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Moderate Level Alcohol During Pregnancy, Prenatal Stress, or Both and Limbic-Hypothalamic-Pituitary-Adrenocortical Axis Response to Stress in Rhesus Monkeys

Mary L. Schneider, Colleen F. Moore, and Gary W. Kraemer

This study examined the relationship between moderate-level prenatal alcohol exposure, prenatal stress, and postnatal response to a challenging event in 6-month-old rhesus monkeys. Forty-one rhesus monkey (*Macaca mulatta*) infants were exposed prenatally to moderate level alcohol, maternal stress, or both. Offspring plasma cortisol and adrenocorticotrophic hormone (ACTH) were determined from blood samples before maternal separation and after separation. Behavioral observations were made repeatedly across separation. Moderate-level prenatal alcohol exposure was associated with significantly higher plasma ACTH response to maternal separation. Offspring exposed to prenatal alcohol, prenatal stress, and prenatal alcohol and stress showed reduced behavioral adaptation to stress compared with controls. Baseline, 2-hr, and 26-hr plasma ACTH levels were intercorrelated and predicted behavior during separation.

During the last two decades, the effects of alcohol use and abuse during pregnancy have received increased attention. Fetal alcohol syndrome (FAS) is characterized by growth retardation, craniofacial anomalies, and central nervous system dysfunction (Jones & Smith, 1973). In animal studies, findings from high-dose alcohol exposure during either gestation or the postnatal brain growth spurt have included physical malformations; overall growth retardation; and cognitive, motor, and behavioral abnormalities (Clarren & Smith, 1978; Hannigan & Riley, 1989; Sulik, Johnston, & Webb, 1981; Weinberg, 1989; West, Parnell, Chen, & Cudd, 2001). Recently, studies have demonstrated that moderate alcohol exposure is associated with similar but less severe deficits in children, including modest but persistent growth deficits (Day et al., 1989; Day, Richardson, Geva, & Robles, 1994), IQ decrements and learning problems (Streissguth, Barr, & Sampson, 1990), and deficits in processing speed (Jacobson & Jacobson, 1999; Jacobson, Jacobson, Sokol, Martier, & Ager, 1993).

A growing body of literature suggests that the list of adverse outcomes from fetal alcohol exposure might be expanded to include emotional disorders, such as increased risk for depression, delinquency, attention deficits, and impaired emotion regulation in affected individuals. As early as the 1970s, Jones and Smith (1973) described repetitive self-stimulating behaviors in children with FAS. More recently, researchers have reported hyperactivity, impulsivity, lack of appropriate inhibition, perseveration, temper tantrums, deficits in social behaviors, and symptoms of depression in individuals with fetal alcohol exposure (Aronson, Hagberg, & Gillberg, 1997; Famy, Streissguth, & Unis, 1998; Mattson & Riley, 2000; O'Connor & Kasari, 2000; Roebuck, Mattson, & Riley, 1999; Steinhausen, Gobel, & Nestler, 1984; Thomas, Kelly, Mattson, & Riley, 1998). Elevated scores on Psychosis and Delinquency Scales (both reflecting higher emotional lability) and poorly developed social skills and social withdrawal were also noted (Nanson & Hiscock, 1990; Roebuck et al., 1999). Aronson et al. (1997) followed 24 children born to mothers who had abused alcohol throughout pregnancy and reported that 10 of the children had attention deficit hyperactivity disorder (ADHD), two had Asperger syndrome (high-functioning autism, characterized by poor motor skills but relatively intact language development compared with autism), and one had an autistic-like condition that

Mary L. Schneider, Departments of Kinesiology and Psychology, University of Wisconsin-Madison; Colleen F. Moore, Department of Psychology, University of Wisconsin-Madison; Gary W. Kraemer, Department of Kinesiology, University of Wisconsin-Madison.

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Correspondence concerning this article should be sent to Mary L. Schneider, Department of Kinesiology, 2175 Medical Sciences Center, 1300 University Avenue, Madison, WI 53706-1532. Electronic mail may be sent to schneider@education.wisc.edu.

failed to meet the criteria for Asperger syndome. Furthermore, Streissguth, Barr, Kogan, and Bookstein (1996) examined 415 individuals with FAS or fetal alcohol effects (FAE), who ranged in age from 6 to 51 years with a median of 14.2 years, and found that most of them experienced mental health problems (90%), disrupted school experience (60%), trouble with the law (60%), confinement for treatment for mental health problems alone or in conjunction with alcohol or drug problems (50%), inappropriate sexual behavior (50%), and alcohol or drug problems (30%). Of course, with studies of humans, even with the best statistical analyses, it is impossible to separate completely the effects of variables often associated with alcohol use, such as tobacco use or chaotic home life, from the effects of fetal alcohol exposure per se.

Although the biological substrates for the apparent risk for emotional disturbances in fetal–alcohol-exposed individuals have not been established, there is some evidence suggesting that dysregulation of the biological systems closely related to stress responses in mammals, specifically the limbic-hypothalamic-pituitary-adrenocortical (LHPA) axis, might play a role (Vazquez, 1998). Several rodent studies have linked prenatal alcohol exposure to either LHPA axis hyper-responsiveness or delayed or deficient recovery of specific LHPA axis parameters in response to a variety of stressful events (Lee, Imaki, Vale, & Rivier, 1990; Nelson et al., 1986; Taylor et al., 1981; Taylor, Branch, Liu, & Kokka, 1982; Taylor, Branch, Van Zuylen, & Redei, 1986; Weinberg, 1992; Weinberg & Gallo, 1982; Weinberg, Kim, & Yu, 1995).

Stressful or challenging conditions that an organism encounters, which could include perceived loss of control or unpredictability, result in an elaborate, integrated pattern of behavioral, autonomic, sensory-motor, cognitive, and neuroendocrine responses including the LHPA axis (Sanchez, Ladd, & Plotsky, 2001; Selye, 1936). More specifically, information relating to stress or challenges is integrated into the paraventricular nucleus of the hypothalamus by neurons expressing corticotropin releasing hormone (CRH; Swanson, Sawchenko, Rivier, & Vale, 1983). CRH subsequently stimlulates the synthesis of the precursor protein proopiomelanocortin and the release of stored adrenocorticotrophic hormone (ACTH) from the anterior pituitary. Other neuroactive peptides, such as arginine vasopressin, are also expressed in CRH neurons and act along with CRH to stimulate ACTH release (Plotsky, 1991). ACTH then stimulates the synthesis and release of glucocorticoids by the adrenal cortex. Glucocorticoids (cortisol in primates and corticosterone in rats) mobilize energy during stress and act as transcriptional regulators from the adrenal cortex. Cortisol is the major hormonal end product synthesized by the LHPA axis in primates. Adaptation to a stressful event depends in part on an individual's ability to produce increased levels of cortisol and to reduce the production of cortisol once the stressor has subsided. Glucocorticoids are part of a negative feedback loop that prevent further LHPA axis activity at pituitary and central sites by way of glucocorticoid receptors (De Kloet, Vreugdenhil, Oitzl, & Joels, 1998; Jacobson & Sapolsky, 1991).

There is some evidence for dysregulation of the LHPA system in infants who were exposed to alcohol prenatally. Jacobson, Bihun, and Chiodo (1999) reported higher poststress cortisol levels, in response to a blood draw, in 13-month-old infants who were heavily exposed to alcohol in utero. Moreover, higher levels of maternal alcohol consumption at conception and during pregnancy were associated with elevated basal cortisol levels at 13 months. Elevated poststress cortisol levels were also related to more infant crying (Jacobson et al., 1999). In another infant study, 2-month-old infants exposed in utero to alcohol or cigarettes showed higher average basal cortisol levels than nonexposed infants (Ramsay, Bendersky, & Lewis, 1996).

Prenatal stress is another factor that has been found to be associated with emotional disturbances as well as altered LHPA axis regulation. Studies with humans have linked prenatal stress to increased risk for preterm delivery and low birth weight (see Wadhwa, 1998, for a review). There are also reports in the literature of an association between prenatal stress and attention deficits, schizophrenia, criminality, autism, and social withdrawal in children (Huttunen & Niskanen, 1978; McIntosh, Mulkins, & Dean, 1995; Meijer, 1985; Ward 1990, 1991). Unfortunately, except for the most recent studies (Glover & O'Connor, 2002; Huizink, de Medina, Mulder, Visser, & Buitelaar, 2002; O'Connor, Golding, Beveridge, & Glover, 2002), most of the human work on prenatal stress and emotional disturbance has been retrospective.

Prospective animal studies have also reported that prenatal stress can alter offspring emotionality. The most likely causal relationship is that maternal stress alters the pre- and post-natal development of neurobiological mechanisms that regulate emotionality in offspring (Barbazanges, Piazza, Le Moal, & Maccari, 1996; Fleming, O'Day, & Kraemer, 1999; Fleming et al., 2002). To support this idea, there is evidence of abnormal regulation of the LHPA axis

after exposure to a stress challenge in prenatally stressed rodents, compared with controls (Barbazanges et al., 1996; Fride, Dan, Feldon, Halevy, & Weinstock, 1986; Maccari et al., 1995; McCormick, Smythe, Sharma, & Meaney, 1995; Peters, 1982; Takahashi, Haglin, & Kalin, 1992; Weinstock, 1997).

Our studies with rhesus monkeys showed heightened responsiveness to stressors among monkeys from prenatally stressed pregnancies that were reared without mothers (see Schneider & Moore, 2000, for a review). Specifically, we reported higher concentrations of plasma cortisol and plasma ACTH in response to stressful events such as social separation, new group formation, and exposure to a noise stressor. We also found increased behavioral reactivity to novelty and challenge during adolescence and early adulthood in nursery-reared prenatally stressed monkeys compared with nursery-reared controls from undisturbed pregnancies (Clarke & Schneider, 1993; Clarke, Soto, Bergholz, & Schneider, 1996; Clarke, Wittwer, Abbott, & Schneider, 1994; Schneider, 1992b; Schneider et al., 1998).

Although the processes underlying fetal alcohol and prenatal stress effects have yet to be fully explicated, it appears likely that the mechanisms by which offspring deficits are induced may overlap. For instance, both maternal alcohol consumption and stress during pregnancy have been linked to increased maternal neuroendocrine activity, which could alter fetal brain development and subsequent offspring postnatal neuroendocrine function. Altered neuroendocrine function in the offspring could mediate, at least in part, other offspring neurodevelopmental problems. Also, at higher exposure levels, both fetal alcohol exposure and prenatal stress are associated with reduced umbilical blood flow (Savoy-Moore, Dombrowski, Cheng, Abel, & Sokol, 1989) and fetal hypoxia (Meyers, 1975; Morishima, Pedersen, & Finster, 1978; Mukherjee & Hodgen, 1982; although see West et al., 2001), which could also contribute to adverse neurodevelopmental outcomes in offspring. It is also possible that fetal alcohol exposure and prenatal stress might act together to enhance or alter the likelihood of adverse consequences beyond that which each variable contributes alone.

The present report is part of a prospective longitudinal study comparing rhesus monkeys from mothers that either consumed moderate level alcohol during pregnancy, underwent daily psychological stress, or both, with undisturbed controls. During neonatal development in this cohort, deficits in attention, motor maturity, and increased drowsiness were demonstrated as a consequence of daily moderate level maternal alcohol consumption (Schneider, Roughton, & Lubach, 1997). Moreover, alcohol accompanied by stress during gestation resulted in 23% fetal losses, and males from the alcohol plus stress condition had reduced birth weights (see Schneider et al., 1997, for details).

In this report, we present behavioral observations and measures of neuroendocrine functioning during a 3-day maternal separation when the monkeys were 6 months old. Maternal separation is a powerful psychobiological stressor—the bond between the mother and infant is one of the strongest social attachments formed by most mammals (Bowlby, 1982). Because the experience of maternal separation engenders both biological and psychological responses, we used not only plasma cortisol and ACTH, physiological indexes of LHPA axis activity, but also a standard separation protocol to evaluate behavior related to adaptive coping in nonhuman primate studies (Mineka & Suomi, 1978). Social separations have been used extensively in nonhuman primate studies to model some types of psychopathology because the responses to social separation include behaviors that resemble depression or anxiety in humans (Harlow, Harlow, & Suomi, 1971; Kraemer, 1986). Understanding the temporal course of the behavioral response to separation is important; thus, we collected repeated behavioral measures across 3 days of separation and compared patterns in the treatment groups with those of the control animals. We also examined the relationship between ACTH and cortisol and the behavioral measures.

Method

Maternal Treatment

Female rhesus monkeys from the breeding colony were identified that consistently and voluntarily consumed an entire 0.6 g/kg, 6% v/v alcohol solution sweetened with Nutrasweet (300 mg/100 ml; Equal Sweetener, Merisant US Inc., Chicago) daily over a 2-week period. Approximately half of the females tested fell into this category. This dosage is comparable to an average-sized woman consuming approximately one to two alcoholic drinks daily, with blood alcohol concentrations (BACs) of 20 to 50 mg/dl 60 min after consumption (blood alcohol levels were tested before pregnancy to minimize additional stress to the pregnant female because prenatal stress was a variable of interest). Alcohol-consuming monkeys were randomly assigned to one

of four groups (control or one of three experimental groups) in a 2×2 factorial design with in utero alcohol prenatal stress, or both, as the independent variables. All breeding females were housed in single cages to minimize introducing variance due to differential maternal or infant social experiences.

One experimental group (alcohol exposed) voluntarily consumed 0.6 g/kg in a 6% (v/v) alcohol solution sweetened with NutraSweet (300 mg/100 ml) daily throughout gestation at 4:00 p.m. Alcohol consumption was begun 5 days before breeding and ended at parturition. All animals were fed Purina Monkey Chow (St. Louis, MO) daily at 6:00 a.m. and were given a fresh fruit supplement on Monday, Wednesday, and Friday at 1:00 p.m. The animals had no Chow left by the time the alcohol solution was given. Water was available ad libitum, including during the time of day when the alcohol solution was available. A second experimental group (alcohol plus stress) consisted of females who voluntarily consumed the alcohol solution daily throughout gestation and were exposed to a mild 10-min psychological stressor during mid to late gestation, Day 90 through Day 145 postconception (see the following discussion). A third experimental group (prenatal stress) consisted of females exposed to a mild 10-min psychological stressor during mid to late gestation, Day 90 through Day 145 postconception. The fourth group consisted of control subjects that voluntarily consumed a sucrose solution that was approximately equivolemic and equicaloric (8g/100 ml water) to the alcohol solution. During the remainder of the day, all females were housed under identical conditions, undisturbed except for necessary routine animal husbandry.

The stress treatment was administered five times per week at approximately 3:30 p.m. for females in the prenatal stress and prenatal stress plus alcohol groups. It involved removing the pregnant female from the home cage, placing her in a transport cage and taking her to a darkened room where three noise bursts were randomly administered over a 10-min period. The noise burst consisted of an alarm horn that produced a 1300 Hz sound of 115 dB intensity at 1 m. Previous studies showed this manipulation raised cortisol levels from approximately 25 to 35 μg/dl (Schneider & Moore, 2000).

Table 1
Sample Size and Gender Distribution as a Function of Prenatal Treatment Conditions

Condition	Sample size	Females	Males
Alcohol – Exposed	10	7	3
Prenatal – Stressed	8	2	6
Alcohol + Stress	10	4	6
Controls	13	10	3
Total	41	23	18

Subjects

The subjects in this study were 41 six-month-old rhesus monkey (*Macaca mulatta*) offspring, 23 females and 18 males, born to the female rhesus monkeys from one of four groups described earlier (see Table 1). All of the monkeys were born between May 1993 and May 1996. They were housed with their mothers in individual cages during the first 6 months of life. All infants were separated from their mothers four times during the first month of life to conduct examinations of early neurobehavioral development and monitor growth (Schneider & Suomi, 1992). Behavioral observations of mother–infant dyads were made weekly throughout the first 6 months of life. At 6 months, the infants were permanently separated from their mothers and housed alone for 3 days to assess behavior and neuroendocrine stress responses and adaptive coping. After the 3-day separation period, they were placed into mixed-sex peer groups consisting of 5 to 6 monkeys from similar prenatal conditions until they reached sexual maturity (approximately 30 months of age), after which they were housed in same-sex pairs. They were maintained on a diet of commercial Purina Monkey Chow (St. Louis, MO) given at 6:00 a.m. and supplemented three times weekly at 1:00 p.m. with fresh fruit. Water was available ad libitum. All housing conditions were light (16-hr light and 8-hr dark) and temperature (21 ± 0.5 degree C) controlled.

Separation Procedure

At 6 months of age, all subjects were separated from their mothers for weaning. During the first 3 days of separation, the monkeys were housed in individual cages. After 3 days of individual housing, the monkeys were placed in peer groups. All subjects were separated from mothers at 9:00 a.m. on Separation Day 1 and moved to another room where they were housed individually in wire-mesh cages. The observations and physiological data reported here occurred during the 3-day period of individual housing. The monkeys could hear other monkeys in the room, but physical and visual contact was prevented.

Blood samples. Blood samples were collected 1 week before separation at 11:00 a.m. (baseline), on

Separation Day 1 at 11:00 a.m. (2 hr after separation), and on Separation Day 2 at 11:00 a.m. (26 hr after separation). Samples were collected within 2 to 3 min of room entry by experimenters. Samples (1 ml) were collected by femoral venipuncture into ethylenediamine tetra-acetic acid (EDTA) -treated vacutainers. Following centrifuging, plasma was harvested and stored at −70 degrees C until assay. Baseline samples for 3 monkeys (2 control females, 1 control male) were lost (6/246 samples overall) due to technical difficulties. In these cases the treatment group mean was used.

Cortisol radioimmunoassay (RIA). Rhesus serum cortisol levels were measured using an antibody coated I^{125} RIA kit, GammaCoat™ Cat. CA-1549 from DiaSorin, Stillwater, MN, in the Assay Services Lab of the Wisconsin Regional Primate Research Center (WRPRC), University of Wisconsin-Madison. The kit protocol was modified to extend the range of the standard curve 2 points higher by pipetting 20 μL and 30 μL of the highest standard vial.

The interassay coefficient of variation for eight assays (32 quality control tubes) was 9.43% and the mean intra-assay coefficient of variation was 3.44%. RIA tubes were counted in a TmAnalytic gamma counter model 1290. Data were captured in a Macintosh computer and uploaded to a Sun OS computer at WRPRC where analysis of RIA data reduction was computed by weighted least squares regression analysis (Rodbard & Lewald, 1970).

ACTH. Rhesus serum ACTH levels were measured using an in-house WRPRC assay. Standards were purchased from Bachem Corporation, Torrance, CA; primary and secondary antibodies were purchased from IgG Corporation, Nashville, TN; and the I-125 trace and precipitating solution was purchased from Diagnostic Products Corporation, Los Angeles, CA. The interassay coefficient of variation for six assays (36 quality control tubes) was 21.17% and the mean intra-assay coefficient of variation was 6.77%.

Behavioral scoring. Behaviors of all infants were videotaped for later coding three times daily (9:00 a.m., 1:00 p.m., and 5:00 p.m.) on Days 1 to 3 of the separation. Behaviors coded from the videotapes were standard for our laboratory and included: (a) environmental manipulation: oral, manual, or pedal exploration or manipulation of the environment, including eating and drinking; (b) locomotion: any self-induced change in physical location of self; (c) vocalization: any sound emitted by the subject with the exception of sneezes or coughs (frequency only); (d) stereotypy: any repetitive, patterned, or rhythmic movement including rocking behavior (scored after three repetitions); (e) passive: absence of all exploratory, locomotor, or other simultaneous scored activity (except vocalizations); (f) self-groom: nonoral grooming or scratching self; and (g) self-mouth: oral contact with any part of the body. Data from 4 of 41 monkeys (1 alcohol female; 1 alcohol + stress male; and 2 mid to late gestation stress: 1 male, 1 female) were lost because of equipment failure. These subjects were dropped from the behavioral analyses.

Data Analyses

To reduce the skewness of the ACTH and cortisol values, logarithm (base 10) transformations were used in mixed analyses of covariance (ANCOVAs) for prenatal stress, fetal alcohol exposure, and sex, with time (2 hr after separation, 26 hr after separation) treated as a repeated measure and preseparation baseline measure as the covariate.

Behavioral data were analyzed by analyses of variance (ANOVAS) with prenatal stress, fetal alcohol exposure, and sex as between-subject variables, and with time (9:00 a.m., 1:00 p.m., 4:00 p.m.) and day (1, 2, 3) treated as repeated measures. The significance levels of the reported results for repeated measures were adjusted by the Huyhn-Feldt correction. Two categories of behavior—self-groom and self-mouth—were observed too rarely to be subjected to statistical analysis.

Results

Hormone Levels

Plasma ACTH. Figure 1 shows the means and standard errors for the plasma ACTH levels in the four treatment groups relative to baseline at 2 hr and 26 hr after maternal separation. The 2 (prenatal alcohol) × 2 (prenatal stress) × Sex × Time (2 hr and 26 hr after separation) ANCOVA with baseline log ACTH as the covariate and log ACTH at 2 and 26 hr as the dependent variable revealed a significant Alcohol × Time interaction, $F(1, 33) = 7.17$, $p < .02$. Alcohol-exposed monkeys (i.e., those in the alcohol and alcohol+stress groups combined) showed the greatest increase in ACTH at 2 hr after separation but were approximately equivalent to the other groups at 26 hr after separation. A main effect of time, $F(1, 33) = 48.5$, $p < .001$ was also significant, with ACTH values at 26 hr after separation being lower than those observed at 2 hr after separation, as expected. There were no significant main effects or interactions of sex or prenatal stress.

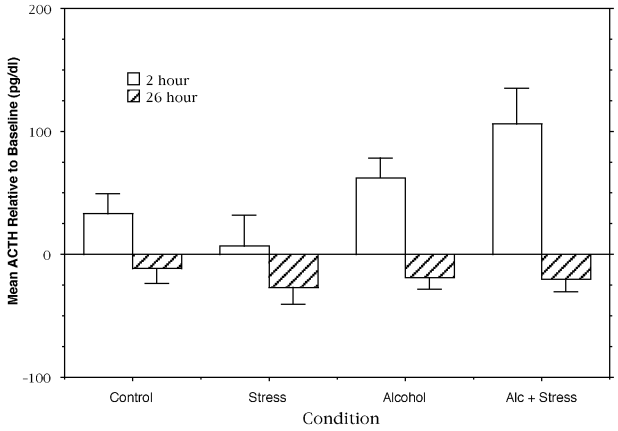

Figure 1. Mean plasma adrenocorticotrophic hormone (ACTH) response (pg/ml) relative to baseline (1 week before separation) at 2 hr and 26 hr after maternal separation as a function of prenatal treatment condition (control $n = 13$, prenatal stress $n = 8$, fetal alcohol exposed $n = 10$, and fetal alcohol + stress exposed $n = 10$). Bars represent SE's.

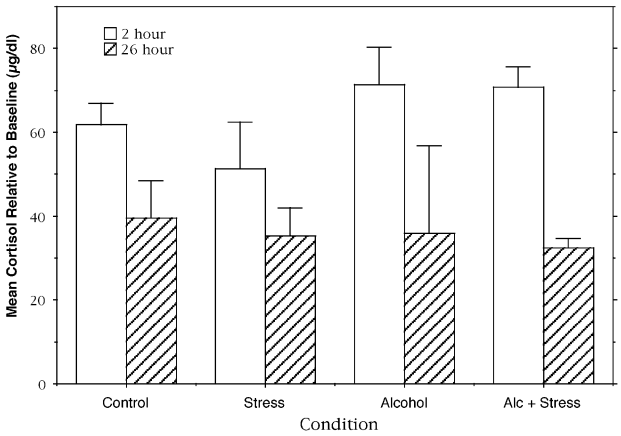

Figure 2. Mean plasma cortisol levels (µg/dl) relative to baseline (1 week before separation) at 2 hr and 26 hr after maternal separation as a function of prenatal treatment condition (control $n = 13$, prenatal stress $n = 8$, fetal alcohol exposed $n = 10$, and fetal alcohol +stress exposed $n = 10$). Bars represent SE's.

Plasma cortisol. Figure 2 depicts the plasma cortisol levels relative to baseline in the four treatment groups as a function of time (2 hr after separation and 26 hr after separation). ANCOVA of log cortisol levels at 2 hr and 26 hr with log baseline cortisol as covariate indicated a marginal trend for an Alcohol × Time interaction, $F(1, 33) = 2.87$, $p < .10$, which was similar to the Alcohol × Time interaction on the ACTH results. The alcohol groups showed the largest increase in cortisol 2 hr after separation but were approximately equivalent to the others 26 hr after separation. There was also a marginal trend toward an effect for prenatal stress, $F(1, 32) = 2.83$, $p = .10$, with prenatally stressed monkeys showing slightly lower cortisol levels overall. There was a slight trend toward females having higher plasma cortisol levels than males, $F(1, 32) = 2.76$, $p < .11$. The ANCOVA also showed a main effect of time, $F(1, 33) = 22.32$, $p = .0001$.

Behavioral Effects

Locomotion. Figure 3 depicts the Day × Prenatal stress interaction, indicating that nonprenatally stressed monkeys showed increased levels of locomotion across days, suggesting adaptation, whereas prenatal stressed monkeys showed a relatively flat pattern, $F(2, 54) = 3.46$, $p < .05$. A Day × Time interaction indicated that at the 9:00 a.m. and 1:00 p.m. observations, monkeys demonstrated an increase in locomotion across Days 1, 2, and 3, whereas at the 4:00 p.m. observation there was no change over days, $F(4, 108) = 2.48$, $p < .05$.

Stereotypies. A main effect of days indicated that stereotypies increased markedly across days, $F(2, 54) = 11.57$, $p < .001$ (see Figure 4). A Day × Time interaction indicated that although there was a significant increase across days at all periods, this was most marked at 9:00 a.m., $F(4, 108) = 2.75$, $p = .04$. A Time × Alcohol × Prenatal Stress interaction indicated stereotypies were highest at 1:00 p.m. in prenatally stressed monkeys, $F(2, 54) = 4.19$, $p = .02$. For the other groups, there was no effect of time.

Environmental manipulation. A Time × Day interaction indicated that levels of environmental manipulation, which is defined as oral, manual, or pedal manipulation of the environment, was highest directly after the maternal separation, at 9:00 a.m. on Day 1, $F(4, 108) = 3.43$, $p < .02$, suggesting that this category of behavior—in a relatively constrained environment—is a response to either a novel or stressful situation. A Prenatal Stress × Day × Time interaction, $F(4, 108) = 3.74$, $p < .01$, indicated that although nonstressed monkeys showed a decrease in environmental manipulation across days at the 9:00 a.m. observation, suggesting adaptation, prenatally stressed monkeys showed relatively high but consistent levels of environmental manipulation at 9:00 a.m. across days, suggesting lack of adaptation to the stressful event. The other times did not show this pattern. A marginal main effect of stress showed a trend for higher environmental manipulation in the prenatally stressed compared with nonstressed animals, M (SE) = 36.4 (4.1) for stressed monkeys compared with 23.1 (2.5) for nonstressed animals, $F(2, 54) = 3.00$, $p < .06$. A marginal Alcohol

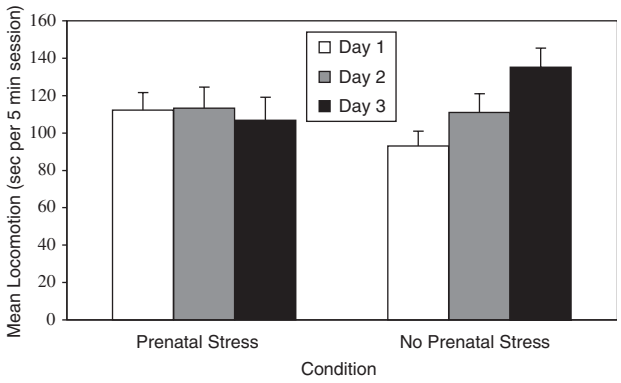

Figure 3. Mean seconds of locomotion across 3 days following maternal separation as a function of prenatal stress treatment. Bars represent SE's.

× Day interaction suggested that non-alcohol-exposed monkeys showed the most environmental manipulation on Day 1 and less on Days 2 and 3, suggesting that they were adjusting to the maternal separation. Alcohol-exposed monkeys showed an increase in environmental manipulation on Day 2 relative to no-alcohol monkeys, but on Day 3 the groups were comparable, $F(2, 54) = 2.996$, $p < .06$.

Vocalizations. There was evidence of high levels of vocalizations on Day 1 at 9:00 a.m., directly after the maternal separation, as expected. There were no main or interactive effects of treatment at this time point. At the other time points, there were too few vocalizations to allow for parametric analysis. Correlations: we examined relationships between plasma ACTH and cortisol levels across sampling times using Spearman rank-order correlations. Figure 5 shows high consistency in ACTH across the three time points (all ps $< .01$). Cortisol measures

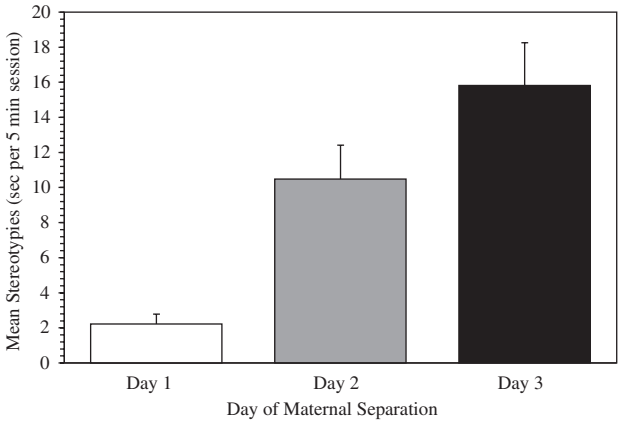

Figure 4. Mean motor stereotypies (seconds per 5-min scoring session) across 3 days following maternal separation. Bars represent SE's.

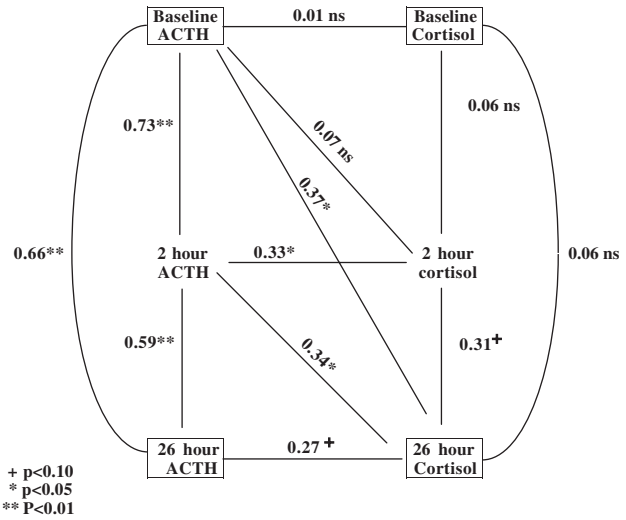

Figure 5. Relationships among plasma adrenocorticotrophic hormone (ACTH) and cortisol values across sampling times using Spearman rank-order correlations. $+p < .10$. $^{*}p < .05$. $^{**}p < .01$.

showed more lability across the three sample times than did ACTH measures (ps $> .05$). Two-hr postseparation cortisol values showed a weak relationship to 26-hr postseparation cortisol values ($p < .10$).

ACTH and cortisol were significantly related to each other at some time points. Two-hr ACTH was positively correlated with both 2-hr cortisol and 26-hr cortisol. This was expected because of the role of ACTH in stimulating cortisol secretion. Twenty-six-hr ACTH and 26-hr cortisol were marginally correlated ($p < .10$). Baseline ACTH (1 week before separation) was positively correlated with 26-hr cortisol.

Figure 6 displays the correlations of separation behaviors with ACTH and cortisol. ACTH and cortisol values were correlated with behaviors for Days 1, 2, and 3, with behavioral data collapsed across time of day. The pattern that emerged was that baseline ACTH levels and 26-hr cortisol levels correlated positively with behavioral measures of activity—stereotypy and locomotion—whereas 26-hr cortisol correlated negatively with environmental manipulation during the early part of the separation period. Figure 6 shows that baseline ACTH levels correlated positively with stereotypies and locomotion on Days 1, 2, and 3, whereas baseline cortisol showed only one significant correlation with behavior. Cortisol levels 26 hr after maternal separation were positively associated with stereotypies on Days 1, 2, and 3 and with locomotion on Days 2 and 3, and negatively correlated with environmental manipulation on Days 1 and 2, whereas 26-hr ACTH levels showed no

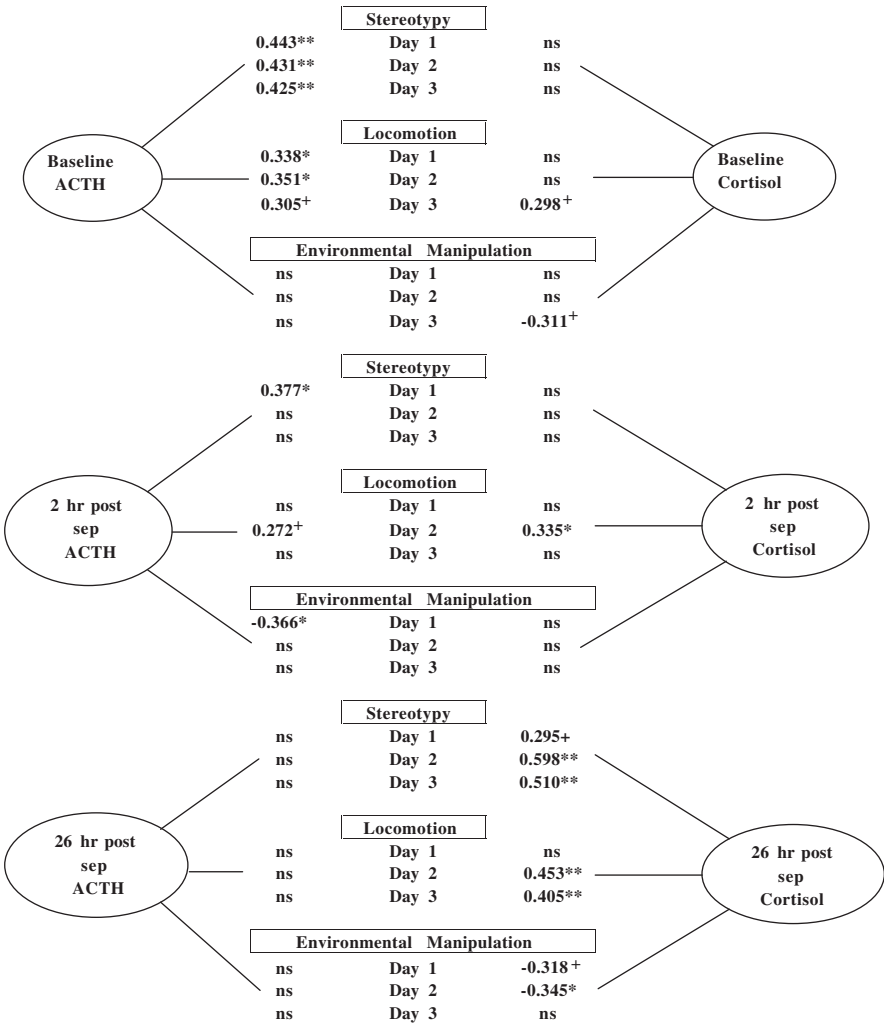

Figure 6. Relationships among adrenocorticotrophic hormone (ACTH), cortisol, and separation behaviors on Days 1, 2, and 3 of maternal separation. $+p<.10$. $^{*}p<.5$, $^{**}p<.01$.

significant correlations with behavior. Plasma ACTH levels 2 hr after maternal separation correlated positively with stereotypies on Day 1 and negatively with environmental manipulation on Day 1, and 2-hr cortisol levels correlated with locomotion on Day 2.

Discussion

There were four principal findings of the present study. First, fetal alcohol-exposed offspring showed significantly different patterns of plasma ACTH across time compared with offspring that were not exposed to alcohol in utero. Second, the temporal course of behavioral responses differed across groups. Nonprenatally stressed monkeys showed a pattern of increasing locomotion across days, whereas prenatally stressed monkeys showed flat patterns across days. Third, there was a weaker relationship of cortisol to prenatal treatment and a different relationship to behavior during the 3-day separation than there was for ACTH. Fourth, baseline ACTH and 26-hr post-separation cortisol levels were significantly associated with high levels of activity (locomotion and stereotypies) during Days 1, 2, and 3 of separation.

The first finding, that fetal alcohol exposure affects ACTH levels across time during a stress episode in monkeys, parallels a large rodent literature suggesting that fetal alcohol exposure has long-lasting effects on LHPA axis regulation (Taylor et al., 1982; Weinberg & Gallo, 1982). The mechanism underlying HPA axis hyper-responsiveness in rats exposed to alcohol in utero has not been completely determined (Glavas, Hofmann, Yu, & Waingerg, 2001). Rodent studies suggest, however, that these effects are mediated at several central nervous system (CNS) levels, including the hippocampus and septal region (Kelly, 1996). The hippocampus is sensitive to both fetal alcohol exposure (Savage et al.,

1992; Sutherland, McDonald, & Savage, 1997) and prenatal fetal and maternal increased levels of glucocorticoids (McEwen, 1994; Sapolsky, 1993). The hippocampal glucocorticoid receptors partially regulate the negative feedback of glucocorticoids on the HPA axis in adult animals (De Kloet & Reul, 1987; McEwen, De Kloet, & Rostene, 1986). Adverse effects of acute and chronic glucocorticoid hypersecretion on cognitive performance, including attention and memory, have been found in several human studies (McEwen & Sapolsky, 1995). These effects are thought to be mediated by glucocorticoid effects on hippocampal neuronal or glial mechanisms, or both (Sapolsky, 2000). Indeed, reduced hippocampal volume has been found not only in combat veterans with post-traumatic stress syndrome but also in adults who reported past physical or sexual abuse (Stein, Koverola, Hanna, Torchia, & McClarty, 1997).

Blood concentrations of ACTH and cortisol measured in this study probably reflect different aspects of neuroendocrine stress responsiveness in rhesus monkeys. Studies of human children's responses to stress have used primarily cortisol alone as a dependent measure because cortisol can be measured in saliva. Cortisol and ACTH can be measured together only in blood samples. Human studies report lower saliva cortisol levels in maltreated nursery school children on high-conflict days (Hart, Gunnar, & Cichetti, 1995), in nursery school children with less experience in peer groups (de Haan, Gunnar, Tout, Hart, & Stansbury, 1998), in children living near the epicenter of an Armenian earthquake in the 1980s (Goenjian et al., 1996), and in boys referred for disruptive aggressive behavior (McBurnett, Lahey, Rathouz, & Loeber, 2000). Elevated urinary cortisol or urinary catecholamines have been found in children living in areas of high transportation noise (Evans, Hygge, & Bullinger, 1995; Evans, Lercher, Meis, Ising, & Kofler, 2001). Reasoning back from salivary or urinary cortisol levels alone to ACTH or other aspects of LHPA neuroendocrine function is probably not possible, however.

Although ACTH promotes cortisol release, the amount of cortisol released is not necessarily proportional to ACTH concentration because maximum levels of cortisol release can be attained by less than maximal levels of ACTH release (Keller-Wood, Shinsako, & Dallman, 1983). Also, higher levels of ACTH may not necessarily produce proportionally higher levels of cortisol but may prolong the duration of cortisol release (Kim, Gilberson, Yu, Thomas-Zoeller, & Weinberg, 1999). Either or both of these effects could affect behavioral adaptation through feedback effects on brain LHPA mechanisms. Because the brain is a target organ for ACTH as well as the adrenals (Bohus & De Wied, 1980; De Wied, 1977), ACTH can modulate behavioral responses involved in adaptation such as increased grooming or stress-induced sedation (Galina, Sutherland, & Amit, 1983). ACTH or fragments of its amino acid structure have also been found to facilitate motivation and vigilance, enhance concentration and visual attention, and promote learning in rats (De Wied & Jolles, 1982). Moreover, ACTH or its fragments have been found to modulate adaptive behavioral response to stress (De Wied & Jolles, 1982). Prenatal alcohol and stress appear to be able to affect aspects of brain and peripheral function related to the LHPA axis at multiple levels. For example, alcohol-exposed rodents exhibit increased stress-induced ACTH but not increased cortisol levels compared with controls (Lee et al., 1990; Weinberg, Taylor, & Gianoulakis, 1996).

A second finding was that there was a weaker relationship of cortisol than ACTH to prenatal treatment, and cortisol and ACTH showed a different time course in their relationships to behavior during the 3-day separation. Prenatally stressed monkeys did not adapt behaviorally in the same way as nonprenatally stressed monkeys in terms of relationships among locomotor behavior, cortisol, and ACTH levels. These results parallel findings of significant reductions in activity and increased ACTH levels immediately after stressful situations in rats (Galina et al., 1983).

The third finding, that is, that locomotion increased over days in nonprenatally stressed animals, but not in prenatally stressed monkeys, suggests that one effect of prenatal stress is impaired behavioral adaptation or difficulty with return to homeostasis. In rhesus monkeys, separation from the mother represents a significant change in the environment, with which the animal must cope or to which it must adapt. Behaviors and endocrine responses exhibited immediately after a stressful situation may return the organism to both physiological and psychological homeostasis (Selye, 1936; see also Kraemer, 1986).

The fourth finding was that high levels of baseline ACTH, per se, were associated with high levels of activity (locomotion and stereotypies) during the separation period (Days 1, 2, and 3), whereas 26-hr cortisol but not baseline or 2-hr cortisol showed hormone–behavior relationships. The postseparation measurement times for ACTH and cortisol were chosen to capture the initial stress responses (2 hr after separation) and the return toward equilibrium (26 hr after separation). The baseline ACTH levels were taken a full week before the separation and yet

were predictive of active behaviors on all three days of separation, accounting for between 9% and 20% of the variance. That a relationship between behaviors during separation and baseline ACTH was detected may be due to a preexisting set of LHPA mechanisms. The 2-hr and 26-hr ACTH levels that were taken during the stressful event were less predictive of separation behaviors than was baseline ACTH, taken 1 week earlier. In contrast, 26-hr cortisol showed a relatively strong relationship with separation behaviors, accounting for between 9% and 36% of the variance. Cortisol is also known to have the brain as a target organ. For example, there is a high density of glucocorticoid receptors in certain cortical areas, such as the prefrontal cortex, in the nonhuman primate brain (Lopez, Akil, & Watson, 1999). Activity may also function as part of the feedback mechanisms during stress because a major function of cortisol is to mobilize energy resources. Thus, cortisol would be expected to have a significant relationship with activity under stressful conditions. However the mechanisms and interplay of ACTH, cortisol, and behavior under stress are not well understood, even in rodents. The interesting timing difference in the relationships of ACTH and cortisol with separation behaviors in the present study provides an important new finding that needs further research.

A theoretical view that might help integrate findings on LHPA axis and behavioral responses to stress is that of Mayes (2000), who proposed the concept of arousal, indexed by the level of CNS activation, or behavioral state in response to novelty, as a central organizing idea for understanding cognitive, social, and stress regulation functioning in children who experienced prenatal perturbations or are experiencing postnatal challenges. According to this view, a significant increase in arousal could result not only in physiological changes in ACTH and cortisol but could lead to less optimal attention, anxiety or avoidance, or poor self-regulation. In our study, therefore, the high levels of ACTH in response to stress in fetal alcohol-exposed monkeys, the compromised behavioral adaptation in the prenatally stressed monkeys, and the numerous significant correlations between physiological and behavioral responses to stress could represent an altered level of overall arousal or CNS activation in fetal alcohol-exposed and prenatal stressed monkeys. According to this view, nonexposed counterparts, on the other hand, could have used adaptive behavior to reduce hormone levels and inhibit automatic behaviors related to hyperarousal (perhaps locomotion and stereotypies in this study) and therefore could have achieved a more optimal level of arousal. Further work is needed to test this hypothesis.

Our study suggests that prenatal alcohol exposure in monkeys yields poor regulation. Not only did these monkeys show altered ACTH responses to stressful situations, compared with controls, but they demonstrated behavioral dysregulation, or a reduced pattern of adaptation. Previous reports from this cohort of fetal alcohol-exposed monkeys indicated other signs of poor regulation, including impaired attention and reduced motor maturity in infancy (Schneider, 1992a; Schneider et al., 1997). When tested on standard primate cognitive tasks, monkeys that were prenatally exposed to alcohol took more trials to learn a nonmatch-to-sample task, and monkeys exposed to alcohol and stress monkeys showed poor behavioral regulation during cognitive testing, as evidenced by increased activity and stereotypies (Schneider, Moore, & Kraemer, 2001).

Taken together, the results of studies on the effects of moderate-level fetal alcohol exposure and prenatal stress on behavioral and physiological measures in monkeys are consistent with the notion that fetal alcohol exposure, even at a relatively low level, can have an effect on the organization of the stress response of the LHPA axis and behavioral adaptation to stressful events. Recent studies, largely with rodents, have linked neonatal maternal separation to several changes in neurological feedback loops, resulting in reorganization of neuronal pathways that regulate neuroendocrine function, arousal, and vigilance behavior. Our results suggest that similar factors could be at work as a result of prenatal factors in primates. Although the mechanisms for these effects are not well understood at this time, these studies suggest an influence of maternal alcohol consumption and prenatal stress on the organization of the LHPA axis before birth, which could have cascading effects on development. Such cascading effects may be the source of increased vulnerability to psychiatric disorders reported in humans who are prenatally exposed to alcohol or stress (Huttunen & Niskanen, 1978; Mattson & Riley, 2000; Meijer, 1985; Streissguth et al., 1996).

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