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Early deprivation and behavioral and physiological responses to social separation/novelty in the marmoset

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Abstract

Long-term effects of adverse early environment on neurobehavioral development have been reported for rodents and primates. The present study used daily early deprivation (ED), a paradigm developed for rats, for the first time in a nonhuman primate, the common marmoset, and investigated its effects on the behavioral and physiological responses to social separation/novelty (SSN) challenge tests in juveniles. On postnatal days (PNDs) 2–28, infants ($n = 5$ twin pairs) were removed from the parents and placed alone in an isolation chamber for 30–120 min (9 h/week). Parents and control subjects ($n = 5$ twin pairs) were briefly restrained (CON). At Weeks 18–20, behavioral responses of ED and CON juveniles to six 60-min SSN tests in an isolated cage, comprising 45 min alone and 15 min reunion with the father, were measured. Baseline and post-test urine samples were collected for measurement of cortisol. ED subjects exhibited significantly lower basal SSN urinary cortisol than CON, whilst SSN response cortisol values were similar in ED and CON. When alone, ED subjects were significantly less mobile and emitted significantly less contact calls than CON. Following reunion, ED subjects were significantly less in contact with or being carried by the father than CON and demonstrated significantly more tail piloerection. Although they require validation by additional parameters (e.g. cardiovascular), these data strongly suggest that early-life stress alters endocrine and behavioral responsiveness to psychosocial challenge in this primate and in a direction that could model important changes in disorders of human affective state. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Marmoset; Early deprivation; Social separation/novelty; Social reunion; Cortisol response; Behavioral arousal; Affective state

1. Introduction

A large body of animal and human evidence on adverse early environments and their long-term consequences indicates that certain aspects of the infant environment are crucial for typical neurobehavioral development (Clarke, 1993; Heim et al., 1997; Kendler et al., 1993; Lyons et al., 2000; Matthews et al., 2001; Meaney et al., 1996; Suomi, 1991; Wong and Licinio, 2001). In mammals, infant–parent relationships form the main aspect of the early environment, with children and young animals having species-specific "expectations" about parental care (Greenough et al., 1987). Accordingly, deviation from that expectation can result in the disturbance of neurobiological, physiological and behavioral development. The majority of this evidence has been obtained from studies in rats, but some very important primate studies have also been conducted. Below we summarize some of this evidence and then introduce our approach of conducting a primate study using a repeated postnatal manipulation originally developed in the rat.

Studies in rats have demonstrated that spontaneous differences in maternal care are related to, and specific manipulations of the pup–dam relationship lead to, specific long-term effects on offspring neurobehavioral development (Caldji et al., 2000a,b; Lehmann and Feldon, 2000; Levine, 2000; Liu et al., 1997, 2000a,b; Matthews et al., 2001; Meaney et al., 1996). Several research groups have investigated the long-term effects of specific manipulations of the infant–mother relationship in primates. In their classical experiments, Harlow et al. separated rhesus macaque infants from the mother at birth and reared them with inanimate surrogate mothers in the complete absence of conspecifics (for summary, see Gandelman, 1992). In a complementary approach, rhesus macaque infants are separated from the

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mother shortly after birth and then either nursery (i.e. human) reared alone for several months followed by group rearing with age-matched peers or nursery reared together with peers from the outset (Champoux et al., 1989, 1991; Clarke, 1993; Clarke et al., 1996; Coe et al., 1992; Higley et al., 1992; Kraemer et al., 1989). Rosenblum et al. with bonnet macaques and Lyons et al. with squirrel monkeys manipulated mother–infant interactions by subjecting mothers with older semi-independent infants to an unpredictable feeding schedule that resulted in infants experiencing less tolerant and more rejecting maternal care (Coplan et al., 1996; Lyons et al., 1998, 2000; Rosenblum and Andrews, 1994; Rosenblum et al., 1994). Taken together, these studies demonstrate that in several primate species a wide range of early-life manipulations exerts chronic effects at the neuroendocrine, physiological or behavioral levels.

Primates that experience adverse early environments often function species-typically in a stable environment but deviate from controls in their behavioral and physiological responses when confronted with a challenging uncontrollable situation (Rosenblum and Andrews, 1994; Suomi, 1991). Whereas maternal separation in infancy has been used to induce adverse early environments, separation from parents/other close social partners and/or confrontation with a novel physical environment are well established as challenge tests in primates and have also been used to investigate the long-term impact of maternal separation. Independent of the early environment, it has been shown that the behavioral and physiological responses to social separation/novelty (SSN) depend on various factors, including species, sex, separation duration (from 30 min to several weeks) and the separation environment (novel vs. familiar cage, alone vs. with group members) (e.g. Mineka and Suomi, 1978). The initial behavioral reaction of primates to separation is often a protest response characterized by increased locomotion and distress or contact vocalization (Berman et al., 1994; Coe et al., 1983; Hinde and Spencer-Booth, 1971; Hoffman et al., 1994; Kalin et al., 1988; Kraemer et al., 1991; Laudenslager et al., 1990, 1995; Levine and Wiener, 1988). If the separation period is prolonged to several days, the protest response can be followed by a despair response that is reflected by a high degree of inactivity, withdrawal and huddle or slouch posturing, referred to as the depression phase (Hinde et al., 1966; Laudenslager et al., 1990, 1995; Rosenblum and Andrews, 1994). In addition to behavioral responses, separation also induces physiological stress responses including increased activity of the HPA system (Bailey and Coe, 1999; Clarke, 1993; Coe et al., 1983; Dettling et al., 1998; Hennessy et al., 1979; Laudenslager et al., 1995; Levine and Wiener, 1988), with the response magnitude depending on social support during separation (Clarke, 1993; Levine and Wiener, 1988), visual accessibility of the separation partner during separation (Levine and Wiener, 1988) or duration of separation (Levine and Wiener, 1988). At the neurobiological level moreover, in juvenile rhesus macaques, separation from the biological or surrogate mother induces increased CSF norepinephrine levels (Kraemer et al., 1991) and PET scans have identified altered cortical activation following separation from the biological mother (Rilling et al., 2001).

A small number of primate studies have explored the effects of the early environment on subsequent social-separation responsiveness. When separated for 4 weeks from their attachment figure (surrogate vs. real mother), surrogate-reared rhesus monkeys exhibited relatively reduced vocalization and locomotion. In addition, they demonstrated despair behaviors such as huddling, rocking and self-directed nonaggressive behavior that were barely observed in mother-reared subjects. The level of despair behavior was negatively correlated with CSF norepinephrine (Kraemer et al., 1991). In response to social separation and new group formation, peer-reared rhesus monkeys demonstrated lower HPA system reactivity in terms of ACTH levels, relative to mother-reared subjects (Clarke, 1993). In squirrel monkeys reared by mothers under different feeding demand conditions, the cortisol responses to 24-h separation was unaffected by rearing condition (Champoux et al., 2001). In Goeldi's monkey, the cortisol response to parental separation correlated positively with spontaneous variation in parental aggression (Dettling et al., 1998). Also, in rhesus macaques, the cortisol response to maternal separation was associated with spontaneous variation in the mother–infant relationship (Gunnar et al., 1981).

In rats, different paradigms manipulating early environment and differing in the length of separation (from 15 min to 24 h) and the social environment (alone vs. litter mates) during separation have been established and their immediate and long-term consequences were investigated. The observed effects depend strongly on the type of early manipulation: Early Handling (EH), Maternal Separation (MS) and Early Deprivation (ED). The paradigm selected for the present study in the common marmoset and used for comparison with the rat findings is ED, involving daily separation of pups from the mother and littermates for a prolonged period (1–6 h) followed by reunion. ED in rats leads to sensitization of the pup's HPA axis such that pups exposed to ED on postnatal days (PNDs) 1–8 demonstrate increased plasma corticosterone levels following ED on PND 9, whereas pups experiencing their first ED on PND 9 do not (McCormick et al., 1998). The long-term effects of ED have been studied relative to pups experiencing non-handling (NH) (minimal environmental disturbance) or normal animal facility rearing (AFR). Challenged with a stressful situation, ED adults demonstrate reduced stress-related endocrine and behavioral responses relative to NH (Kosten et al., 2000; Pryce et al., 2001) and to AFR (Ogawa et al., 1994) and altered mesolimbic dopamine activity and its behavioral expression relative to both NH and AFR (Hall et al., 1999; Kehoe et al., 1998; Pryce et al., 2001; Zimmerberg and Shartrand, 1992).

We are currently conducting a long-term study of the effects of ED in the common marmoset, a small-bodied New World primate, that is characterized by twinning and high levels of maternal and paternal care. The common marmoset has become increasingly popular as a laboratory primate and its relatively short developmental period makes it particularly suitable for long-term studies. Rat and primate infants differ in their developmental stage at birth, with rats being described as altricial and nonhuman primates as precocial. Moreover, differences are also observed in the postnatal development of the HPA system. Undisturbed rat pups are characterized by an HPA hyporesponsive period (PNDs 2–14) including insensitivity of the neonatal adrenal to ACTH (Rosenfeld et al., 1992). Nonhuman primates and humans, in contrast, demonstrate a cortisol response to a stressful situation as early as the first few days of life (Bowman and Wolf, 1965; Dettling et al., in press; Gunnar, 1991). Common marmoset infants additionally exhibit high basal levels of ACTH and cortisol relative to older conspecifics (Pryce et al., 2002). In the common marmoset, ED performed for 30–120 min/day across the first 4 weeks of life elicits an acute stress response in terms of cortisol, epinephrine and norepinephrine. At PND 28, basal cortisol is reduced in ED infants relative to control infants (CON) (brief daily restraint on parent), and ED infants weigh less than CON infants (brief daily restraint on parent) at PND 28. Behaviorally, ED infants spend a higher proportion of the time in the suckling position and spend a greater proportion of their playing time in solitary rather than social play (Dettling et al., in press). In the present study, we investigated the effects of ED on behavioral and physiological responses to SSN challenge and reunion at the juvenile stage of development.

2. Material and methods

2.1. Subjects and ED

The study was conducted under experimental permit in accordance with the Animal Protection Act of 1978, Switzerland. Ten unrelated breeding pairs of common marmosets each contributed one set of twin offspring, which were the subjects of the study. Family groups, which consisted of the breeding female, breeding male and the study twin infants, were maintained in cages measuring 3–4 m^3, each equipped with natural branches, shelves, a removable sleeping box (also used as a transport box) and sawdust on the cage floor. Colony rooms were each occupied by two to six such groups, with groups in auditory and olfactory but not visual or tactual access to each other, and were illuminated by artificial lighting on an 11:13-h L/D cycle. Daily feeding comprised high-protein porridge containing vitamin and mineral supplements, crickets scattered in the sawdust, with commercial high-protein pellets and drinking water available ad libitum. Twice weekly, monkeys were fed fruit and eggs.

At birth, twin litters were allocated equally and at random to either the ED ($n=5$) or the CON group ($n=5$). The five CON litters constituted three male–female and two male–male twins (seven males, three females) and the five ED litters constituted four male–female twins and one female–female twin pair (four males, six females). On PNDs 2–28 (4 weeks), ED was conducted daily according to a fixed schedule for between 30 and 120 min/day, beginning at different time points from 08:30 to 17:00 h. For each of Weeks 1–4, a total of 9-h ED was administered, comprising 2 × 30-, 1 × 60-, 2 × 90- and 2 × 120-min ED sessions. An additional condition was that ED duration on PNDs 2 and 3 was set at 30 min/day. Variable durations of ED and variable times of day were chosen to add a factor of unpredictability. In contrast to rat pups, marmoset infants are in continuous body contact (i.e. being carried) with a caregiver for 24 h/day throughout the first 2–3 weeks of life (Pryce, 1993). For several weeks thereafter, time spent alone between bouts of caregiving is of only several minutes duration. Therefore, for the young marmoset infant, even a short period of deprivation is a nonbiological event and is likely to constitute a stressful condition.

ED began with the infant being removed from the carrying parent's back while restraining the parent in the home cage. The infant was then taken to the procedures room where it was placed alone in a plastic mouse cage (Macrolon, 25 × 19 × 14 cm) with an aluminum mesh top to which the infant could cling. This cage was placed in an isolation chamber fitted with a 4-W light. Temperature within the isolation chamber was 23–25 °C. The procedures room was auditory isolated from the colony rooms. ED was conducted consecutively with each infant in each twin pair, so that one infant remained with the parents at all times. With respect to the separation order, the separation of each twin subject occurred randomly. At the end of the ED session, the infant was returned to the sleeping box attached to the front of the home cage, from where it was retrieved immediately by one of the parents. In the case of CON infants, the carrying parent was restrained briefly and then released in order to control for this component of the ED procedure.

Thereafter, the majority of the 20 subjects remained undisturbed with their family group until the SSN test was performed at age 18–20 weeks, which is the juvenile stage in this monkey. Six subjects (one female–male CON twin pair, one male–male CON twin pair and one female–male ED twin pair) were also included in a telemetry study of the effects of ED on cardiovascular function and were implanted at age 15 weeks with a 4-g blood pressure transmitter, placed in the peritoneal cavity under general anesthetic. One CON subject (from the male–male twin pair) had to be euthanized due to postsurgery lameness in the hind limbs, so that 9 CON subjects (six males, three females) and 10 ED subjects (four males, six females) were available for the present study.

2.2. SSN challenge test

At age 18 weeks, the effects of ED on behavioral and physiological responses to a SSN challenge were tested. SSN was performed in a test cage that was auditory isolated from the colony rooms and the test room and cage were novel to the subject at the first SSN test. The test cage measured 2 m^3, was similarly equipped to the home cage and had been demarcated into eight sectors of equal volume. Favored foods (raisins, banana pellets) were available to the subject during testing.

On each day of testing, at 08:00 h, a baseline urine sample was collected by one of us entering the home cage and collecting the test subject's first void urine sample into a beaker. All subjects were familiar with this procedure. After baseline sampling, at either 08:30 or 09:45 h, the subject was captured and transferred in the sleeping/transport box to the test cage. The subject's behavior was scored for 45 min by the experimenter located in a one-way viewing cabin, using Observer software (Noldus Information Technology, Wageningen, NL) and compatible hardware. The behavioral elements of interest were categorized under Activity, Exploration, Distress, Care and Social and are listed in Table 1. States were scored using 30-s instantaneous sampling and events using continuous sampling.

Following the 45-min observation of subject alone, the father was introduced into the test cage and subject and father were observed for an additional 15 min following reunion. In the common marmoset, infants are primarily carried by the father and, if available, by other nonbreeding group members. The design of the reunion phase of the SSN was stimulated by the child strange-situation test developed by Ainsworth and Wittig (1969) to investigate children's responses to separation from and reunion with the parent and has been used by us previously in a study of Goeldi's monkey (Dettling et al., 1998). The reunion phase was not aimed at identifying different attachment types, as is a frequent aim of child studies, but was rather aimed at investigating the extent of stress attenuation following introduction of the father to the subject in the SSN environment. Moreover, the duration of the separation phases of the strange situation test deployed in children (3–6 min) and that deployed here for the common marmoset (45 min) differ markedly, suggesting that the severity of the separation stress was more marked in this nonhuman primate test than in the human test. At the end of the reunion phase, now approximately 70 min after the subject was captured, it was placed in a urine collection chamber and the urine voided over the following 60 min was collected (post-SSN test value). In Goeldi's monkey, we demonstrated that urinary cortisol values were maximal 60 min after stressor (handling and blood sampling) termination (Jurke et al., 1995). In the present study, 60 min was sufficient to obtain post-SSN test urine samples in approximately 95% of cases. After urine sampling, the subject was returned to the home cage.

Each subject was exposed to six SSN tests with 1–3 days between tests. Subjects within twin pairs were tested on alternate days and starting times for SSN tests were bal-

Table 1
Mean ± S.E.M. behavior scores for the alone phase and the reunion phase, split by early condition and session number

		ED				CON			
		Alone		Reunion		Alone		Reunion	
	Effects	SSN 1–3	SSN 4–6	SSN 1–3	SSN 4–6	SSN 1–3	SSN 4–6	SSN 1–3	SSN 4–6
Activity									
Being mobile (%)	a, c	19.8 ± 4.2	21.2 ± 3.9	22.0 ± 6.7	29.2 ± 5.7	26.8 ± 5.1	36.2 ± 9.3	24.9 ± 7.9	17.4 ± 4.8
Crossing sector (f/h)	a, d, e	45.2 ± 11.5	65.7 ± 17.8	24.9 ± 11.0	23.5 ± 6.9	57.4 ± 12.4	94.6 ± 24.6	27.7 ± 13.0	18.7 ± 5.9
Contact calls (f/h)	a, b, d, e	52.5 ± 14.3	56.9 ± 15.7	0.7 ± 0.5	0.9 ± 0.7	91.1 ± 21.4	138.5 ± 26.5	0.4 ± 0.3	1.5 ± 1.1
Exploration									
Enter main box (f/h)	*a*	2.4 ± 0.9	2.8 ± 1.5	6.3 ± 2.6	7.1 ± 2.0	1.5 ± 0.7	4.6 ± 1.9	6.1 ± 4.2	2.4 ± 1.4
Enter novel box (f/h)	d, *g*	1.5 ± 0.9	2.0 ± 1.1	4.4 ± 2.1	5.6 ± 2.8	4.7 ± 2.4	2.4 ± 1.0	1.8 ± 0.9	11.11 ± 5.6
Explore main box (f/h)	d, *f*	0.4 ± 0.3	0.3 ± 0.1	2.1 ± 0.9	3.7 ± 1.5	0.9 ± 0.1	0.5 ± 0.2	1.3 ± 0.9	0.4 ± 0.3
Explore novel box (f/h)	*b*, *d*, e	0.8 ± 0.5	0.3 ± 0.2	2.0 ± 1.2	5.6 ± 2.3	0.3 ± 0.2	0.0 ± 0.0	0.2 ± 0.2	0.4 ± 0.4
Explore other substrate (f/h)	*d*	1.9 ± 1.0	0.9 ± 0.3	2 ± 0.6	4.3 ± 2.2	1.5 ± 0.6	1.0 ± 0.4	0.9 ± 0.7	2.1 ± 1.5
Distress									
Tail-hair piloerection (%)	a, d, f	79.6 ± 7.4	63.9 ± 7.7	35.0 ± 6.8	18.2 ± 4.8	67.4 ± 10.0	59.5 ± 10.4	15.9 ± 5.5	6.1 ± 3.0
Distressed vocalization (f/h)		7.2 ± 2.7	4.4 ± 2.6	–	–	10.9 ± 3.4	8.4 ± 3.6	–	–
Care and social behaviors									
Approach parent (f/h)		–	–	20.9 ± 7.9	25.2 ± 6.8	–	–	20.6 ± 7.3	14.9 ± 4.5
Leave parent (f/h)		–	–	2.5 ± 0.9	3.7 ± 1.4	–	–	6.1 ± 3.6	4.9 ± 2.7
Being carried (%)	f	–	–	0.3 ± 0.2	0.0 ± 0.0	–	–	37.9 ± 14.7	27.3 ± 13.4
Being in contact (%)	e	–	–	53.9 ± 9.9	71.4 ± 6.7	–	–	41.0 ± 11.1	63.7 ± 11.3
In contact or carried (%)	f	–	–	54.2 ± 10.0	71.4 ± 6.7			78.9 ± 5.9	90.9 ± 2.8

a denotes main effect of session number whilst alone. b denotes main effect of early condition whilst alone. c denotes effect of interaction term of Early condition × Session number whilst alone. d denotes main effect of phase (alone vs. reunion). e denotes effect of interaction term of Phase × Session number. f denotes main effect of early condition during reunion. g denotes effect of interaction term of Early condition × Session number during reunion. Normal case letter denotes $P < .05$. Italic letter denotes $.05 < P < .1$. f/h = frequency per hour.

anced within and between subjects to control for any effect of circadian rhythm on cortisol response values. As a discrete measure of subjects' behavioral response to novelty during the SSN test, two boxes (30 × 20 × 20 cm, accessible via the front) were placed according to a preset schedule in two fixed locations, at the same height and at the back of the cage. The boxes were identical except in color, one being bright blue and the other bright yellow. These colors are visible and distinguishable for New World monkeys (Rosenberg, 1994). On SSN 1–6, the same colored box (main box) was presented in the same location. Color and location of the main box were counterbalanced between treatment groups to cancel out any effects of innate color or place preference. For SSN 3 and 6, the second box was introduced as a novel object (novel box) and placed in the vacant location.

2.3. Measurement of urinary cortisol

Urine samples were stored immediately at -20 °C and transferred to -80 °C within 48 h. Determination of total urinary cortisol concentration was performed by RIA following enzyme hydrolysis of 5-μl urine, as described in Dettling et al. (1998) with the only protocol change being that ^{3}H-cortisol ([1,2,6,7-^{3}H] cortisol, specific activity 80–105 Ci/mmol; Amersham International, Amersham, UK) was used as tracer. Intra-assay precision was 3% ($n=6$) and inter-assay precision was 14% ($n=7$). Urinary cortisol concentrations were expressed relative to urinary creatinine (Cr) content to control for variation in urine volume/concentration. Creatinine was measured using a commercially available kit (Beckman Creatinine Kit 555A, Sigma-Aldrich, Schnelldorf, Germany). Intra-assay precision was 1% ($n=4$) and inter-assay precision was 15% ($n=7$).

2.4. Data analysis

Statistics were performed using The Statistical Package for the Social Sciences (SPSS, Chicago, USA). In order to accommodate the nonindependence of twins in the statistical model, i.e. to control for "litter effect," a nested design was used with twins nested within early condition (ED, CON) as the appropriate error term for the early condition effect (Denenberg, 1977). To investigate potential effects of telemetry surgery on subjects' body weight, cortisol response or behavior during SSN testing, subjects that experienced surgery ($n=5$) were compared with a random subsample of nonsurgery animals ($n=6$) using unpaired *t* tests. Only 1 of 54 comparisons reached significance, justifying inclusion of transmitter-implanted subjects in the overall analysis. To explore the effect of ED on body weight during the SSN test period, a nested design 2 × 2 × 2 general linear model ANOVA was performed, with session number (SSN 1, SSN 6) as within-subject factor and early condition (ED, CON) and sex (female, male) as between-subject factors. For behavioral measures and urinary cortisol, mean values for SSN 1–3 and 4–6 were calculated and used for analyses. For cortisol values, the convention of $\log_{10}$ transformation was further employed to normalize distribution and reduce heterogeneity of variance. Urinary cortisol values were then subjected to a nested design 2 × 2 × 2 × 2 ANOVA with session number (SSN 1–3, SSN 4–6) and sampling time (baseline value, post-test value) as within-subject factors and early condition (ED, CON) and sex (female, male) as between-subject factors. For behaviors (Table 1), alone phase or reunion phase behaviors were analyzed by means of a nested design 2 × 2 × 2 ANOVA with session number (SSN 1–3, SSN 4–6) as within-subject factor and early condition (ED, CON) and sex (female, male) as between-subject factors. Behavioral differences between the alone phase and the reunion phase were investigated using a 2 × 2 ANOVA with session number (SSN 1–3, SSN 4–6) and phase (alone, reunion) as within-subject factors. A nested design 2 × 2 ANOVA with session number (SSN 1–3, SSN 4–6) as within-subject factor and early condition (ED, CON) as between-subject factor was employed to investigate fathers' behavior (specifically, the elements crossing sectors, initiate body contact with subject, break body contact with subject). Univariate nested design ANOVA or the paired *t* test was used for a posteriori testing. Almost universally, sex was without significant effects on body weight, cortisol concentrations or behavior and is therefore not reported in Section 3. The significance level was set at $P<.05$.

3. Results

3.1. Body weight

The nested design ANOVA revealed neither a main effect of Early condition [$F(1,7)=0.84$, $P>.39$] nor an Early condition × Session number interaction [$F(1,7)=2.26$, $P>.17$] on body weight. However, there was a main effect of Session number on body weight [$F(1,7)=58.58$, $P<.001$], revealing that all subjects increased their body weight significantly between SSN 1 and 6 (SSN 1 = 185 ± 4 g, SSN 6 = 196 ± 4 g, mean ± S.E.M.).

3.2. Urinary cortisol

Urinary cortisol values demonstrated a significant Sampling time × Early condition interaction [$F(1,7)=34.43$, $P=.001$] (Table 2). A posteriori reanalysis of the two sampling times separately revealed that ED subjects demonstrated significantly lower basal urinary cortisol values than CON subjects, both at SSN 1–3 [$F(1,9)=42.53$, $P<.001$] and at SSN 4–6 [$F(1,9)=8.14$, $P=.05$], whereas post-test cortisol values were not significantly different in ED and CON at either session number [$F(1,9)=3.01$, $P>.11$]. The main effect of sampling time indicated that, overall, post-test cortisol values were significantly greater

Table 2
Mean ± S.E.M. baseline and post-test urinary cortisol concentrations (μg per mg creatinine), split by early condition and session number

	ED		CON	
	SSN 1–3	SSN 4–6	SSN 1–3	SSN 4–6
Baseline	75.9 ± 9.0	75.1 ± 6.6	113.9 ± 8.3	101.3 ± 11.0
Post-test	262.9 ± 23.4	227.1 ± 19.3	275.0 ± 28.7	254.3 ± 15.7

ED exhibited lower baseline cortisol concentrations than CON ($P<.05$) during SSN 1–6. There was no effect of early condition on post-test cortisol concentrations. Post-test cortisol concentrations were significantly higher than baseline concentrations ($P<.001$) and demonstrated a trend towards a significant decrease from SSN 1–3 to 4–6 ($P<.08$).

than basal values [$F(1,7)=1356.21$, $P<.0001$]. There was also a significant main effect of Session number [$F(1,7)=11.12$, $P<.02$] and paired t tests performed a posteriori revealed a trend to significantly lower post-test cortisol at SSN 4–6 than SSN 1–3 [$t(9)=1.99$, $P<.08$]. There was also a significant main effect of litter on urinary cortisol values [$F(8,7)=4.98$, $P<.03$].

3.3. Behavior

3.3.1. Alone phase

For the time period that subjects were alone in the test cage, the behavior elements of interest, categorized within Activity, Exploration and Distress, were analyzed for effects of Early condition and Session number (Table 1). Significant main effects of Session number appeared to reflect behavioral adaptation to repeated SSN. Therefore, between SSN 1–3 and 4–6, there was a significant increase in time spent in a state of mobility [$F(1,7)=7.33$, $P=.03$], a significant increase in the frequency of sector crossing [$F(1,7)=20.98$, $P<.001$], a significant increase in the frequency of contact calling [$F(1,7)=14.34$, $P<.001$] and a significant decrease in time spent with piloerection of the tail hair [$F(1,7)=18.82$, $P=.003$]. There was a trend to a significant increase in the frequency of entering the main box [$F(1,7)=4.90$, $P<.07$].

The following effects of early condition were obtained (Table 1). There was a significant Session number × ED interaction for time spent in a state of mobility [$F(1,7)=5.65$, $P<.05$]. ED marmosets were significantly less mobile compared with CON during SSN 4–6 [$F(1,9)=6.48$, $P<.04$]. ED marmosets emitted significantly less contact calls than CON [$F(1,7)=14.78$, $P<.006$]. ED marmosets demonstrated a trend to a significantly higher frequency of exploration of the novel box than CON [$F(1,7)=3.30$, $P=.10$].

Finally, there were significant main effects of litter for the behaviors of contact calling, tail-hair piloerection and sector crossing ($P<.04$).

3.3.2. Reunion phase

Following reunion with the father, additional behavioral elements categorized as Care and Social were also analyzed (Table 1). The ANOVA, with Phase (alone vs. reunion) and Session number (SSN 1–3 vs. SSN 4–6) as within-subject factors, revealed that subjects responded to the presence of the father (Table 1). Therefore, following reunion, as indicated by the main effect of Phase, subjects demonstrated a significant decrease in the frequency of sector crossing [$F(1,7)=11.56$, $P=.003$], a significant decrease in the proportion of time that they exhibited tail-hair piloerection [$F(1,7)=79.18$, $P<.001$] and a significant decrease in the frequency of contact calling [$F(1,7)=32.83$, $P<.001$]. Reunion also impacted upon behaviors categorized as exploration: Subjects explored the main box at a significantly higher frequency following reunion [$F(1,7)=5.99$, $P<.03$]. Subjects entered the novel box at a significantly higher frequency following reunion [$F(1,7)=5.73$, $P<.03$]. For exploration of the novel box, there was a significant Session number × Phase interaction [$F(1,7)=11.11$, $P=.004$], with an a posteriori t test indicating that there was a trend to a significant increase in novel box exploration following reunion at SSN 4–6 [$t(9)=-2.07$, $P<.07$].

During reunion, the following effects of early condition were obtained (Table 1). ED marmosets were carried by their fathers significantly less than CON marmosets, as revealed by the main effect of Early condition [$F(1,7)=31.38$, $P<.001$]. Indeed, most ED subjects were never carried during SSN reunion, whereas for CON subjects being carried was common. ED subjects also obtained significantly lower scores on the combined care measure of either being carried or in body contact [$F(1,7)=31.85$, $P<.001$]. Reunited ED subjects demonstrated significantly more tail-hair piloerection than CON subjects, as revealed by the main effect of Early condition [$F(1,7)=13.79$, $P<.01$]. Reunited ED subjects demonstrated a trend to significantly more frequent exploration of the main box compared with reunited CON subjects [$F(1,7)=3.95$, $P<.08$] and there was also a trend to a significant interaction of Session number × Early condition for exploration of the novel box [$F(1,7)=5.38$, $P<.06$]. A posteriori testing revealed that ED marmosets tended to explore the novel box significantly more than CON during SSN 4–6 [$F(1,9)=3.95$, $P<.08$].

There were significant main effects of litter for the behaviors of sector crossing ($P<.002$), entering the main box ($P<.02$) and approaching the father ($P<.04$).

Not only subjects' behavior but also behavior of fathers differed significantly according to early condition. Significant main effects of Early condition on fathers' behavior scores were obtained for sector crossing [$F(1,7)=24.21$, $P=.002$], initiating body contact [$F(1,7)=6.36$, $P=.04$] and breaking body contact [$F(1,7)=8.28$, $P<.03$]. Accordingly, fathers of ED subjects performed more sector crossing and initiated but also terminated body contact with subjects at higher frequency than did fathers of CON subjects. These three behaviors were highly correlated with each other ($r>.82$, $df=17$, $P<.001$). Furthermore, significant main effects of litter were obtained for all father behaviors analyzed ($P < .03$).

4. Discussion

In line with the existing nonhuman primate literature, juvenile common marmosets demonstrated marked HPA and behavioral responses to separation from the social group and placement in an unfamiliar physical environment. On several important measures, repeated exposure to this stressor led to adaptation of responsiveness to it. Subjects demonstrated an increase in urinary cortisol levels following SSN as well as adaptation of this cortisol response to repeated SSN. ED marmosets exhibited lower basal urinary cortisol levels than did CON during the SSN testing period, although the acute cortisol stress response to SSN was very similar to that of CON. Behaviorally, during the alone phase of the initial SSN tests, ED and CON subjects were relatively immobile, performed little contact calling and were often in a state of tail-hair piloerection compared with later tests. Compared with CON, ED resulted in reduced mobility and contact calling during the alone phase of the SSN tests. Reunion with the father resulted in reduced piloerection, sector crossing and contact calling and increased box exploration. Reunited ED marmosets, almost without exception, failed to initiate a carrying bout by the father, whereas carrying of CON subjects by their fathers was commonplace. Reunited ED subjects demonstrated more tail-hair piloerection than did their reunited CON counterparts and also tended to explore the familiar and novel boxes more. These findings are discussed below in the order that they have just been summarized.

4.1. Affective states induced by social separation in a novel environment

In ED and CON juvenile marmosets, the post-test increase in urinary cortisol was marked, providing further evidence that this noninvasive method of monitoring pituitary–adrenal reactivity is of experimental value (Jurke et al., 1995; Smith and French, 1997). However, the merits of urinary cortisol relative to blood cortisol in quantifying treatment effects on pituitary–adrenal reactivity remain to be investigated. By comparing both activity and distress behaviors between the alone and the reunion phases of SSN tests and in doing so in conjunction with their pituitary–adrenal stress response, we would conclude that juvenile common marmosets displayed high levels of stress-related behavior or anxiety, when alone under SSN challenge. Thus, when alone, subjects demonstrated more tail-hair piloerection. Although they were not more or less mobile than during reunion, whilst alone, subjects crossed sectors more and explored less, which is consistent with a state of generally high motor activity and arousal with little attention to specific substrates. This co-occurred with a high frequency of contact calling, probably also stress related (Wiener et al., 1990). We (Dettling et al., 1998) and others (von Holst, 1969, 1998) have proposed that tail-hair piloerection can be used in various species as a noninvasive marker of activity in the sympathetic autonomic nervous system. The experiment we are performing currently, in which we are measuring stress-responsive cardiovascular parameters via telemetry in addition to all the parameters used in this study, will allow us to test this proposal directly for the common marmoset. In line with the human literature on children's responses to separation from their attachment figures, behavioral agitation following social separation in nonhuman primates has been described as the protest reaction and is recognized as the typical response pattern common to a wide range of primate species (Mineka and Suomi, 1978). In the present study, the postreunion reduction in crossing sectors, contact calling, tail-hair piloerection and increase in exploration all indicate that it was the social separation per se rather than the novel environment that challenged subjects most and that elicited the protest reaction.

When subjects were alone, they demonstrated behavioral adaptation to repeated SSN testing. The urinary cortisol response also adapted across successive tests, although this measure did not differentiate between alone and reunion phases. This is in line with previous findings in the closely related Goeldi's monkey where adaptation of both urinary cortisol values and tail-hair piloerection to repeated SSN tests was demonstrated in juveniles (Dettling et al., 1998). In the present study, juvenile marmosets also demonstrated adaptation in terms of activity, contact calling and exploration. It is well established that prolonged activation of the HPA system participates in catabolic processes and eventually results in a reduction in body weight (Chrousos and Gold, 1992). In the present study, however, baseline urinary cortisol concentrations remained stable across SSN sessions, suggesting consistent post-test return to homeostasis. Furthermore, rather than body weight loss, ED and CON subjects demonstrated a development-related increase across the SSN period.

4.2. Effects of repeated ED on affective responses to social separation in a novel environment

The basal cortisol values in urine samples collected across the period of SSN testing were reduced in ED compared with CON subjects. Despite this, the post-SSN urinary cortisol response was equivalent in the two treatment groups. An association between adverse early environment and lower basal cortisol levels, either during development or in adulthood, has been proposed for humans (Gunnar and Vazques, 2001). Basal adrenocortical hypofunction is also a characteristic of specific mood disorders, including posttraumatic stress disorder (Heim et al., 1997). Low basal plasma cortisol has been reported in women that experienced early-life stress in the form of physical or sexual abuse (Heim et al., 2001), both in those suffering from major depression and in those without clinical diagnosis at the time of testing. The present evidence for basal adrenocortical hypofunction during a period of repeated stress in ED juvenile marmosets represents an important addition to research in this area and one intriguing possibility is that early-life stress increases HPA inhibitory tone in the marmoset. Sanchez et al. (2001) provides a recent review of the evidence for the effects of early adverse environment on HPA function in rodents and nonhuman primates and it is clear that additional studies are required in this area. In the rhesus macaque, there is a report that maternally prived peer-reared juveniles and adolescents exhibit lower basal and stress-related ACTH but not cortisol levels compared with mother-reared controls (Clarke, 1993). In the rat, 6 h of ED per day at nest temperature led in adulthood to increased basal and stress-related ACTH levels relative to AFR but did not affect corticosterone levels (Ladd et al., 1996). Maternal separation of intact litters for 3 h/day at room temperature did not affect adult basal or stress-related corticosterone levels compared with NH controls (Plotsky and Meaney, 1993). In a study from our laboratory, ED for 4 h/day at nest temperature did not affect adult corticosterone levels compared with AFR and reduced the corticosterone stress response relative to adults that experienced NH in infancy (Pryce et al., 2001).

In terms of behavior, whilst alone under SSN challenge, ED resulted in reduced mobility and contact calling compared with CON. Whilst high levels of these two behaviors very probably represent expression of anxiety-related agitation in CON, we would not interpret the ED–CON difference as evidence that ED were less responsive to SSN. It could well be that ED resulted in increased anxiety-related inactivity during SSN. Indeed, ED–CON differences at reunion provide support for this interpretation (see below). Furthermore, the forthcoming cardiovascular data will provide a very important complement to the present data and should greatly assist accurate interpretation of the meaning of the behavioral differences we have identified. In the rat, ED for 4 h/day increased adulthood activity in the open field compared with AFR and NH (Pryce et al., 2001), suggesting that ED can lead to the opposite motoric effects in the marmoset and the rat.

4.3. Effects of repeated ED on affective responses to reunion in a novel environment

ED juveniles also differed from CON juveniles in their behavior during reunion in terms of ED exhibiting a striking reduction in (indeed near absence of) being carried, markedly higher piloerection and a tendency to increased exploration of the boxes (main and novel) compared with CON. Interpretation of the difference in carrying is difficult and requires consideration of the species-typical features of this behavior. As infants, marmoset offspring are typically carried more by their fathers than mothers (Pryce, 1993). This was the case with the subjects of the present study and what is also relevant here is the finding that, as infants, the ED and CON subjects of the present study were carried by their respective fathers for very similar amounts of time (Dettling et al., in press). Carrying of juveniles by fathers or mothers is a rare event in the home cage, but juveniles do, or at least do attempt, to initiate carrying as a defense mechanism, e.g. in response to novelty. Such carrying episodes require the juvenile to attempt to climb on to the parent's back and also require that the parent does not reject (via aggression) the attempt to initiate carrying. In the present study, paternal carrying of ED subjects may have been low because their fathers were more rejecting, because the ED subjects did not attempt to initiate carrying or some combination of these two. Further studies are therefore necessary to determine if ED leads to a fundamental change in the juvenile marmoset's social response to its caregiver in a stressful situation. Altered social interactions and relationships can of course be an important manifestation of several distinct psychiatric disorders, including anxiety, depression and schizophrenia (DSM-IV, 1994).

The relatively high amount of piloerection following reunion in ED compared with CON strongly suggests that the sympathetic arousal induced by the alone phase of SSN was maintained for longer following reunion in ED compared with CON subjects. Again, cardiovascular data will be very important for the confirmation, or otherwise, of this interpretation. If high postreunion piloerection is indeed indicative of sustained arousal in ED subjects, then this might be related to the near absence of their being carried. ED subjects did spend considerable time during the reunion phase in contact with the father (although their combined carrying or contact scores were lower than those of CON) and it is therefore noteworthy that they demonstrated considerable piloerection although they were in contact with the father and yet they did not attempt to initiate carrying, the latter being the species-typical response of juvenile marmosets in anxiogenic situations in the home cage and also the response of the CON subjects in the SSN. These putative effects of ED on the sympathetic ANS could be either directly due to ED-induced individual differences or be mediated by ED-induced differences in offspring–caregiver relationships. Importantly, fathers' behavior during reunion was also associated with early condition, with ED fathers being more active and breaking but also establishing body contact more often. Therefore, differences in juvenile behavior during reunion may also relate to differences in the fathers' behavior, either chance differences unrelated to ED or differences related to these males being exposed to their ED offspring.

Exploratory behavior was exhibited at a low level by all subjects. Despite this and despite their lower mobility and higher tail-hair piloerection, ED subjects tended to demonstrate more exploration than CON subjects. Although these differences were at trend level, they are still worthy of discussion. ED led to a tendency to exhibit increased exploratory behavior during both the alone and the reunion phases of SSN. This was specific to the conspicuous colored boxes rather than the general substrate and in the case of the alone phase was specific to the novel colored box (indeed, this box was not strictly novel when it was presented during the reunion phase). In rats, it has been demonstrated that novelty-seeking behavior is associated with relatively high mesolimbic dopaminergic activity (Dellu et al., 1996). Furthermore, using acute amphetamine challenge of locomotion in an open-field test, we have demonstrated that 4-h ED in rats leads to increased presynaptic mesolimbic dopamine responsiveness in adulthood (Pryce et al., 2001). Applying this adult rat ED evidence to juvenile marmosets, it is possible that the tendency to exhibit increased exploratory responsiveness to novelty in ED marmosets is related to altered functioning of the mesolimbic dopaminergic system. Finally here, it is important to point out that the trend to higher exploration during reunion might have been related to the absence of time spent being carried (if a subject was being carried and the father entered the main or novel box, this would give the subject the opportunity to explore but would not be scored as exploring). We also cannot exclude the possibility that the greater amount of ED piloerection was due to sympathetic arousal elicited by exploration.

This study has revealed some marked effects of repeated ED on endocrine and behavioral status of juvenile marmosets when challenged with social separation followed by reunion in a novel environment. The study was conducted with siblings from the same litter, as are many similar studies in rodents, and the fact that we obtained many significant litter effects that could have otherwise confounded the statistical significance of treatment effects (i.e. false-positive effects) should serve to emphasize the importance of correct statistical design whenever siblings or littermates are studied in this manner (Denenberg, 1977). The treatment effects obtained indicate that ED juveniles exhibited lower basal cortisol levels in their morning urine. They were less mobile and did less calling when alone in the SSN test, suggesting that they experienced more negative affect than did CON. At reunion with the father, they did not attempt/succeed to initiate carrying although their maintenance of high levels of piloerection suggests that they continued to experience a state of high negative affect. These effects of ED in the juvenile marmoset under conditions of challenge are similar to some of the endocrine and behavioral changes that occur in given psychiatric disorders, most notably mood disorders and the negative symptoms of psychotic disorders. Given the epidemiological evidence that adverse early-life events constitute a major group of long-term risk factors for the development of mood-related psychiatric disorders, then the present study has provided experimental evidence that ED in the common marmoset could well constitute an important animal model for the study of the etiology, neurobiology and pharmacology of these disorders.

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