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Development of Pituitary-Adrenal Endocrine Function in the Marmoset Monkey: Infant Hypercortisolism Is the Norm

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Early life stress, involving activation of the hypothalamic-pituitary-adrenal (HPA) system, is associated with altered functioning of stress-related systems in adulthood. In the rat, postnatal development is characterized by low basal HPA activity and stress hyporesponsiveness, and infant exposure to atypical glucocorticoid levels leads to chronic alteration of HPA function and HPA-dependent peripheral and central processes. There have been few studies of primate HPA ontogeny, and here we report a study of changes in pituitary-adrenal function between birth and adulthood in the common marmoset monkey. In this simian primate, basal plasma ACTH and cortisol levels were actually elevated in neonates (ACTH, 141 ± 28 pg/ml; cortisol, 1903 ± 326 μg/dl) and wk 4 infants (ACTH, 114 ± 9 pg/ml; cortisol, 290 ± 8 μg/dl) relative to month 2 infants, juveniles (month 6), subadults (month 12), and adults (>2 yr; ACTH, 37 ± 4 to 61 ± 8 pg/ml; cortisol, 101 ± 2 to 195 ± 4 μg/dl). In contrast to older life stages, neonates lacked circadian change in their plasma cortisol levels, and this state of consistently high cortisol was associated with large adrenal glands in addition to high ACTH levels. Cerebrospinal fluid cortisol levels were, in accord with plasma levels, higher in wk 4 infants than in juveniles and subadults. In terms of stress response, month 2 infants demonstrated ACTH and cortisol peak stress responses similar to those at older life stages (infant stress cortisol, 185 ± 36% of basal; subadult stress cortisol, 174 ± 6% of basal); whereas infant ACTH recovery was also similar to that in older subjects, their cortisol poststress recovery was retarded. This primate, it is proposed, provides an excellent complementary model in which to test hypotheses derived from the rat model relating to HPA system ontogeny and the chronic effects and biomedical implications of hypercorticoidism during early life. (*J Clin Endocrinol Metab* 87: 691–699, 2002)

THE HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) system regulates physiological processes in the periphery and brain that serve to maintain homeostasis in a stable environment and facilitate adaptation to environmental stress. The bioactive glucocorticoid (GC) hormones (corticosterone and cortisol) synthesized and secreted by the cortex of the adrenal gland mediate many of these essential functions, primarily by the regulation of *de novo* protein synthesis after binding of GC to transcription factor receptors (1). The ontogeny of the HPA system and that of its endocrine activity, basal and stress-related, are of considerable biomedical importance, and a central hypothesis is that 1) stress-related activation of the HPA system during specific stages of ontogeny can have important effects on the peripheral organs and brain regions that are GC sensitive at this stage, such that 2) this acute ontogenetic exposure alters the set-point of cellular and/or molecular structure-function in GC-responsive organs throughout life (1–3). Animal evidence in support of this hypothesis includes reports that prenatal exposure of the rat to elevated GC results in an adult phenotype of increased CRF and GR mRNA expression in the hypothalamus and increased basal plasma corticosterone (CORT) levels (4). Postnatal exposure of the rat pup to 3-h daily maternal separation leads in adulthood to increased hypothalamic CRF mRNA expression (5) and reduced hypothalamic and hippocampal GR binding (6), although postnatal exposure of the rat pup to elevated CORT taken in with maternal milk leads in adulthood to an attenuated HPA stress response and reduced behavioral fearfulness (7). In the bonnet macaque monkey (*Macaca radiata*), infants reared by mothers in an unpredictable environment experienced the stress of frequent interruptions in maternal care and as adults exhibited elevated cerebrospinal fluid (CSF) levels of CRF (8, 9). Human epidemiological studies have yielded evidence that adult depression, characterized by HPA hyperactivity, is one of the most frequent consequences of childhood abuse, and that lifetime stressors, including childhood abuse and neglect, predict the likelihood of adult depression (10, 11).

The most comprehensive description of the ontogeny of the HPA system and its neuroendocrine activity is that existing for the rat. Although adult-like levels of GC are present on postnatal d 1 (PND 1), PND 2–14 are characterized by low basal levels of ACTH and GC in the peripheral circulation, followed by a gradual increase to adult-like levels beginning on PND 15 (12, 13). Furthermore, rats at this ontogenetic stage of low basal pituitary-adrenal activity are also hyporesponsive in terms of the pituitary-adrenal hormone response to environmental events that elicit a marked stress response in older conspecifics (*e.g.* saline injection, ether, and electric shock) (14, 15). Importantly, the expression of this

Abbreviations: CBG, Corticosteroid-binding globulin; CORT, corticosterone; CSF, cerebrospinal fluid; GC, glucocorticoid; HPA, hypothalamic-pituitary-adrenal; 11βHSD-2, 11β-hydroxysteroid dehydrogenase-2; NW, New World; PND, postnatal day; RP-HPLC, reverse phase HPLC; SHRP, stress-hyporesponsive period.

stress-hyporesponsive period (SHRP) is dependent on an intact infant-mother relationship (14), with litter-dam separation for periods of 8 h or more leading to a marked increase in CORT levels (*i.e.* there is a CORT response to maternal deprivation *per se*) and to a marked disinhibition of the infant rat's hyporesponsive adrenocortical response to physical stressors. The demonstration of the SHRP in the rat adds considerable weight to the hypothesis, detailed above, that early life stress activation of the HPA system can induce important chronic effects.

Similar detailed, systematic descriptions of the ontogenetic profile of the HPA system are lacking for other mammals. There is a detailed description of the ontogenetic profile of urinary cortisol levels for the tree shrew (*Tupaia belangeri*), in which maximal values are present on PND 1–20, followed by a decline and then a gradual increase during puberty until adulthood (16). For the nonhuman simian primates [New World (NW) monkeys and Old World monkeys and apes], studies in the Old World rhesus macaque (*M. mulatta*) have measured ACTH and/or cortisol at specific periods of development, but these have been aimed primarily at investigating the effects of infant-mother separation, and the ontogenetic period studied in each case has been short. Integrating these studies, to the extent that this is possible, reveals that in mother-reared rhesus macaques, basal plasma ACTH and cortisol levels are either stable or decrease slightly across the period at least from PND 14 to month 6 (17–19). In terms of stress response ontogeny, a study of rhesus macaques on PND 2 revealed that an adult-like adrenocortical stress response is already present in such primate neonates; the stressor used was physical rotation conducted immediately after a brief separation from the mother (20). In the NW squirrel monkey (*Saimiri sciureus*), an adrenocortical stress response to maternal separation has been demonstrated in subjects aged 12 wk (21). In humans, healthy neonates (PND 1–7) have basal cortisol levels slightly lower than those in infants aged 3 months or more (22). Young human infants lack a circadian rhythm in their basal adrenocortical activity, and this becomes established by months 3–6 (23, 24). From this age, basal plasma cortisol levels remain stable into adulthood. Human neonates are already capable of a robust cortisol stress response (25).

Against a background of evidence describing important long-term effects of stress or high GC exposure in the rat and the existence of the SHRP in the rat, but a paucity of systematic long-term studies even describing the typical ontogeny of HPA system activity in primates, here we report on age-specific basal and stressor-induced pituitary-adrenal hormone levels in the NW common marmoset monkey (*Callithrix jacchus*), by comparing neonates, infants, juveniles, subadults, and adults. In adulthood, the marmoset and some other NW simian primates (*e.g.* squirrel monkey) are characterized by high absolute basal levels of steroid hormones, including vitamin D, sex steroids, mineralocorticoids, and GC (26). Furthermore in the case of GC the high levels co-occur with low levels of corticosteroid-binding globulin (CBG) (27, 28). Regarding the ontogenetic study of the marmoset HPA system providing information of potential biomedical relevance, detailed studies in adult marmosets demonstrate that this hypercortisolism compensates for steroid resistance without compromising typical cortisol function. For example, the marmoset does not exhibit clinical chemical signs of cortisol excess and does exhibit typical HPA system characteristics, including dexamethasone suppression of pituitary-adrenal activity (29, 30). An earlier study of development changes in marmoset adrenal function reported that basal cortisol levels are actually markedly elevated in marmoset neonates (31). Based on this very important finding, we undertook a detailed developmental study of pituitary-adrenal endocrine function in this simian primate.

Materials and Methods

Animals

The study was conducted under experimental permit and in accordance with the regulations of the Swiss Federal Veterinary Office. Eight breeding pairs of common marmosets provided 16 litters, 6 sets of triplets and 10 sets of twins, yielding a total of 38 offspring, 21 male and 17 female, which were the subjects of this study (Fig. 1). Subjects remained with their parents throughout the study period. Cages were 2.5–4.0 m^3 and were equipped with branches, a sleeping box that also served as a transport box, and sawdust as floor substrate. The light/dark cycle was 11 h of light, 13 h of darkness, with lights on from 0800–1900 h, and conditions were maintained at 23 ± 1 C and 55 ± 5% humidity. NW monkey pellet feed (Moulin Kliba SA, Kaiseraugst, Switzerland) and water were available continuously; a protein-rich hash with vitamin and mineral supplements was provided at 0900 h, and crickets and fruit were given at 1630 h. A weekly first void urine sample was collected from each breeding female for the determination of urinary estrogen and creatinine values; this allowed, as described by Nievergelt and Pryce (32), for estimation of day of conception and, therefore, duration of subject gestation periods. Estimates of the latter were 143 ± 2 d (mean ± SEM; n = 16), which is the species norm (33). Body weight on PND 1–2 (see below) was 30.9 ± 0.5 g (n = 16, 1 neonate/study litter), which is also species-typical (34).

Figure 1 provides an overview of the experimental design in terms of allocation of subjects to specific research questions and sample collection against stage of development. In the common marmoset weaning occurs typically at wk 10–12, and sexual maturation at age 15–18 months (35, 36). Subjects 1 wk of age are referred to throughout as neonates, and those 4 wk and 8 wk/month 2 are referred to as infants; the age range from 3–9 months is referred to as the juvenile stage, and that from 10–12 months as subadult.

Blood and CSF sampling in the basal endocrine state

Neonates and infants (Fig. 1). In six triplet litters and five of the twin litters, at 0800 h on PND 1 or 2 the neonates were removed from the parents, and sex and weight were determined. Time from removal to return to the parent and then the home cage was 2 min maximum. In triplet litters, immediately after weighing one neonate was selected at random, and a blood sample of 0.3 ml was withdrawn from the femoral vein using a 27-gauge needle and a chilled EDTA-primed syringe. The neonate was then sedated using Saffan (10 mg/kg, im) and killed by an overdose of sodium pentobarbital. The peritoneum was opened, and the adrenal glands and kidneys were removed, dried, and weighed (the brain was also removed for a separate study); time from anesthesia to completion of sampling and organ removal was 5 min maximum. At wk 4 in one of the remaining twins from the six triplet litters, at 0800 h a blood sample was withdrawn, and animals were sedated. The dorsal cervical-occipital region was shaved, and the head was flexed, followed by puncture of the fourth ventricle at the cysterna magna using a 25-gauge needle and withdrawal of 50–100 μl clear CSF. After death, the adrenals and kidneys (and brain) were isolated and processed. The remaining triplet in each of these litters had blood sampled at 0800 h in wk 8 and was then studied again at month 6 or 12 (see below). In the remaining five twin litters blood was sampled in one neonate at 0800 or 1600 h on PND 1 and in the other at 0800 or 1600 h on PND 2 for analysis of circadian change in cortisol levels. These subjects were studied again at months 6 and 12. Blood and CSF samples were placed immediately on ice; blood was

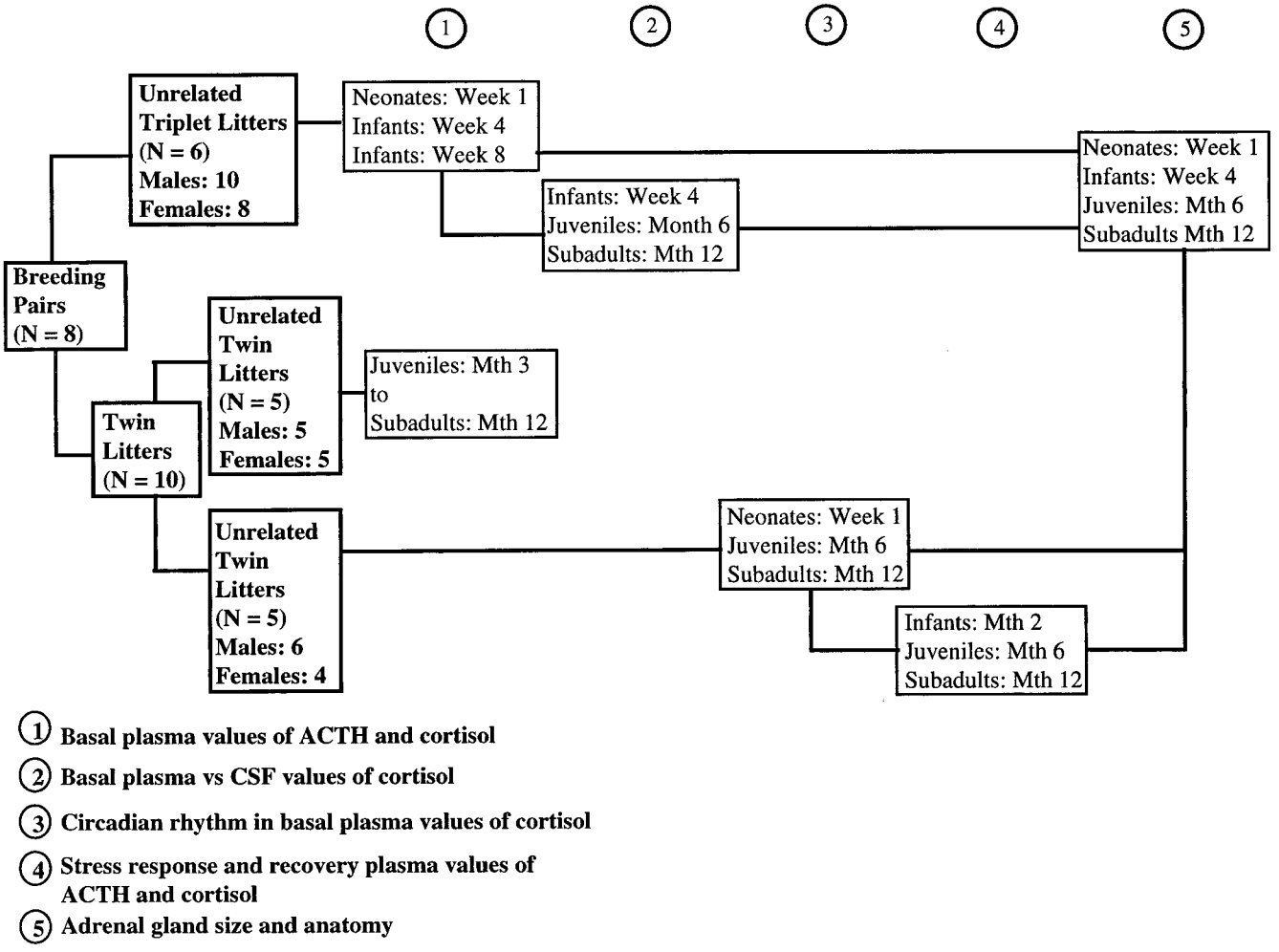

FIG. 1. Overview of allocation of subjects to specific developmental research questions.

centrifuged, and plasma and CSF samples were stored at −80 C before hormone determinations.

Juveniles and subadults (Fig. 1). At month 6 or 12, the six remaining subjects from the triplet births were blood sampled, anesthetized for CSF sampling, and killed before organ sampling. In the five twin litters that were blood sampled as neonates, either a morning or an evening sample was collected at 6 and 12 months of age. At the latter age some subjects were killed for organ analysis. Beginning at wk 9–10 the remaining five twin litters (so far sexed and weighed only) were caught at 0800 h and blood was sampled (0.4 ml) at 4-wk intervals up to month 12. All blood samples were centrifuged, and 200 μl plasma were stored at −80 C before hormone analysis. For direct comparison of subjects with adults in terms of basal ACTH and cortisol values, eight adult common marmosets, aged 2–5 yr (five males and three nonpregnant females), were also bled at 0800 h.

Stress-related endocrine response

The endocrine response to a composite stressor of catching, blood sampling, and novel isolation was determined and compared in infants aged 2 months, juveniles aged 6 months, and subadults aged 12 months; the subjects were some of those studied in terms of ontogeny of basal circadian rhythmicity (Fig. 1). Beginning at 1400 h, the subject was caught in its home cage, transferred to the procedures room, and bled. It was then transferred alone to a novel cage for 15 min, removed, bled a second time, and then returned to the home cage for 120 min, followed by recatching to obtain a third blood sample. The first sample (−15 min) provided the estimate of basal plasma hormone levels, the second sample (0 min) provided the estimate of peak stress response, and the third sample (120 min) provided the estimate of poststress recovery. Only one subject per d was studied in this procedure.

Hormone measurement

Plasma samples were analyzed for their immunoreactive content of ACTH and cortisol, and CSF samples were analyzed for cortisol. All hormone determinations were conducted in duplicate. Plasma ACTH was measured using a commercial RIA kit (KACD1, Diagnostic Products, Los Angeles, CA) developed for human ACTH determination and validated in-house for use with marmoset EDTA plasma. The kit was a double antibody, competitive RIA with a highly specific ACTH antiserum, [^{125}I]ACTH tracer, and goat antirabbit γ-globulin for separation of bound from free hormone and was recommended for direct (without prior extraction) use with EDTA plasma samples. One quantitative deviation from the kit protocol was that 50 μl plasma/tube, rather than the recommended 100 μl plasma/tube, were assayed. The assay sensitivity was 12 pg/ml (n = 4). Accuracy was estimated at 108 ± 11% (mean ± SD; n = 6) over the range of 0.6–70 pg/50 μl added to a marmoset plasma pool of 60 pg/ml. Serial dilutions of a marmoset plasma pool (5–120 μl) gave a displacement curve parallel to that obtained with the ACTH reference standards (1.2–140 pg/μl). Intraassay precision was estimated as 11% (n = 10), and interassay precision as 16% (n = 4).

Plasma and CSF cortisol were measured by an in-house RIA with rabbit antiserum raised against cortisol-3-BSA (Cambridge Medical Technology, Billerica, MA), [1,2,6,7-^{3} H]cortisol tracer (SA, 82.0 Ci/

mmol; TRK 407, Amersham International, Little Chalfont, UK), and cortisol (H-4001, Sigma, Buchs, Switzerland) as reference standard (39–2500 pg/100 μl). EDTA plasma (5 μl) was diluted 1:100, and 10 μl (0.1 μl plasma) were taken to be assayed in duplicate. For CSF, 1-μl aliquots were taken to assay in duplicate. Although CBG levels are negligible in marmoset blood, plasma, and CSF, standard RIA tubes were heated for 10 min at 90 C to denature proteins in the former before RIA. Separation of the antibody-bound and -unbound cortisol/tracer was achieved using dextran-coated charcoal. The cross-reactivity of the antiserum was less than 0.02 with CORT and less than 0.01 with 11-deoxycortisol and sex steroids. The assay sensitivity was 250 pg/ml; accuracy was 97 ± 10% (n = 14); serial dilutions of marmoset plasma pools (0.04–2.5 μl) yielded displacement curves parallel to that obtained with cortisol standard. Intraassay precision was estimated as 10% (n = 10), and interassay precision as 11% (n = 4). As an additional validation step, included to determine whether the immunoreactivity detected in the cortisol RIA was indeed attributable to cortisol, plasma samples from marmosets at different ages were subjected to reverse phase HPLC (RP-HPLC) for ether-soluble steroids (37), followed by analysis in the cortisol RIA. Briefly, 200 μl plasma were extracted with 10 vol redistilled diethyl ether, and the extractants were reconstituted in dichloromethane and injected onto a Novapak C_{18} column (3.9 × 150 mm) fitted with a Mini-Guard column (C_{18}, Waters Corp., Milford, MA). The flow rate was 1 ml/min, and a methanol/water mixture was used as the mobile phase for RP-HPLC. The initial methanol concentration was 50% and was increased linearly up to 75% within 40 min and thereafter up to 100% within a further 5 min. Fractions were collected at a rate of 3 min^{-1} and reconstituted in RIA buffer for assay.

Data analysis

After logarithmic transformation to stabilize variance, data were subjected to ANOVA, followed by determination of main effects and interactions, with statistical significance set at $P < 0.05$. Significant effects were subjected to *post-hoc* pairwise analysis using Fisher's protected test of least significant difference. The effect of age on basal morning plasma ACTH and cortisol values in triplets aged 1, 4, or 8 wk was tested using one-way ANOVA with litter included in the measure of error variance. Age effects on the same parameters in twin litters across 3–12 months of age were first assessed using two-way ANOVA with the between-subject factor of litter and the within-subject factor of age; in the absence of a significant effect of litter, the data were reanalyzed with the between-subject factor of sex and the within-subject factor of age. The effect of age on morning *vs.* evening cortisol values was assessed in twins aged 1 wk, 6 months, and 12 months using one-way ANOVA with litter included in the measure of error variance. Stress response was analyzed using two-way ANOVA with the between-subject factor of age and the within-subject factor of time relative to stressor. The relationship between plasma and CSF cortisol values was described using Pearson's correlation coefficient and was analyzed using simple regression. In *Results*, all data are expressed as the actual (*i.e.* untransformed) values (mean ± SEM).

Results

Basal ACTH and cortisol

The basal plasma ACTH and cortisol values determined in morning blood samples from otherwise nonmanipulated marmoset monkeys, covering the ontogenetic period of 1 wk to 12 months, are given in Fig. 2. For plasma ACTH values, comparison of triplets aged 1, 4, or 8 wk demonstrated a significant main effect of age [F(2,10) = 4.1; $P < 0.05$]; *post-hoc* pairwise comparisons revealed that plasma ACTH values did not differ between wk 1 and 4, but were significantly higher at both of these ages than at wk 8 ($P < 0.03$ and $P < 0.05$, respectively; Fig. 2A). A *t* test demonstrated that there was not a significant difference between ACTH values in marmosets at wk 8 *vs.* month 3 ($t = -0.66$; df = 14; $P > 0.51$). In five litters of male-female twins, aged 3–12 months, there was no significant difference between male and female

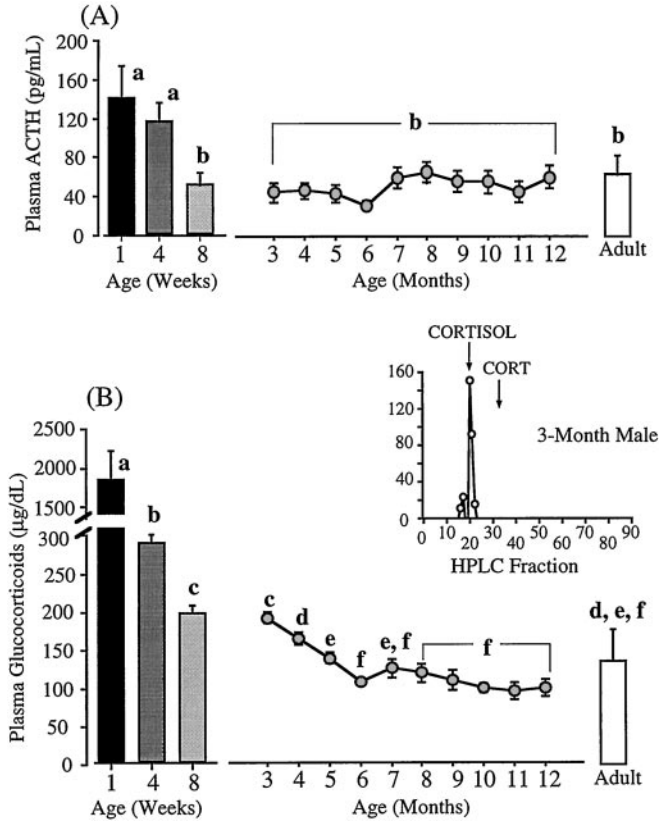

FIG. 2. Mean ± SEM basal plasma values of ACTH (A) and cortisol (B) in marmosets from neonate to adulthood. Data were obtained from six monkeys each for ages 1 (3 males and 3 females), 4 (3 males and 3 females), and 8 (4 males and 2 females) wk; 10 monkeys for ages 3–12 months (5 males and 5 females); and 8 adult monkeys (5 males and 3 females). The *inset* in B provides the immunoreactivity profile of RP-HPLC fractions run in the cortisol RIA from a plasma sample obtained from a male aged 3 months. In A and B, *histogram bars denoted by different letters* are significantly different from each other at the following probabilities: A:, a *vs.* b, $P < 0.05$; B: a *vs.* b–f, $P < 0.0001$; b *vs.* c, $P < 0.001$; c *vs.* d, $P < 0.05$; d *vs.* e, $P < 0.05$; e *vs.* f, $P < 0.05$.

ACTH values [F(1,8) < 1; $P > 0.77$], and ACTH values did not differ significantly across this age period [F(9,72) = 1.26; $P > 0.27$]. A *t* test demonstrated that there was no significant difference between the basal plasma ACTH values demonstrated by subadults (month 12) *vs.* adults ($t = 0.75$; df = 16; $P > 0.49$).

For plasma cortisol (Fig. 2B), the comparison of triplets aged 1, 4, or 8 wk demonstrated a significant main effect of age [F(2,10) = 30.6; $P < 0.0001$]. *Post-hoc* tests confirmed the markedly higher plasma cortisol values at wk 1 *vs.* wk 4 and 8 ($P < 0.0001$), and that cortisol concentrations were also higher at wk 4 than wk 8 ($P < 0.001$). As with ACTH, there was not a significant difference in cortisol in 2- *vs.* 3-month-old marmosets ($t = -1.02$; df = 14; $P > 0.34$). However, during months 3–6, when ACTH values were stable, there was a consistent decline in cortisol values in males and females, with values then being stable from month 7 until the end of the first year of life. These findings were demonstrated by the absence of a main effect of sex [F(1,8) = 2.3; $P > 0.16$), a significant main effect of age [F(9,72) = 31.6; $P < 0.001$], and

the *post-hoc* pairwise comparisons (Fig. 2B). Cortisol values at month 12 were not significantly different from those measured in adults ($t = 1.57$; df = 16; $P > 0.19$), although adult females did have relatively high cortisol values (note the relatively large SE value for adults in Fig. 2B). Evidence that the majority of the immunoreactivity detected in the cortisol RIA was indeed attributable to cortisol was provided by RP-HPLC fractionation of plasma samples collected from different subjects representing several ages and determination of fraction-specific immunoreactivity in the cortisol RIA. As shown in Table 1 and Fig. 2B (*inset*), there was reasonable agreement between immunoreactive values of plasma samples measured directly and, for the same samples, the sum of the immunoreactivity in those fractions (20–22) corresponding to the fractions in which cortisol standard was eluted; nearly all of the immunoreactivity detected was confined to these fractions. There was some immunoreactivity in the fractions eluted immediately before those containing cortisol in samples from a 1-wk-old male and a 3-month-old male.

The relationship in the marmoset between cortisol values in matched morning plasma and CSF as well as age-related changes in CSF cortisol are given in Fig. 3. Matched blood and clear CSF samples were obtained from marmosets aged 4 wk, 6 months, or 12 months. CSF cortisol values were highly correlated with plasma cortisol (r = 0.91), as confirmed by ANOVA of the linear regression of CSF values against plasma values [$F(1,7) = 63.9$; $P < 0.001$]. As would be predicted from this high correlation, 4-wk-old marmosets demonstrated significantly higher morning CSF cortisol values than 6- and 12-month-old marmosets [$F(2,6) = 38.7$; $P < 0.001$]; as was the case for plasma, the two older age groups did not differ significantly in their CSF cortisol values.

Figure 4 presents the findings for the comparison of morning and evening basal cortisol values at 1 wk, 6 months, and 12 months. Subjects were five unrelated twin litters. At each age one twin provided a morning sample, and the other provided an evening sample. There was a significant interaction between age and time of day [$F(2,8) = 33.7$; $P < 0.001$] as well as significant main effects of time of day [$F(1,4) = 42.9$; $P < 0.003$] and age [$F(2,8) = 592.9$; $P < 0.0001$]. Paired t tests conducted *a posteriori* demonstrated that morning and evening plasma cortisol values did not differ significantly in 1-wk-old marmosets ($t = 1.1$; df = 4; $P > 0.33$), and that they were significantly higher in the morning at months 6 ($t = 8.9$; df = 4; $P < 0.001$) and 12 ($t = 8.5$; df = 4; $P < 0.001$).

To summarize the findings on the ontogenetic profile of basal pituitary-adrenal hormone values in this primate, morning values of ACTH and cortisol were significantly higher at 1 and 4 wk of age than across the age range of 2–12 months inclusive. ACTH did not decline between wk 1 and 4, whereas cortisol decreased by about a factor of 10 during this period. ACTH values were consistent across months 2–12, whereas cortisol values decreased consistently across months 3–6. At ages 6 and 12 months a clear circadian rhythm in cortisol values was apparent, whereas this was not the case at 1 wk of age; at this earliest stage of postnatal development the very high basal morning cortisol values also pertained in the evening period of the light cycle.

TABLE 1. Comparison of plasma corticosteroid values in marmoset samples measured directly and after RP-HPLC

Sample no.	Subject sex and age	Immunoreactive HPLC fractions[a]		Direct to RIA (μg/dl)
		Cortisol (μg/dl)[b]	Unidentified (μg/dl)[c]	
1	Male, wk 1	1842	98	1792
2	Male, wk 4	372	ND	267
3	Male, month 3	293	34	238
4	Male, month 11	74	ND	79
5	Male, adult	114	ND	96

ND, No immunoreactivity detected.

[a] HPLC elution fractions were collected at a rate of 3 min^{-1} and were run in the cortisol RIA (see *Materials and Methods*).

[b] Values represent the sum of the three elution fractions corresponding to those containing cortisol standard.

[c] Values represent the sum of elution fractions other than those corresponding to cortisol. There was no immunoreactivity corresponding to corticosterone (see inset Fig. 2B).

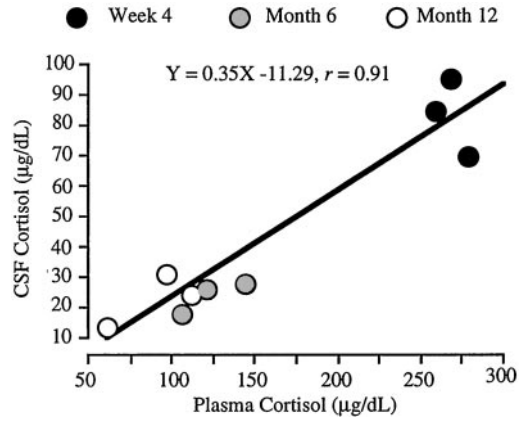

FIG. 3. Relationship between CSF and plasma cortisol values in matched basal samples obtained from marmosets at different ages. n = 2 males and 1 female at each age.

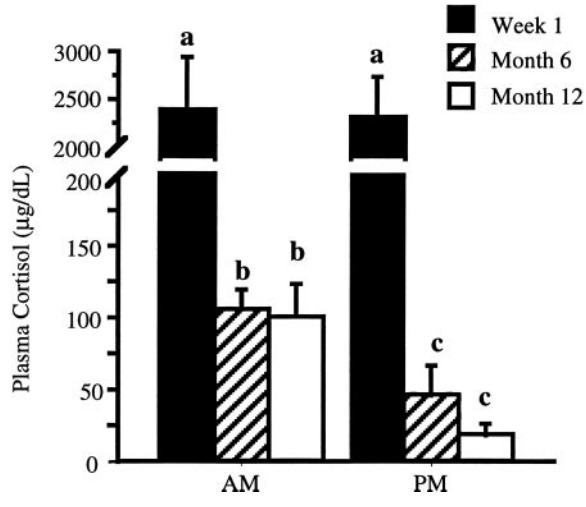

FIG. 4. Effect of age on the relationship between time of day and basal plasma cortisol in marmosets. Values are the mean ± SEM (n = 5). *Histogram bars denoted by different letters* are significantly different from each other. n = 6 males and 4 females.

Stress ACTH and cortisol

Observed effects of age on stress-related ACTH and cortisol responses are provided in Fig. 5. Stress response and poststress recovery of evening values were analyzed in marmosets aged 2, 6, or 12 months. In the case of ACTH values, there was a significant age × time interaction [$F(4,12) = 6.6$; $P < 0.005$] and a significant main effect of time [$F(2,12) = 64.7$; $P < 0.001$; Fig. 5A, *left panel*]. At each age, plasma ACTH values were significantly higher at 0 min than at −15 and 120 min, as indicated by significant within-subject one-way ANOVAs [$F(2,4) \geq 10.9$; $P < 0.03$] and *post-hoc* tests. In addition, at 0 min plasma ACTH was significantly higher in subjects aged 2 months than in those aged 6 months [$F(2,6) = 5.7$; $P < 0.05$]. When ACTH values were reanalyzed in terms of percent change from basal values, two-way ANOVA revealed a significant age × time interaction [$F(4,12) = 8.3$; $P < 0.002$]; one-way ANOVAs at individual time points revealed that, as for absolute values, this was due to the relatively high percent increase in ACTH at 0 min at age 2 months compared with levels at 6 and 12 months of age (Fig. 5A, *right panel*).

For cortisol absolute stress values (Fig. 5B, *left panel*), there was a significant interaction between age and time [$F(4,12) = 5.2$; $P < 0.02$] as well as significant main effects of time [$F(2,12) = 22.4$; $P < 0.001$] and age [$F(2,6) = 75.5$; $P < 0.001$]. Reanalysis *a posteriori* revealed that at −15, 0, and 120 min, marmosets aged 2 months had significantly higher plasma cortisol values than marmosets aged 6 or 12 months. In 6- and 12-month-old subjects, there was a significant increase in cortisol at 0 min *vs.* −15 and 120 min [$F(2,4) \geq 28.8$; $P < 0.005$], whereas in 2-month-old subjects cortisol values were significantly increased at both 0 and 120 min relative to −15 min [$F(2,4) = 9.4$; $P < 0.04$]. Analysis according to percent change from the basal value (Fig. 5B, *right panel*) revealed a trend for a significant age × time interaction [$F(4,12) = 3.0$; $P < 0.06$], attributable to the relatively high cortisol values at 120 min in the 2-month-old subjects only.

Adrenal gland size

Adrenal gland weights (neonates, 31 ± 4 mg; infants, 24 ± 2 mg; juveniles, 36 ± 4 mg; subadults, 86 ± 7 mg), expressed as a percentage of kidney or total body weight, were analyzed for an effect of age using between-subject, one-way ANOVA. There was a significant effect of age on percent adrenal/kidney weight [$F(3,15) = 22.1$; $P < 0.001$], with *post-hoc* tests demonstrating that, according to this relative measure, neonates had significantly larger adrenals than was the case in each of the older age groups, with no significant differences among the latter. As shown in Fig. 6, there was a significant effect of age on percent adrenal gland weight relative to total body weight [$F(3,15) = 44.4$; $P < 0.001$], attributable to neonates possessing relatively large adrenals compared with older age groups and also to infants aged 4 wk possessing relatively large adrenals *vs.* 6-month-old juveniles.

Discussion

This detailed analysis of the ontogeny of the HPA system in a nonhuman primate, the common marmoset monkey, is

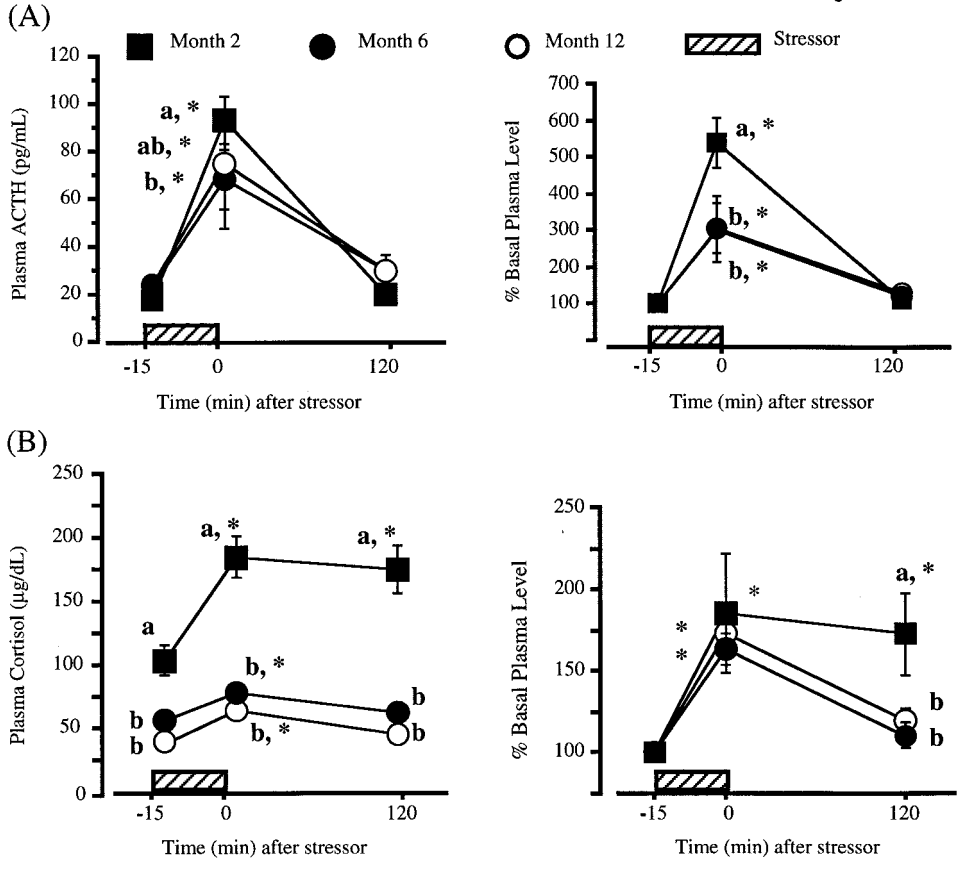

FIG. 5. Effect of age on the stress response in marmosets in terms of plasma ACTH (A) and plasma cortisol (B). Values on the *left* depict the absolute mean ± SEM plasma hormone values, and those on the *right* show the mean ± SEM percent change relative to basal (=100%). *, Significant within-age difference between the hormone values at that time point and basal values. Values at the same time point denoted by *different letters* indicate significant differences between age groups.

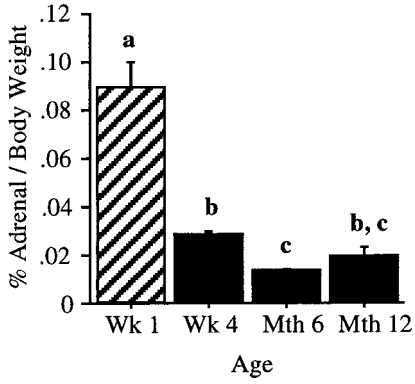

FIG. 6. The effects of age on adrenal gland weight expressed as a percentage of body weight. Values are derived from the average of left and right adrenal gland weights obtained from four to six marmoset monkeys per age group. *Histogram bars denoted by different letters* are significantly different from each other.

an important complement to the comprehensive rat studies. The major finding is that, exactly opposite to the rat as well as in contrast to the rhesus macaque and human, basal plasma ACTH and cortisol values are higher during early infancy than at any other life stage from late infancy to adulthood. As such, this primate represents an important model in which to investigate research questions related to the hypothesis, based on the rat evidence and the human epidemiological evidence, that high GC exposure during postnatal ontogeny can lead to chronic alteration of GC-dependent processes, including hepatic glucose dynamics, cardiovascular function, and affective and cognitive brain-behavior systems (1, 38, 39). When comparing the findings of the present study with those in the rat, macaque, and human, it is important to consider the relative stage of offspring development at birth across these species. According to the altricial-precocial neonate classification of Portman (40) and Martin (41), rats are an altricial species, characterized by neonates that have poorly developed sensory and motor systems and small brains that grow considerably postnatally; marmosets and macaques are precocial species, characterized by neonates that have well developed sensory and motor systems and large brains that grow moderately postnatally; and humans are uniquely secondarily altricial, characterized by neonates that are well developed in terms of sensory systems and poorly developed in terms of the motor system and have large brains that grow considerably postnatally. As viewed against this comparative background, it is clear that a combined rodent-primate approach to the biomedical study of the ontogeny of the HPA system and its importance for adult stress-associated disorders might well provide considerably more information than the study of one species alone.

Marmoset neonates aged PND 1–2 and infants aged 4 wk exhibit high basal levels of both ACTH and cortisol in the peripheral circulation. This strongly suggests both that the immature marmoset's postnatal HPA system is hyperactive at the level of the pituitary and adrenals, and that there exists a state of reduced GC negative feedback on this system relative to later life stages. In comparison, rat pups demonstrate adult-like levels of corticosterone on PND 1–2, followed by very low levels during the SHRP and then a sustained increase (15); infant/juvenile rhesus macaques demonstrate a slight decrease in ACTH and cortisol levels across months 1–6, but these levels are very similar to those in adulthood (18), and in humans cortisol increases gradually to adult-like levels between wk 1 and month 3 (22). Furthermore, 4-wk-old marmoset infants demonstrate correspondingly high levels of cortisol in their CSF, suggesting that the blood-brain barrier does not function differently in terms of cortisol penetrance during the postnatal hypercortisolic period relative to older life stages. CSF levels might not accurately indicate specific brain tissue exposure to cortisol, given the potential for neuronal level factors and ontogenetic shifts thereof to confound any direct relationship between levels of CSF cortisol and GC intracellular receptor binding. The prereceptor metabolism of cortisol by the catabolic enzyme 11β-hydroxysteroid dehydrogenase-2 (11βHSD-2) to inert 11 keto-products represents one such potential age-dependent regulator of cortisol action at the neuronal level (for review, see Ref. 42). Furthermore, in the rat at least, the expression of 11βHSD-2 is developmental stage dependent: midgestation fetal brain levels of 11βHSD-2 activity are high relative to late gestation and adult levels (42, 43). Given this situation and the present findings, an ontogenetic analysis of 11βHSD-2 brain expression in the marmoset is warranted.

Plasma ACTH levels do not differ significantly between neonates and young infants and decline to a stable level by month 2, whereas cortisol levels are 10-fold higher in neonates than in young infants, and the decline to stable levels does not occur until month 6 of life. Therefore, there is an ACTH-GC discrepancy in terms of relative ontogenetic change between wk 1 and 4 and also months 2 and 6. The extremely high cortisol levels in neonates, which confirm and expand on the findings of an earlier study by Levine *et al.* (31), relative to wk 4 and older life stages, are probably attributable to their composite phenotype of high ACTH levels and relatively large adrenal glands. Levine *et al.* (31) propose that the marmoset neonatal adrenal includes a large fetal zone that regresses dramatically during the first week of life; if this interpretation is correct, it would suggest that the fetal zone synthesizes massive levels of GC and that the loss of the fetal zone accounts for the marked reduction in absolute and relative adrenal size and, despite consistent ACTH, in cortisol levels between wk 1 and 4. The reduction in ACTH levels between wk 4 and month 2 could well contribute to the reduction in cortisol levels across the same period, but the continuing decline in cortisol only between months 2 and 6 requires a different explanation, and age-related changes in cortisol metabolism might be responsible (see below).

That marmoset neonates do not demonstrate circadian cyclicity in their blood cortisol levels provides an interesting analogy, or even homology, to the same phenomenon in human neonates and young infants (23, 24). To our knowledge, the existence of circadian changes in GC levels have not been investigated in the macaque neonate. In the infant rat the unperturbable levels of CORT during the SHRP are also characterized by the absence of circadian cyclicity (39). By the month 6 juvenile stage we observed a clear morning to evening drop in marmoset blood cortisol values, and comparison of the wk 8 data in the morning basal study (Fig. 2)

with the month 2 basal data in the evening stress study indicates that a circadian rhythm in both ACTH and cortisol levels already exists in the marmoset by this old infant stage. One further point that is very noteworthy is that the only previous published study of morning and evening cortisol values in the marmoset found no evidence of a circadian cycle in adults (29). The marked circadian effect we observed at 6 and 12 months also pertains in adults (unpublished data); this interlaboratory difference is unlikely to be a genetic effect, so environmental/husbandry differences provide the most likely explanation, possibly reflecting just how sensitive even fundamental biological processes are to captive conditions in primate species.

Ontogeny of the marmoset's pituitary-adrenal stress response was first studied at age 2 months, when subjects were frequently locomoting independently. These infants expressed ACTH peak stress response and poststressor recovery similar to those of juveniles and subadults. The peak stress cortisol response of the infants was of the same magnitude, despite their higher basal levels, as that of juveniles and subadults, whereas their cortisol poststressor recovery was retarded, such that at 2 h poststressor there was still no reduction from peak stress levels. This combination of ACTH and cortisol findings strongly suggests that peripheral (at least) cortisol metabolism is inefficient in young marmosets. Indeed, a longer half-life of cortisol in the circulation in infant-juvenile marmosets could also be a major contributing factor to the discrepancy in the ontogenetic profiles of basal blood levels of ACTH *vs.* cortisol; specifically, the slow developmental decline to adult-like basal values of cortisol compared with ACTH might well reflect slow cortisol metabolism at the immature life stages. It needs to be noted here that in the rat the rate of corticosterone metabolism actually decreases across the SHRP and beyond, that is, the opposite chronological relationship to that proposed here for the marmoset; however, this might well be related to the increase in CBG levels with age in the rat (39), which is unlikely to be an issue in the marmoset (27, 28).

Therefore, as are macaque neonates and infants (18, 20) and human neonates and infants (25), older marmoset infants at least are capable of demonstrating an adult-like pituitary-adrenal stress response during a single short separation from the parents. This is in contrast to the rat infant, in which a single prolonged pup-dam separation across several hours (14) or repeated pup-dam separations for 1 h/d (44) are required before a marked pituitary-adrenal stress response is detectable. At the time of conducting this experiment we had not investigated the tolerance of younger marmosets, or indeed their parents, to a separation/reunion experience of greater than the 2 min required for a single blood sampling, and therefore we did not perform the stress study with neonates/young infants. We have since validated a protocol for daily 1- to 2-h parental separation of marmoset infants beginning on PND 2, and are using this to study the acute and chronic effects of repeated parental deprivation on HPA function in this primate (45). Finally, it is important to note that regardless of age the magnitude of the stress response was low for cortisol compared with ACTH. This discrepancy does not pertain in the rat (6) or rhesus macaque (18) and might indicate a ceiling effect related to high basal GC activity in the marmoset monkey.

It will be very interesting to examine the relationship between the ontogeny of pituitary-adrenal endocrine activity in the marmoset monkey, as reported here, and the ontogeny of this primate's CRF, MR, and GR expression and activity in limbic and hypothalamic brain structures. In the altricial rat, the only species studied in this respect to date, the SHRP pup exhibits, in addition to the low basal and stress levels of ACTH and CORT, low basal and stress levels of CRF in the hypothalamus (reviewed in Ref. 39), adult-like levels of MR, and low levels of GR expression (46), with the latter not reaching an adult-like state in the hippocampus or hypothalamus until about postnatal wk 3 (47). It has been proposed that MRs are pivotal in the maintenance of basal HPA activity, including the levels observed in the SHRP rat pup, via proactive negative feedback (1). This hypothesis can be examined directly in the marmoset in terms of whether in neonates and young infants the high ACTH and cortisol levels reported here co-occur with low MR levels in the hippocampus and/or hypothalamus. There are no reports to date on the brain ontogeny of levels of CRF, MR, GR, or their respective transcripts in any primate species.

In summary, this study has revealed that the marmoset is characterized by high pituitary-adrenal basal activity during early postnatal life, and that adult-like stress reactivity is established by older infancy at least. As such, this primate provides a model to investigate a number of important specific hypotheses concerning the effects of postnatal GC levels on long-term development, including those relating to postnatal stress predisposing to GC-related disease in human adulthood (1, 2, 10, 11). These include the following: 1) high postnatal ACTH and GC levels can be tolerated by some species and not others dependent on their stage of development at birth; 2) high postnatal ACTH and GC values are the consequence of a postnatal-specific profile of corticosteroid receptor density (MR and/or GR, or MR:GR balance) at the limbic and hypothalamic levels; 3) high GC values and target tissue exposure are only detrimental under certain conditions of MR, GR, and MR:GR density, and these conditions do not pertain postnatally in certain species; 4) high postnatal GC values are not necessarily detrimental under any conditions of target tissue MR and/or GR density if postnatal-specific protective factors [*e.g.* 11βHSD-2 conversion of bioactive GCs to inactive forms (42) or antiglucocorticoid effects of dehydroepiandrosterone (31, 48)] are in place; 5) even in a species in which high postnatal target tissue GC exposure is the norm, there is still a threshold of maximal exposure, and exceeding this threshold, for example during parental deprivation, is detrimental to long-term functioning of GC-dependent processes. Given the rat experimental evidence and the human epidemiological evidence for the chronic effects of high GC exposure during early stages of development, this marmoset HPA system model is clearly of marked importance.

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