On the mechanism of lactational anovulation in the rhesus monkey

TAMÁS ÖRDÖG, MING-DAO CHEN, KEVIN T. O’BRYNE, JASON R. GOLDSMITH, MARTIN A. CONNAUGHTON, JULANE HOTCHKISS, AND ERNST KNOBIL

Laboratory for Neuroendocrinology and Department of Integrative Biology, Pharmacology, and Physiology, The University of Texas-Houston Medical School, Houston, Texas 77225

Ördög, Tamás, Ming-Dao Chen, Kevin T. O’Byrne, Jason R. Goldsmith, Martin A. Connaughton, Julane Hotchkiss, and Ernst Knobil. On the mechanism of lactational anovulation in the rhesus monkey. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E665–E676, 1998.—The relative roles of infant suckling and of maternal prolactin (PRL) secretion in lactational anovulation were studied in ovary-intact and ovariectomized rhesus monkeys nursing young that had been removed from their natural mothers. Hypothalamic gonadotropin-releasing hormone (GnRH) pulse generator activity was monitored electrophysiologically in freely behaving animals by radiotelemetry. Serum luteinizing hormone, PRL, estradiol, and progesterone were also measured. Suckling inhibited GnRH pulse generator activity and ovarian cyclicity in all ovary-intact females but had no such effect on the pulse generator in long-term ovariectomized animals. When PRL secretion was suppressed by daily bromocriptine administration, GnRH pulse generator activity remained significantly inhibited and ovulation was prevented in four monkeys (6 trials), whereas in two females (6 trials) a rapid increase in pulse generator frequency and the resumption of ovarian cyclicity were observed although suckling activity was maintained. One monkey displayed both response types. Although these results indicate that suckling per se is able to restrain GnRH pulse generator activity in the absence of PRL, they also suggest that the relative importance of these determinants is variable depending on factors that remain to be determined. The present study also confirms the permissive role of the ovary in the lactational suppression of GnRH pulse generator activity.

Suckling inhibits folliculogenesis and ovulation in most mammalian species including Old World monkeys and humans (see Ref. 22 for review). In all species for which there are adequate data, the major limiting factor with regard to follicular development appears to be the suppression of pulsatile luteinizing hormone (LH) secretion (22), suggesting an inhibition of the gonadotropin-releasing hormone (GnRH) pulse generator, the central neuronal oscillator in the mediobasal hypothalamus that controls pituitary gonadotropin secretion (reviewed in Ref. 17). The mechanisms underlying this suppression of the hypothalamo-pituitary-ovarian (HPO) axis are uncertain. Clinical and experimental data suggest that high levels of prolactin (PRL), the pituitary hormone secreted in response to suckling, can result in hypothalamic amenorrhea (9) and the inhibition of pulsatile LH secretion (31) and of GnRH release both in vivo (18) and in vitro (24). However, earlier studies in rhesus monkeys utilizing indirect indexes of the functioning of the HPO axis suggested that this suckling-induced process could not be accounted for simply by the associated hyperprolactinemia (32). That the suckling stimulus per se may play a significant role has also been suggested in other species including humans (reviewed in Ref. 22). However, the importance of elevated PRL levels in the lactational suppression of GnRH pulse generator activity remains unclear. The goal of this study was to reexamine in the rhesus monkey the relative roles of suckling per se and of suckling-induced hyperprolactinemia in this phenomenon by using more direct approaches to assess GnRH pulse generator activity than were previously possible.

MATERIALS AND METHODS

Animals

Five adult, regularly cycling and five ovariectomized rhesus monkeys (Macaca mulatta, age 8-16 yr, wt 7–9.5 kg) were studied. The animals were housed singly in rooms with controlled temperature and illumination (lights on 0700–1900). Under these conditions, none of the ovary-intact females selected for the present study displayed seasonal anovulation. The animals were fed once daily with a ration of Purina monkey chow (Ralston Purina, St. Louis, MO) or High Protein Monkey Diet (PMI Feeds, St. Louis, MO) supplemented with fresh fruit three times weekly. Water was available ad libitum. Body weight was measured biweekly. The animals were fitted with bilateral recording electrode arrays, each consisting of nine nichrome wires, 50 µm in diameter, chronically implanted in the mediobasal hypothalamus as described previously (27) and with chronic indwelling cardiac catheters connected to subcutaneously implanted access ports (27). The animals were maintained and all experiments were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the Animal Welfare and Use Committee of the University of Texas-Houston Health Science Center.

Assessment of GnRH Pulse Generator Activity

In four ovary-intact and the five ovariectomized monkeys, GnRH pulse generator activity was assessed electrophysiologically by the frequency of the associated volleys of multunit electrical activity (MUA volleys) recorded from the mediobasal hypothalamus (27). The correlation between LH pulses, as determined by bioassay of LH (28) in blood samples taken every 10 min, and hypothalamic MUA volleys was assessed in each animal before the study. A unitary correlation between MUA volleys and LH pulses has been absolute and invariable (Fig. 1) under a great variety of physiological and experimental circumstances, permitting the conclusion that LH pulses and MUA volleys both represent manifestations of the activity of the GnRH pulse generator (27–29). An identical conclu-
A rhesus monkey.

The heparin contained in these samples did not affect the perforation. Samples were stored at 20°C until assay (see Hormone Assays below). The serum was used as a separate control and used as a diluent when serum drawn 24–48 h later, which was included in all assays. The intra-assay coefficient of variation of 5–7% as determined by the bioassay of the LH content of the same serum pool at 3 volumes in every assay (37). This more arduous approach yielded GnRH pulse generator frequency patterns that were in agreement with those obtained with radiotelemetry.

Hormone Assays

Blood samples were taken by femoral venipuncture, a procedure the animals had been habituated to, once or twice a day (between 0800 and 0900 and between 1600 and 1700) for the measurement of LH, PRL, 17β-estradiol (estradiol), and progesterone in the serum. Samples were allowed to clot overnight at 4°C, and the sera were separated and stored at −20°C until assay. LH was measured by bioassay as described previously (28) using the NIH monkey LH reference preparation (NICHHD RP-1, WD-XV-20) as a standard. Purified monkey pituitary follicle-stimulating hormone (FSH) and human or monkey PRL are devoid of biological activity in the gerbil Leydig cell LH bioassay. This assay has a sensitivity of 3.1 pg LH/tube (1.0 ng/ml when 3 µl serum is assayed), with intra- and interassay coefficients of variation of 10.0 and 14.0%, respectively.

PRL levels in serum were measured with a modified human double-antibody PRL RIA kit (Diagnostic Products, Los Angeles, CA). The product literature reports no cross-reactivity with growth hormone, FSH, LH, chorionic gonadotropin, thyrotropin, or placental lactogen. We found that a rhesus monkey PRL standard (WDP-XI-49–29) cross-reacted in this assay identically with the human PRL standard. A pool of monkey serum with high PRL levels was assayed at 5, 10, 20, and 50 µl, with results parallel to the human and rhesus standard curves. The reactivity of purified rhesus monkey pituitary hormones was as follows: LH (WDP-XV-20, NICHHD RP1), 0.3% FSH (WDP-XIII-24–24), 0.003% growth hormone (AFP-5282B), 1%, and thyrotropin (WP-X-103–30), not detectable. All assays were performed using the human PRL kit standards and a quality control pool at 20 and 50 µl. BSA (4%, in 0.1 M PBS, pH 7.0) was used as an assay diluent. The working range of the assay was 2.5–250 ng PRL/ml. Treatment of monkeys with Z-bromo-a-ergocryptine (ergocryptine, Sigma Chemical, St. Louis, MO) dissolved with an equal weight of tartaric acid (Fisher Scientific, Houston, TX) in warmed polyethylene glycol (Fisher Scientific) containing 6.25% of 70% ethanol to give a final concentration of 30 mg bromocriptine/ml administered as a single intramuscular injection (30 mg/animal), provided PRL-free serum drawn 24–48 h later, which was included in all assays as a separate control and used as a diluent when serum samples <50 µl were assayed. In 20 assays the standard pool of human PRL, when tested at 20 µl, read at 50.9 ng/ml, with intra- and interassay coefficients of variation of 5.3 and 13.2%, respectively. In the same assays, this standard pool was tested at 50 µl, read at 59.0 ng/ml with intra- and interassay coefficients of variation of 3.6 and 12.8%, respectively.

Estradiol levels in serum were measured using a double-antibody RIA kit (Diagnostic Products) modified for use in the rhesus monkey. The product literature reports significant cross-reactivity with estrone (12.5%) and 17β-estradiol-3b-D-glucuronide (6.0%) but insignificant cross-reactivity (<5%) for 40 additional steroids or steroid conjugates. Estradiol standards (3–1,400 pg/ml) were prepared in ovariectomized rhesus monkey serum, and 100-µl aliquots of the standards and experimental serum samples were assayed without extraction. Replicates of five pooled sera (with estradiol concentra-

Fig. 1. Multiunit electrical activity (MUA) volleys recorded from mediodorsal hypothalamus and luteinizing hormone (LH) pulses measured in peripheral circulation on day 3 of menstrual cycle of a rhesus monkey.

The corticosterone was determined by inspection and by submitting the LH data to the ULTRA pulse detection algorithm (threshold 3 times the intra-assay coefficient of variation of 5–7% as determined by the bioassay of the LH content of the same serum pool at 3 volumes in every assay) (37). This more arduous approach yielded GnRH pulse generator frequency patterns that were in agreement with those obtained with radiotelemetry.
Suckling, PRL, and GnRH Pulse Generator Activity

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tions ranging from 80 to 800 pg/ml) were included in each assay. In 20 assays the intra- and interassay coefficients of variation for these five pools ranged from 1.3 to 2.0% and 6.7% to 9.3%, respectively.

Progesterone concentrations in duplicate 50-µl aliquots of rhine serum were measured using a double-antibody RIA with reagents purchased from ICN Biomedicals (Costa Mesa, CA; rabbit anti-progesterone, 125I-labeled progesterone derivative, and goat anti-rabbit gamma-globulin second antibody solution). Progesterone standards (0.2–16 ng/ml) were prepared in ovariectomized monkey serum. Four replicates of a monkey serum pool were included in each assay. In 20 assays the serum pool read 2.99 ± 0.20 (SD) ng/ml, with intra- and interassay coefficients of variation of 3.58 and 6.78%, respectively. Levels <2 ng/ml were considered as an indication that ovulation had occurred.

Adoption of Infants

After a control period that included at least two ovulatory menstrual cycles in the intact females, the instrumented animals were given infants (n = 11) born to other monkeys. In an earlier study from our laboratory, this approach was shown to result in a suppression of the HPO axis in intact monkeys indistinguishable from that seen in postpartum lactating females (30). Although most infants were 1–11 days old at the time of adoption (n = 9), foster pairs could also be successfully established using older infants (up to 105 days old). Interestingly, only females that had previously nursed young of their own would accept (adopt) infants, whereas monkeys with no prior maternal experience invariably rejected or ignored them. However, prior nursing experience did not necessarily predict a favorable adoption because two animals with a history of nursing their own young did not accept alien infants. The initiation and progress of lactation were monitored by manual expression of milk, a procedure that was performed daily until copious amounts of milk were detected and daily or weekly thereafter. The body weight of the adopted infants was measured at least weekly. To accomplish this the mothers were sedated with 3–5 mg/kg ketamine hydrochloride (Ketaset; American Vet Supply, San Antonio, TX) usually before the evening blood sample was taken. The administration of this low dose of ketamine was maintained throughout the entire study, even in the absence of the infant, as a control measure, but was found to have no effect on circulating PRL levels. After one or more bromocriptine trials (see Bromocriptine Treatment and Supplemental Feeding below), the young were weaned and the intact foster mothers monitored until normal menstrual cycles were re-established.

Assessment of Suckling Behavior

The magnitude of sensory inputs associated with suckling was estimated by determining the percentage of time the infant was attached to the nipple (total suckling activity) (1, 38, 42) and by the frequency of the suckling episodes (10). To this end, behavioral observations were made of five mother-infant pairs from 09:00 to 17:00 on 3–5 consecutive days, both before and during bromocriptine treatments (total 9 pairs of 3- to 5-day series) by seven different observers in 2-h shifts. The animals were familiar with all seven observers and were apparently not disturbed by their presence in the recording room, as indicated by the observation that between suckling episodes the foster mothers let the infants roam and play in the cage. In these sessions the clock time at which attachment and detachment from the nipple occurred was recorded along with the duration of the periods when no assessment could be made because the observer’s view of the infant was obstructed. The duration of these “no assessment” intervals never exceeded 10% of the total observation time and was subtracted from the latter before the percentage values were calculated. A suckling bout was considered to be completed when the infant interrupted nipple contact for ≥60 s, whereas interruptions shorter than 60 s were treated as within-episode breaks (10). Nutritive suckling could not be distinguished from nonnutritive suckling.

Because nighttime suckling has been reported to play an important role in the stimulation of PRL release and in the maintenance of the suppression of the HPO axis (1, 22), we have attempted to extend our observations beyond the day by using a surveillance camera connected to a videotape recorder and a dim red light source. Although the single infant so observed appeared to spend virtually the entire night attached to the nipple, no reliable assessments could be made because a clear view of the infant’s face could only be obtained for a small fraction of the total observation time, a finding that forced us to abandon this approach.

Bromocriptine Treatment and Supplemental Feeding

The role of PRL in the lactational suppression of GnRH pulse generator activity was studied by daily bromocriptine administration (0.1 mg·kg⁻¹·day⁻¹ bromocriptine given subcutaneously immediately after the blood sample was taken between 1600 and 1700). The bromocriptine was dissolved in a propylene glycol-based solution as described previously except that the final concentration of the drug was 2.5 mg/ml. This regimen rendered PRL undetectable in the blood samples routinely taken 24 h after the injections in all animals and did not inhibit GnRH pulse generator activity or affect reproductive hormone levels in cycling nonlactating animals (e.g., see Fig. 3, days 121–149). That the blockade of PRL secretion was also complete at night was verified in a lactating monkey by taking blood samples once every 3 h for 24 h. The duration of these treatments was 35 days in 10 of the 12 trials performed and at 48 and 54 days in the remaining two studies.

Because lactation was also inhibited by the administration of bromocriptine, the infants were bottle fed with a commercially available liquid infant primate diet (Primilac; Bio-Serv, Frenchtown, NJ). The formula was offered in a volume of 25 ml twice a day (32). As a control measure this feeding regimen was continued throughout the entire study whether or not the animals were lactating.

Data Analysis

All statistical analyses were performed using the Sigma-Stat Statistical Analysis System, Version 1.01 (Jandel Scientific, Sausalito, CA). Nonparametric tests were used when the assumptions for the parametric test were violated. The following analyses were used: Student’s t-test (paired and unpaired), Mann-Whitney rank-sum test, one-way repeated measures ANOVA followed by all-pairwise multiple comparisons (Student-Newman-Keuls method), and Pearson product moment correlation. The probability value of P < 0.05 was used as a cutoff for statistical significance. For the purpose of data presentation and statistical analysis, serum hormone concentrations below the limit of detectability were assigned the value of the minimum detectable level.

RESULTS

Ovary-Intact Animals

Control cycles. Before adoption, GnRH pulse generator activity, pituitary and gonadal hormone secretion in
all five intact rhesus monkeys studied, displayed time courses characteristic of ovulatory menstrual cycles in this species (15, 28). These patterns are illustrated in Figs. 2 (days 22–44), 3 (days −64–0), and 4 (days −34–1), beginning with the first day of a control menstrual cycle. MUA volley frequency increased during luteolysis and achieved a plateau frequency of 1.00–1.50/h by midfollicular phase. The rise in serum estradiol concentrations late in the follicular phase arrested GnRH pulse generator activity for ~12–24 h and elicited the midcycle LH surge. After this sharp decline, MUA volley frequency resumed at a low rate (0.17–0.42/h) under the influence of progesterone secreted by the corpus luteum. GnRH pulse generator activity was lower at night in the follicular but not in the luteal phase. Circulating PRL was undetectable or very low (<10 ng/ml) throughout the cycle.

Suckling and PRL levels during lactation. The young started suckling within 1–2 h after they were introduced to and accepted by the foster mothers. PRL levels rose dramatically within 24 h (Fig. 2, day 45) and milk secretion became detectable within 1–6 days. When blood was taken both in the morning and in the evening (3 mothers, 7 trials), PRL was significantly higher in the evening samples [78.1 ± 8.8 (evening) vs. 54.3 ± 11.5 (SE) ng/ml (morning); P < 0.001, paired t-test]. The infants started to eat solid food between 32 and 71 days of age, but in most cases their access to the chow was limited by the restrictive behavior of the foster mothers. Between 26 and 227 days of infant age (the time frame within which most of our bromocriptine trials were performed), daytime suckling activity declined from 76% (63–86%, median and 25–75 percentiles) of time spent on the nipple to 40% (38–41%) (P < 0.001, Mann-Whitney rank-sum test), whereas the frequency of suckling episodes remained unchanged [4.0/h (2.0–6.1/h) vs. 4.3/h (3.6–5.0/h); median 25–75 percentiles]; P > 0.05; Mann-Whitney rank-sum test]. The morning and evening PRL levels remained relatively stable over the same period [Fig. 2, days 45–170 (morning); Fig. 4, days 1–205 (evening)]. When PRL concentrations during the first 50 days of infant age were compared with those measured during another 50- to 60-day interval around the age of 180 days, no significant differences were found [73.8 ± 12.7 vs. 81.3 ± 7.1 (SE) ng/ml; P > 0.05; paired t-test]. This constancy of PRL levels with time in the face of falling daytime suckling activity probably reflects a constant

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**Fig. 2.** Daytime (i.e., lights on 0700–1900) MUA volley frequency, morning (0800–0900) prolactin (PRL), LH, estradiol (E2), and progesterone (P4) concentrations during lactational suppression of hypothalamo-pituitary-ovarian (HPO) axis in a representative ovary-intact rhesus monkey. Open symbols, hormone levels at or below limit of detectability. Figure starts with day 1 of control menstrual cycle. Presence of suckling infant is indicated by open horizontal bar on top of figure; abscissa denotes age of infant (i.e., infant age was 44 days at time of adoption).
high nighttime suckling activity (1), a notion that is also supported by our observation that even relatively old babies remain attached to the nipple whenever asleep. In two foster mothers, PRL levels were found to decline after 300 days of infant age.

Effects of nursing on GnRH pulse generator activity, LH, and gonadal hormone levels. The effects of nursing on GnRH pulse generator activity, LH, and gonadal hormone levels are illustrated in Fig. 2 and summarized in Table 1. In all ovary-intact animals, suckling significantly suppressed GnRH pulse generator activity relative to the frequencies observed during both the follicular and the luteal phases of the control menstrual cycles (Table 1). MUA volley frequency remained significantly reduced during both the day and night. Pearson product moment correlation indicated a significant negative correlation between daytime pulse generator activity and morning PRL levels ($r = -0.606, P < 0.02$), the correlation between nighttime MUA volley frequencies and evening PRL concentrations did not reach statistical significance ($r = -0.479, P > 0.05$).

Although the inhibition of pulse generator activity could be observed within a couple of days after the initiation of suckling in both the luteal (Fig. 2, from day 44) and the follicular phases (Fig. 3, days 1–34), in some experiments the introduction of the infant in the late luteal or early follicular phase did not cause a suppression of MUA volley frequency until the subsequent luteal phase. In these instances ($n = 3$, not shown), the nursing animals underwent an apparently normal follicular phase and had an LH surge, and the inhibition of pulse generator activity specifically due to suckling could be recognized by the fact that despite an early decline in progesterone secretion, MUA volley frequency did not increase.

The inhibition of GnRH pulse generator activity was reflected in an obvious but statistically insignificant reduction of LH levels in the daily blood samples (Table 1). Estradiol was significantly suppressed but never became undetectable, whereas progesterone re-

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1 In a single animal that had been artificially inseminated, GnRH pulse generator activity was continuously monitored by radiotelemetry for 16 mo throughout pregnancy, postpartum lactation, and the recovery of ovarian cyclicity after weaning. MUA volleys disappeared 18 days after insemination, and pulse generator activity remained completely suppressed during pregnancy (177 days, day 1 being the day of insemination) except for 5 days when one or two MUA volleys/12 h were observed. During lactation, pulse generator activity remained within the range of 0 and 0.25 MUA volleys/h. After weaning the infant on postpartum day 193, MUA volley frequency increased to normal follicular phase levels within 4 days.
mained under the limit of detection in most experiments (Table 1). In the two foster mothers that were monitored beyond ~300 days of infant age, both GnRH pulse generator activity and ovarian cyclicity recovered spontaneously at ~350 days (see Fig. 2, from day 340).

Effects of bromocriptine inhibition of PRL secretion during suckling. Figures 3 and 4 illustrate the typical responses to bromocriptine treatments, and the results are summarized in Fig. 5 and in Tables 2 and 3. Although daily bromocriptine administration suppressed serum PRL levels to or below the limit of detectability, neither suckling activity (62 ± 9% of time on the nipple vs. 63 ± 6%, means ± SE, bromocriptine vs. prebromocriptine, P > 0.05, paired t-test) nor the frequency of suckling episodes (3.2 ± 0.6 vs. 4.3 ± 0.6/h, P > 0.05, paired t-test) changed significantly relative to prebromocriptine levels despite the fact that lactation was suspended and the infants had already begun eating solid food by the time of these observations. In three control experiments performed in three cycling animals, this bromocriptine regimen did not inhibit day- or nighttime GnRH pulse generator activity (P > 0.05; Student’s t-test; e.g., Fig. 3, days 121–149), had no effect on LH, estradiol, and progesterone levels (P > 0.05, Student’s t-test, or Mann-Whitney rank-sum test), and did not seem to affect the duration of the menstrual cycle.

In six trials involving four individual mother-infant pairs, despite the bromocriptine-induced fall of PRL secretion below the limit of detectability, GnRH pulse generator activity remained unambiguously and significantly inhibited for the duration of the six bromocriptine treatments (24–35 days) relative to the frequencies observed during the follicular phase of control menstrual cycles [Fig. 3 (days 34–66), Fig. 5A, and Table 2] and no ovulation occurred. Although it was evident, by observation, that GnRH pulse generator activity increased in each bromocriptine trial relative to the levels recorded during the preceding lactation (Fig. 5A), statistically significant differences were only detected at night (Table 2). In any case, this rise in pulse generator activity was insufficient to support ovulation.

In another six trials involving two foster mothers and four infants, the suppression of PRL secretion and lactation by bromocriptine administration resulted in a rapid increase in GnRH pulse generator activity peaking 4–19 days after the initiation of the daily injections and a consequent resumption of folliculogenesis and ovulation [Fig. 4 (days 34–49, 74–98, and 147–176), Fig. 5B, and Table 2]. Maximum MUA volley frequen-
Panel no. 1730, Fig. 5, yr apart (responses in experiments performed approximately 1 to support ovulation.

Table 1. Effect of nursing on hypothalamic-pituitary-ovarian axis

<table>
<thead>
<tr>
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<th>Control Menstrual Cycle</th>
<th></th>
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<th>Lactation</th>
<th>P</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Follicular</td>
<td>Luteal</td>
<td>phase</td>
<td>phase</td>
</tr>
<tr>
<td>GnRH pulse generator activity, MUA volleys/h or LH pulses/h†</td>
<td>1.17±0.05a</td>
<td>0.24±0.02a</td>
<td>0.09±0.04f</td>
<td>&lt;0.001</td>
<td>0.24±0.02a</td>
</tr>
<tr>
<td>Day</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Night</td>
<td>0.94±0.04a</td>
<td>0.23±0.03a</td>
<td>0.07±0.02a</td>
<td>&lt;0.001</td>
<td>0.23±0.03a</td>
</tr>
<tr>
<td>LH, ng/ml†</td>
<td>15.9±2.7</td>
<td>22.6±6.0</td>
<td>7.4±3.4</td>
<td>NS</td>
<td>22.6±6.0</td>
</tr>
<tr>
<td>PRL, ng/ml†</td>
<td>3.7±0.9</td>
<td>4.2±0.7</td>
<td>48.0±16.3</td>
<td>&lt;0.001</td>
<td>4.2±0.7</td>
</tr>
<tr>
<td>Morning sample</td>
<td>(0800–0900)</td>
<td>(4)</td>
<td>(4)</td>
<td></td>
<td></td>
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<tr>
<td>Evening sample</td>
<td>(1600–1700)</td>
<td>(3)</td>
<td>(3)</td>
<td></td>
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<tr>
<td>Estradiol, pg/ml</td>
<td>85.2±7.0</td>
<td>57.6±5.3</td>
<td>27.5±3.2</td>
<td>&lt;0.001</td>
<td>57.6±5.3</td>
</tr>
<tr>
<td>Progesterone,</td>
<td>NA</td>
<td>4.3±0.6</td>
<td>0.3±0.0</td>
<td>&lt;0.002</td>
<td>4.3±0.6</td>
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<tr>
<td>ng/ml†</td>
<td>(5)</td>
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Values are means ± SE; n = no. of foster mothers. In each adopting female mean values were calculated for each study phase as described. *Because gonadotropin-releasing hormone (GnRH) pulse generator activity fluctuates during normal cycle (see results) multiunit electrical activity (MUA) valley frequency was determined in follicular phase for 5 days before beginning of estradiol peak and in luteal phase for 5 days beginning 3 days after luteinizing hormone (LH) peak. When pulse generator activity was determined from LH pulses in the serum, the average pulse frequency (in pulses/h) during one or more 10-h blood sampling time courses during each cycle phase was used. During lactation, data were included starting with day when suppression of GnRH pulse generator activity could no longer be attributed to inhibition by either estradiol or progesterone (see results for more detailed explanation). †PRL data encompass the entire follicular or luteal phase [LH, prolactin (PRL), estradiol] or luteal phase only (progesterone), excluding days of midcycle LH and estradiol peaks. During lactation, data from day of adoption + 1 or from last day of bromocriptine administration + 2 to end of untreated suckling period was used (range 20–126 days). Paired t-test (progesterone) or 1-way repeated-measures ANOVA (all other). a,b,c,Columns not sharing the same superscript are significantly different (P < 0.05; all-pairwise multiple comparisons, Student-Newman-Keuls method). NS, not significant; NA, not analyzed.

Ovariectomized Animals

Suckling in long-term ovariectomized animals (n = 5) did not have any effect on GnRH pulse generator activity and LH levels (Fig. 6). Serum PRL concentrations during lactation in the gonadectomized monkeys did not differ significantly from those in the ovari-intact animals (34.1 ± 8.9 vs. 50.4 ± 14.1, respectively; means ± SE; morning samples only; P > 0.05; Student’s t-test).

One of the ovariectomized foster mothers was given an infant soon after ovariectomy (35 days). Suckling initially suppressed GnRH pulse generator activity to ~30% of control levels and LH also decreased (Fig. 7, days 11–35), but this response later became attenuated (Fig. 7, days 65–102) and was completely lost ~150–160 days after gonadectomy (around day 130 in Fig. 7). During the initial suppression, bromocriptine treatment restored MUA volley frequency and increased LH secretion (days 36–60).

DISCUSSION

The results obtained with bromocriptine administration to nursing animals demonstrate that in the rhesus monkey suckling alone can inhibit GnRH pulse generator activity and ovarian cyclicity even in the absence of maternal PRL secretion. That the bromocriptine regimen used in this study did not inhibit the pulse generator and LH secretion (9, 19) was clearly demonstrated in control experiments using nonnursing monkeys. Nevertheless, in some of the suckled animals, GnRH pulse generator frequency promptly increased and ovarian cyclicity resumed when PRL secretion was abolished by bromocriptine administration. Behavioral observations performed during the day hours in three of these six trials clearly indicated that the recovery of the HPO axis in these instances was not due to reduced suckling activity. Because nursing restricted to the daytime only has been reported to maintain the suppression of the HPO axis for >80 days in monkeys (11),
there is no reason to believe that an unrecognized decrease in nighttime suckling activity, an unlikely scenario in this species (1), could have been responsible for this response. A causal relationship between the decline in PRL and the increase in GnRH pulse generator activity was also indicated by the promptness of the latter after the suppression of PRL secretion. Therefore, it can be concluded that, whereas suckling-induced suppression of pulse generator activity can occur in the absence of PRL in some circumstances, PRL may play a critical role in the induction of lactational anovulation in others, but it should be emphasized that the small number of animals involved in the present study does not permit generalization regarding the relative frequency of the two response types in this species.

Further analysis of the effect of the bromocriptine-induced reduction in PRL secretion revealed that the response of the HPO axis was not an all-or-none phenomenon. GnRH pulse generator activity being obviously (and at night significantly, see Table 2) increased even in the trials that did not result in ovulation and that peak GnRH pulse generator frequencies seemed to form a continuum across the two ovarian response types (see Fig. 5). In the animals that did not ovulate, these moderate elevations in pulse generator frequency did support some follicular development as indicated by a significant increase in serum estradiol levels (data not shown). These findings therefore strongly suggest that in this species both PRL-dependent and -independent factors participate in suckling-induced suppression of GnRH pulse generator activity and ovarian cyclicity albeit the relative importance of these factors can vary from case to case and even in the same animal. Because it has been shown that the inhibition of gonadotropin secretion by nursing...
Table 2. Effect of bromocriptine-induced inhibition of PRL secretion during suckling on GnRH pulse generator activity in rhesus monkeys that did not ovulate and that ovulated during treatment

<table>
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<tr>
<th>Control Menstrual Cycle</th>
<th>Luteal phase</th>
<th>Lactation</th>
<th>Bromocriptine</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monkeys that did not ovulate during treatment</td>
<td>Folllicular phase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GnRH pulse generator activity, MUA volleys/h or LH pulses/h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day (n)</td>
<td>1.26 ± 0.03 a</td>
<td>0.23 ± 0.01 b</td>
<td>0.09 ± 0.05 b</td>
<td>0.21 ± 0.07 b</td>
</tr>
<tr>
<td>Night (n)</td>
<td>0.98 ± 0.06 a</td>
<td>0.23 ± 0.04 b</td>
<td>0.06 ± 0.03 b</td>
<td>0.15 ± 0.03 b</td>
</tr>
<tr>
<td>Monkeys that did ovulate during treatment</td>
<td>Folllicular phase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GnRH pulse generator activity, MUA volleys/h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day (n)</td>
<td>0.99 ± 0.08 a</td>
<td>0.27 ± 0.07 b</td>
<td>0.05 ± 0.02 b</td>
<td>0.69 ± 0.14 c</td>
</tr>
<tr>
<td>Night (n)</td>
<td>0.85 ± 0.10 a</td>
<td>0.24 ± 0.07 b</td>
<td>0.10 ± 0.02 b</td>
<td>0.63 ± 0.11 c</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = nos. of mother-infant pairs. Note that one foster mother showed both response types suckling 2 different infants. Another adopting female displayed the same response (ovulation) with 3 different infants. For additional data see legend for Table 1. In the case of bromocriptine treatment without revival of ovarian activity, data were included starting with the day when PRL levels became undetectable. P values are 1-way repeated-measures ANOVA. a-b Columns not sharing the same superscript are significantly different (P < 0.05; all-pairwise multiple comparisons, Student-Newman-Keuls method).

is independent of antecedent events such as pregnancy and delivery (30), the conclusions of the present study performed in foster mothers are also applicable to normal postpartum lactation.

We have attempted to identify the cause of the aforementioned variability in the role of PRL by comparing some uncontrolled variables relevant to suckling-induced anovulation (10, 34, 43) between the two groups with different ovarian responses to bromocriptine administration (see Table 3). These analyses, however, failed to reveal compelling explanations for this phenomenon (see RESULTS). Of particular interest is the observation that, unlike in the rat (13, 21, 34), the stage of lactation does not seem to affect the sensitivity of the HPO axis to bromocriptine inhibition of PRL secretion. Although older foster mothers tended to be more likely to ovulate in response to bromocriptine treatments, this association did not reach statistical significance. Interestingly, the same monkeys also tended to suckle the infants with fewer interruptions, a finding that confirms a report by Gomendio (10) but conflicts with another by Wilson et al. (43). The differ-

Table 3. Comparison of some parameters between groups of adopting mothers that displayed different ovarian responses to inhibition of PRL secretion by bromocriptine administration during suckling

<table>
<thead>
<tr>
<th>Did Not Ovulate</th>
<th>n</th>
<th>Ovulated</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adopting mothers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, yr</td>
<td>9.8 (9.5–10.3)</td>
<td>5 a</td>
<td>14.5 (13.5–15.0)</td>
<td>6</td>
</tr>
<tr>
<td>Time from adoption to bromocriptine treatment, days</td>
<td>53.2 ± 6.4</td>
<td>6</td>
<td>79.5 ± 26.0</td>
<td>6</td>
</tr>
<tr>
<td>Serum PRL before bromocriptine treatment, ng/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morning sample (0800–0900)</td>
<td>33.6 (16.1–54.9)</td>
<td>3</td>
<td>78.5 (71–88.2)</td>
<td>4</td>
</tr>
<tr>
<td>Evening sample (1600–1700)</td>
<td>62.1 ± 9.6</td>
<td>4</td>
<td>94.7</td>
<td>1</td>
</tr>
<tr>
<td>Serum PRL during bromocriptine treatment, ng/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morning sample (0800–0900)</td>
<td>2.5 ± 0.0</td>
<td>2</td>
<td>2.8 ± 0.2</td>
<td>4</td>
</tr>
<tr>
<td>Evening sample (1600–1700)</td>
<td>2.6 ± 0.1</td>
<td>4</td>
<td>2.7</td>
<td>1</td>
</tr>
<tr>
<td>Nursing history</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infants</td>
<td>2, 1, 1, 1, &gt;1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total suckling activity during bromocriptine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute values, % time on nipple</td>
<td>59.1 ± 11.1</td>
<td>6</td>
<td>69.4 ± 18.5</td>
<td>3</td>
</tr>
<tr>
<td>% of prebromocriptine</td>
<td>93.0 ± 11.9</td>
<td>6</td>
<td>106.3 ± 6.7</td>
<td>3</td>
</tr>
<tr>
<td>Suckling frequency during bromocriptine, bouts/h</td>
<td>4.1 (3.6–4.6)</td>
<td>6</td>
<td>2.5 (0.8–4.2)</td>
<td>3</td>
</tr>
<tr>
<td>Sex</td>
<td>1F, 3M</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Values are means ± SE or median (25–75 percentiles); n = no. of mother-infant pairs (PRL, see explanation in legend to Table 2) or number of bromocriptine trials (all other). Latter method became necessary because, due to nature of some parameters (age, time from adoption to bromocriptine) or to existence of trends that appeared to be nonlinear when bromocriptine treatments were repeated in same mother-infant pair (suckling parameters), it was inappropriate to calculate a mean across bromocriptine trials. a On first day of each bromocriptine treatment. b Number of infants mothers delivered and nursed before adopting babies. c Number of suckling episodes preceded by period without nipple contact > 60 s. d One animal’s birth record is unavailable. e Mann-Whitney rank-sum test. f Student’s t-test. g Same animal displaying 2 different responses with 2 adopted infants. h Full history unavailable.
ence between the two reports is probably due to the use of a different statistical procedure to define a “suckling bout” (10). Whether these statistically insignificant differences in suckling frequency and in the age of the foster mother are relevant to the degree of recovery of the GnRH pulse generator and of the ovary in response to the inhibition of PRL secretion remains to be determined.

Our observation that PRL can be a critical factor in lactational anovulation is at variance with an earlier view (22, 32) that this hormone plays but a minor role in this phenomenon. In our previous experiments (32) none of six bromocriptine-treated nursing animals with undetectable circulating PRL responded to an estradiol benzoate-challenge with an LH surge, whereas all four of the weaned, bromocriptine-treated controls did, suggesting that PRL is inconsequential in the lactational suppression of the HPO axis. At present, we have no compelling explanation for the discrepancy between our earlier finding and the findings of the present study, but considering that a full recovery of the HPO axis in response to the suppression of PRL secretion may be relatively infrequent and given the small number of animals involved, it is possible that such a response could simply have been missed by chance.

The role of PRL in lactational anovulation is also unclear in women (22). A causative role has been suggested on the basis of an association between the duration of hyperprolactinemia and the duration of amenorrhea (7), although this relationship could be indirect, with PRL levels reflecting the intensity of suckling (23). More convincing is the observation that high bioactive PRL levels can forecast a prolonged lactational anovulation even though suckling activity in the group with long amenorrhea is not different from that in mothers who experience a shorter period of suppressed ovarian cyclicity (3). Similarly, an increased release of PRL in response to suckling was observed in women with prolonged lactational amenorrhea (8), although this was not confirmed by another group (36). Among nonprimate species considerable differences seem to exist with respect to the relative roles of suckling and PRL. In lactating cows and sows PRL does not seem to play a major role in postpartum anovulation because bromocriptine treatment did not appear to influence LH secretion (40, 41) or has but marginal effects compared with those of weaning (2). In lactating cows partial suppression of PRL secretion by bromocriptine administration also did not advance the onset of estrus (6, 25). That suckling may be a more important factor in lactational anovulation is suggested by the observation that bromocriptine treatment did not advance the onset of estrus (6, 25).

Fig. 6. MUA volley frequency, PRL, and LH concentrations during lactation in a representative long-term ovariectomized rhesus monkey; lack of inhibition of GnRH pulse generator activity. See Fig. 2 for details.

Fig. 7. MUA volley frequency, PRL, and LH concentrations during lactation; gradual loss of lactational inhibition of GnRH pulse generator activity after ovariectomy. Day 0 corresponds to day 24 after castration. Cf. Figs. 2 and 3 for further details.
factor than PRL is suggested by the finding that suckling markedly increases the postpartum interval to first ovulation when compared with machine milking, although PRL levels are the same in both circumstances (5). On the other hand, in lactating sheep, the reflex discharge of PRL in response to the suckling stimulus, but not its basal release, has been implicated in the suppression of the HPO axis (16). In rats, unlike in monkeys (present results), the relative importance of suckling per se and of PRL is clearly dependent on the stage of lactation (13, 21, 34). During early and midlactation, the inhibition of PRL secretion does not result in the rapid return of estrus (13) or recovery of LH secretion (20) seen after pup removal. Similarly, bromocriptine inhibition of PRL secretion does not completely (34) or even partially (21) prevent the suppression of the postcastration rise in LH or the inhibition of LH secretion in response to the reintroduction of suckling after a period of pup separation (20, 33). On the other hand, in late lactation, when suckling activity is reduced, the lowering of PRL levels increases LH secretion (20), advances estrus (13, 20), and abolishes the inhibition of the postcastration rise in circulating gonadotropin concentrations (21, 34).

The present results, obtained either with the monitoring of electrophysiological correlates of GnRH pulse generator activity or by detecting LH pulses in the peripheral circulation, are in complete agreement with the generally accepted notion that the suppression of the HPO axis by suckling per se and/or by PRL is primarily due to the inhibition of this hypothalamic signal generator (reviewed in Ref. 22). In some species lactation, in addition to inhibiting pulse generator activity, can also affect the functionality of the gonadotropes by attenuating their responsiveness to GnRH (reviewed in Ref. 22). This mechanism was clearly demonstrated by a study employing simultaneous sampling of pituitary portal and jugular blood in lactating ewes with suppressed pulse generator activity, showing the occurrence of considerably more GnRH than LH pulses in the same animals (44). Indeed, in rats, the decrease in pituitary responsiveness to endogenous GnRH during lactation has been attributed to a downregulation of GnRH receptors subsequent to the decrease of GnRH secretion per se (35). On the other hand, a recent report by Cardenas and Ramirez (4) showed an insignificant decrease in pulse generator activity and in the rate of GnRH secretion during lactation as measured by detecting pulses of GnRH in push-pull perfusates of pituitary glands of rats suckling 4–15 pups, suggesting a “decoupling” of GnRH and LH secretion by mechanisms independent of changes in GnRH release. Although it is unclear at present what underlies the striking discrepancy between this and other studies in the same species (22, 35), this finding underscores the importance of direct monitoring of GnRH pulse generator activity in the investigation of such complex physiological situations.

Our data confirm the observation by Gordon et al. (12) that in long-term ovariectomized cynomolgus monkeys, suckling is unable to suppress GnRH pulse generator activity and LH secretion. We have also confirmed their finding that there are no significant differences in PRL levels between the suckled ovary-intact and long-term ovariectomized groups (12), indicating that the striking dissimilarity of the hypothalamic response to nursing in the presence and absence of the ovaries cannot be explained on this basis. Whether ovarian estradiol production or other ovarian factors are involved in this phenomenon remains to be investigated.

Contrary to the absence of an inhibitory effect of nursing on the hypothalamic-hypophysial apparatus in long-term ovariectomized monkeys, suckling is initially able to suppress the rise in gonadotropin secretion that follows castration (12, 39), but this suppression eventually declines despite the maintenance of suckling (12). Our present finding in a recently ovariectomized foster mother that suckling-inhibited GnRH pulse generator activity can be demonstrated for up to 150–160 days after castration (Fig. 7), but not thereafter, is in accord with these earlier observations. That this decline in the suppression of pulse generator activity cannot be attributed to a decrease in suckling intensity with time is demonstrated by the finding that the adoption of newborns, whose suckling is the most intense (see results), does not inhibit hypothalamic activity in chronically ovariectomized monkeys (see Fig. 6).

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Address for reprints: E. Knobil, Lab. for Neuroendocrinol.

REFERENCES

SUCKLING, PRL, AND GnRH PULSE GENERATOR ACTIVITY


