Biol Res 29: 259-265 (1996)

Maintenance of hypothalamic GnRH release during lactation in the rat: a push-pull perfusion study

HUGO CARDENAS[1] and VICTOR D RAMIREZ[2]

[1] Departamento de Ciencias Biológicas,
Universidad de Santiago de Chile, Santiago, Chile
[2]Department of Physiology and Biophysics, University
of Illinois at Urbana-Champaign, Urbana, IL, USA

The activity of the gonadotropin-releasing hormone (GnRH) pulse generator during lactation was assessed by direct determination of GnRH levels impinging upon the pituitary gland. Sprague-Dawley rats were implanted on day 15 of pregnancy with a push-pull perfusion cannula directed to the anterior pituitary. All implanted animals showed normal parturition, maternal behavior and lactation. Push-pull perfusions were performed in 15 rats suckling 11.0 ± 0.8 pups (range 4-15) on day 7-20 of lactation and repeated on diestrous 1 after weaning in some of the same animals. GnRH content of the samples was assayed by RIA. GnRH pulses were clearly detected during lactation. Mean GnRH secretion rate was 1.9 ± 0.3 pg/10 min (x ± SE, range between 0.5 and 3 pg/10 min) and interpulse interval was 37.5 ± 1.7 min (range between 27 and 50 min). There was a significant decrease of about 19% in the interpulse interval after weaning. There was no significant difference in GnRH pulse amplitude nor in GnRH secretion rate between lactation and diestrous. These results demonstrate that nursing does not suppress the GnRH pulse generator in the rat.

Key terms: *GnRH, hypothalamus, lactation, pituitary, push-pull perfusion, rat*

INTRODUCTION

As in most mammals, lactation in the rat serves the dual role of supplying nutrition for the young and spacing out the interval between successive births (Short, 1984; McNeilly, 1994). During lactation in this species there is a decrease in pituitary gonadotropin-releasing hormone (GnRH) receptor levels coincident with a sharp decrease in pituitary luteinizing hormone (LH) mRNA levels and total suppression of pulsatile LH in the peripheral circulation. These processes are restored promptly upon removal of the suckling stimulus (Lee *et al*, 1989a; Smith and Lee, 1989). Pulsatile GnRH administration restores pituitary GnRH receptors and pulsatile LH secretion in the presence of the suckling stimulus and hyperprolactinemia (Lee *et al*, 1989b), implying that decreased pituitary LH production during lactation might reflect decreased hypothalamic GnRH release (Lee *et al*, 1989b, c). However, hypothalamic GnRH content and GnRH mRNA levels are not suppressed during lactation (Marks *et al*, 1992) which raises the possibility that reduced levels of pituitary GnRH receptors do not reflect reduced outflow of hypothalamic GnRH. To ascertain whether this interpretation is correct we decided to directly measure the hypothalamic GnRH release during lactation using the push-pull perfusion (PPP) technique.

Correspondence to: Dr Hugo Cárdenas, Departamento de Ciencias Biológicas, Universidad de Santiago de Chile, Casilla 307, Santiago 2, Chile. Phone: (56-2) 681-8511. Fax: (56-2) 633-6204. E-mail: hcardena@lauca.usach.cl.

MATERIAL AND METHODS

Animals

Sprague-Dawley rats weighing 250-300 g (Holtzman, Madison, WI) were housed in a temperature controlled (22° C) room under a 14:10 light:dark cycle with lights on at 05:00 h. Food and water were available at libitum. Rats were implanted with a push-pull cannula (PPC) directed towards the anterior pituitary on day 15-16 of pregnancy, as previously described (Park and Ramírez, 1989). Preliminary experiments indicate that this procedure does not interrupt pregnancy nor affects subsequent lactation. Some of the implanted animals were not submitted to PPPs because they lost their PPC, and others were not included in the final report because autopsy revealed that the tip of the PPC was not located in the anterior pituitary (see below).

Push-pull cannula implantation

A push-pull cannula was constructed according to Park and Ramirez (1989) with some modifications. To increase the GnRH levels in the perfusates outer cannulae of bigger diameter than previously reported by Park and Ramirez were used. Outer cannulae were 20 gauge and 12 mm long, with a removable stylet (29 gauge) protruding about 0.5 mm whereas the push cannulae (29 gauge) protruded only about 0.2 mm. On day 15-16 of pregnancy, rats were anesthetized with a ketamine-acepromazine solution (10:1 v/v) at 9 mg/100 g body weight. Chronic outer cannulae with a removable stylet were stereotaxically implanted with the tip directed toward the anterior pituitary according to coordinates of DeGroot (1959); the cannula was positioned along the midline 3.5 mm posterior to the bregma and 0.5 mm to the right from midline, lowered to the base of the skull, raised 0.6 mm and secured with dental acrylic. The rats were autopsied after the experiments and the position of the cannulae verified by visual examination of the pituitary gland under a dissecting microscope. Only data from rats whose cannulae were properly implanted were included in the experiment.

Push-pull perfusion

Rats were transported from the animal facilities to the laboratory and handled during at least 4 consecutive days before the experiments to habituate them to the procedures. The PPPs began at around noon. For the perfusion the stylet was replaced by an inner cannula assembly connected to two peristaltic pumps with equal flow rates. Push-pull perfusions were performed using a modified Krebs-Ringer-Phosphate solution: 126 mM NaCl, 4.9 mM KCl, 0.28 mM $CaCl_2$, 1.23 mM $MgSO_4$ and 12.3 mM sodium phosphate buffer. Bacitracin was added at a concentration of 14.2 mg/100 ml and the pH was adjusted to 7.4. The flow rate of the PPPs varied from 15 to 20 µl/min between the different experiments but it was constant within each experiment. There was no relationship between the perfusion flow and the GnRH levels in the perfusates (data not shown). Samples were collected every 10 min and immediately prepared for storage (see below). Upon completion of the PPP the inner cannula assembly was replaced by the stylet and the animal returned to the animal quarters.

One successful PPP was performed during days 7-20 of lactation in each animal. Following weaning and after at least one normal postweaning cycle an additional PPP was performed on diestrous 1. Between both PPPs the animals were submitted to handling and transportation to the laboratory to maintain their habituation to the experimental procedures. Pups born from mothers submitted to PPPs were slightly larger than those from intact controls (data not shown). The adequacy of lactation in the animals implanted with PPC and submitted to perfusions was verified by the weight of the pups at day 10 and at weaning. Pups nursed by implanted mothers grew faster than pups nursed by intact control rats. On day 10 the body weight of pups nursed by intact rats was 23.1 ± 0.4 g (n = 36 pups, 3 mothers) versus 24.8 ± 0.4 g (n = 24, 3 mothers) in pups born from implanted rats ($p = 0.028$, Wilcoxon test). On day 21 (the day of weaning) pups reared by intact rats weighed 53.9 ± 1.0 g (n = 36 pups, 3 mothers) versus 64.2 ± 0.8 g in the experimental group (n =

63 pups, 7 mothers) ($p = 0.0001$, Wilcoxon test).

On the other hand, in most animals there were regular oestrous cycles after weaning, an additional evidence of normal pituitary function in the rats submitted to PPPs. It has previously been shown that repeated PPPs in the same rat do not disrupt oestrous cyclicity or GnRH release (Park and Ramírez, 1989).

Characterization of nursing activity

Nursing during the experiments occurred in episodes interrupted sometimes by the eating and drinking activities of the mother. At the beginning of the nursing episodes most of the pups engaged in frenetic sucking. However, as the episode progressed sucking usually became asynchronous with different pups loosing and regaining connection with the nipples at different times. Because it was not possible to assess the exact number of pups connected to nipples without overt disturbance of the mother and pups, a nursing episode was defined as a period of mother-pups contact in which at least one pup was sucking, and both the starting and ending times of the episodes were recorded.

Radioimmunoassay (RIA)

GnRH was determined by RIA using a highly specific polyclonal antibody developed by Ramirez *et al* (Chen-Ramirez Ab R11 B73). Samples were acidified to 0.1 N HCl immediately after collection, centrifuged at 3000 RPM during 20 min, and the supernatant kept frozen until the assay. Before the RIA, the pH of the samples was adjusted to 6.0-7.4 with 1 N NaOH and 30 µl of 0.01 M phosphate buffer (pH = 7.4). The sensitivity of the assay varied between 0.2 and 1.0 pg/tube. The intra-assay coefficient of variation was about 5%, and all the samples collected from a given animal were measured in a single assay. The inter-assay coefficient of variation was about 15%.

Analysis of data

The GnRH pulsatile pattern was described using the PULSAR program (Merriam and Watcher, 1982) adapted for a microcomputer (Gitzen and Ramírez, 1987). The SAS Statistical System version 6.07 was used for the statistical analysis. Differences were considered significant at $p < 0.05$. Results are given as mean ± SE.

RESULTS

GnRH release during lactation

Fifteen out of 20 implanted rats were successfully perfused once on different days of lactation. The other five rats lost their implants presumably because the PPC sometimes became entrapped in the metallic cover of the cages. Most rats displayed exploratory behavior during the first 30-60 min after replacement of the stylet by the PPP assembly. After that initial period the mothers resumed their nursing activity with sporadic spontaneous interruptions to drink and eat.

The mean number of pups was 11.0 ± 0.8 (range 4-15) and nursing activity encompassed 51 ± 8 % (n = 15) of the duration of the PPPs. GnRH pulses were clearly discernible during lactation, their mean interpulse interval was 37.5 ± 1.7 min. Results from two representative cases are shown in Figure 1. Despite of suckling during most of both perfusions, a decrease in the frequency of pulses or mean GnRH secretion rate along the experiments was not observed. One of the rats nursed 10 pups (Fig 1A) and had an average interpulse interval of 36 min, the other nursed 15 pups and showed an average interpulse interval of 47 min (Fig 1B). The lack of acute effects of suckling upon the pattern of GnRH release is further illustrated in Figure 2 which shows GnRH levels in two rats that displayed 3 short nursing episodes each during the perfusions. Despite the brief initial period of hyperactivity associated with PPP cannula placement there was no obvious difference in the GnRH release pattern between the first and second half of the PPPs (for example see Figs 1 and 2). The mean GnRH secretion rate was 1.9 ± 0.3 pg/10 min during lactation.

There was a statistically significant lengthening of the GnRH interpulse interval as the number of pups increased (Kendall's τ

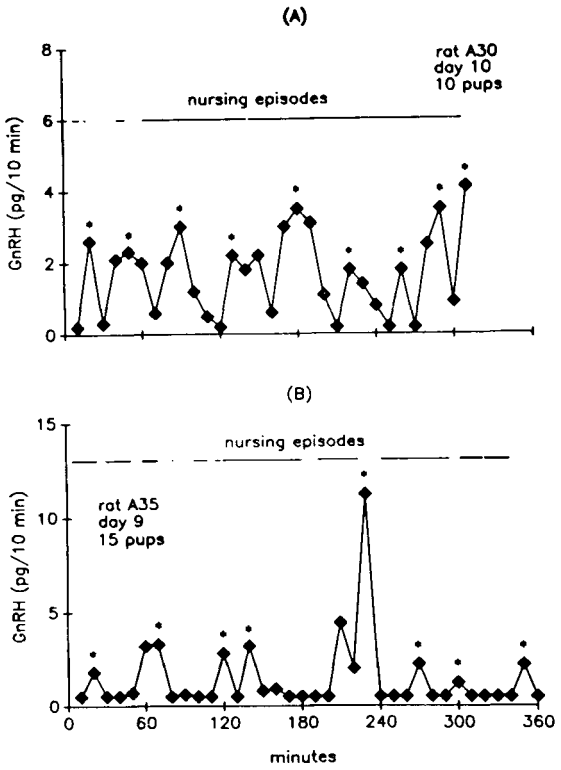

Fig 1. GnRH levels in pituitary perfusates from two lactating rats. Each horizontal line over the experimental points indicates a nursing episode. A) Rat A30 was on day 10 of lactation and nursing 10 pups. Average interpulse interval and mean GnRH secretion rate were 36 min and 1.67 pg/10 min, respectively. B) Rat A35 was on day 9 of lactation and nursing 15 pups. Average interpulse interval and mean GnRH secretion rate were 47 min and 1.42 pg/10 min, respectively. Note the different scales in both graphs. In this and subsequent figures, asterisks indicate pulses identified by Pulsar program.

= 0.410; p = 0.039) (Fig 3A), rats suckling 4-5 pups had an interpulse interval of about 30 min which increased to about 50 min in rats nursing 15 pups. However there was no significant influence of the number of pups upon the mean GnRH secretion rate (Fig 3B).

The frequency of nursing episodes during the experiments did not influence the GnRH interpulse interval (Fig 4A), but showed a significant negative influence on the mean GnRH secretion rate (Kendall's τ = -0.529; p = 0.006) (Fig 4B).

The GnRH interpulse interval and secretion rate were not correlated with the total nursing time or day of lactation (data not shown).

GnRH release during diestrous

Six out of the 15 rats perfused during lactation were also perfused during diestrous 1 after weaning. In these animals, the GnRH interpulse interval decreased by 19% from 38.3 ± 3.3 min (n = 6) during lactation to 30.6 ± 2.7 min during diestrous 1 (paired t = 2.786; d.f. = 5; p = 0.019). The mean GnRH secretion rate was 3.1 ± 0.9 pg/10 min during diestrous 1, that is 63% higher than during lactation but the difference did not reach statistical significance. The pulse amplitude was 2.1 ± 0.7 pg during lactation and 2.3 ± 0.6 pg during diestrous. GnRH levels in pituitary perfusates from two rats perfused during lactation and diestrous are shown in Fig 5.

DISCUSSION

The results presented above provide the first report on the pattern of GnRH release during

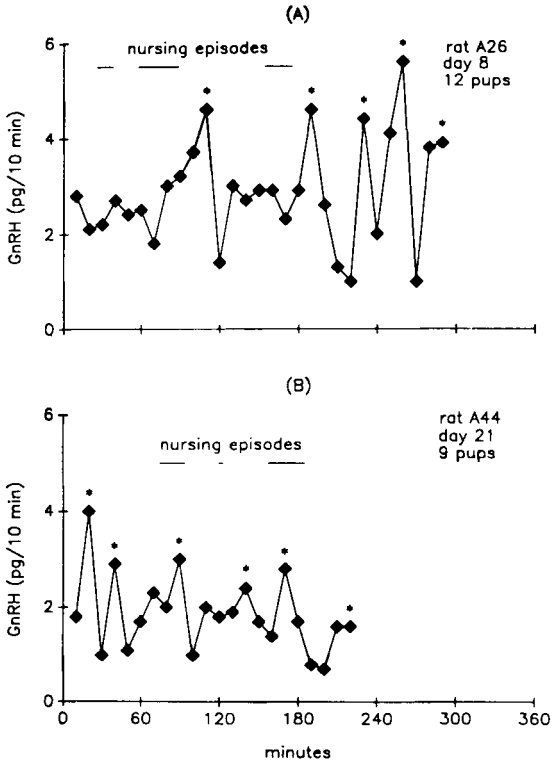

Fig 2. Lack of acute effect of short nursing episodes upon GnRH secretion rate. Each horizontal line over the experimental points indicates a nursing episode. A) Rat A26 was on day 8 of lactation and nursing 12 pups. Note GnRH pulses beginning at the end of 2nd and 3rd nursing episodes. B) Rat A44 was on day 21 of lactation and nursing 9 pups. Note that GnRH pulses occurred during 1st and 3rd nursing episodes.

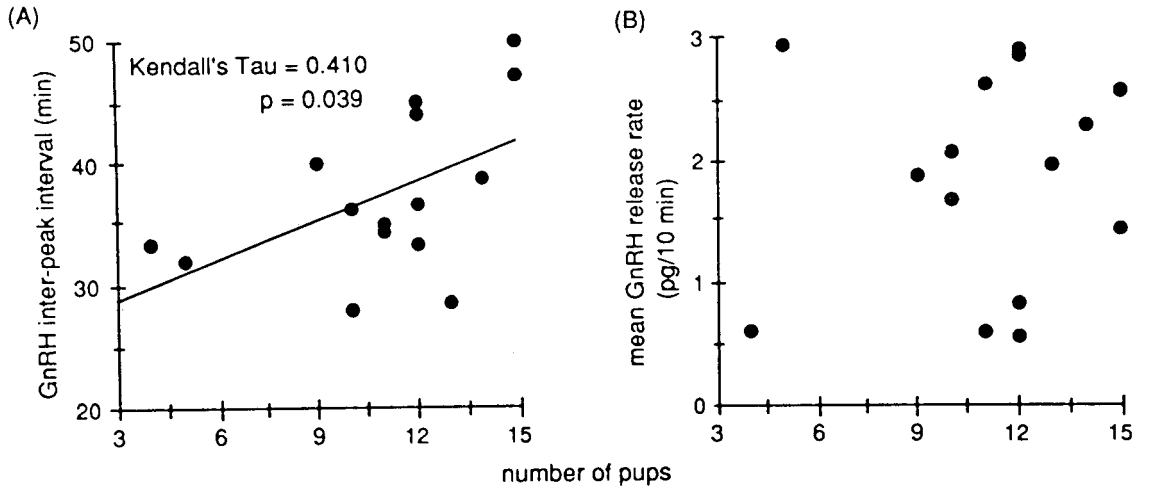

Fig 3. Relationships between number of pups and GnRH interpulse interval (A) and mean GnRH secretion rate (B). Number of pups was positively correlated with length of interpulse interval, but it had no correlation with mean GnRH secretion rate. Least squares line in A drawn by standard linear regression, but statistical significance evaluated by non-parametric Kendall's τ. Each point represents data obtained from different animals.

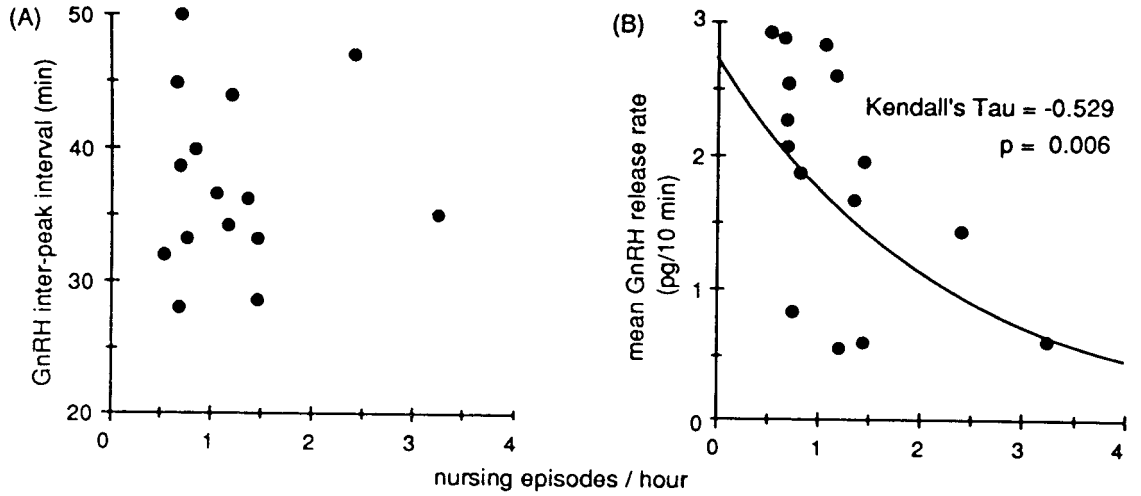

Fig 4. Relationships between frequency of nursing episodes and GnRH interpulse interval (A) and mean GnRH secretion rate (B). Frequency of nursing episodes had no correlation with GnRH interpulse interval, but it was negatively correlated with mean GnRH secretion rate. Least squares line in B drawn by standard exponential regression, but statistical significance evaluated by non-parametric Kendall's τ.

lactation and the characteristics of the suckling stimulus affecting GnRH secretion in the rat. Lactation was associated with high activity of the GnRH pulse generator with an average interpulse interval of 38 min ranging from 35 to 50 min in rats nursing 4-5 to 15 pups, respectively.

The number of pups, *i.e.*, the strength of sucking, was negatively correlated with the frequency of GnRH pulses but had no relationship with the mean secretion rate. On the other hand, the frequency of nursing episodes was negatively correlated with the mean GnRH secretion rate but not with the GnRH pulse frequency. To our best knowledge this is the first evidence of influence of frequency of nursing upon hypothalamic GnRH secretion.

After weaning, the interpulse interval decreased slightly by 19%, from 38.3 ± 3.3 min to 30.6 ± 2.7 min, without significant changes in mean GnRH secretion rate. Three

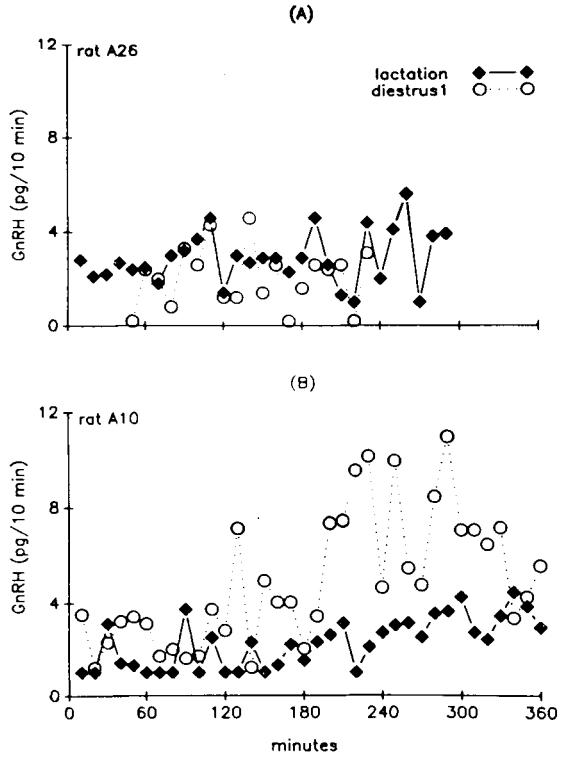

Fig 5. GnRH levels in pituitary perfusates from two rats perfused during lactation and diestrous 1. A) Rat A26 was on day 8 of lactation and nursing 12 pups. This is a representative case of 3 rats showing no significant changes in GnRH secretion rate after weaning. Mean GnRH secretion rates during lactation and diestrous 1 were 2.88 pg/10 min and 2.56 pg/10 min, respectively. B) Rat A10 was on day 7 of lactation and nursing 14 pups. This is a representative case of 4 rats showing increases in GnRH secretion rate during diestrous 1 compared with lactation. Mean GnRH secretion rates during lactation and diestrous 1 were 2.27 pg/10 min and 4.88 pg/10 min, respectively.

of the lactating rats in our experiments had 9-10 pups each with a GnRH interpulse interval of 34.8 ± 3.5 min (x ± SE) which is not substantially different from the interval observed in diestrous 1 rats. However, Fox and Smith (1984) reported that 8 sucking pups completely suppressed LH plasma levels both in intact and ovariectomized rats. Other factor(s) then must contribute to the hypogonadotropism observed in rats lactating 8 or more pups.

One of those factors could be hypothalamic galanin, recently shown to be a modulator of GnRH function. There are galanin-immunoreactive neurons in the mediobasal hypothalamus of the rat projecting towards the median eminence (Merchenthaler *et al*, 1990) and 63% of GnRH-positive neurons in the proestrus rat are also immunopositive for galanin (Merchenthaler *et al*, 1991). This peptide is released in pulses which are coincident or precede GnRH pulses into the pituitary-portal circulation (Lopez *et al*, 1991), produces a dose-dependent release of GnRH from hypothalamic fragments *in vitro* (Merchenthaler *et al*, 1990), and more importantly, it stimulates LH secretion by itself and potentiates the GnRH-induced release of LH from *in vitro* pituitary cells (Lopez *et al*, 1991). It has recently been shown that, contrary to what is observed in the levels of GnRH and GnRH mRNA in the hypothalamus, both galanin and its mRNA are significantly reduced in the lactating rat as compared with diestrous (Marks *et al*, 1992).

The previously reported LH interpulse interval for the diestrous rat of 40-60 min (Gallo, 1981; Higuchi and Kawakami, 1982) is longer than the GnRH interpulse interval we found for rats in the same stage of the oestrous cycle. However, the existence of 'silent' GnRH pulses, not associated with LH pulses, has been reported in the ovariectomized sheep (Clarke and Cummins, 1982; Levine *et al*, 1982), the long-term castrated ram (Caraty and Locatelly, 1988), the ovariectomized rhesus monkey (Terasawa *et al*, 1988) and the male rat (Ramírez *et al*, 1991). In the intact male rat about half of the GnRH pulses were silent whereas almost all GnRH pulses were coincident with LH pulses after orchiectomy (Levine and Duffy, 1988; Ramírez *et al*, 1991).

We did not observe acute decreases in GnRH release as result of nursing episodes. Acute decreases of plasma LH levels associated with suckling have been reported in the rat (Sirinathsinghji and Martini, 1984; Baumann and Rabii, 1991). In most studies of acute effects of suckling upon peripheral hormone levels the pups have been first separated from, and then returned to, their mother, which makes difficult to dissociate the non-specific stress of pup removal-return by the researcher from the effects of suckling *per se*. In our experiments we recorded spontaneous mother-pups separation and reinitiation of suckling which provides a more physiological approach to assess the impact of suckling, and we did not find evidence of

suckling-induced acute inhibition of GnRH release.

In summary, these experiments show that the release of hypothalamic GnRH is not suppressed during lactation in the rat, which is consistent with the lack of suppression of hypothalamic GnRH content and GnRH mRNA levels in the lactating rat (Marks *et al*, 1992), supporting the conclusion that other mechanism(s) must be involved in lactational hypogonadotropism in this species.

ACKNOWLEDGEMENTS

The support of The Rockefeller Foundation, The Lalor Foundation and the DICYT of the University of Santiago of Chile is greatly appreciated.

REFERENCES

BAUMANN MH, RABII J (1991) Inhibition of suckling-induced prolactin release by mu- and kappa-opioid antagonists. Brain Res 567: 224-230

CARATY A, LOCATELLY A (1988) Effect of time after castration on secretion of LHRH and LH in the ram. J Reprod Fertil 82: 263-269

CLARKE IJ, CUMMINS JT (1982) The temporal relationship between gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) secretion in ovariectomized ewes. Endocrinology 111: 1737-1739

DE GROOT J (1959) The rat forebrain in stereotaxic coordinates. Verhandel Koninklijke Nederl Akad Wetenschappen 52: 1-40

FOX SR, SMITH MS (1984) The suppression of pulsatile luteinizing hormone secretion during lactation in the rat. Endocrinology 115: 2045-2051

GALLO RV (1981) Pulsatile LH release during periods of low level LH secretion in the rat estrous cycle. Biol Reprod 24: 771-777

GITZEN JF, RAMIREZ VD (1987) PC-Pulsar: pulsar pulse analysis for the IBM-PC. Psychoneuroendocrinology 12: 1

HIGUCHI T, KAWAKAMI M (1982) Changes in the characteristics of pulsatile luteinizing hormone secretion during the oestrous cycle and after ovariectomy and oestrogen treatment in female rats. J Endocrinol 94: 177-182

LEE LR, HAISENLEDER DJ, MARSHALL JC, SMITH MS (1989a) Expression of alpha-subunit and luteinizing hormone (LH) beta messenger ribonucleic acid in the rat during lactation and after pup removal: relationship to pituitary gonadotropin-releasing hormone receptors and pulsatile LH secretion. Endocrinology 124: 776-782

LEE LR, PAUL SJ, SMITH MS (1989b) Dose response effects of pulsatile GnRH administration on restoration of pituitary GnRH receptors and pulsatile LH secretion during lactation. Neuroendocrinology 49: 664-668

LEE LR, HAISENLEDER DJ, MARSHALL JC, SMITH MS (1989c) Effects of progesterone on pulsatile luteinizing hormone (LH) secretion and LH subunit messenger ribonucleic acid during lactation in the rat. Endocrinology 124: 2128-2134

LEVINE JE, DUFFY MT (1988) Simultaneous measurement of luteinizing hormone (LH)-releasing hormone, LH, and follicle-stimulating hormone release in intact and short-term castrate rats. Endocrinology 122: 2211-2221

LEVINE JE, PAU K-YF, RAMIREZ VD, JACKSON GL (1982) Simultaneous measurement of luteinizing hormone-releasing hormone and luteinizing hormone release in unanesthetized, ovariectomized sheep. Endocrinology 111: 1449-1455

LOPEZ FJ, MERCHENTHALER I, CHING M, WISNIEWSKI MG, NEGRO-VILAR A (1991) Galanin: a hypothalamic-hypophysiotropic hormone modulating reproductive functions. Proc Natl Acad Sci USA 88: 4508-4512

MARKS DL, SMITH MS, CLIFTON DK, STEINER RA (1992) Regulation of gonadotropin-releasing hormone (GnRH) and galanin gene expression in GnRH neurons during lactation in the rat. Endocrinology 133: 1450-1458

McNEILLY AS (1994) Suckling and the Control of Gonadotropin Secretion. In: KNOBIL E, NEILL JD, GREENWALD GS, MARKERT CL, PFAFF DW (eds) The Physiology of Reproduction. New York: Raven Press, pp 1179-1212

MERCHENTHALER I, LOPEZ FJ, NEGRO-VILAR A (1990) Colocalization of galanin and luteinizing hormone-releasing hormone in a subset of preoptic hypothalamic neurons: anatomical and functional correlates. Proc Natl Acad Sci USA 87: 6326-6330

MERCHENTHALER I, LOPEZ FJ, LENNARD DE, NEGRO-VILAR A (1991) Sexual differences in the distribution of neurons coexpressing galanin and luteinizing hormone-releasing hormone in the rat brain. Endocrinology 129: 1977-1986

MERRIAM GR, WATCHER KW (1982) Algorithms for the study of episodic hormone secretion. Am J Physiol 243: E310-E318

PARK O-K, RAMIREZ VD (1989) Spontaneous changes in LHRH release during the rat estrous cycle, as measured with repetitive push-pull perfusions of the pituitary gland in the same female rats. Neuroendocrinology 50: 66-72

RAMIREZ VD, PICKLE RL, LIN WW (1991) In vivo models for the study of gonadotropin and LHRH secretion. J Steroid Biochem Mol Biol 40: 143-154

SHORT RV (1984) Breast Feeding. Sci Am 250: 35-41

SIRINATHSINGHJI DJS, MARTINI L (1984) Effects of bromocriptine and naloxone on plasma levels of prolactin, LH and FSH during suckling in the female rat: responses to gonadotrophin releasing hormone. J Endocrinol 100: 175-182

SMITH MS, LEE LR (1989) Modulation of pituitary gonadotropin-releasing hormone receptors during lactation in the rat. Endocrinology 124: 1456-1461

TERASAWA E, KROOK C, HEI DL, GEARING M, SCHULTZ NJ, DAVIS GA (1988) Norepinephrine is a possible neurotransmitter stimulating pulsatile release of luteinizing hormone-releasing hormone in the rhesus monkey. Endocrinology 123: 1808-1816