioral scores for each week were averaged across D1 to produce the Acute phase and across days 2-4 for the Chronic phase. The primary behavior measured was withdrawal or freezing behavior, defined as the amount of time the infant remained inactive and typically withdrawn, not engaging their environment or interacting with other individuals. Freezing or behavioral withdrawal is widely accepted as a measure of anxiety or fear (Bethea et al., 2005; Biederman et al., 1993; Kalin & Shelton, 1989; Kalin, Shelton, Rickman, & Davidson, 1998; Rosenbaum et al., 1993). The time spent withdrawn was recorded in seconds over a five-minute observation period. Behavioral coders were generally not aware of the rearing conditions of the subjects.

Maternal quality of care. To assess for differences in the treatment of the two groups of infants, maternal behaviors were collected from a subset of the mothers during less stressful home-cage reunions. The mothers' behaviors (*n*=130, adopted=16, biological mother reared=114), were collected using a behavior coding system designed to capture the quality of maternal care (Barr et al., 2008). Three 5-minute behavior coding sessions were collected: two immediately following the mothers' return to the social group and one on Monday morning just before the next separation began or following the last reunion. The three scores were averaged and the mean score for the reunions was used to assess maternal treatment. Behaviors collected and studied included duration or frequency of mutual break, mutual ventral, leave by infant, leave by mother, mother grooming infant, play, and receive groom – see Table 1 for definitions.

Table 1

Behavioral Definitions

Behavior	Definition
Withdrawal	Time spent freezing or unengaged in social or meaningful behavior
Mutual Break	Separation of mother and infant that is deemed mutual
Mutual Ventral	Mother and infant are belly to belly and/or on the nipple. Infant cannot be in social contact with any other animal or in locomotion but mother can
Leave by Infant	Infant leaves the mothers contact
Leave by Mother	Mother leaves infant contact
Mother Grooming Infant	Grooming of infant by mother; can include scratching, licking, or rubbing
Receive Groom	Any grooming behavior toward the mother from other group members or her infant. Can include scratching, licking, or rubbing.
Play	High activity interaction (chase, rough and tumble play, wrestling, with other individuals). This can include nonaggressive physical contact, can occur at a distance, swinging by the feet, or visually checking/coordinating with other play members

Physiological data collection from infants. To establish response differences in the HPA axis activity during acute and chronic stress, blood samples were obtained on the first and fourth day of each social separation- see Table 2 for ketamine conditions. Unlike the Chronic phases where the subjects were anesthetized, on the first day of the separation, a 5-minute baseline (5m) sample and two acute samples, obtained one (h1), and two hours (h2) after mother-infant separation, a time of maximal cortisol/ACTH output, were drawn while the infant was awake and restrained immediately following capture (Gunnar, Gonzalez, Goodlin, & Levine, 1981; Levine, Johnson, & Gonzalez, 1985). To obtain the three D1 blood samples, the infants were hand captured and manually restrained during the blood draws. Blood samples were obtained while they were awake to simultaneously assess group differences in behavior and the HPA axis response to the acute separation stress. All three D1 blood samples were obtained between 1300 and 1500 hours. The Chronic phase blood samples were taken on day-four (D4), Thursday, of each separation as an indication of chronic stress. To collect these four samples the infants were hand captured in their home cage and anesthetized within a 1-2 minutes of entering the cage using ketamine hydrochloride (15 mg/kg, IM). Blood samples were then taken from the femoral vein within ten minutes of anesthesia. Both the acute and chronic samples were assayed for plasma cortisol and ACTH.

Table 2

Blood Draws and Ketamine Anesthesia Received

Blood sample phase and time	Ketamine anesthesia
Preseparation baseline	Yes
Acute (D1)- 5 minutes	No
Acute (D1)- hour 1	No
Acute (D1)- hour 2	No
Chronic (D4)	Yes

After the blood samples were obtained, they were immediately placed on wet ice and centrifuged at 4^0 C for 20 minutes. The plasma was aliquoted and frozen in liquid nitrogen, after which the plasma samples were stored in a freezer at -70^0 C until they were assayed. Plasma samples were assayed for ACTH and cortisol using standard radioimmunoassay described elsewhere (Higley et al., 1992), with an intra- and interassay reliability greater than .90 for cortisol and .85 for ACTH.

Data analysis for infant behavior and hormones. Preliminary analyses showed no significant main effects or interactions for sex. Thus, this variable was not included in later analyses, except for the mother treatment analysis. Plasma cortisol and ACTH data were analyzed using a three-way mixed design, repeated measures analysis of variance. Independent variables were rearing condition (adopted or biological mother reared), sample taken (5m, h1, h2, d4), and week of separation (1-4). ACTH and cortisol means for Acute, and Chronic phases of separation were used as the dependent variables and run as separate ANOVA's. Acute and Chronic phase behavior measures of withdrawal were analyzed separately also using a three-way mixed design, repeated measures ANOVA. Independent variables were rearing condition (adopted or biological mother reared), observation time (Acute/Chronic) and week of separation (1-4); dependent variable was duration of behavior withdrawal. Because of illness or technical problems, in some of the weeks subjects were missing one or more data points and were excluded from the analysis resulting in unequal sample sizes across some of the analyses.

Maternal treatment analysis. Mothers' behavior with her infant during reunion periods was analyzed using a 3-way ANCOVA. Independent variables included rearing condition (adopted or biological mother reared), week of reunion (1-4), and infant sex (male (n=79)/female (n=51)), in addition the covariate of mothers' age was included in analysis (M=7.9 years, SD=3.45 years). Dependent variables were the mothers' behaviors described above.

Experiment 2—Intruder Challenge Test

Subjects and rearing. Subjects for the Intruder Challenge Test were 110 socially-housed rhesus macaques with 86 subjects (48 female and 38 male) raised in a social group by their biological mother, and the remaining 24 adoptees (15 female and 9 male) were the same adopted subjects from study one. Age of subjects at testing varied, with a mean of 58.6 months (*SD*=31.02).

Intruder Challenge Test. Behavioral responses to an unfamiliar conspecific were measured using a slightly modified version of the Intruder Challenge Test developed by Fairbanks (2001). In this test, subjects are exposed to an unfamiliar conspecific in a controlled manner by placing the 'intruder' animal into a cage designed for monkeys to be housed alone, with an open front and top mesh so the intruder is visible from all directions. The cage is on wheels is positioned adjacent to the test subjects' home enclosure. Subjects may approach the intruder and interact through the mesh of the cage, but full body contact is prevented to avoid injury to any of the animals. The Intruder Test typically elicits high rates of aggression in the residents, both toward the intruder, as well other animals in the home cage. During the Intruder

Test, animals also show a variety of stress-related behaviors, including behavioral withdrawal. Intruder animals are unfamiliar to the test subjects and are selected to match the test subjects' age, sex, and relative body size.

Before the test, the intruder animal was placed into the individual living cage (.76x.63x.91 m), for a 30-min acclimation period. Three randomly selected test subjects from the same social group were separated from the larger social group and placed into the outdoor portion of their home enclosure for a 10-min acclimation period. The test began when the intruder animal's cage was rolled directly to the front of the outdoor enclosure containing the three subjects. Test subjects' behavior was recorded for 30 minutes, with a separate observer assigned to each of the three subjects. Testing was divided into three ten-minute segments (M10, M20, M30). Recording of behavior was performed using handheld computers equipped with Observer software (Noldus, Leesburg, Virginia). The software allows each observer to record the frequency and duration of various behaviors performed by the test subject. Behavioral withdrawal or freezing behavior was recorded based on a standard behavioral ethogram developed by our laboratory, which has been used in previous studies (Schwandt et al., 2010). Interobserver reliability was established at greater than $r = 0.85$, and all observers were blind to the subjects' rearing condition at the time of data collection.

Intruder Challenge data analyses. Data were analyzed using a mixed design, repeated measures ANCOVA, with mean seconds engaging behavioral withdrawal as the dependent variable. Rearing was used as the fixed independent variable, and the repeated measure was the three segments behavioral observation (10, 20, or 30 minute mark). The covariate of test subject age was also included in analysis. No main effect or interactions were found with sex in initial analysis and therefore it was excluded from further analysis.

Experiment 3—Alcohol Intake

Subjects. Data measuring free access, alcohol intake under controlled conditions was available from 54 of the original subjects, obtained when they were adolescents. Forty-nine subjects (38 female and 11 male) were raised by their biological mother and 16 (10 female and 6 male) were adopted and raised by their adoptive mother. Rearing conditions were identical to experiment one and two.

Oral alcohol exposure. At adolescence (*M*= 47 months, *SD*=4 months), rhesus macaque subjects were tested for alcohol intake and preferences. To test for alcohol intake, the subjects were given access to an aspartame-sweetened 8.4% (vol/vol) alcohol solution five days a week (M-F) in their home cage. To assure equal access for all subjects, three drinking stations were provided with a protective Plexiglas™ chamber surrounding each drinking nipple. Each station was designed as a monkey-sized 'phone booth' that the subjects could enter from the bottom. To identify which subject was drinking, a chip reader located inside the drinking booth read a microchip located in a collar worn by each subject and a computer automatically recorded the volume consumed. (See (Flory, Chen, Woltz, Magleby, & Higley, 2006) for a more detailed description of the apparatus). Alcohol was available to freely consume for one hour a day between the hours of 1300 and 1500 and intake was measured across 6 weeks. In order to allow the subjects to become accustomed to the alcohol solution, the process of alcohol administration was performed in three phases. During phase one, spout training, animals were trained to drink from nipple-like spouts that dispensed aspartame-sweetened water. This phase typically lasted for five days, with the subjects quickly consuming the bulk of the aspartame solution each day. Initial alcohol exposure, phase two, was designed to ensure all monkeys experienced the pharmacological effects of alcohol before the experimental phase begun. The color of the sweetened mixture was changed and sufficient alcohol was added to produce an 8.4% (vol/vol) solution. Animals were given free access to the solution for one hour a day until each animal met a pre-established criterion of consuming more than .67 g/kg body weight of alcohol on two or more occasions. After the criterion was achieved, phase three, the test phase began. During the test phase, subjects were allowed to consume the alcohol solution freely for the next 8-10 weeks. As in the above phases, the alcohol solution was available in the home cage for one hour a day, five days a week. No special methods, such as water or food deprivation, were used to induce consumption of the ethanol solution. All subjects had ample opportunity to consume the alcohol solution as evidenced by periods when the three stations remained empty, particularly during the second half-hour. The National Institutes on Alcohol Abuse and Alcoholism and the National institute of Child Health and Human Development Animal Care and use Committees approved all procedures.

Data analysis of alcohol intake. Previous studies show that week-to-week alcohol intake is highly correlated, and average alcohol intake was likewise stable across weeks in this study. Therefore, alcohol intake data was first averaged across weeks for each of the two testing phases to produce a mean alcohol intake across six weeks for each subject. A one-way ANOVA was used to test for group differences. The independent variable was rearing condition (adopted vs. biological mother reared) and the dependent variable was the amount of alcohol (grams) consumed per kg body weight for each subject. Again, no main effects or interactions were found with sex so it was left out of subsequent analysis.

Results

Experiment 1—Separations

Infant behavior. The three-way repeated measures ANOVA showed that across the four separation periods there was a significant main effect of rearing on behavioral withdrawal ($F=8.58$, $df=1,135$, $P<0.004$). This main effect was consistent across both the Acute ($F= 7.55$, $df=1,137$, $P<0.007$) and Chronic ($F=6.78$, $df=1,137$, $P<0.01$) phases of separation. In both phases, infants reared by an adopted mother engaged in longer durations of behavioral withdrawal across all four weeks of separation. However, there was no difference between the two groups when comparing the initial baseline levels before the stressful separations. As seen in other studies, for both rearing groups, behavioral withdrawal was higher for the Acute (D1) phase than the Chronic phase ($F=25.268$, $df=1,143$, $P<0.001$). An effect for weeks was also seen for behavior withdrawal ($F=5.013$, $df= 3,143$, $P<0.003$). There was a progressive decrease in the time in behavioral withdrawal across the four weeks of separation. There were no significant interactions.

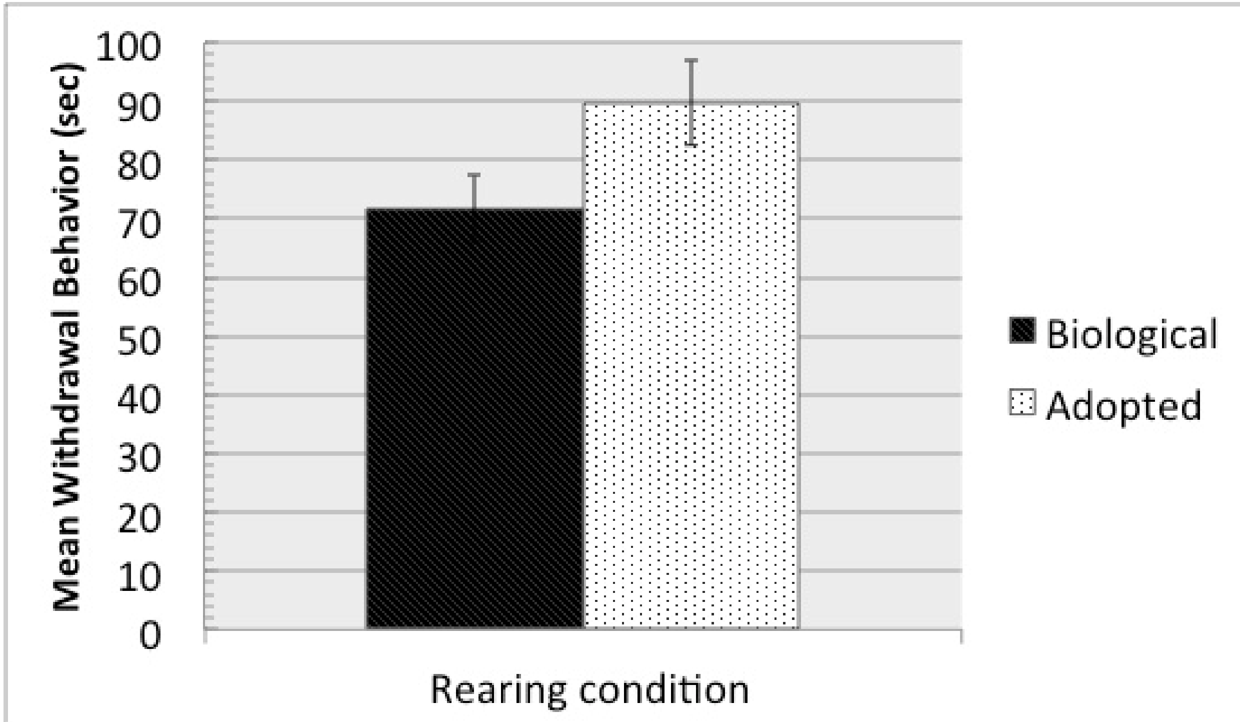

Figure 1. Main effect of rearing on behavioral withdrawal during mother-infant separation. Mean seconds in behavior withdrawal for infant rhesus macaques reared by their biological mother (Biological) or adopted mother (Adopted). When compared to the infants reared by their biological mothers, adopted infants displayed more withdrawal behavior in both separation phases and across all four separations.

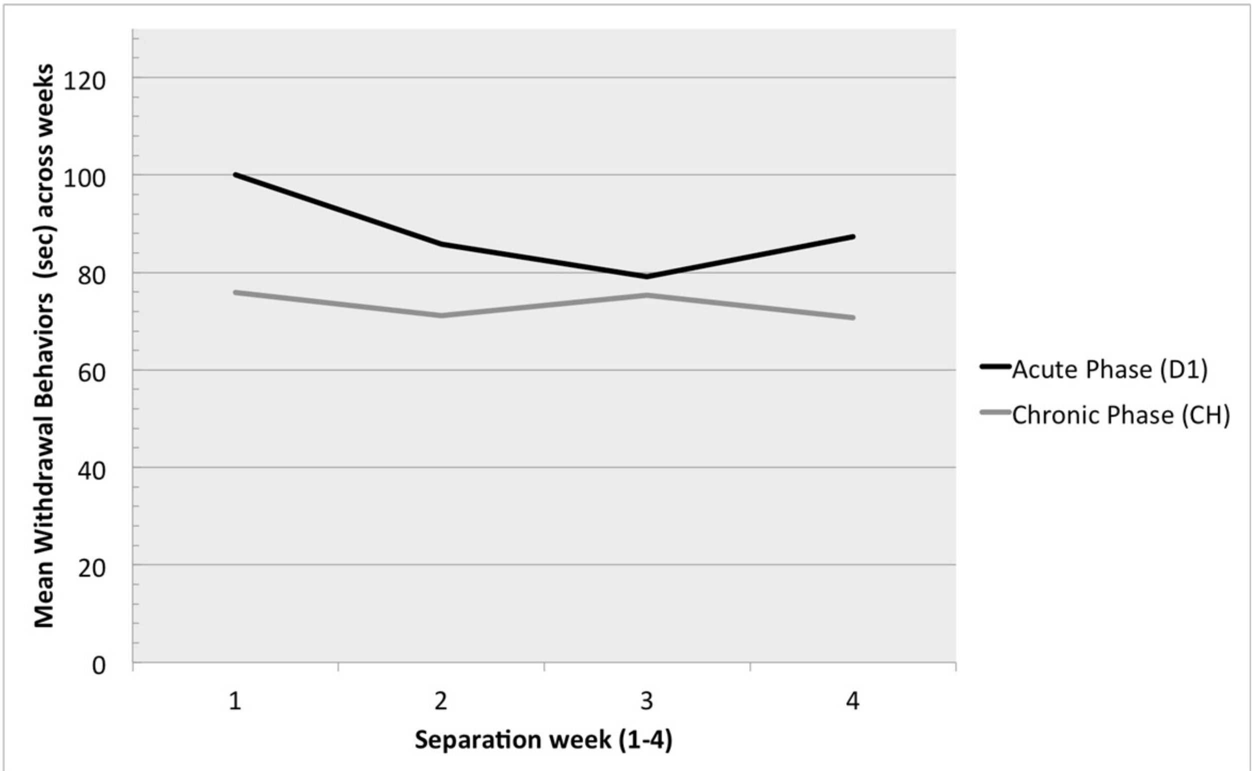

Figure 2. Effect across time of infant behavioral withdrawal for the Acute and Chronic phases. Behavior withdrawal was measured across the four separations and means for the Acute (Day 1 - D1) and Chronic (Days 2-4 - CH) phases are compared across four weeks. Seconds in behavioral withdrawal was higher during the Acute phase than the Chronic phase for both groups.

Maternal treatment. Analyses of maternal behavior during the reunions showed a significant main effect for several behaviors indicative of increased independence between mother and infant for the adopted infant. There was a significant rearing main effect for mutual break ($F=4.24$, $df=3,130$, $P<0.05$). Adopted mothers and infants showed more mutual moving away from each other when compared to the mothers rearing their biological infants. There was also a significant interaction between week and rearing condition for the behavior leave by infant ($F=5.33$, $df=3,130$, $P<0.003$). Infants reared by an adopted mother left their mothers' side more often than those infants reared by their biological mothers. This difference between the two groups was more pronounced in week one than weeks 2-4. There was a significant three-way interaction for time in close, intimate mutual ventral cradling of infants (F=2.99, *df=3,*130, P<0.04). Mothers with their biological female infants showed significantly more time in mutual ventral contact. Week 3 was lower in mutual ventral across compared to weeks 1,2 and 4. The three-way interaction for play was significant (F=4.819, *df=3,*130, P<0.003). Mothers with adopted female infants exhibited higher play especially during week one, when compared to the infants reared by their biological mother. A significant three way interaction was also revealed for receive groom (F=3.94, *df*=3,130, P<0.03). Mothers who raised adopted female infants also received more grooming from their offspring when compared to their counterparts reared by their biological mother, and this effect increased over the four weeks. There were no significant interactions for leave by mother, and maternal grooming of infant.

Table 3

Maternal Treatment Repeated Measures ANOVA Results During a Nonstressful Reunion Period

	df	F Ratio	P Value: Rearing	Mean/SE	P value: week x rearing	P value: week x sex of infant x rearing	Mean/SD Reunion 1	Reunion 2	Reunion 3	Reunion 4
Mutual Break	3	4.24	**.042***		.098	.867				
XF				.615/.1473			.625/.739	.708/1.060	.604/.611	.281/.657
Bio				.360/.036			.301/.496	.345/.513	.345/.490	.400/.816
Mutual Ventral	3	2.99	.394		.507	**.030***				
XF				203.695/13.29			191.489/72.03	210.995/62.958	186.671/81.946	226.162/80.833
Bio				214.433/5.954			222.374/76.46	214.482/74.594	201.791/82.561	214.121/94.853
Leave by Infant	3	5.33	.841		**.004***	.188				
XF				.561/.161			1.042/1.424	.437/.617	.313/.523	.313/.602
Bio				.581/.054			.520/.958	.569/.660	.672/.721	.513/.816
Receive Groom	3	3.94	**.017***		.408	**.009***				
XF				56.706/10.011			27.517/34.903	49.933/49.788	49.737/36.091	99.637/104.938
Bio				33.542/4.430			18.997/31.893	32.088/44.344	19.179/30.527	44.109/63.362
Play	3	4.819	.050		**.031***	**.003***				
XF				.525/.304			.945/3.687	.845/3.383	.058/.233	.381/.901
Bio				.165/.058			.086/.341	.113/.607	.291/2.317	.214/1.21

****p<.05 significance***
XF- adoptive mother -reared, Bio- biological mother-reared

Physiology. While the adopted infants showed increased independence and play, there was evidence that they showed a more responsive pituitary response. There were no rearing differences for cortisol during any of the phases of the separation stressor. However, robust effects for rearing were seen for ACTH during the Acute but not the Chronic separation phase. Analyses of the Acute phase of separation (5m, h1, h2) showed a significant main effect for rearing (F=16.02, *df*=1,157, P<0.001) –see Figure 2. Infants raised by an adopted mother exhibited higher levels of ACTH than those raised by a biological mother. There was also a main effect of weeks, with both adopted infants and infants reared by their biological mother showing a decrease in ACTH across the four weeks of separation. Hour-one measures within each separation were higher than the five-minute and hour-two measures. This trend was seen across both rearing conditions.

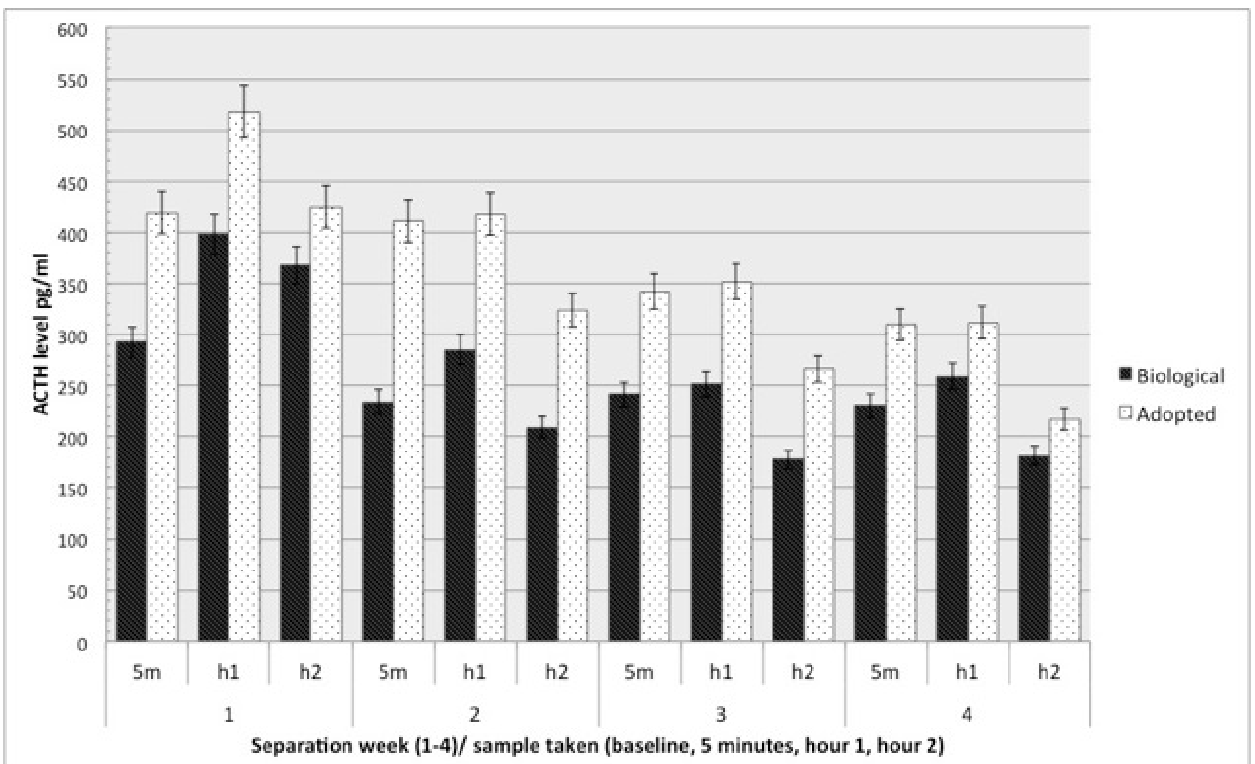

Figure 3. ACTH response to mother-infant separation. ACTH levels of infant rhesus macaques subjected to four, sequential, mother-infant separation stressors. Infants were reared by their biological or adopted mother. ACTH levels are in picograms/ml and were compared across the four weeks and graphed as three blood draws for each week: a 5-minute (5m), hour 1 (h1), and hour 2 (h2). Adopted infants showed higher levels of plasma ACTH across every period relative to the infants reared by their biological mother. A linear decrease in plasma ACTH was also seen across the four weeks for both rearing groups. Hour-one measures within each separation were higher than the five-minute and hour-two measures.

Experiment 2—Intruder Challenge Test

As with the separation stressor, during the Intruder Challenge Test there was a significant main effect for rearing on behavioral withdrawal across all three time points (10, 20, and 30 minutes) of the observations (F=4.80, *df*=1,157, P<0.03)—see Figure 3. Infants reared by an adoptive mother displayed higher levels of behavioral withdrawal across all of the observation periods.

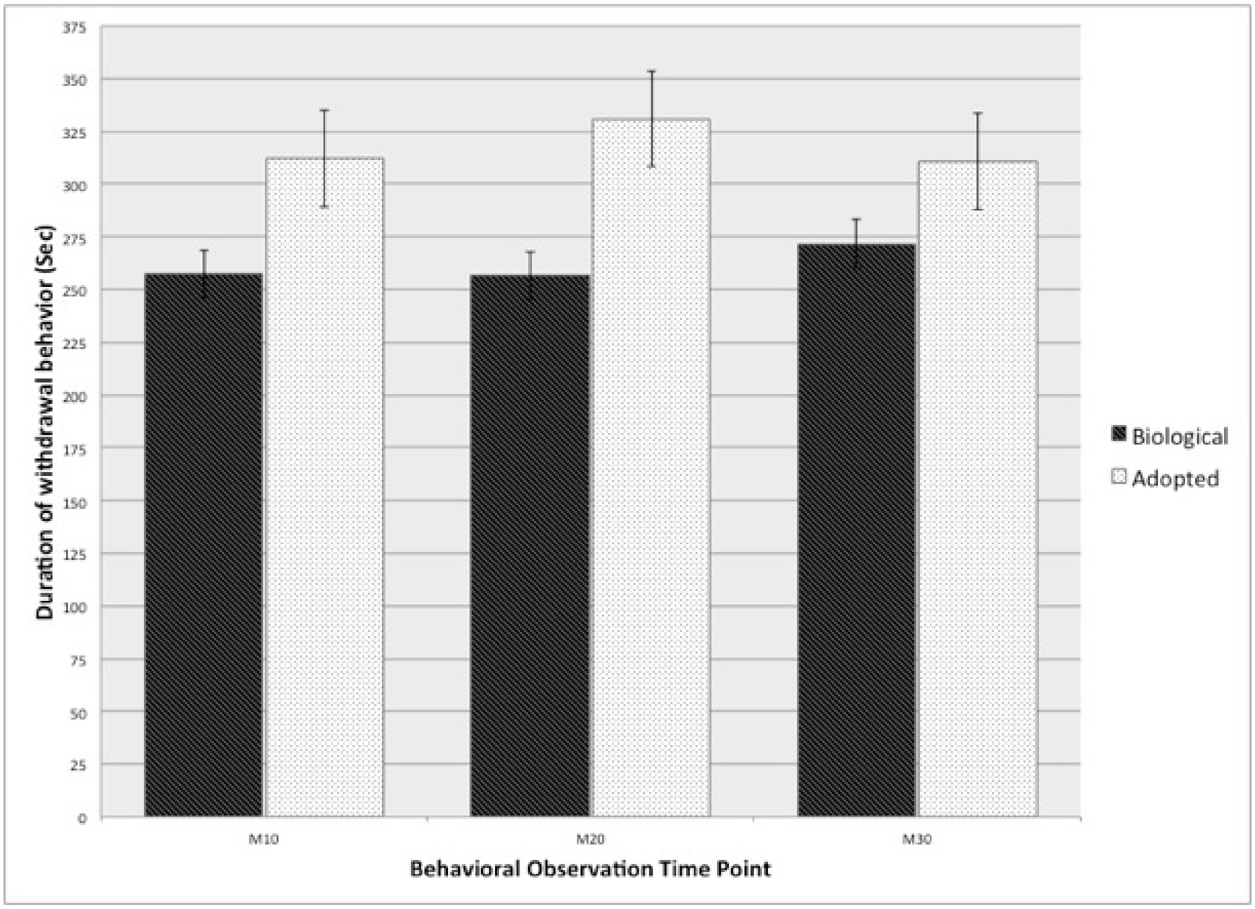

Figure 4. Behavioral withdrawal during the intruder challenge. Mean seconds in behavior withdrawal exhibited during exposure to the stressor of a similar age, unknown intruder monkey in subjects reared by their biological mother or adopted mother. Behavioral withdrawal was measured in seconds across a 30-minute observation period. Behavioral observations are split up into three, 10-minute segments. Adopted infants showed increased seconds in behavior withdrawal when compared to the subjects reared by their biological mother during the stressor of an unknown rhesus macaque intruder.

Experiment 3—Alcohol Intake

On average, subjects showed a stable drinking rate across weeks, with an average correlation across weeks of $r = 0.721$, $p < 0.01$. As in the first experiment, no main effects or interactions with sex were observed. However, univariate ANOVA revealed significant effects of rearing on alcohol consumption ($F=8.80$, $df=1,65$, $P<0.004$). Adolescents reared by an adoptive mother consumed significantly less alcohol than adolescents reared by their biological mother—see Figure 4. As is typical of mother-reared subjects in other studies (Higley et al., 1991), intake was modest in both groups, with the subjects that as infants had been reared by their biological mother consuming on average the human equivalent of about two drinks a day and the adopted subjects consuming on average about one drink a day. Both groups exhibited daily variability showing days with higher intake, punctuated with days of low intake.

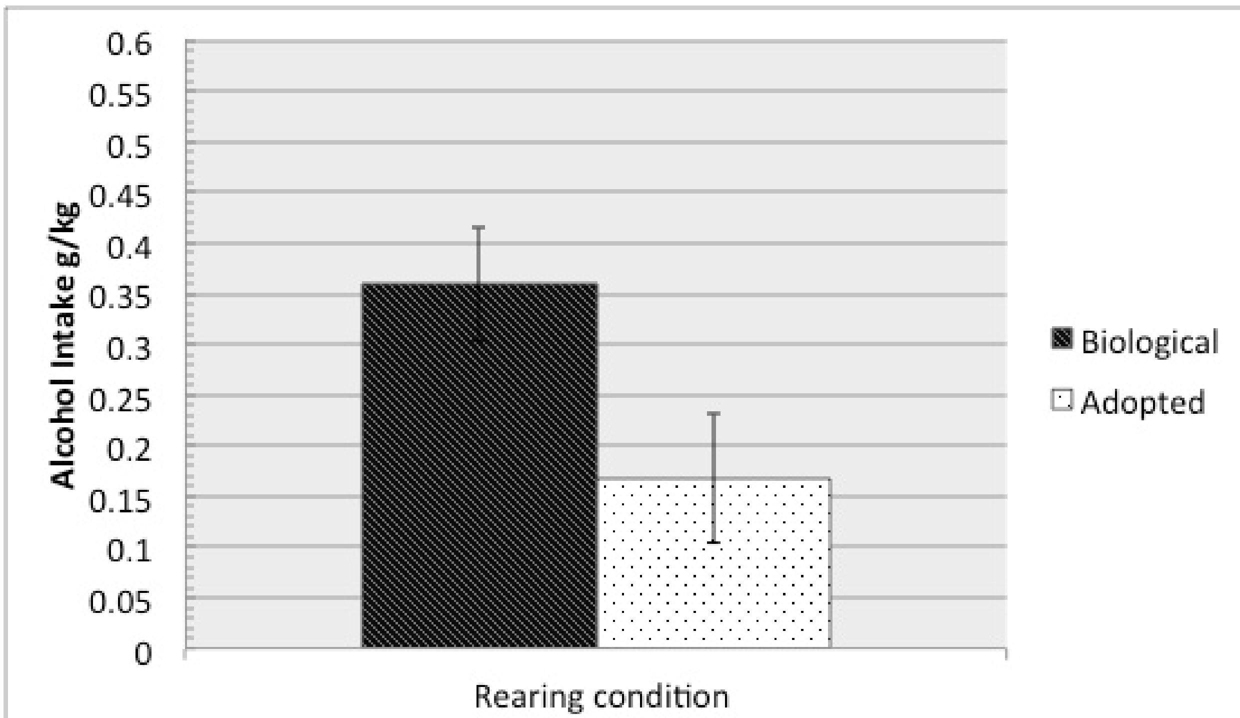

Figure 5. Alcohol intake by rearing groups. Comparison of mean alcohol intake in grams of ETOH per kilogram for subjects reared by their adopted or biological mothers averaged across 8-10 weeks of alcohol access. Adopted infants show significantly lower rates of alcohol consumption.

Discussion

Experiment 1—Separations

Mother-infant social separation typically elicits high levels of stress in infant macaques (Barr et al., 2004; Mineka & Suomi, 1978). The results revealed that when the two groups of infants were compared during the highly stressful situation of mother-infant separation, adopted infants displayed more behavioral and physiological signs of stress, such as anxiety-like behavioral withdrawal and high plasma ACTH levels than infants reared by their biological mother. Behavioral withdrawal and high plasma ACTH are both indicators of anxiety in rhesus macaques (Kalin et al., 1998; Rogers, Shelton, Shelledy, Garcia, & Kalin, 2008), as well as in human toddlers and preschoolers (Biederman et al., 2001; Kagan & Snidman, 1999; Rosenbaum et al., 1993). Adopted infants showed higher levels of behavioral withdrawal than infants reared by their biological mothers, particularly in the Acute phase (first two hours) of the separation, but also during the Chronic phase (days 2-4 of the separation). Despite decreases in behavioral withdrawal from the Acute to the Chronic phase, time spent in behavioral withdrawal remained higher in the adopted infants across both phases of separation. This may indicate that even though both groups of infants remained in the familiar setting of their home cage, in the absence of their mother, adopted infants showed less evidence of adaptation, as indicated by increased time in behavioral withdrawal during separation but not baseline, when compared to the infants reared by their biological mothers. These findings are consistent with previous research in kindergarten age children, showing increased HPA axis response and a parallel increase in withdrawal behaviors in a socially stressful environment (Smider et al., 2002), but also suggests that differences between infants reared by their biological mother and those that are adopted are only present during stressful challenges. This was pattern was also present when considering the behaviors between the mother and infant during the less stressful reunion, where play levels were higher and time in close proximity to mother was lower in the adopted infants.

In addition to withdrawal behaviors, adopted infants also showed greater response to stress in the HPA pituitary level. Plasma ACTH levels were higher for infants reared by an adoptive mother at the 5-minute, hour 1, and hour 2 stress samples, but returned to the levels of the infants reared by their biological mother during the Chronic phase (d4). Unlike ACTH, there was no difference between the two groups in plasma cortisol levels, which is likely an indication that adopted infants are more responsive at the pituitary and possibly the central level. Because of its relatively immediate response and its close proximity to the brain, ACTH is considered a more central measure of stress responsiveness. Alternatively, the lack of rearing condition effects on cortisol may be because we did not measure cortisol at the optimal time points, although this is doubtful given that we measured it at the two time points that other studies have shown a robust stress response and group differences between peer-reared and mother-reared infant monkeys. Alternatively, the effects of adoption may produce chronic ACTH hypersecretion across the day and across different situations, which may have been present for a long enough time that cortisol became more or less down regulated at the adrenal cortex level. Regardless of the reason for the cortisol/ACTH dissimilarities, it is clear, that the effects of adoption on ACTH were robust. ACTH was higher in the adopted infants across all measurements in the Acute phase. As noted above, this may indicate that adopted infants have a reduced ability to regulate their stress response.

Experiment 2—Intruder Challenge Test

The effect of adoption appears to produce long-term, possibly permanent developmental changes, as shown by the persistence of high levels of behavioral withdrawal during the stressor of the Intruder Challenge Test, which occurred 1-2 years after the separation study for most of the subjects. In the Intruder Challenge Test, when faced with the challenge of an unknown same age intruder, behavioral withdrawal was higher for the adolescents and young adults raised by an adopted mother when compared to subjects reared by their biological mother across all of the segments—minute 10, 20, 30. These findings are interesting because the infants from both groups were permanently removed from their mother shortly after their separations at age six-months, and thereafter both groups received identical treatment. Such findings indicate that the effects of adoption on the adopted subjects continue long after the infant is no longer with its mother, demonstrating that differences are not simply a result of ongoing maternal treatment, support, or lack thereof. That adopted subjects showed high behavioral withdrawal during the Intruder Challenge Test which occurred one to two years later may indicate a long-term sensitivity to stress, with a propensity to respond acutely and chronically with higher anxiety, which may reflect increased social anxiety in the adopted subjects, a trend similar to that seen in adopted humans (Essex, Klein, Slattery, Goldsmith, & Kalin, 2010).

Given the random assignment of our subjects, our data suggests that mothers with adopted infants treat their infant in a manner that the response of the HPA axis to stress, as well as the behavioral stress systems, does not develop identical to infants raised by biological mothers. Overall, the results from experiment one and two show that adoption reduces an infant's ability to modulate stress-induced anxiety and arousal. As indicated in Ainsworth's classic attachment research, a sensitive and responsive mother reliably acts to reduce an infant's arousal, and overtime, a securely attached infant becomes self-reliant in reducing its arousal when faced with stressful situations (Ainsworth, 1979, 1989). There is evidence that adopted infants have a less secure attachment with their mothers. In a recent masters thesis studying these animals early in life, Bogh (2010) found that beginning in the first couple of months of life, adopted infants had more difficult and conflictual interactions with their adopted mothers than infants reared by their biological mothers. Bogh noted that the adoptive mothers show no evidence that they perceive the infant as not their biological infant, and that because on average the infants from the adopted mothers share fewer genes than the infants reared by their biological mother, there is less likelihood that in the adoptive mother-infant dyads, mother and infant temperaments match. Thomas and Chess called matching temperaments between mother and infant, goodness of fit and suggest that mothers have more difficulty and conflict when rearing infants whose temperaments do not fit their own personality, and as a result have outcomes that are more negative and more conflictual interactions. Thus, one explanation for our results is that temperamentally there is less goodness of fit between mother and infant in the adopted mother-infant dyads.

On the other hand, Bogh found evidence, similar to our own, that in the less stressful homecage environment, adopted infants appeared to be more independent. In Bogh's research adopted infants spend more time away from the secure base and protective eyes of their mother, receive less defense from their mother, and perhaps as a consequence, adopted infants elicit and receive more aggression from other group members. In the correct study, these behaviors continue as the adopted infants spend more time apart from mother as well as more time playing. Such early life experiences as infant adoption appear to have a profound influence on development and adolescent behavior. To the extent our results generalize to humans, the results suggest that the increased risk for behavioral pathology and other developmental outcomes in adopted infants may be due to early treatment and not just differences in genetic background.

Experiment 3—Alcohol Intake

Results of study three revealed an interesting, but unexpected effect of adoption on alcohol intake. When the adopted, now adolescent, subjects were given free access to alcohol in a social setting, those reared by an adoptive mother showed reduced alcohol consumption when compared to those reared by their biological mother. Studies show that anxiety increases alcohol intake particularly in stress-prone peer-reared monkey (Higley et al., 1991) and individual differences in HPA output are positively predictive of high alcohol intake (Fahlke et al., 2000; Higley et al., 1996a, 1996b). Given their higher levels of anxiety and high ACTH during the stressful situations of social separation and social challenge, we would have expected increased intake in the adopted infants. Adoption appears to have decreased alcohol intake to levels that previous studies show is sufficient to produce a pharmacological effect, with intake at about the level of one drink. This average level of intake is lower than we have seen across other studies for mother-reared subjects drinking in social settings (Roberg, 2011). Although a number of studies show higher intake in stress-prone peer-reared monkeys, a recent study with a large number of subjects show that when drinking in social settings, differences in alcohol intake between the highly anxious peer-reared subjects and the less anxious mother-reared subjects disappear, with both groups drinking similar amounts. Primarily, the effect of rearing on alcohol intake disappears when these two groups, who vary dramatically in both behavioral and HPA axis stress response, are allowed to drink in social settings, but differences between groups are robust when subjects are tested in highly stressful situations such as social separation or isolation where alcohol consumption increases (Roberg, 2011). While speculative, it may be that the lower level of behavioral stress exhibited by the adopted infants in the low stress (when reunited with mom), home-cage setting is reflected later in life when they are tested for alcohol consumption while in their home-cage. Unfortunately, both groups of our subjects were tested for alcohol intake while they were in their less stressful homecage in their social groups, so it is difficult to know how more stressful settings might have affected intake in the adopted infants. What is clear is that one must consider subtle early rearing differences in understanding the value of reinforcers such as alcohol.

Overall Discussion

Adopted infants showed behavioral and ACTH differences during conditions of high stress when compared to infants reared by their biological mothers. Differences were seen in the response to stress across infancy and behavioral measures of stress continued into adolescence. In addition, rearing differences in infancy continue to affect behavior later in life, as was shown when the adopted subjects, compared to the subjects reared by their biological mothers, were tested in the Intruder Challenge Test, and again when they were allowed to consume alcohol, indicating long-term effects of early in life adoptions. The results assessing the mother's behavior toward her infant indicate that the differences in the behavior of these two groups can be attributed to differences in mother-infant relationship early in life and at least for separation and Intruder Challenge, results in a reduced ability to control and regulate internal arousal in the adopted subjects. On the other hand, data from previous studies of these subjects, as well as the data collected when the adopted subjects were with their mothers indicate that perhaps during nonstressful situations, at birth adoptions may lead to increased independence and possibly better social adaptation during normal day-to-day interactions. It should be noted that this model is does not generalize to all types of adoption, but is limited to at birth or very early adoptions. Unlike later-life adoptions, such orphanage adoptions or childhood adoptions of children abused and neglected by their parents, all of our infants received high quality care right from the start of life. Our peer-reared subjects may better model such early-life neglect in later in life adoptions. To the extent that our findings from at birth adoptions generalize to humans, these results are important in addressing our understanding of the differences in adopted vs. children reared by their biological parents, and suggest that the increased risk for psychopathology in adopted humans may not necessarily just be the result increased genetic loading.

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