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RESEARCH ARTICLE

Pattern of Maternal Circulating CRH in Laboratory-Housed Squirrel and Owl Monkeys

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The anthropoid primate placenta appears to be unique in producing corticotropin-releasing hormone (CRH). Placental CRH is involved in an endocrine circuit key to the production of estrogens during pregnancy. CRH induces cortisol production by the maternal and fetal adrenal glands, leading to further placental CRH production. CRH also stimulates the fetal adrenal glands to produce dehydroepiandrostendione sulfate (DHEAS), which the placenta converts into estrogens. There are at least two patterns of maternal circulating CRH across gestation among anthropoids. Monkeys examined to date (*Papio* and *Callithrix*) have an early-to-mid gestational peak of circulating CRH, followed by a steady decline to a plateau level, with a possible rise near parturition. In contrast, humans and great apes have an exponential rise in circulating CRH peaking at parturition. To further document and compare patterns of maternal circulating CRH in anthropoid primates, we collected monthly blood samples from 14 squirrel monkeys (*Saimiri boliviensis*) and ten owl monkeys (*Aotus nancymaae*) during pregnancy. CRH immunoreactivity was measured from extracted plasma by using solid-phase radioimmunoassay. Both squirrel and owl monkeys displayed a mid-gestational peak in circulating CRH: days 45–65 of the 152-day gestation for squirrel monkeys (mean $\pm$ SEM CRH = 2,694 $\pm$ 276 pg/ml) and days 60–80 of the 133-day gestation for owl monkeys (9,871 $\pm$ 974 pg/ml). In squirrel monkeys, circulating CRH declined to 36% of mean peak value by 2 weeks before parturition and then appeared to increase; the best model for circulating CRH over gestation in squirrel monkeys was a cubic function, similar to previous results for baboons and marmosets. In owl monkeys, circulating CRH appeared to reach plateau with no subsequent significant decline approaching parturition, although a cubic function was the best fit. This study provides additional evidence for a mid-gestational peak of maternal circulating CRH in ancestral anthropoids that has been lost in the hominoid lineage. Am. J. Primatol. 72:1004–1012, 2010.

Key words: placenta; anthropoid; corticotropin-releasing hormone; gestation

INTRODUCTION

Placental production of corticotropin-releasing hormone (CRH) appears to be an anthropoid primate adaptation [Bowman et al., 2001; Power & Schulkin, 2006; Robinson et al., 1989]. Shortly after CRH was first isolated from sheep hypothalamus [Vale et al., 1981], the peptide was detected in serum obtained from pregnant women [Sasaki et al., 1984]. The source of the CRH in maternal circulation was determined to be the placenta [Grino et al., 1987; Frim et al., 1988]. Subsequently, circulating CRH was detected in pregnant chimpanzees [Smith et al., 1999], gorillas [Robinson et al., 1989; Smith et al., 1999], baboons [Goland et al., 1992; Smith et al., 1993], macaques [Bowman et al., 2001; Giussani et al., 1998; Robinson et al., 1989], and common marmosets [Bowman et al., 2001; Power et al., 2006]; but not in rats or guinea pigs [Robinson et al., 1989] nor in several species of Malagasy prosimian primates [Bowman et al., 2001; Robinson et al., 1989]. African and Asian prosimians (lorises and tarsiers) have yet to be examined. Thus, the

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placentas of humans and apes, Old World monkeys, and New World monkeys all appear to synthesize CRH and release it into maternal circulation; but no other primates nor any non-primate mammals are known to do so (Fig. 1).

One function of placental CRH is to stimulate the maternal and fetal adrenal glands to synthesize and release steroids. This maternal–placental–fetal endocrine circuit serves to produce the increasing concentration of maternal circulating estrogens as pregnancy progresses. In most mammalian species, the increase in circulating estrogens across pregnancy is accomplished by placental conversion of progestins to estrogens [Smith et al., 2005]. The anthropoid primate placenta does not express to any appreciable extent a key enzyme (17α-hydoxylase-17,20-lyase) required for this process [Kallen, 2004; Rainey et al., 2004]. Thus, in anthropoids circulating progesterone does not decrease as estrogen increases over pregnancy [Smith et al., 2005]; rather both continually rise in concentration. In anthropoids, placental production of estrogens is dependent instead on the substrate steroid dehydroepiandrostendione sulfate (DHEAS), produced by the fetal adrenal in response to stimulation by placental CRH [Rainey et al., 2004].

The temporal profile of maternal circulating CRH in humans is very consistent. Circulating CRH is undetectable until approximately the end of the first trimester. From then on, circulating concentrations of CRH increase exponentially over pregnancy, peaking at parturition [Campbell et al., 1987; Goland et al., 1986; McLean et al., 1995; Sasaki et al., 1987]. After the placenta is expelled from the uterus, circulating CRH rapidly returns to undetectable levels. This pattern is consistent with the proposed endocrine feedback system between maternal and fetal adrenals and the placenta. Similar to its feed-forward effect on CRH levels in certain brain regions such as the amygdala [Makino et al., 1994; Watts & Sanchez-Watts, 1995], cortisol induces CRH production in placenta [Jones et al., 1989; Robinson et al., 1988]. Placental CRH acts on the maternal and fetal adrenal glands to induce production of cortisol (predominantly from the maternal adrenals) and DHEAS (predominantly from the fetal adrenals). Cortisol released by the maternal adrenal glands and to a lesser extent by fetal adrenal glands acts on the placenta to induce greater CRH production. This positive feedback system results in increasing the production by fetal adrenal glands of DHEAS, which is converted by the placenta into estrogens [Rainey et al., 2004]. The system is putatively essential for the appropriate timing of human parturition [Lockwood, 2004].

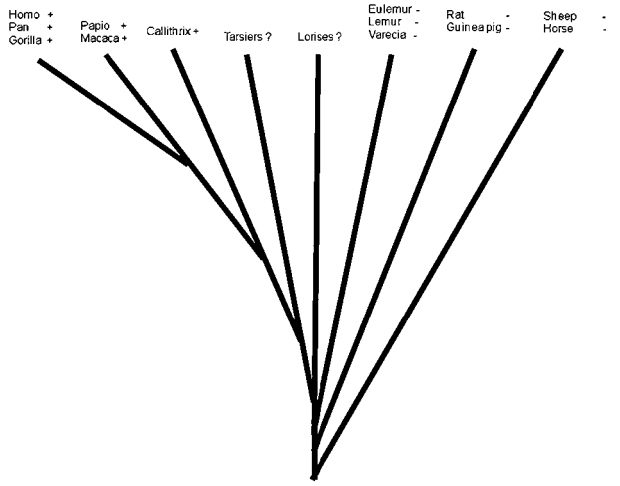

Fig. 1. Simple phylogenetic representation of species tested for presence/absence of maternal circulating CRH during pregnancy. + indicates CRH detected; – indicates CRH not detected; ? means no species in these taxa have been tested. Data from Power and Schulkin, 2006.

The temporal profile of maternal circulating CRH over gestation in the great apes is qualitatively the same as that of humans [Power & Schulkin, 2006; Smith et al., 1999]. However, the temporal profile seen in pregnant monkeys (baboons and common marmosets) appears to be fundamentally different [Power & Schulkin, 2006]. Despite an exponential increase in circulating CRH across gestation, these monkey species exhibit a pattern best described by a cubic function, with an early-to-mid gestational rise to a peak value, followed by a decline in circulating CRH concentration until shortly before parturition when it appears to rise again [Power & Schulkin, 2006]. The fact that an Old World monkey and a New World monkey share a qualitatively similar pattern of maternal circulating CRH across gestation that differs from the common pattern seen in humans and apes suggests that the monkey pattern may be the ancestral pattern whereas the human and ape pattern is derived.

However, results from only two species do not constitute overwhelming evidence for a consistent pattern of CRH expression during gestation in monkeys. Until more taxa can be examined, the full picture of the anthropoid patterns of maternal circulating CRH remains uncertain. Accordingly, the present research was designed to investigate the pattern of maternal circulating CRH in two additional New World monkey species: the squirrel monkey (*Saimiri boliviensis*) and the owl monkey (*Aotus nancymaae*).

METHODS

Subjects

This research was carried out under the authorization of existing protocols approved by the Institutional Animal Care and Use Committee of the University of South Alabama and was in compliance with all applicable US laws and the ASP Principles for the Ethical Treatment of Non Human Primates.

Squirrel monkeys (*Saimiri boliviensis boliviensis*) in this study were part of a national resource for Neotropical Primate Research and Resources maintained at the University of South Alabama Center. The squirrel monkeys were housed in social groups of between 15 and 35 animals, containing one adult male and between 10 and 15 adult females with their offspring. Housing consisted of indoor pens measuring approximately $4.5\,m \times 2.5\,m \times 1.5\,m$, connected by two round, 14-inch diameter doors. Social groups had access to two to three pens depending on its group size. All animals were fed a commercial New World primate diet that had a guaranteed analysis of crude fat $\geq 9\%$. The diet was supplemented three times weekly with chopped vegetables, including celery, bell peppers, squash, and beans. Grapes, peanuts, and meal worms were fed sparingly as positive reinforcers when animals presented for clinical observations. Water was available *ad libitum*. The light/dark schedule was maintained to track the local sunrise and sunset; hence, the animals were exposed to long and short days annually.

The owl monkeys were housed indoors at the University of South Alabama vivarium, an Association for the Assessment and Accreditation of Laboratory Animal Care, International- accredited facility. The female owl monkeys in this report were pair-housed with a mate and offspring <6 months of age, if present, in stainless steel vertical cages $0.81\,m \times 0.7\,m \times 1.8\,m$ equipped with a $25\,cm \times 20\,cm \times 27.5\,cm$ nestbox. The monkeys were fed a mixed diet consisting of ZuPreem® Primate Diet canned (Mission, KS), 1/4 orange, and Laboratory Fiber-Plus® Monkey Diet (PMI®; Nutrition International, St. Louis, MO) daily. The diet was supplemented with PRIMA-Treats® (Bio-Serve, Frenchtown, NJ), fruits, and vegetables, and water was available *ad libitum*. Light/dark cycle was offset with lights coming on at 3 am and going off at 3 pm. Red lights came on during the dark cycle to enable observation of these nocturnal primates during their more active phase. Temperature was maintained at 26–27°C. All of the owl monkey dams had had a previous live birth.

Blood samples were obtained from 14 pregnant squirrel monkeys (median age = 6.5 years, range = 4–11 years; median parity = 1, range = 0–6) and ten pregnant owl monkeys (median age = 6 years, range = 4 to 14 years; median parity = 4, range = 3 to 11) across gestation (2–5 samples per female). Blood was collected from manually restrained animals via the femoral vein. The restraint was for <3 min, and has been shown not to affect measured values of other reproductive hormones [Yoeman et al., 1988]. Samples were collected in EDTA, centrifuged (3,000 RPM for 10 min) and then plasma was pipetted off. All blood samples were obtained between 8 am and 10 am. All samples from squirrel monkeys were taken during gestation; for nine of the ten owl monkeys, there were samples taken either before or after pregnancy in addition to the pregnancy samples. All females produced a term birth. All owl monkey births were live births; one female produced twins. In the squirrel monkeys, four of the 14 births were term stillbirths.

Gestational age was estimated for each sample from the date of birth, assuming a 152-day gestation for squirrel monkeys [LEW, unpublished data] and a 133-day gestation for owl monkeys [Hunter et al., 1979]. In the cases of stillbirths in squirrel monkeys, a determination was made that the births were term based on an examination of the dead infant. There were 46 blood samples collected from the 14 squirrel monkey dams (2–4 samples from each dam) ranging from an estimated 11 to 145 days of gestation. There were 39 blood samples collected from the ten owl monkey dams (2–4 samples from each dam) ranging from an estimated 4 to 128 days of gestation. An additional sample from each of nine of the owl monkey samples collected outside of gestation was assayed.

CRH Extraction and Radioimmunoassay

CRH peptide was extracted using the established method described in Power et al. [2006]. Plasma samples were thawed at room temperature, centrifuged ($900 \times g$, 5 min, 4°C), and returned to ice. Supernatant (0.2 ml) was transferred to low-binding, screw-cap polypropylene tubes (Axygen, Union City, CA), to which ice-cold 100% methanol (0.8 ml) was added. After vortexing, samples were rotated (15 min, 4°C) and centrifuged ($2{,}000 \times g$, 20 min, RT). Then, the supernatant was transferred into a polypropylene tube containing 2 µl of 1% Triton X-100, dried in a Savant Speed Vac Concentrator overnight, and then stored at −80°C until immunoassay.

For assay, samples were reconstituted in 0.2 ml of gelatin assay buffer (0.15 M K_2HPO_4, 0.2 mM ascorbic acid, 0.1% gelatin, pH 7.5). Plasma CRH immunoreactivity content was quantified with a sensitive and specific solid-phase radioimmunoassay adapted from Zorrilla et al. [2001]. Immulon-4 HBX Removawell 96-well plates (Thermo Fisher Scientific, Rochester, NY) were coated with protein A/G (1 mg/100 µl of 1 M $NaHC0_3$/well, pH 9.0; Pierce Biotechnology, Rockford, IL) for 48 hr. Plates were rinsed with wash buffer (0.15 M KH_2PO_4 supplemented with 0.2 mM ascorbic acid and 0.1% Tween-20, pH 7.5) to dislodge loose protein A/G. Wells were incubated for 7 days at 4°C with 40 µl of anti-CRF serum (rC70, generously provided by W. Vale, The Salk Institute, La Jolla, CA) at a titer of 1:400,000 in gelatin assay buffer. After three rinses to dislodge loose antibody, 40 µl of sample or standard (6 to 20,000 pg/ml) in duplicate was incubated for 4 days at 4°C. After incubation, 40 µl of [^{125}I-Tyr^0]-r/h corticotropin-releasing factor (~10,000 cpm/40 µl; Perkin Elmer, Boston, MA) was added to each well and incubated for an additional 24 hr at 4°C. The wells were rinsed with wash buffer,

blotted dry, and separated. Residual radioactivity was counted by using an Apex Automatic Gamma Counter. A four-parameter logistic curve fit model was used for interpolation of the standard curves (SigmaPlot 10.0; Systat Software, Point Richmond, CA).

The rC70 antiserum, raised in rabbit against full-length synthetic rat/human CRF by the Vale laboratory, has been well-characterized for radioimmunoassay [Vale et al., 1983], and used extensively in previous studies to measure CRF immunoreactivity in non-human primate species [Hsu & Price, 2009] including squirrel monkeys [Bassett & Foote, 1992; Cha & Foote, 1988; Foote & Cha, 1988]. In this study, ln dose–ln cpm response dilution analysis demonstrated parallelism of *Aotus* monkey samples with human CRF standards across 600–20,000 pg/ml concentrations, which spanned the range of samples. Even dilution analysis of *Saimiri sciureus* samples around the assay's limit of sensitivity (6–600 pg/ml) still demonstrated parallelism with the rat/human CRF standards (albeit both yielding regressions of shallow slope). The assay's mean limit of sensitivity, defined as two standard deviations of cpm from the zero concentration, was 499 pg/ml (~25 pg/well). The mean intra-assay coefficient of variation across all samples was 19–21 and 16% for samples with more typical concentrations of between 500 and 2,000 pg/ml. Typical inter-assay coefficients of variation for this assay are 20% or less [Cottone et al., 2009]. To reduce the influence of plate-to-plate variability on results, most samples of a given species were run on a single plate (78 and 67% for squirrel and owl monkey, respectively), all samples from a given subject were analyzed within the same plate, and gestational age was equally represented across the two plates required for each species.

Statistical Analysis

Mean ± SEM are given for CRH values at key time points. Differences were tested by ANOVA. The patterns of maternal CRH across gestation were modeled using the SPSS® curve estimation function (SPSS® 16.0; SPSS Inc, Chicago, IL). The data were tested for linear, quadratic, cubic, and exponential relationships with gestational age. The mean residual values for each female of maternal circulating CRH by gestational age between squirrel monkey dams that produced a live infant versus a stillborn infant were tested by ANOVA. The pattern of the residuals (i.e. always positive, always negative, and mixed) between mothers of live and stillborn infants was tested by χ^2 analysis.

RESULTS

The pattern of maternal circulating CRH across pregnancy in squirrel monkeys was qualitatively similar to that found in the common marmoset by Power et al. [2006]. Circulating CRH concentration rapidly increased to a peak between days 45 and 70 of gestation (mean CRH = 2,694 ± SEM 276 pg/ml), and then declined to a mean of 961 ± SEM 213 pg/ml between days 110 and 130 of gestation, after which it appeared to begin to increase again. The pattern was best described by a cubic function ($R^2 = 0.481$) with a peak at about day 55 of gestational age, an inflection point at day 95 of gestational age, followed by a local minimum about 2 weeks before parturition, or around day 136 of gestational age (Fig. 2). Although the curve estimations for linear ($R^2 = 0.135$), quadratic ($R^2 = 0.234$), and exponential ($R^2 = 0.085$) functions were all significant, there were significantly less good fits to the data than was the cubic function.

Infant birth weight was not associated with residual deviations of circulating CRH from the fit cubic function in squirrel monkeys. There also appeared to be no qualitative difference in the gestational pattern of circulating CRH between *Saimiri* females producing live or stillbirths (Fig. 2), with no significant mean difference between the residuals from the cubic function estimation between live (−84.4 ± 151.1 pg/ml) and still (171.2 ± 201.9 pg/ml) births (ANOVA: $F = 0.881$, df = 1,12, $P = 0.367$). However, three females had residual values for circulating CRH always below zero (one stillbirth and two live births), nine females had residual values that were both negative and positive (one stillbirth and eight live births), and two females had residual values that were always positive (two stillbirths). This pattern was significantly different from chance (χ^2: $P = 0.041$) with stillbirths overrepresented in those females that showed consistently higher values than the mean cubic function (2 of 2 births, 100%) when compared with those with both negative and positive residuals or consistently lower values (2 of 12 births, 17%; χ^2: $P < 0.02$).

The pattern of circulating CRH concentration was not as clear in the owl monkeys (Fig. 3). First, nonpregnant values for CRH immunoreactivity were

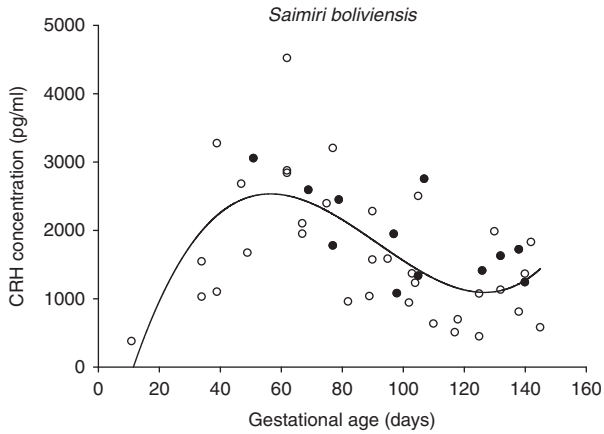

Fig. 2. Maternal circulating CRH in pregnant squirrel monkeys; open circles identify samples from live births and filled circles identify samples from term stillbirths. The best fit curve was a cubic function.

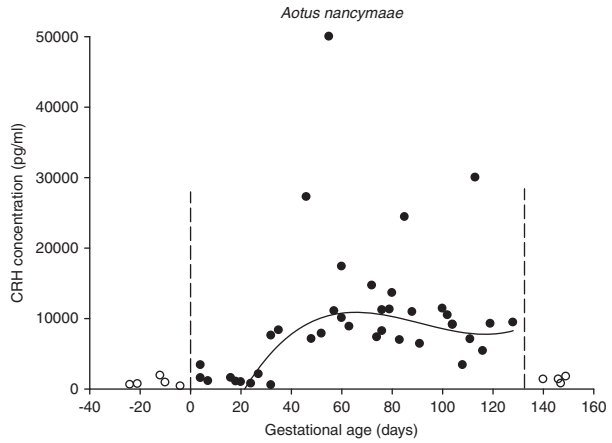

Fig. 3. Maternal circulating CRH in pregnant owl monkeys. Open circles identify samples from before or after gestation; closed circles identify samples from during gestation. Vertical dashed lines indicate the beginning and end of gestation. Excluding the four highest values during gestation and all values before day 20 of gestation resulted in the best-fit curve for maternal circulating CRH across gestation being a cubic function (shown).

not zero, although the values were consistent and much lower than pregnant values (1,078 ± SE 177 pg/ml versus 9,949 ± SE 1,514 pg/ml, $P = 0.008$). Before day 30 of gestation, maternal circulating CRH concentration was not significantly different from baseline, nonpregnant values (1,559 ± SE 299 pg/ml, $P = 0.176$). After day 30 of gestation, mean CRH concentration was significantly higher than nonpregnant or early (before day 30) values (12,114 ± SE 1,699 pg/ml, $P = 0.001$). There was no clear peak, however, and later gestation samples did not differ from mid-gestation samples. A linear regression of CRH after day 30 of gestation was not significant. Although the quadratic, cubic, and exponential curve estimations all returned significant results, none of the R^2 values were greater than 0.332. There were four samples with very high values for CRH (>20,000 pg/ml); eliminating these outliers and running the curve estimation from day 20 of gestation to eliminate part of the long tail of baseline values in early gestation dramatically improved the fit for the cubic functions ($R^2 = 0.551$), as illustrated in Figure 3. The female that gave birth to twins did not have exceptional values for circulating CRH.

DISCUSSION

Placental expression of CRH appears to be a unique reproductive adaptation for anthropoid primates. A key function of placental CRH expression is to stimulate the fetal adrenal zone to synthesize and release DHEAS, which the placenta then converts to estrogens. Another function is to stimulate the maternal adrenal gland and the transitional zone of the fetal adrenal gland (both directly and through stimulation of the fetal pituitary) to synthesize and

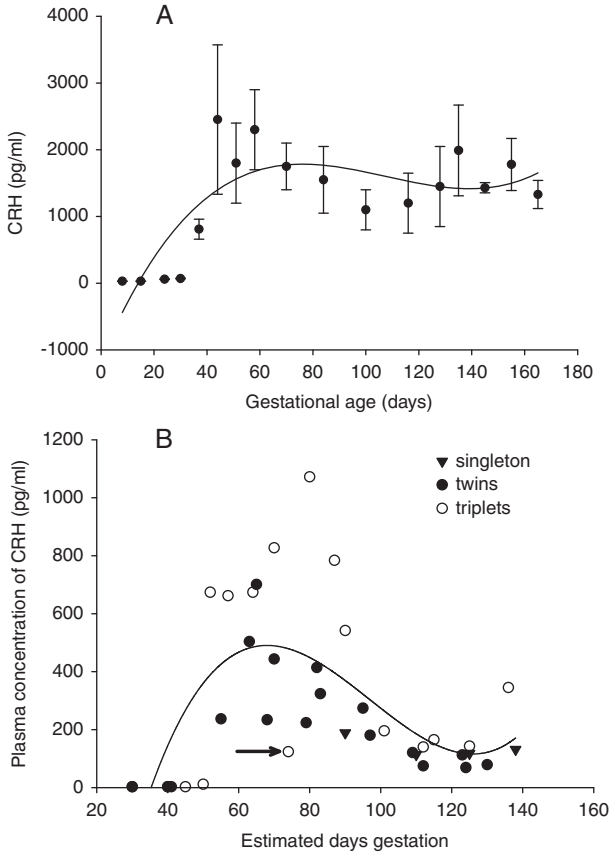

Fig. 4. The patterns of maternal circulating CRH across pregnancy in (**A**) baboons (*Papio hamydryas*), data from Goland et al., 1992; and (**B**) common marmosets (*Callithrix jacchus*), data from Power et al., 2006. The arrow indicates a value from a triplet pregnancy that subsequently aborted.

release cortisol. Cortisol serves to drive the maternal–placental–fetal adrenal axis through a positive feedback system [Robinson et al., 1988], which results in increasing estrogen production over gestation [Lockwood, 2004; Rainey et al., 2004]. Maternal cortisol also is likely involved in many of the metabolic adjustments necessary for successful pregnancy, such as the increase in maternal metabolic rate and insulin resistance [Damjanovic et al., 2009]. Circulating cortisol in the fetus serves to mature fetal organs. Thus, the progression of gestation is appropriately linked to the timing of fetal development, and infants are born with organs (especially lungs) appropriately matured for extra-uterine life [Challis et al., 2000; McLean et al., 1995].

The results from this study provide further evidence that there are two patterns of placental CRH expression and release among anthropoid primates, with monkeys differing from the ape–human pattern. Maternal circulating CRH in squirrel (Fig. 2) and owl monkeys (Fig. 3) is qualitatively similar to that of baboons and common marmosets (Fig. 4), with a peak in mid gestation.

The function of the early peak in maternal circulating CRH common to all monkeys so far studied is unknown. In common marmosets (*Callithrix jacchus*), peak maternal circulating CRH coincides with a period of significant fetal growth and development [Power et al., 2006], implying that placental CRH performs additional important functions beyond the endocrine circuit mentioned above that coordinates the progression and ending of pregnancy. These other possible functions may be lineage-specific (i.e. monkeys differ from apes), but also might reflect different functions for CRH at different times during gestation, or for different target tissues. Placental CRH produced during gestation is likely to have multiple effects on many maternal and fetal organs besides the pituitary and adrenals, and these effects will likely be tissue-specific.

Maternal excretion of estrogens and cortisol has been shown to increase in early-to-mid gestation in monkey species that have been studied. For example, maternal urinary excretion of cortisol significantly increased in successful common marmoset pregnancies starting around the tenth week of gestation, reached a peak value by the 14th week and then was relatively constant until parturition [Tardif et al., 2005]. The timing of the increase in urinary cortisol corresponds to peak maternal circulating CRH in this species (Fig. 4). In several New World monkeys, maternal urinary estrogen excretion is known to increase early in gestation and then to decline after mid-gestation, though, of course, remaining elevated relative to the nonpregnant state: *C. jacchus* [Eastman et al., 1984; Saltzman et al., 2008; Tardif et al., 2005], *Callimico goeldii* [Ziegler et al., 1990], *Saguinus fuscicollis* [Heistermann & Hodges, 1995], and *Saguinus oedipus* [Ziegler et al., 1987]. In *C. jacchus*, the increase in excreted estrogen to a peak value again roughly corresponds to the time of peak maternal CRH. CRH has been shown to stimulate estrogen production in cultured human placental cells [You et al., 2006]. Urinary chorionic gonadotropin (CG) excretion peaks in *C. jacchus* at about 60 days of gestation and then rapidly declines [Saltzman et al., 2008]; thus, urinary CG excretion declines as maternal CRH increases. Finally, maternal urinary androgen excretion in another marmoset (*C. geoffroyi*) steadily increases to a peak between days 70 and 100 of gestation and then rapidly declines [French et al., 2009], again mirroring to a large extent the pattern of maternal circulating CRH in the closely related *C. jacchus*. Thus, the early-to-mid gestational peak in maternal circulating CRH may indeed be associated with maternal–placental–fetal steroid production circuits in monkeys.

Interpretation of the two known different patterns of anthropoid placental CRH expression is complicated by the fact that most, though possibly not all, anthropoid primates exhibit detectable levels of circulating CRH binding protein (CRH-BP) of either hepatic or placental origin [Bowman et al., 2001]. Circulating CRH-BP has been detected in pregnant and nonpregnant humans and chimpanzees, pregnant gorillas, and in nonpregnant orangutans, gibbons, lion-tailed macaques, common marmosets and squirrel monkeys, but not in ruffed lemur, horse or sheep. Surprisingly, CRH-BP was not detected in nonpregnant mandrill, baboon, and spider monkey [Bowman et al., 2001]. Thus, hepatic expression of CRH-BP is not consistent among anthropoids, being expressed in apes, but variably among monkeys. Human placenta expresses CRH-BP [Petraglia et al., 2005]; studies of pregnant monkeys are required to determine whether circulating CRH-BP is a common feature of anthropoid pregnancy. If so, the time course of circulating maternal CRH-BP will be important to determine in non-human primates, given its putative regulation of ''free'' CRH levels in human pregnancy [Linton et al., 1993; McLean et al., 1995].

The pattern of maternal circulating CRH-BP during gestation is known for humans [Linton et al., 1993] and gorillas [Smith et al., 1999] (stable until the third trimester, then a decrease in maternal serum CRH-BP concentration), and chimpanzees [Smith et al., 1999] (stable throughout gestation), but remains to be determined in other anthropoids. In humans, the concentration of maternal serum CRH-BP in early pregnancy is probably sufficient that most placental CRH secreted into the maternal compartment is bound. Placental CRH is thought to have its major effects on maternal physiology in the third trimester, as the concentration of CRH begins to equal and then exceed that of CRH-BP. It is unknown whether the early-to-mid gestational peak in placental CRH expression in baboons, common marmosets, squirrel monkeys, and owl monkeys results in substantial free CRH in maternal circulation.

Maternal physiology and behavior can affect placental CRH expression [Erickson et al., 2001; Herrmann et al., 2001], and it is reasonable to hypothesize that placental CRH released into the maternal compartment can and will have significant effects on maternal physiology. The same is true for fetal physiology. In human pregnancy, maternal circulating CRH has been suggested to serve as a signal of adversity [Schulkin, 1999]. Pregnancies destined to be delivered preterm have early and higher increases of maternal circulating CRH [Hobel et al., 1999; Majzoub et al., 1999; McLean et al., 1995; Smith et al., 2009]. Maternal circulating CRH is higher in preeclampsia [Goland et al., 1995] and several other pregnancy complications including multiple gestations [Warren et al., 1990]. In this small sample, the timing of maternal CRH was not different between live births and stillbirths in squirrel monkeys. However, the two females that consistently had circulating CRH values above the mean values of the cubic function both delivered

a still born infant, a frequency significantly greater than those of females with more mixed or consistently low CRH values across pregnancy. The owl monkey who delivered twins did not exhibit any difference in maternal circulating CRH. In contrast, common marmoset peak maternal circulating CRH depended on litter size, being highest in triplet pregnancies (Fig. 4). Aborted pregnancies in common marmosets are associated with a lack of an increase of maternal urinary cortisol excretion [Tardif et al., 2005]. A single case of an aborted triplet pregnancy was associated with a low value for maternal circulating CRH during the expected peak time period [Power et al., 2006]. However, the low values for cortisol and CRH likely represented placental failure, rather than a specific regulatory disruption. Overall, the data are not yet sufficient to specify whether or how disruptions in the pattern of maternal circulating CRH associate with pregnancy complications or poor birth outcomes in monkeys, but the present report advances the hypothesis that consistently high maternal CRH during pregnancy may be associated with increased risk of stillbirth.

The non-zero values for CRH immunoreactivity outside of pregnancy in *Aotus* is puzzling. It is possible that CRH from some organ other than placenta is reaching peripheral circulation in owl monkeys; a low level of circulating CRH immunoreactivity has been found in the horse, with no effect of pregnancy [Ellis et al., 1994]. Alternatively, there may be a circulating factor in *Aotus* blood that cross-reacts with CRH antibodies. The CRH protein family consists of at least four ligands (CRH and urocortins I, II, and III), two receptors with multiple splice variants, and the binding protein. CRH immunoreactivity values were not different from those outside of pregnancy in early owl monkey gestation; but after day 30 of gestation, values were significantly elevated until parturition. Moreover, dilution analysis demonstrated parallelism of estimated CRH signal in *Aotus* samples across the range of pregnancy-associated elevations with signal from authentic human CRH standard. Thus, we have confidence in the existence of a pregnancy-associated rise in circulating CRH in *Aotus*, which we hypothesize derives from placental CRH production.

The consistency in the qualitative pattern of maternal circulating CRH found in an Old World monkey (Fig. 4) and now in three species of New World monkey (Figs. 2–4) suggests that it represents the ancestral pattern that arose before the last common ancestor of Old and New World monkeys. The pattern seen in great apes and humans thus is likely derived. Data from the smaller ape species (hylobatids) and from tarsiers and lorises would be helpful to further elucidate the evolutionary path of placental CRH expression in primates, as would data from more Old World monkey species, especially colobines.

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