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The Mechanism for the Suppression of Gonadal Function during Lactation

by

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Abstract

The present dissertation describes the results of experiments demonstrating the neuroendocrine mechanism by which the suckling stimulus suppresses pulsatile luteinizing hormone (LH) secretion in lactating rats used as a model for other animals and women.

In Chapter 3, changes in pulsatile secretion of LH after removal of pups and subsequent resuckling were examined in ovariectomized lactating rats, and the change after removal of pups was compared with that after removal of ovaries in cyclic female rats. The mean LH level and the frequency and amplitude of LH pulses gradually increase after removal of pups, until after 45 h of separation the frequency reached the high level observed 6 days after ovariectomy in cyclic rats. The subsequent resuckling by pups after a 24-h separation decreased these three parameters of LH pulses rapidly. In contrast, the frequency of LH pulses was unchanged after ovariectomy in cyclic rats, although the mean LH level and the amplitude of LH pulses increased. These results suggest that the suckling stimulus suppresses pulsatile LH secretion in a different manner from that of ovarian steroids.

In Chapter 4, effects of the suckling stimulus on the daily LH surge induced by chronic oestrogen treatment were examined in ovariectomized lactating rats. Daily LH surges occurred in the late afternoon in both lactating and non-lactating rats implanted with oestradiol on day 6 (day 0 = day of parturition) or 15. The amplitude of daily LH surges in lactating rats implanted on day 6 declined much more rapidly than in non-lactating rats implanted on day 6, but no significant difference was found in the profile of the LH surge between lactating and non-lactating rats implanted on day 15. Pituitary LH contents just before the daily LH surge (12.00-12.30 h) 4 days after implantation in lactating rats implanted with oestradiol on day 6 were significantly less than those in non-lactating rats implanted with oestradiol on day 6 or 15 and in lactating rats implanted

on day 15. These results suggest that the mechanisms responsible for the oestradiol-induced LH surge were not impaired by the suckling stimulus, and that a rapid decline of the amplitude of LH surges observed in mid-lactation could be ascribed to the small amount of LH stored in the pituitary.

In Chapter 5, effects of the reduction of prolactin (PRL) release by bromocriptine (CB-154) treatment on the suppression of LH pulses were examined in ovariectomized lactating rats at mid-lactation. Pulsatile LH secretion was strongly suppressed in all lactating rats in spite of the treatment of CB-154, which abolished PRL secretion. Frequent LH pulses were observed in all non-lactating animals, indicating no direct action of CB-154 or ovine PRL on LH secretion at the doses employed. These results suggest that PRL secretion does not mediate the suppressing effect of the suckling stimulus on pulsatile LH secretion in mid-lactation.

In Chapter 6, the pulsatile LH secretion in ovariectomized lactating rats bearing complete (CD), anterior (AD), anterolateral (ALD), posterior (PD), or roof deafferentation (RD) of the hypothalamus was determined. The loss of LH pulses associated with lactation was still apparent following AD, PD and sham-deafferentation (SD); pulsatile LH secretion was, however, present in rats with CD, ALD and RD despite continued suckling. The only significant difference in plasma PRL concentrations among the various groups was a reduction in the PRL level in rats with RD in comparison to those with SD. I conclude that the neural signal responsible for the inhibition of pulsatile LH release by suckling is conveyed through the dorsal part of the hypothalamus and PRL does not mediate the suppression of LH pulses in mid-lactation.

In Chapter 7, to determine the afferent pathway of the suckling stimulus suppressing the pulsatile LH secretion, various types of roof deafferentation, i.e. large anterior (LARD), large posterior (LPRD), small anterior (SARD) and middle (MRD), or

electrolytic lesions of the paraventricular nucleus (PVN) were made in the ovariectomized lactating rats. Pulsatile LH secretion with high frequency and amplitude appeared in rats with LARD or LPRD in spite of the continuous suckling. In rats with MRD, LH pulses with small amplitude were observed when the cut was on or under the ventral margin of the PVN, but there were few LH pulses when the cut passed through the PVN. Pulsatile secretion remained suppressed in animals bearing the lesion sparing the periventricular nucleus ventral to the PVN, but the pulse with small amplitude was apparent in animals with the lesion destroying the periventricular nucleus. These results suggest that the periventricular region ventral to the PVN is the crucial pathway conveying the inhibitory inputs of the suckling stimulus toward the mediobasal hypothalamus (MBH).

The results in this dissertation demonstrate the neuroendocrine mechanism by which the suckling stimulus suppresses LH secretion during lactation; 1) the suckling stimulus suppresses pulsatile LH release in the absence of the negative feedback effect of the ovarian steroid, but not daily LH surges induced by chronic treatment of oestradiol. 2) PRL does not mediate the suppressing effect of the suckling stimulus on LH pulses. 3) the inhibitory signal emanating from the mother's teat is conveyed dorsally to the MBH through the periventricular nucleus and suppresses pulsatile LH secretion.

CONTENTS

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Finally, I am deeply grateful to my parents for their encouragement and support.

CHAPTER 1

General introduction

Reproduction is, as described by Monod (1971), a decisive aspect which discriminate between the creatures and non-creatures; the former has the reproductive ability, but the latter does not. Thus, reproduction is the essential event for all organisms including the mammal to maintain their own species. Reproductive function is affected by environmental factors surrounding the organisms. Even the paramecium, a kind of unicellular organisms, is able to change the strategy for reproduction according to the environment. Cell division is the principal strategy under the appropriate environmental condition, while the sexual reproduction is used under the unsuitable condition such as the mal-nutrition, because the sexual reproduction has the advantage of producing better posterity (Brock, 1970). It has been also known that various environmental factors (eg. pH, nutrition, temperature) affect reproduction of the bacteria (Hobson, 1969).

The reproductive function in the vertebrate is also affected by the environmental factor. In mammals, many factors originated from the circumstances have been reported to affect the activity of the reproductive system. Exposure to stress, i.e. social stimuli, immobilization and electric shock, is accompanied by disruption of reproductive functions in many species (rhesus monkey: Rose *et al.*, 1972; rat: Rivier *et al.* 1986; Briski & Sylvester, 1988), and the gonadal activity is depressed under the condition of restricted feeding (Campbell *et al.*, 1977; Pirke & Spyra, 1981; McClure & Saunders, 1985), since animals must keep their own body first until environmental conditions turn to be suitable for raising the following generation. Changes in the daylength also alter the gonadal activity of seasonal breeders; the long-day condition reduces the reproductive activity in short-day breeders, i.e. sheep, goats and rhesus monkeys, while it increases the activity in long-day breeders, i.e. hamsters, horses and birds (Turek & Campbell, 1979). The gonadal function is also affected by the physiological

condition of the animal; lactation is one of the conditions suppressing the reproductive activity.

Lactation is one of the essential phases of reproductive cycles for all mammals. The principal role of lactation is to supply milk to the newborn which is completely dependent on maternal milk during the neonatal period. Various pituitary hormones, i.e. prolactin (PRL), growth hormone, adrenocorticotrophic hormone (ACTH), oxytocin etc. (Tucker, 1988; Voogt *et al.*, 1969; Wakerley *et al.*, 1988), are released to the circulation to maintain milk production and to remove milk from the mammary gland. The other important feature of lactation is suppression of the reproductive function of mothers. Since lactation forces mothers to spend a large amount of energy and nutrients for the milk production, mothers must avoid the other reproductive events causing an expense of excessive energy. For this purpose, the animal adopts the blockade of ovulation as a strategy for the survival of the species. Actually, ovulation is blocked as long as lactation lasts in many mammalian species and it recurs immediately after weaning of pups.

There has been a considerable body of evidence which indicates that the suckling stimulus is a dominant factor inhibiting ovulation during lactation in many mammalian species. In women, the restoration of the ovarian activity and recurrence of ovulation in the post-partum period are delayed in the early puerperium (Howie & McNeilly, 1982). Similarly, the puerperium is associated with anovulation and amenorrhea in monkeys, the length of which depends on the duration and intensity of suckling (Goodman & Hodgen, 1978). In the dairy and beef cow, the inhibitory effect of suckling on the return of oestrus is also well documented (Bluntzer *et al.*, 1989). La Voie *et al.* (1981) reported that the interval between parturition and the first oestrus was shorter in cows without calves than in those with calves. In the postpartum sow, reducing the

litter size to three 5 days before weaning resulted in earlier post-weaning oestrus than that in sows weaning 8 piglets (Stevenson & Britt, 1981), and suckling increased the interval parturition (Grinwich & Mckay, 1985). The overlapping of seasonal anoestrus and lactation delayed the resumption of ovarian and oestrous activity in ewes (Mandiki, *et al.*, 1990).

The blockade of ovulation in lactating mothers has been reported to be due to suppression of the release of gonadotrophin, especially luteinizing hormone (LH). In women, secretion of LH in breast-feeding mothers is more strongly suppressed than that in bottle-feeding mothers (Glasier *et al.*, 1983). Weiss *et al.* (1976a & b) reported that the LH secretion was suppressed in intact or ovariectomized rhesus monkeys during lactation. Similarly, suckled beef cows showed lower serum LH concentrations in the early postpartum period than non-suckled beef cows (Short *et al.*, 1972; Randel *et al.*, 1976; Carruthers *et al.*, 1980), and deprivation of calves for 48 or 96 h increased basal levels of LH in mothers (Smith *et al.*, 1977). Plasma LH concentrations in postpartum sows nursing 7-11 piglets for 4 weeks were significantly lower than those nursing 2-4 piglets (Kunavongkrit, 1984). On the other hand, secretion of follicle-stimulating hormone (FSH) is not suppressed in lactating animals (Foxcroft *et al.*, 1987; Schirar *et al.*, 1990). Plasma concentrations of FSH show only a minor change after the removal of pups in rats (Taya & Sasamoto, 1980) and ovariectomy increases FSH secretion without any effect on LH secretion in lactating sows (Stevenson *et al.*, 1981). Therefore, the suckling stimulus suppresses the gonadal activity through controlling LH secretion. FSH secretion could be primarily controlled by ovarian factors (e.g. inhibin) during lactation.

In lactating rats nursing an adequate number of pups, cyclic ovulation is interrupted and vaginal dioestrus is maintained after post-partum ovulation for about 20 days

(Rothchild, 1960; Tomogane *et al.*, 1976), since maturation of ovarian follicles is suppressed during lactation (Taya & Sasamoto, 1988; Taya *et al.*, 1989). Moreover, the removal of pups or reduction of the litter size results in immediate follicular development (Taya & Sasamoto, 1980) and the subsequent LH surge (Taya & Sasamoto, 1987). When the rat is used as an experimental animal for studying the mechanism underlying the suppression of LH secretion in lactating animals, we must deserve to consider the role or roles played by the corpus luteum formed after the post-partum ovulation. The corpus luteum secreting progesterone, which can suppress LH secretion, persists in rats as long as lactation lasts (Tomogane *et al.*, 1969). Previous findings, however, suggest that the suckling stimulus can suppress the tonic LH release at early and mid-lactation in ovariectomized rats in the absence of the negative-feedback effect of ovarian steroids on LH release (Hammons *et al.*, 1973; Fox & Smith, 1984; Maeda *et al.*, 1987). These findings indicate that lactating rats ovariectomized immediately after parturition are a useful model for human and other domestic animals which have no lactational corpus luteum in investigating the mechanism of the suppression of LH secretion during lactation.

LH is known to be secreted in a pulsatile manner, reflecting the pulse of LH-releasing hormone (LHRH). Pulsatile secretion of LH is significant for the follicular maturation and occurrence of oestrous cyclicity (Knobil, 1980; Rojanasthien *et al.*, 1985). Many workers have reported that suppression of pulsatile LH secretion by the suckling stimulus in many mammalian species (rat: Fox & Smith, 1984; Maeda *et al.*, 1987; cow: Garcia-Winder *et al.*, 1984; Edwards, 1985; Hinshelwood *et al.*, 1985; Williams *et al.*, 1987; Shively & Williams, 1989; sow: Foxcroft *et al.*, 1987; Newton *et al.*, 1987; ewe: Schirar *et al.*, 1990), suggesting that the suckling stimulus directly inhibits the release of LHRH pulses at the hypothalamic level. However, little is

known about the mechanism of this suppression. It could be useful to compare the mechanism of suppression of LH secretion by the suckling stimulus with that by other factors, i.e. stress, fasting etc., to know whether or not the suckling stimulus is a specific factor for suppressing LH secretion.

The aim of the present study is to determine the mechanism by which the suckling stimulus suppresses the LH secretion in lactating animals by using ovariectomized rats as a model of the human and/or domestic animals. In Chapter 3, I demonstrated the direct suppression of the pulsatile LH release by the suckling stimulus, and compare the pattern of this suppression with that by the ovarian steroids. In Chapter 4, I attempted to answer the question whether or not the suckling stimulus could inhibit the LH surge causing ovulation as well as pulsatile LH secretion. Chapter 5 revealed that PRL did not mediate the suppressing effect of the suckling stimulus on LH secretion. In Chapters 6 and 7, I suggested the neural pathway in the hypothalamus which was involved in suppression of pulsatile LH secretion by the suckling stimulus.

CHAPTER 2

General procedures

Animals

Female Wistar-Imamichi strain rats (250-300 g) were kept under conditions of 14 h light: 10 h darkness (lights on at 05.00 h) and 22±2°C with free access to food (Labo-MR-RO; Nihon Nosan Co., Yokohama, Japan) and water. Pro-oestrous females were each placed with a male overnight and the resulting pregnant females were housed in individual maternity cages. The day of parturition was designated day 0 of the experiment. Litter size was adjusted to eight on day 1, and bilateral ovariectomy was performed on day 2. All rats were cannulated with silicon tubing (inner diameter 0.5 mm, outer diameter 1.0 mm; Shinetsu Polymer Co., Tokyo, Japan) inserted into the right jugular vein (Maeda & Tsukamura, 1989) by the day before the onset of blood sampling. The cannula was filled with 40 % polyvinylpyrrolidone (PVP, M.W. 10,000, Sigma, St. Louis, MO, U.S.A.) dissolved in saline, containing heparin sodium (200 U/ml, Shimizu Pharmaceutical Co., Ltd., Shimizu, Japan) and cephalosporin (2 mg/ml, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan), for preventing of blood coagulation and bacterial infection, respectively. All operation and surgical procedures were performed under ether anaesthesia and aseptic conditions.

Blood sampling

Blood samples were collected through the indwelling atrial cannula with a heparinized syringe. When the sampling was performed at 6-min intervals for 3 h, red blood cells, which had been taken from other female rats and washed with and resuspended in saline, were replaced following each sampling to maintain the hematocrit value constant. The plasma was separated by centrifugation immediately after blood collection and stored at -20°C until assayed for hormones.

Hormone assays

LH:

Contents of LH in 50 μl of plasma were determined by double-antibody radioimmunoassay (RIA) using a kit provided by the National Hormone and Pituitary Program (NHPP, Baltimore, MS, U.S.A.) and the antiserum to ovine LH (GDN No. 15) provided by Dr. G.D. Niswender of Colorado State University, Fort Collins, CO, U.S.A. (Niswender *et al.*, 1968). GDN No. 15 was used for plasma samples taken every 6 min for 3 h. LH concentrations were expressed in terms of the NIDDK-rLH-RP-2. The least detectable level of LH and intra- and inter-assay coefficients of variation were 1.9 pg/tube for 50 μl plasma sample, 14.4 % at the level of 0.62 ng/ml and 9.2 % at the level of 0.58 ng/ml, respectively, for the assay using GDN No. 15. They were 7.8 pg/tube, 10.7 % at the level of 7.2 ng/ml and 10.3 % at the level of 6.4 ng/ml, respectively, for the assay using the antiserum (S-9) provided by NHPP.

PRL:

Contents of PRL in 10 μl of plasma were determined by double-antibody RIA using a PRL RIA kit supplied by the NHPP and expressed in terms of NIDDK-rPRL-RP-3. The least detectable level of PRL was 0.0625 ng/tube. The intra- and inter-assay coefficients of variation for PRL assay were 5.1 % at the level of 107 ng/ml and 2.7 % at the level of 108 ng/ml, respectively.

Oestradiol:

Oestradiol levels were determined by a double-antibody RIA using ^{125}I-labelled oestradiol as described by Taya *et al.* (1985). Oestradiol was extracted from 50 μl plasma twice with diethylether. The least detectable level of oestradiol was 4.59

fmol/tube, and the intra-assay coefficients of variation at the level of 20.56 fmol/tube was 9.7 %.

Histological procedures

The brains were perfused with saline followed by 10 % formalin immediately after the sampling through the left ventricle under pentobarbitone (Nembutal, Abott Laboratories, North Chicago, IL, U.S.A.) anaesthesia. The brain was kept in a 10 % formalin over 2 days for post fixation, and subsequently kept in 30 % sucrose solution over 2 days. Coronal sections of the brain (30 μm) were made with a cryostat (Cryotome 1600, Lipshaw Manufacturing Co., Detroit, MI, U.S.A.) and stained with cresyl violet to examine the exact location of incision or lesion histologically according to the map for the stereotaxic coordinates (Paxinos & Watson, 1986).

LH pulse analysis

Pulses of LH were identified by the PULSAR computer program supplied by Drs. G.R. Merriam and K.W. Wachter (Merriam & Wachter, 1982). The following criteria were chosen in our pulse analysis. If the difference between a single LH concentration and the baseline concentration was 3.0 times greater than the standard deviation (S.D.) at the level of the baseline concentration, it was considered to be part of a LH pulse. If the differences between 2 or 3 consecutive LH concentrations and the baseline were 2.4 or 1.85 times greater than the S.D. at the baseline concentrations, they were also considered to be part of a LH pulse. The S.D. for each plasma concentration was calculated by the equation $y = (13.04x+1.4)/100$ (Chapter 3), or $y = (2.5x^2 + 2.8x + 2.4)/100$ (Chapters 5, 6 & 7); where x is the LH level and y is the S.D. for each LH level determined by assaying five series of control plasma in ten replicate.

CHAPTER 3

The suckling stimulus: The major factor for suppressing LH secretion during lactation.

-Suppression of tonic secretion of LH in ovariectomized lactating rats-

Introduction

Plasma concentrations of LH were maintained at a low level at early and mid-lactation in lactating rats ovariectomized 2 days after parturition (Fox & Smith, 1984; Maeda *et al.*, 1987), suggesting that pulsatile LH secretion can be suppressed during lactation in the absence of the negative feedback effect of ovarian steroids. Ovariectomized lactating rats could, therefore, be an excellent model in studying the mechanism involved in gonadal suppression during lactation in women and other animals.

The suckling stimulus has been reported to be a dominant factor suppressing LH secretion during lactation. Recurrence of post-partum ovulation in bottle-feeding mothers has been reported to be earlier than in breast-feeding mothers (Howie *et al.*, 1982; Glasier *et al.*, 1983). The removal of pups or reduction of litter size results in an immediate follicular development and an LH surge followed by ovulation (rat: Taya & Sasamoto, 1980; sow: Edwards & Foxcroft, 1983). The initial process, which leads to ovulation after the removal of the suckling stimulus, would be an increase in tonic LH secretion released in a pulsatile manner. However, little is known about the detailed change in pulsatile LH secretion after removal of and subsequent resuckling by pups in lactating animals. In the present chapter, the changes in pulsatile LH secretion occurring after removal of and resuckling by pups were determined using ovariectomized lactating rats at mid-lactation. In addition, the changes in lactating rats after removal of pups were compared with those in cyclic rats after ovariectomy.

Materials and Methods

Changes in LH secretion after removal of pups

Pups were removed from lactating rats for 6, 12, 18, 24 or 45 h before the onset of blood collection. Blood samples (120 μl) were taken from these mothers for 3 h at 6-min intervals on day 8 of lactation. Ovariectomized lactating rats with litters were used as controls.

Changes in LH secretion after resuckling by pups

Pups were attached to lactating rats after 24 h of separation. Blood collection for 3 h at 6-min intervals started 1, 4, 7 or 12 h after initiation of resuckling. These animals were also bled on day 8 of lactation.

Changes in LH secretion after ovariectomy in cyclic rats

Cyclic rats which had shown at least two consecutive regular 4-day oestrous cycles were ovariectomized on day 1 or 2 of dioestrus. Blood collection for 3 h at 6-min intervals started 12, 18, 24 or 48 h or 6 days after ovariectomy. Cyclic rats on day 1 of dioestrus were used as controls.

Statistical analysis

Statistical differences were determined by Duncan's multiple-range test.

Results

Changes in LH secretion after the removal of pups

Figure 3.1 shows the individual pattern of pulsatile LH secretion after the removal of pups. Plasma concentrations were maintained at a low level in control rats with pups on day 8 of lactation. Few LH pulses were observed in rats deprived of their pups for 6 h. LH pulses appeared in some animals 12 h after separation from their pups. In rats that had been deprived of their pups for 18 and 24 h, frequent LH pulses with high amplitude appeared in almost all animals. Typical LH pulses with high amplitude were observed after 45 h of separation from pups.

Mean LH levels during the 3-h sampling period remained low in rats with pups and in those deprived of their pups for 6 of 12 h (Fig. 3.2). The mean LH levels were significantly higher in rats separated from pups for 18, 24 and 45 h than in control lactating rats or those separated from pups for 6 h ($P<0.01$, Duncan's multiple-range test). The frequency and amplitude of LH pulses increased gradually as the period of separation was prolonged. The frequency after 45 h of separation was similar to that in rats 6 days after ovariectomy (Fig. 3.3).

Changes in LH secretion after resuckling

Pulsatile LH secretion disappeared in some animals and LH pulses with small amplitude were observed in the remaining animals that had been resuckled by their pups for 1 or 4 h. LH pulses were rare in animals suckled by their pups for 7 or 12 h (Fig. 3.4).

Suckling for longer than 1 h significantly lowered the mean LH level. The frequency of LH pulses was not affected by resuckling for 1 or 4 h, but was significantly reduced

by 7 or 12 h of resuckling. The amplitude was significantly lowered by resuckling for 4, 7 or 12 h ($P<0.01$,Duncan's multiple-range test). All parameters of LH pulses decreased gradually as the duration of resuckling increased (Fig. 3.5).

Changes in LH secretion after ovariectomy in cyclic rats

The individual patterns of pulsatile LH secretion after ovariectomy in cyclic rats are shown in Fig. 3.6. There were no significant differences in the frequency of LH pulses between groups (Duncan's multiple-range test), although the frequency increased very slightly from 48 h to 6 days after ovariectomy (Figs 3.3 & 3.6). The frequency was kept at a high level even during day 1 of dioestrus. The mean LH level and amplitude of LH pulses were increased significantly from 48 h to 6 days after ovariectomy ($P<0.01$, Duncan's multiple-range test).

Discussion

The results in the present chapter demonstrate that pulsatile LH secretion began to increase from 12 to 18 h after removal of pups from ovariectomized lactating rats. By 18 h after removal of pups, the mean concentration of LH reached the high level that was observed in rats 45 h after removal of pups. The frequency of LH pulses gradually increased from 6 to 45 h after removal of pups. The maximum frequency was reached after the separation of pups for 45 h, since this frequency was very similar to that in rats 6 days after ovariectomy (Figs 3.2 & 3.3). The result of a previous report in intact rats (Taya & Sasamoto, 1980), in which the LH surge occurred 3 days after removal of pups, could be explained well by present findings in ovariectomized lactating rats: the removal of the suckling stimulus induces an abrupt decrease in prolactin secretion (Haggi *et al.*, 1986) and an immediate increase in LH secretion that starts within 24 h after removal of pups, and is followed by luteolysis and follicular development. Fox & Smith (1984) reported that pulsatile LH secretion did not increase in some animals 48 h after removal of pups in Sprague-Dawley rats. Jakubowski & Terkel (1985) reported that oestrus recurred 20 days after the removal of pups in Sprague-Dawley rats. The inconsistency between the present results and theirs might be ascribed to the difference in the strain of rats used.

The resuckling by pups reduced the mean LH level and amplitude of LH pulses more rapidly than the frequency in lactating rats that had been separated from their pups for 24 h (Figs. 3.4 & 3.5). This indicates that the initial effect of the resuckling might be the reduction of the amplitude of LHRH pulses but not the frequency, since the amplitude of LH pulses was reported to be correlated with the amplitude of LHRH pulses in the sheep (Levine *et al.*, 1982). It is noteworthy that resuckling by pups suppressed LH secretion

within only 12 h.

The LH pulse frequency did not increase in cyclic rats after ovariectomy and was kept at a high level before and after ovariectomy (Fig. 3.3). The present results are consistent with previous reports in which the frequency remained unchanged during the oestrous cycle (Fox & Smith, 1985) and after ovariectomy, and the replacement of oestradiol did not affect the frequency (Weick *et al.*, 1981; Higuchi & Kawakami, 1982). The suckling stimulus reduced both the frequency and the amplitude of LH pulses, while ovarian steroids might reduce only the amplitude and not affect the frequency. These results suggest a different mechanism for the suppression of LH release during lactation from that in the suppression of LH release by ovarian steroids. Since the frequency of LH pulses could directly reflect the frequency of LHRH pulses and the activity of the putative LHRH pulse generator (Levine & Duffy, 1988), it is likely that the suckling stimulus suppresses the activity of the LHRH pulse generator more directly than do ovarian steroids. The steroid-independent inhibition of the activity of the pulse generator by the suckling stimulus is supported by findings showing that pulsatile LH secretion was suppressed in ovariectomized lactating rats (Figs. 3.1 & 3.2; Fox & Smith, 1984; Maeda *et al.*, 1987) and sows (Stevenson *et al.*, 1981).

In summary, pulsatile LH secretion appeared within 12-18 h after removing pups from ovariectomized lactating rats at mid-lactation. Parameters of pulsatile LH secretion, i.e. mean level, frequency and amplitude, recovered gradually in ovariectomized lactating rats after the removal of pups. The subsequent resuckling by pups suppressed the mean LH level and the LH pulse amplitude immediately, and the frequency of the LH pulse gradually. In contrast, the frequency of the LH pulse did not show a significant increase after ovariectomy in cyclic rats. These results suggest a different mechanism for the suppression of pulsatile LH secretion by the suckling stimulus from that by ovarian

steroids.

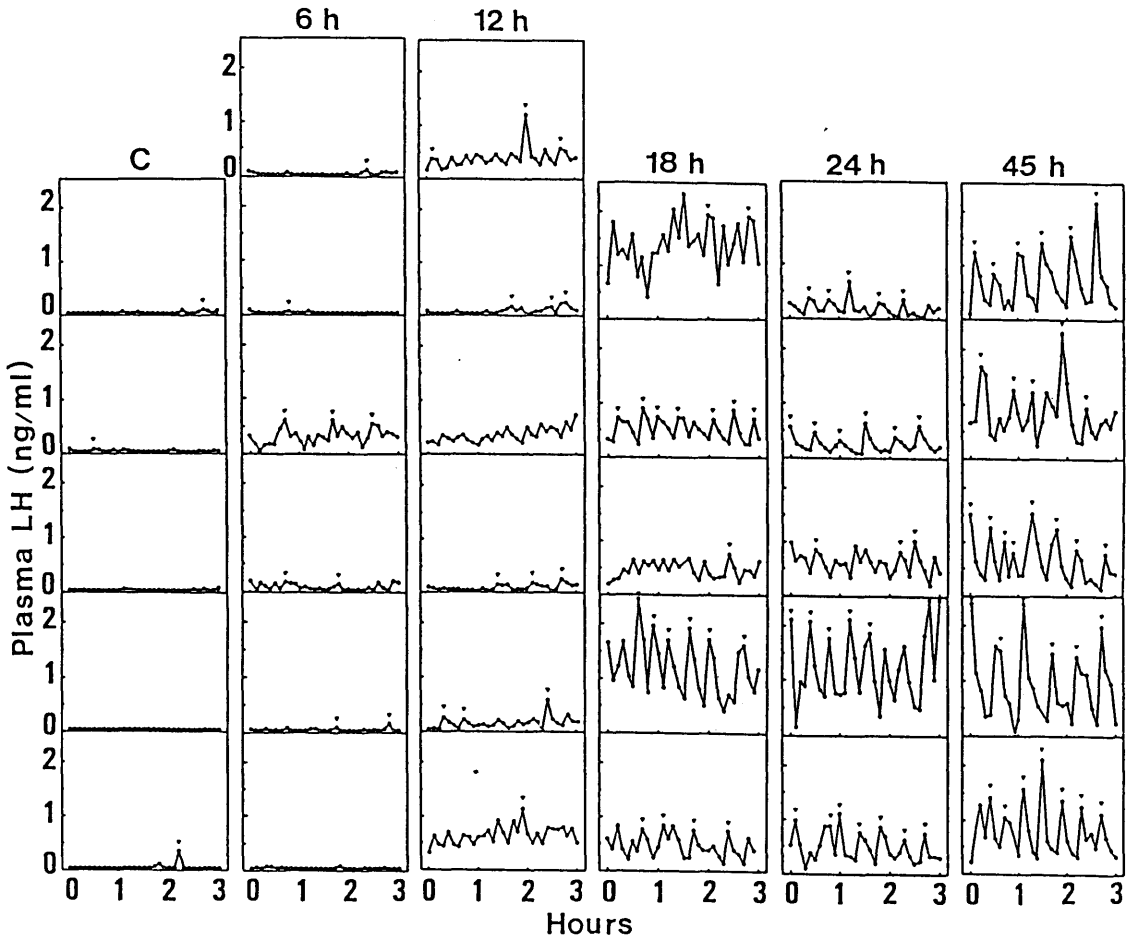


Figure 3.1. Plasma profiles of LH in individual ovariectomized lactating rats deprived of their pups for 6, 12, 18, 24 or 45 h. All rats were ovariectomized on day 2 of lactation (day 0 = the day of parturition) and blood samples were taken on day 8 of lactation at 6-min intervals for 3 h from 11.00 to 13.00 h. Arrowheads indicate the LH pulses identified with the PULSAR computer program. C; control rats which had not been deprived of their pups.

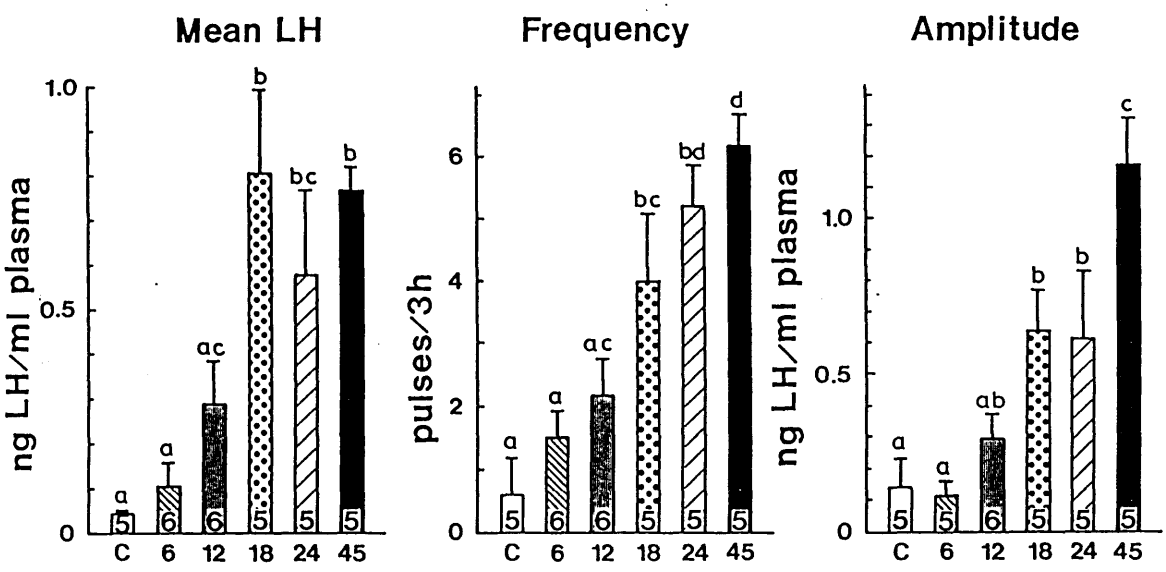


Figure 3.2. The mean LH level and the frequency and amplitude of LH pulses calculated by the PULSAR computer program in ovariectomized lactating rats deprived of their pups for 6, 12, 18, 24 or 45 h. Values are means ± S.E.M. Mean LH levels represent the means for all samples collected over the 3-h period. Numbers in each column represent the numbers of animals used. Values with different letters are significantly different from each other ($P<0.01$, Duncan's multiple-range test). C; control rats which had not been deprived of their pups.

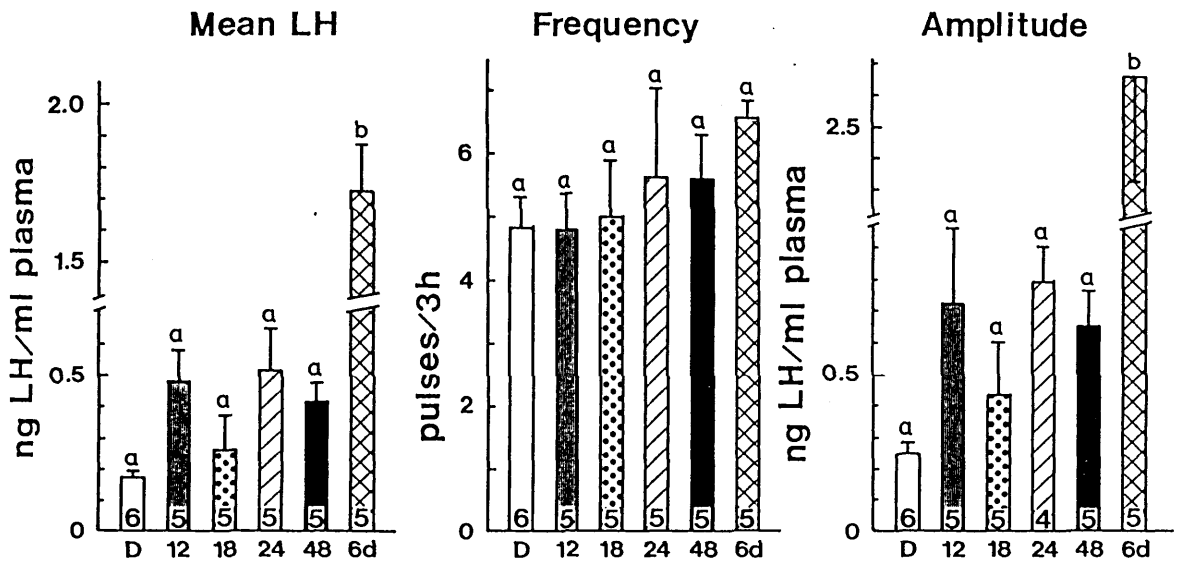


Figure 3.3. The mean LH level and the frequency and amplitude of LH pulses in cyclic rats ovariectomized for 12, 18, 24 h or 6 days. Values are means ± S.E.M. Numbers in each column represent the numbers of animals used. Values with different letters are significantly different from each other ($P<0.01$, Duncan's multiple-range test). D; control rats bled on the day 1 of dioestrus.

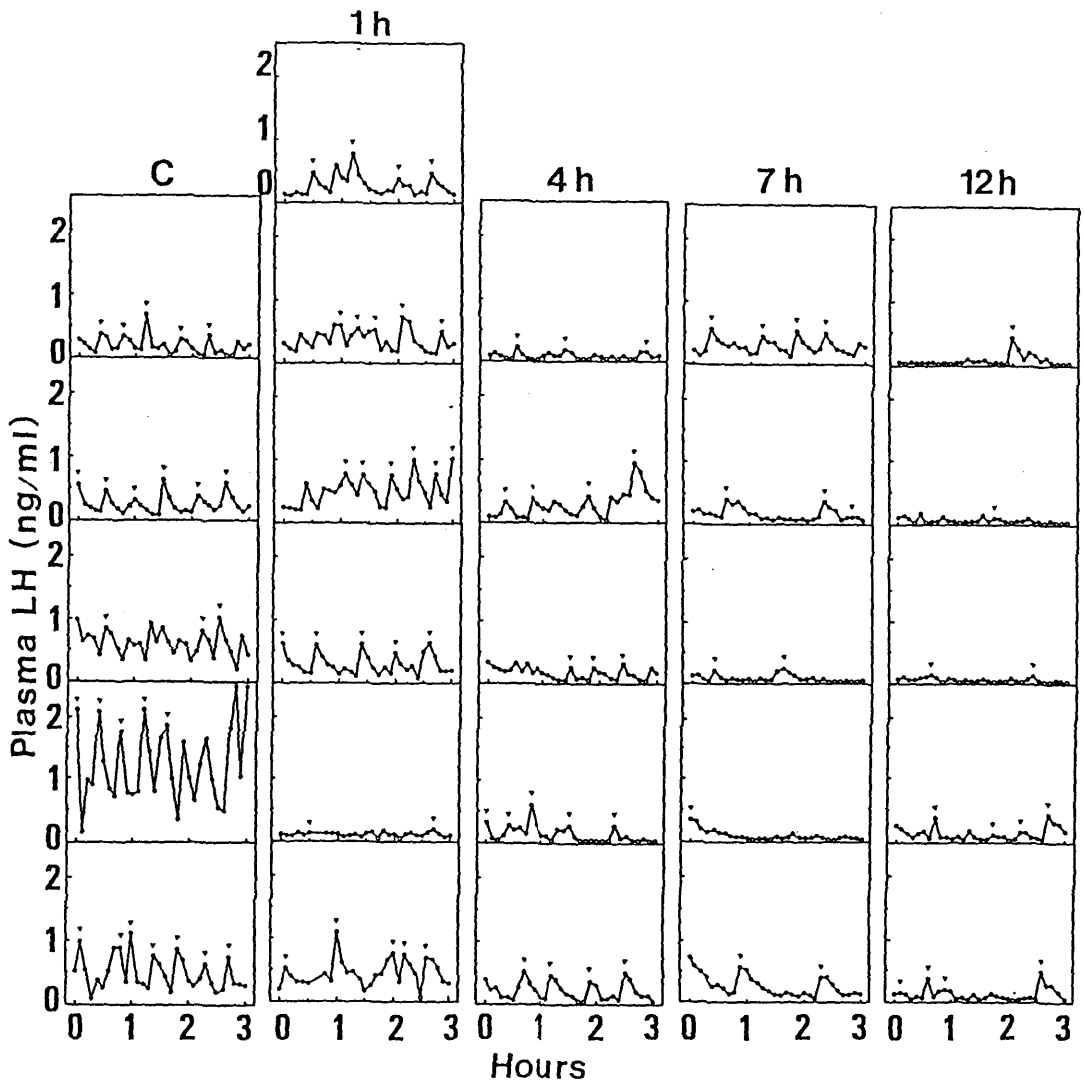


Figure 3.4. Profiles of plasma LH concentrations in individual ovariectomized lactating rats resuckled by their pups for 1, 4, 7 or 12 h after a 24-h separation from pups. All rats were ovariectomized on day 2 of lactation (day 0 = the day of parturition) and blood samples were taken on day 8 of lactation at 6-min intervals for 3 h. Arrowheads indicate the LH pulses identified with the PULSAR computer program. C; control rats which were not resuckled by pups.

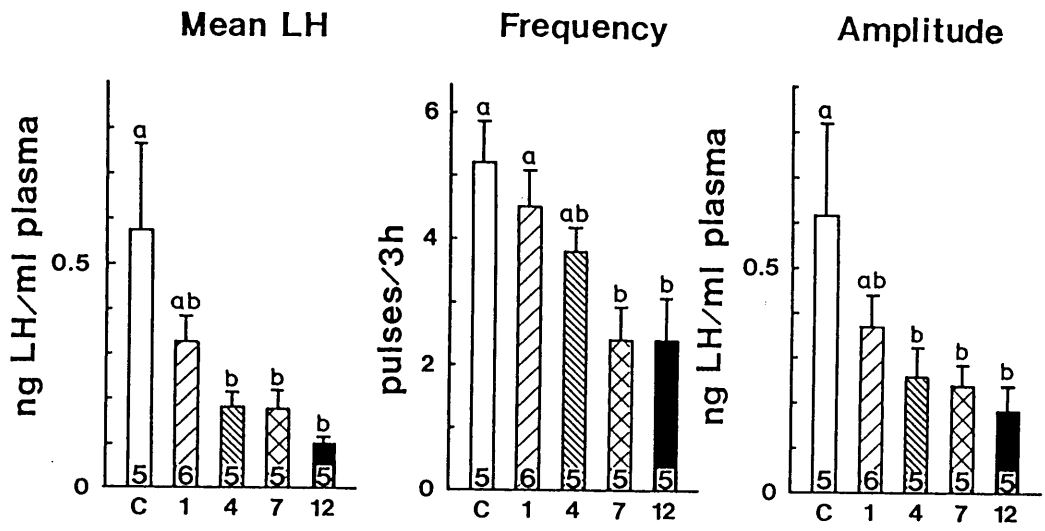


Figure 3.5. The mean LH level and the frequency and amplitude of LH pulses in ovariectomized lactating rats resuckled by their pups for 1, 4, 7 or 12 h after a 24-h separation from pups. Values are means ± S.E.M. Numbers in each column represent the numbers of animals used. Values with different letters are significantly different from each other ($P<0.01$, Duncan's multiple-range test). C; control rats which were not resuckled by pups.

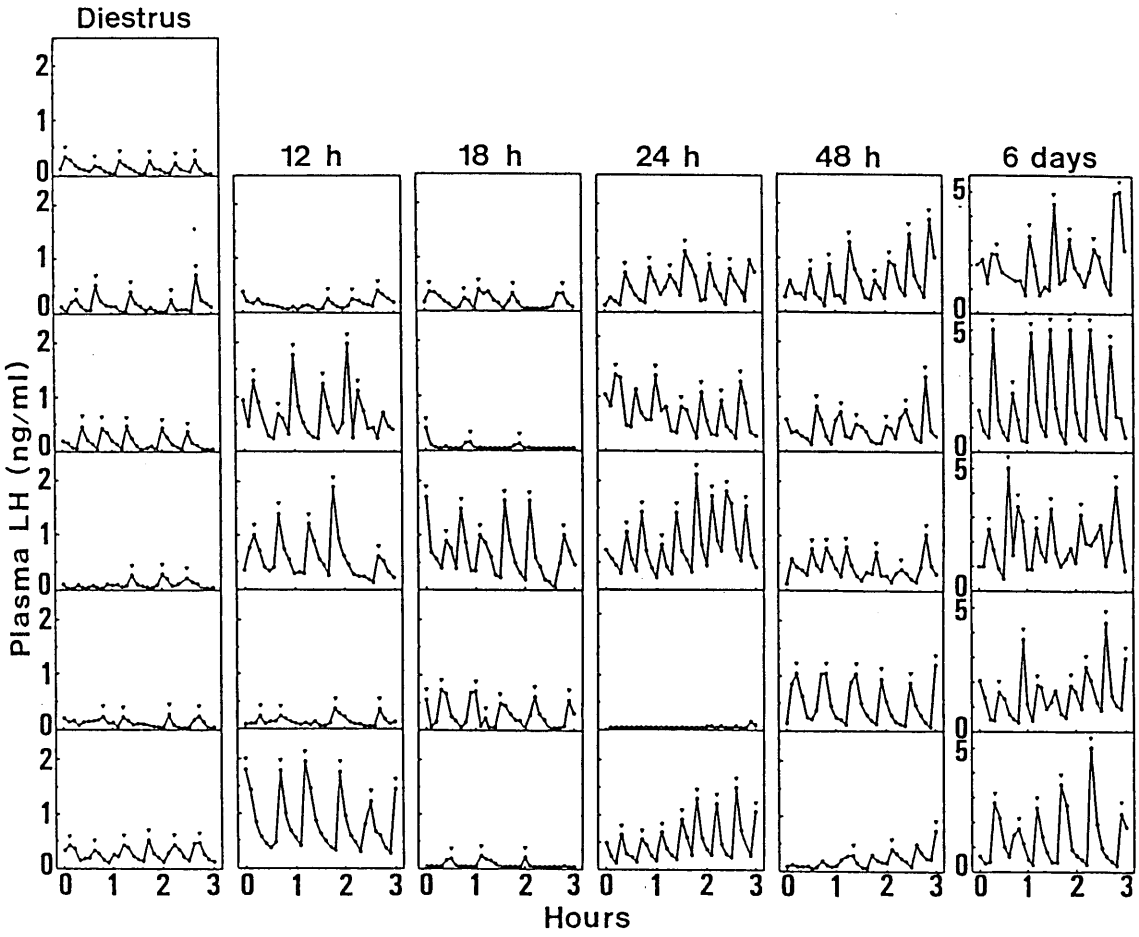


Figure 3.6. Plasma profiles of LH in rats ovariectomized for 12, 18, 24, 48 h or 6 days and in dioestrous rats. Animals were ovariectomized on the day of dioestrus and blood samples were taken at 6-min intervals for 3 h from 11.00 to 13.00 h. Arrowheads indicate the LH pulses identified with the PULSAR computer program.

CHAPTER 4

LH surges in lactating rats

-Effect of the suckling stimulus on daily LH surges induced by chronic oestrogen treatment in ovariectomized lactating rats-

Introduction

The findings in Chapter 3 suggest that the suckling stimulus can suppress tonic LH release at early and mid-lactation in ovariectomized lactating rats in the absence of the negative feedback effect of ovarian steroids on LH release.

It has been well established that there are two types of LH release (tonic and cyclic) in the cyclic female rat. These are regulated by the mediobasal hypothalamus (MBH) and the preoptic area of the hypothalamus, respectively (Gorski, 1968). Induction of an LH surge by oestrogen treatment in ovariectomized lactating rats (Smith, 1978b; Coppings & McCann, 1979) implies the possibility that the mechanism by which LH is released in response to oestrogen is not impaired in lactating animals receiving a strong suckling stimulus. In cyclic rats the biological clock is involved in regulating cyclic LH release, together with high levels of circulating oestrogen, because LH surges occur daily at a fixed time of the day in ovariectomized rats bearing chronic implants of oestrogen (Chazal *et al.*, 1977). Since the removal of the suppressive effect of the suckling stimulus on tonic LH release was observed in late lactation in ovariectomized lactating rats (Smith & Neill, 1977; Maeda *et al.*, 1987), the hypothalamic mechanism controlling the cyclic LH surge could alter its activity with the advancement of lactation.

The aim of the study in this chapter was to determine the effects of the suckling stimulus on the daily LH surge induced by chronic oestradiol treatment in mid- and late lactation in ovariectomized lactating rats.

Materials and Methods

Animals and treatments

Lactating rats deprived of their litter on day 0 served as non-lactating controls. Lactating and non-lactating ovariectomized rats were implanted subcutaneously under the dorsal skin with Silastic tubing (inner diameter 1.0 mm, outer diameter 1.5 mm, 2 cm in length; Dow Corning, Midland, MI, U.S.A.) filled with crystalline oestradiol-17β (Sigma, St Louis, MO, U.S.A.) at 17.00-18.00 h on day 6 (group 1) or day 15 (group 2). Control rats were implanted with empty tubing on day 6. Blood samples (250 μl) were collected through the indwelling atrial cannula every day at 10.00 and 17.00 h to detect the daily LH surge, since the LH concentration in the pro-oestrous rat showed a peak at 17.00 h under the lighting conditions employed in the present study (Fig. 4.1). To determine the detailed profile of LH secretion, blood samples were taken 2 and 8 days after oestradiol implantation at 1-h intervals from 10.00 to 20.00 h in both lactating groups. Trunk blood was collected at decapitation on the last day of the experiment to determine the plasma concentrations of oestradiol.

The anterior pituitary was removed immediately after killing at 12.00-12.30 h 4 days after oestradiol implantation, weighed, homogenized with 2 ml phosphate-buffered saline (pH 7.6) and centrifuged (10,000 g for 30 min). The supernatant was stored at -20°C until assayed for LH.

Data analysis

Statistical differences were determined by Duncan's multiple-range test, Student's *t*-test or two-way ANOVA.

Results

The maternal behaviour of ovariectomized lactating rats with or without oestradiol implants was similar to that of intact lactating rats. The weights of litters were not significantly different among ovariectomized, ovariectomized oestradiol-implanted and intact lactating rats throughout lactation (Duncan's multiple-range test, Table 4.1).

Plasma LH concentrations in lactating rats bearing empty capsules remained low throughout lactation. They started to increase on days 14-15 and continued to increase gradually up to the end of lactation. The LH concentration in non-lactating animals was much higher than that in lactating animals throughout the experimental period. Concentrations of LH in the morning (10.00 h) were similar to those in the afternoon (17.00 h) in both lactating and non-lactating animals implanted with empty capsules (Fig. 4.2).

Daily LH surges occurred every afternoon from the day following oestradiol implantation in both lactating and non-lactating rats of group 1. The amplitude of the daily LH surges declined much more steeply in lactating rats of group 1 than in non-lactating rats of the same group. The amplitude of LH peaks in lactating rats was very similar to that in non-lactating rats for the first 2 days, but it was significantly lower from day 9 onward in the former group than in the latter ($P<0.05$, Student's *t*-test, Fig. 4.3). Peaks of LH surges were observed between 15.00 and 17.00 h in each individual 2 days after implantation, but almost disappeared 8 days after implantation in lactating rats of group 1 (Fig. 4.4a).

In lactating and non-lactating rats of group 2, daily LH surges were observed every afternoon from 2 days after oestradiol implantation. No significant difference in the amplitude of LH peaks was observed between lactating and non-lactating animals in

group 2 until the end of the experiment (Student's *t*-test, Fig. 4.5). In lactating rats of group 2, peaks of LH surges in each individual appeared between 15.00 and 18.00 h and between 14.00 and 16.00 h 2 and 8 days after implantation, respectively ($P<0.05$, two-way ANOVA, Fig. 4.4b).

Pituitary LH contents 4 days after implantation in lactating animals of group 1 were significantly lower than those in both the non-lactating rats in group 1 and the lactating rats in group 2 (Fig. 4.6).

There was no significant difference in mean plasma concentrations of oestradiol between lactating and non-lactating rats in both groups 1 and 2 (Student's *t*-test, Table 4.2).

Discussion

The results of the present chapter clearly demonstrate that in both mid-and late lactation the daily LH surge could be induced by chronic oestradiol treatment in ovariectomized lactating rats. The suckling stimulus given to mothers in this experiment seemed to be as strong as that to the intact animals, since the weights of litters and the maternal behaviour of ovariectomized lactating rats with or without oestrogen implants were very similar to those in intact lactating rats. It is reasonable to consider that the suckling stimulus does not nullify the positive-feedback effect of oestrogen on LH release both in mid- and late lactation; in other words, the high level of oestrogen could induce LH surges at any phase of lactation even if vigorous suckling was provided.

In contrast to the surge-like secretion of LH, tonic LH secretion was strongly suppressed in mid-lactation, and the suppression was gradually withdrawn in late lactation in control rats bearing empty capsules (Fig. 4.2). This finding agrees well with that reported previously (Chapter 3; Smith & Neill, 1977; Maeda *et al.*, 1987) and shows that the suckling stimulus prevented tonic LH secretion in the early and mid-lactating period. The suckling stimulus could therefore block ovulation by disturbing follicular development through the suppression of tonic LH secretion.

Cyclic release of LH in rats is regulated by levels of circulating oestrogen and by the daily signal from the putative biological clock in the suprachiasmatic nucleus (SCN), since LH surges occur daily at a fixed time of the day in ovariectomized rats with Silastic oestrogen implants (Chazal *et al.*, 1977). That the centre for cyclic LH release is located in the medial preoptic area (MPOA) and that it integrates information concerning the level of circulating oestrogen and daily signals from the biological clock has been suggested by many workers. Goodman (1978) reported that the daily LH surge was induced only

when oestrogen was implanted in the MPOA, but not in other brain areas. Blockade of the surge by lesion of the SCN and an increase in the multiple unit activity in the MPOA before the pro-oestrous LH surge were demonstrated by Kawakami *et al.* (1970, 1980). The present results showing induction of the daily LH surge at around 17.00 h by oestrogen implants indicate that the centre for cyclic LH release in the MPOA might operate properly in vigorously suckling rats if oestrogen was provided.

The decline in the amplitude of daily LH surges in lactating animals of group 1 was more rapid than in non-lactating rats (Fig. 4.3). This might be due to more rapid exhaustion of LH from the pituitary in lactating rats compared with that in non-lactating animals, because the LH content in the anterior pituitary just before the daily LH surge (12.00-12.30 h) in lactating animals of group 1 was much less than that in non-lactating animals in the same group (Fig. 4.6). The rapid exhaustion of pituitary LH in lactating rats in mid-lactation obtained in the present experiment could be consistent with the finding of Copping & McCann (1979). They reported that the level of LH release induced by injection of LHRH on the third day of chronic oestrogen treatment in lactating rats was lower than that on the following day of the treatment. In the experiment in which oestrogen was implanted in late lactation (group 2), the profile of the change in the peak level of LH surge in lactating rats was very similar to that in non-lactating rats. The absence of a rapid decline in the amplitude of the LH surge in lactating rats of group 2 might be due to high pituitary LH contents in these rats.

The small amount of pituitary LH in lactating rats of group 1 could be caused by suppression of LHRH release in mid-lactation, since LHRH stimulates the synthesis as well as the release of LH in the pituitary (Redding *et al.*, 1972). The suppression of LHRH release in mid-lactation is deduced from the observation that tonic LH secretion is maintained at a low level in this stage of lactation (Fox & Smith, 1984; Maeda *et al.*,

1987). The increased release of LHRH in late lactation, resulting from withdrawal of the suppressing effect of the suckling stimulus on the hypothalamus, could promote pituitary LH production and pulsatile LH release (Maeda *et al.*, 1987). Thus the LH stored in lactating rats of group 2 increased to compensate for the daily release of large amounts of LH, although the LH stored in lactating rats of group 2 was significantly less than that in non-lactating animals in the same group.

In conclusion, cyclic LH release produced both in mid- and late lactation by chronic oestradiol treatment in ovariectomized lactating rats indicates that the suckling stimulus could not impair the mechanism involved in the cyclic LH surges, and the steeper decline in amplitude of daily LH surges caused by oestrogen implantation in mid-lactating rats might be ascribed to the smaller contents of pituitary LH in these animals compared with those in late lactating animals.

Table 4.1. Weights of litters (of eight pups) nursed by intact rats and by ovariectomized (OVX) rats, with or without oestradiol implants, on days 10, 14 and 18 of lactation.

	Weight of litter (g)		
	Day 10	Day 14	Day 18
OVX + oestradiol	157.8± 8.7[a] (6)[b]	190.8±15.8 (6)	219.6±11.8 (4)
OVX	152.3±12.2 (6)	208.5±14.8 (6)	250.4±33.7 (6)
Intact	144.5±22.9 (5)	208.4±26.4 (5)	251.4±34.3 (4)

The day of parturition was designated day 0 of lactation. Empty implants were given on day 6. There were no significant differences between treatments on each day of lactation (Duncan's multiple-range test). [a]Means ± S.D. [b]Numbers of litters used.

Table 4.2 Plasma oestradiol concentrations (nmol/l) in lactating or non-lactating rats implanted with oestradiol on day 6 (group 1) or 15 (group 2).

	Lactating	Non-lactating
Group 1	1.70±0.83	2.43±1.02
Group 2	1.56±0.31	1.86±0.34

Values are means ± S.D. (n = 8 in each group). Blood samples were collected on day 20-23 (day 0 = the day of parturition). There is no significant difference between lactating and non-lactating rats within the same group (Student's *t*-test)

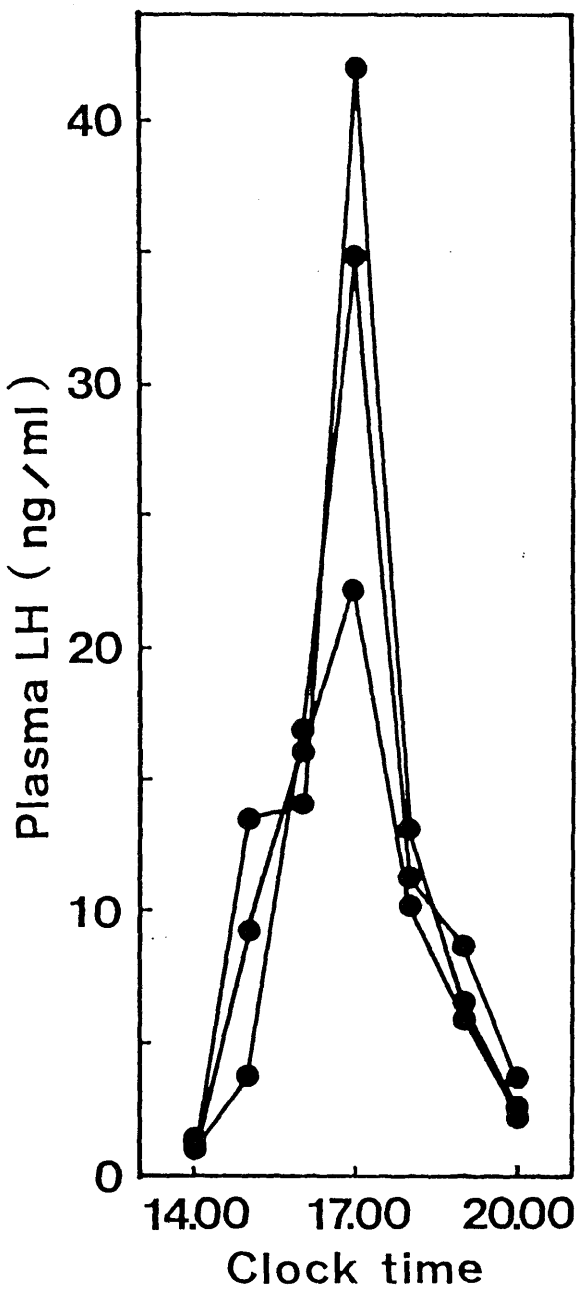


Figure 4.1. Preovulatory LH surges in individual pro-oestrous rats (n = 3). Blood samples were taken at 1-h intervals from 14.00 to 20.00 h on the day of pro-oestrus.

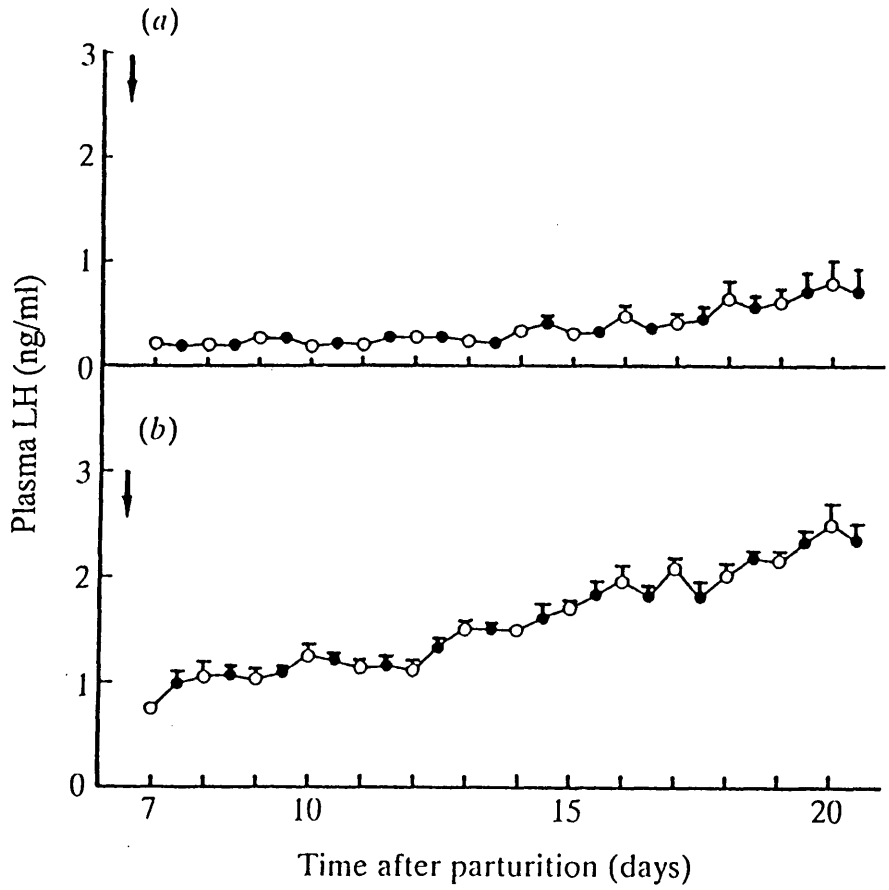


Figure 4.2. Plasma LH concentrations in ovariectomized (a) lactating and (b) non-lactating rats (n = 8 in each group) implanted with empty tubing on day 6 (arrows; day 0 = the day of parturition). In non-lactating rats, pups were removed from their mothers on day 0. Ovariectomy was performed on day 2. Open and closed circles indicate LH concentrations at 10.00 and 17.00 h, respectively. Values are means ± S.E.M.

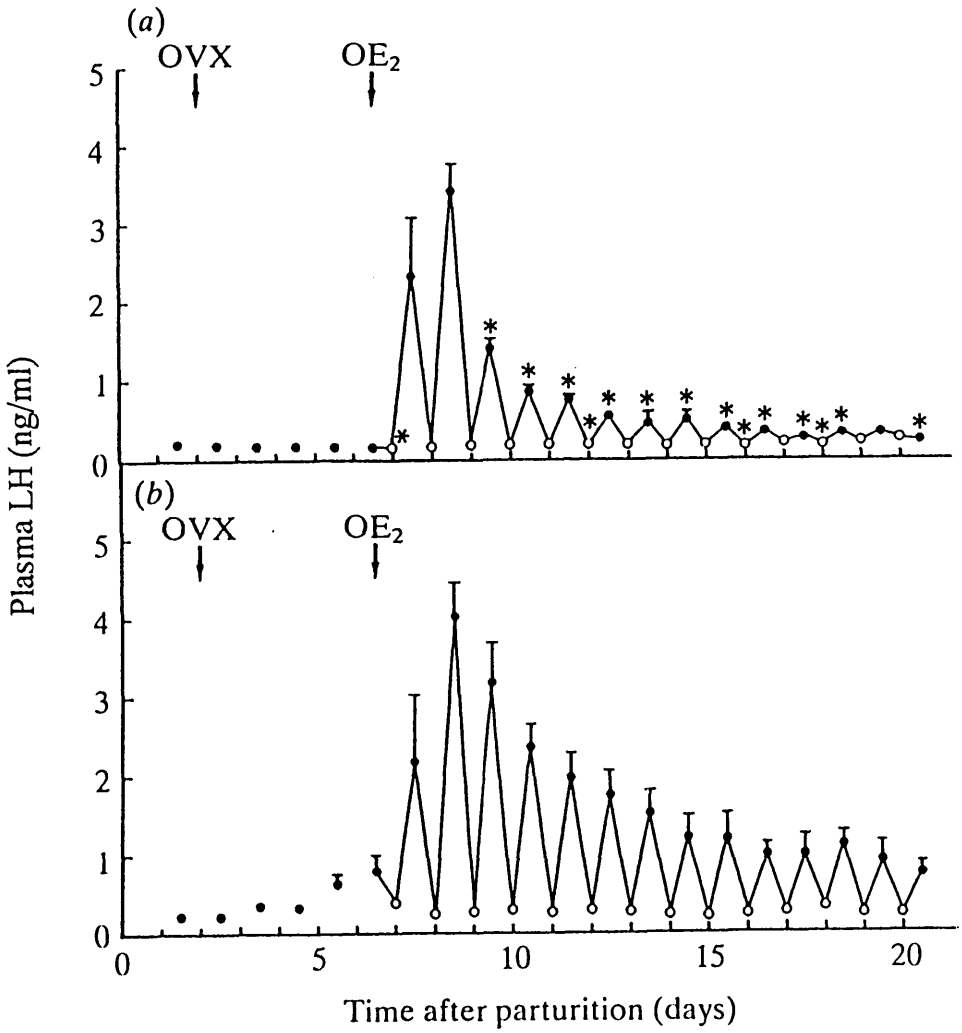


Figure 4.3. Daily LH surges in ovariectomized (a) lactating and (b) non-lactating rats (n = 8 in each group) implanted with oestradiol on day 6. In non-lactating rats, pups were removed from their mothers on day 0. Ovariectomy was performed on day 2. Open and closed circles indicate values at 10.00 and 17.00 h, respectively. *P<0.05 compared with non-lactating rats (Student's *t*-test). Values are means ± S.E.M. Arrows indicate the times of ovariectomy (OVX) and implantation (OE_2).

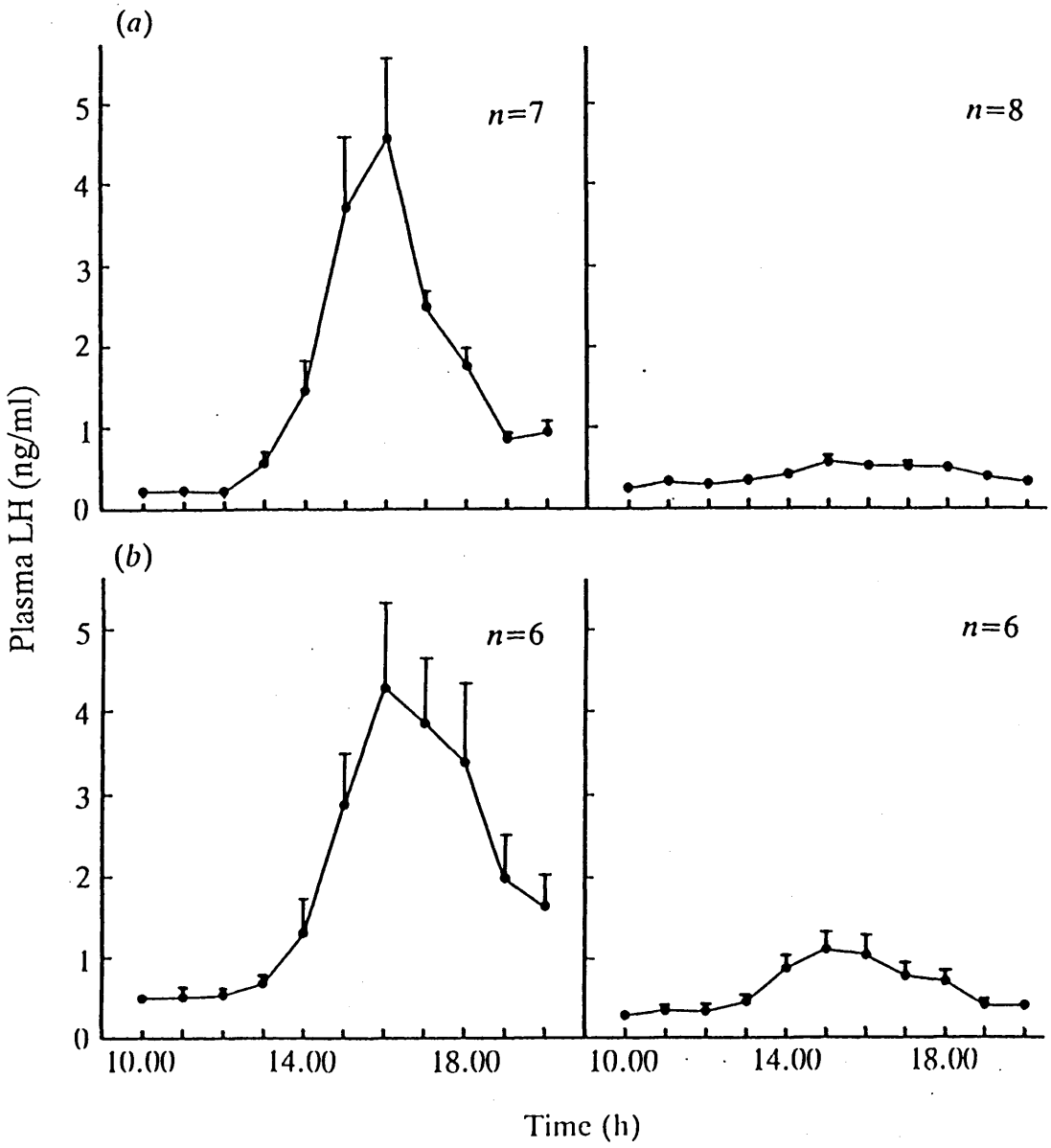


Figure 4.4. Changes in plasma concentrations of LH in ovariectomized lactating rats 2 days (left-hand panels) and 8 days (right-hand panels) after implantation with oestradiol on (a) day 6 and (b) day 15 of lactation. Ovariectomy was performed on day 2. Blood samples were taken at 1-h intervals from 10.00 to 20.00 h. Values are means ± S.E.M.

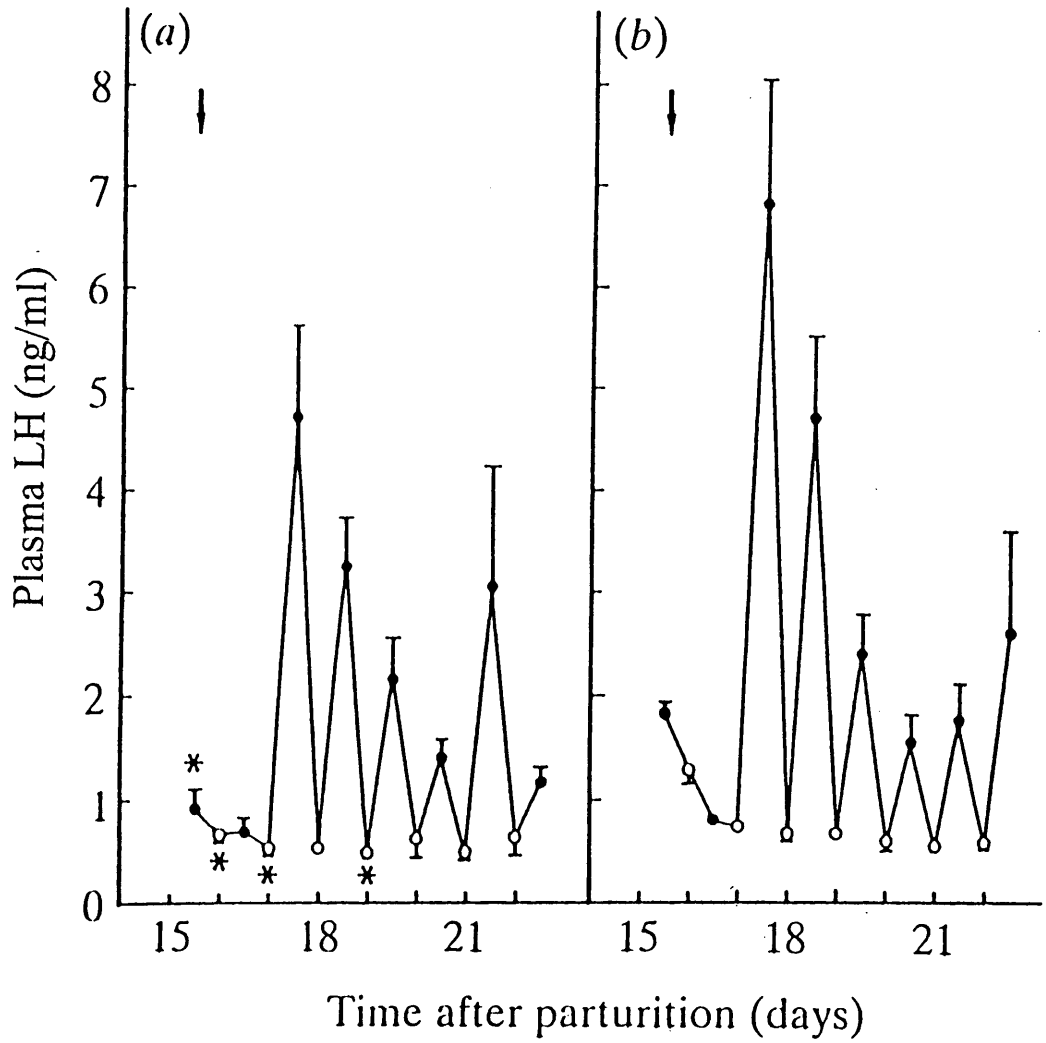


Figure 4.5. Daily LH surges in ovariectomized (a) lactating and (b) non-lactating rats (n = 8 in each case) implanted with oestradiol on day 15 (arrows). In non-lactating rats, pups were removed from their mothers on day 0. Ovariectomy was performed on day 2. Open and closed circles indicate values at 10.00 and 17.00 h, respectively. *P<0.05 compared with non-lactating rats (Student's *t*-test). Values are means ± S.E.M.

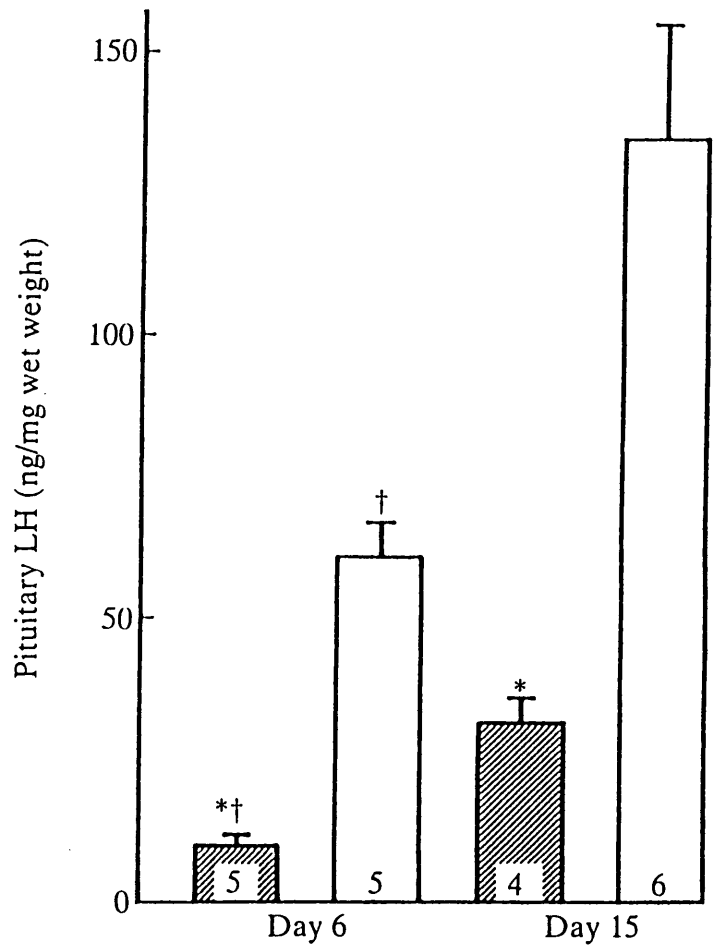


Figure 4.6. Pituitary LH contents 4 days after oestradiol implantation (on day 6 or 15) in ovariectomized lactating (hatched bars) and non-lactating (open bars) rats. All animals were decapitated at 12.00-12.30 h. *P<0.05 compared with non-lactating rats, †P<0.05 compared with rats implanted with oestradiol on day 15 (two-way ANOVA). Values are means ± S.E.M.; numbers in bars indicate numbers of animals used.

CHAPTER 5

Role of prolactin (PRL)

-Does PRL mediate suppression of pulsatile LH secretion in lactating rats?-

Introduction

Since hyperprolactinaemia is often accompanied by the decline of plasma and pituitary levels of LH (Cohen-Becker *et al.*, 1986; Smith & Bartke, 1987), plasma levels of PRL maintained at high level throughout lactation (Amenomori *et al.*, 1970; Kacsoh & Nagy, 1983; Mattheij *et al.*, 1985) could mediate the suppressing effect of the suckling stimulus on the LH secretion. On the other hand, previous works indicated that the reduction of plasma PRL concentrations by the treatment with bromocriptine (CB-154), a dopamine agonist, did not affect LH levels in ovariectomized rats at early and mid-lactation, although this treatment increased the plasma LH levels earlier than the saline treatment at late lactation (Lu *et al.*, 1976; Smith, 1978a; Maeda *et al.*, 1990). However, the effect of reduced PRL secretion on the suppression of pulsatile LH release during lactation is still unknown.

In the present chapter, the effect of the reduction of PRL release induced by CB-154 treatment on suppression of LH pulses in ovariectomized lactating rats was examined to determine whether PRL mediate this suppressive effect of the suckling stimulus in mid-lactation. In addition, replacement with PRL in the CB-154-treated animals was also studied.

Materials and Methods

Animals and treatments

Postpartum rats deprived of their litters on day 2 served as non-lactating controls. Blood samples (100 μl) were taken through the indwelling cannula at 6-min intervals for 3 h on day 7 or 8. A dopamine agonist, 2-Br-α-ergocriptine mesylate (CB-154) provided by Sandoz, Basel, Switzerland was dissolved in a small amount of ethanol and diluted with saline. Ovine PRL for biological use (NIDDK-oPRL-18), provided by the NHPP was dissolved in 0.03 M $NaHCO_3$, diluted with saline and stored at -20°C until use.

CB-154 solution (0.6 mg/day) or saline was injected daily into both lactating and non-lactating animals at 18.00 h from day 2. The half of the rats injected with CB-154 was infused with ovine PRL solution (NIDDK-oPRL-18, 0.3 mg/day) , using a mini-osmotic pump (Alza, CA, U.S.A., model No. 2001) placed under the dorsal skin on day 2. To give the similar strength of the suckling stimulus to each mother, litters were rotated every day among 4 lactating animals in the following order as previously described (Maeda *et al.*, 1990); a CB-154-treated mother, 2 intact mothers and a saline-injected mother. Litters were weighed every day at the time of rotation (18.00 h) and the maternal behaviour was checked visually twice a day.

Statistical analysis

Statistical differences were determined by Student's *t*-test.

Results

Plasma LH concentrations remained low in all lactating animals (Figs. 5.1, 5.2 & 5.3). There was no significant difference in 3 parameters of LH pulses, i.e. mean LH level, LH pulse frequency and LH pulse amplitude, between lactating rats treated with CB-154 or with both CB-154 and ovine PRL and saline-treated controls (Table 5.1, Student's *t*-test). Litters rotated among a CB-154-treated mother, 2 intact mothers and a saline-treated mother and those of CB-154-treated mothers with ovine PRL infusion increased their weight constantly until the day of sampling. The mother in each group of treatment showed a similar maternal behaviour. Frequent LH pulses were observed in all non-lactating animals (Figs. 5.4, 5.5 & 5.6). The treatment with CB-154 did not affect the pulsatile release of LH in non-lactating animals. No additional effect of PRL replacement on pulsatile LH secretion was found in both CB-154 treated lactating and non-lactating animals (Table 5.1).

Discussion

LH secretion was strongly suppressed in all lactating animals (Figs. 5.1, 5.2 & 5.3) as shown in the previous chapters. The CB-154 treatment, at the dosage level used in the present experiment, failed to restore the mean LH level and the frequency and amplitude of LH pulses (Table 5.1). The treatment completely inhibits PRL release to the undetectable level as reported in our previous study (Maeda *et al.*, 1990). Since litters rotated among a CB-154-treated mother, 2 intact mothers and a saline-treated mother increased their weight constantly until the sampling day and since each mother showed a similar maternal behaviour, all mothers seemed to receive the suckling stimulus at similar strength. Moreover, CB-154 itself did not suppress the LH secretion at the dose employed, since the frequency of LH pulses in non-lactating animals treated with CB-154 were comparable to that in saline-treated controls (Table 5.1 & Fig. 5.5). These results indicate that suppression of pulsatile secretion of LH by the suckling stimulus at early or mid-lactation is not mediated by PRL and strongly support the concept that the secretions of LH and PRL during lactation is regulated independently at the hypothalamic level.

Smith & Lee (1989) has reported that the high level of PRL is responsible to reduce the number of LHRH receptors during lactation. However, reduction of the PRL level with the CB-154 treatment, which might increase the number of LHRH receptors, failed to restore the pulsatile LH release in lactating rats as shown in the present experiment. Therefore, the suppression of pulsatile LH secretion observed in lactating rats could primarily be due to the reduction of LHRH release, but not to the decrease in the number of LHRH receptors in the pituitary caused by high plasma level of PRL.

Table 5.1. Mean plasma LH concentrations and frequency and amplitude of LH pulses (means ± S.E.M.) in ovariectomized lactating and non-lactating rats treated with CB-154 or with CB-154 and ovine PRL (oPRL).

	n	Mean LH (ng/ml)	LH pulse frequency (pulses/3 h)	LH pulse amplitude (ng/ml)
Lactating				
Saline	5	0.05±0.01	1.2±0.2	0.13±0.06
CB-154	5	0.11±0.04	2.2±0.9	0.31±0.06
CB-154 +oPRL	5	0.06±0.01	0.8±0.2	0.07±0.01
Non-lactating				
Saline	5	1.32±0.19	8.4±0.2	1.63±0.30
CB-154	5	1.48±0.40	7.8±0.4	1.59±0.35
CB-154 +oPRL	4	0.95±0.11	7.5±0.6	1.34±0.15

n; number of animals used. Values are not significantly different from those in corresponding saline-treated controls.

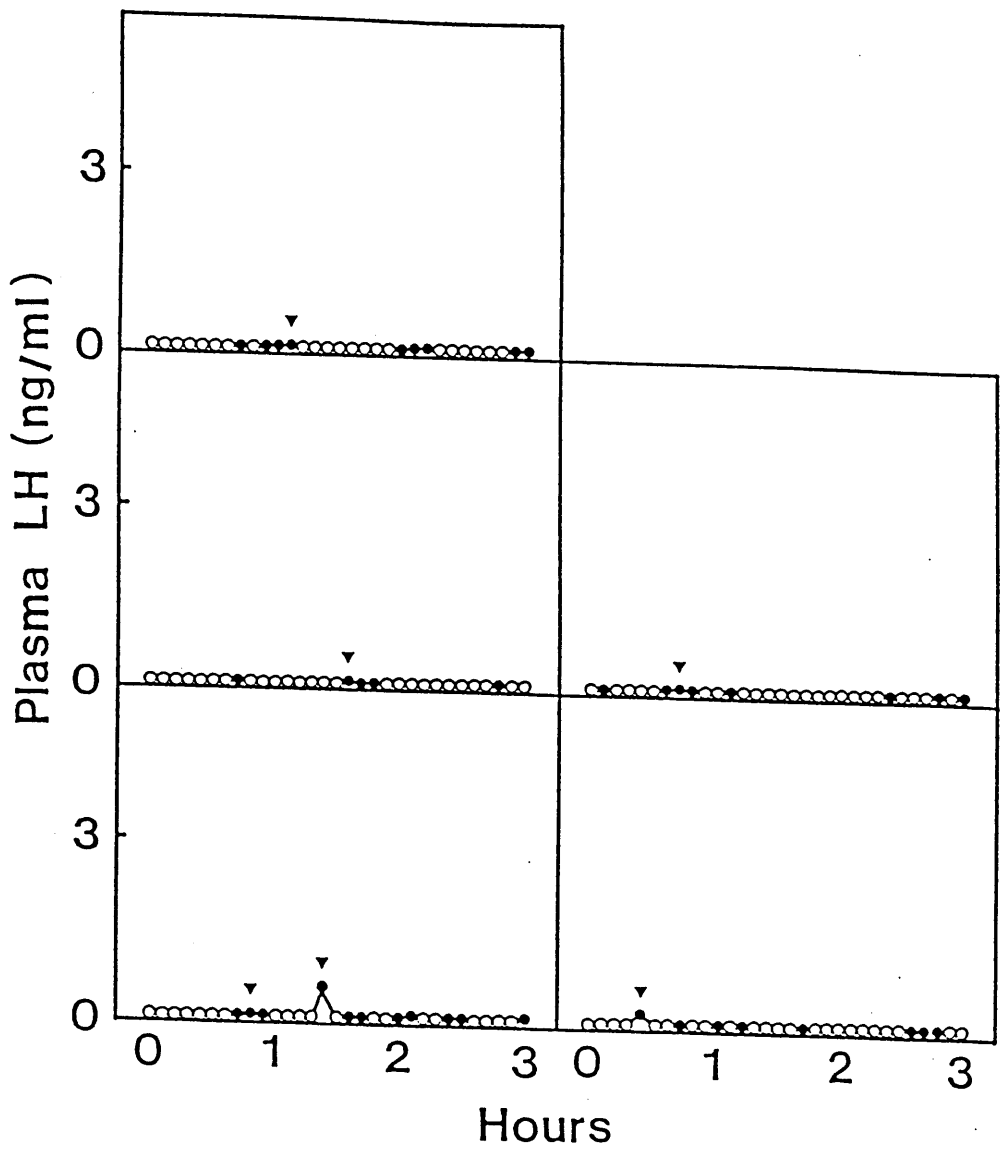


Fig. 5.1. Profiles of plasma LH concentrations in ovariectomized lactating rats injected daily with saline. All rats were ovariectomized on day 2 and blood samples were taken on day 7 or 8 at 6-min intervals for 3 h. Open circles and arrowheads represent the values which were lower than the limit of assay (0.39 ng/ml plasma) and the peak of LH pulses identified with the PULSAR computer program, respectively.

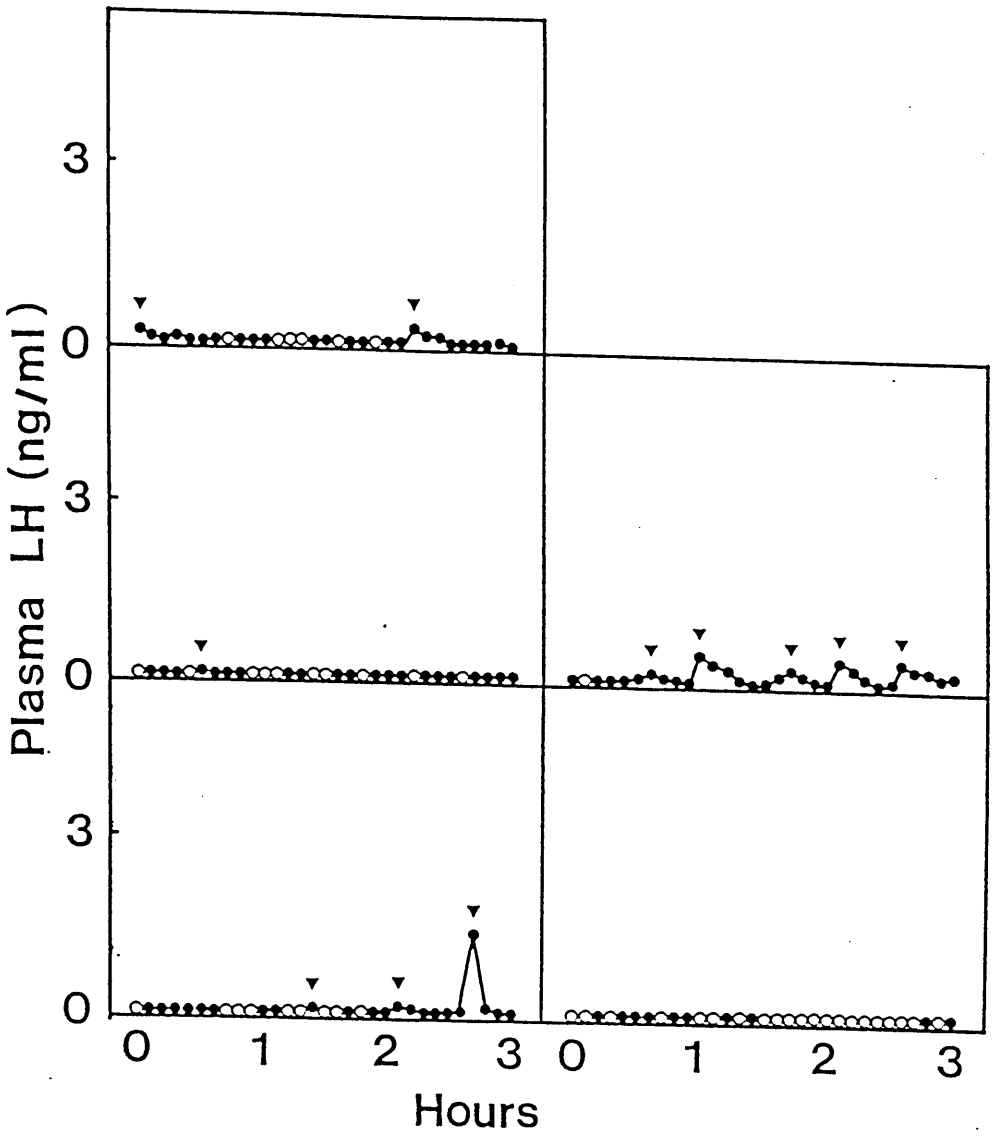


Fig. 5.2. Profiles of plasma LH concentrations in ovariectomized lactating rats injected daily with CB-154. All rats were ovariectomized on day 2 and blood samples were taken on day 7 or 8 at 6-min intervals for 3 h. Open circles and arrowheads represent the values which were lower than the limit of assay (0.39 ng/ml plasma) and the peak of LH pulses identified with the PULSAR computer program, respectively.

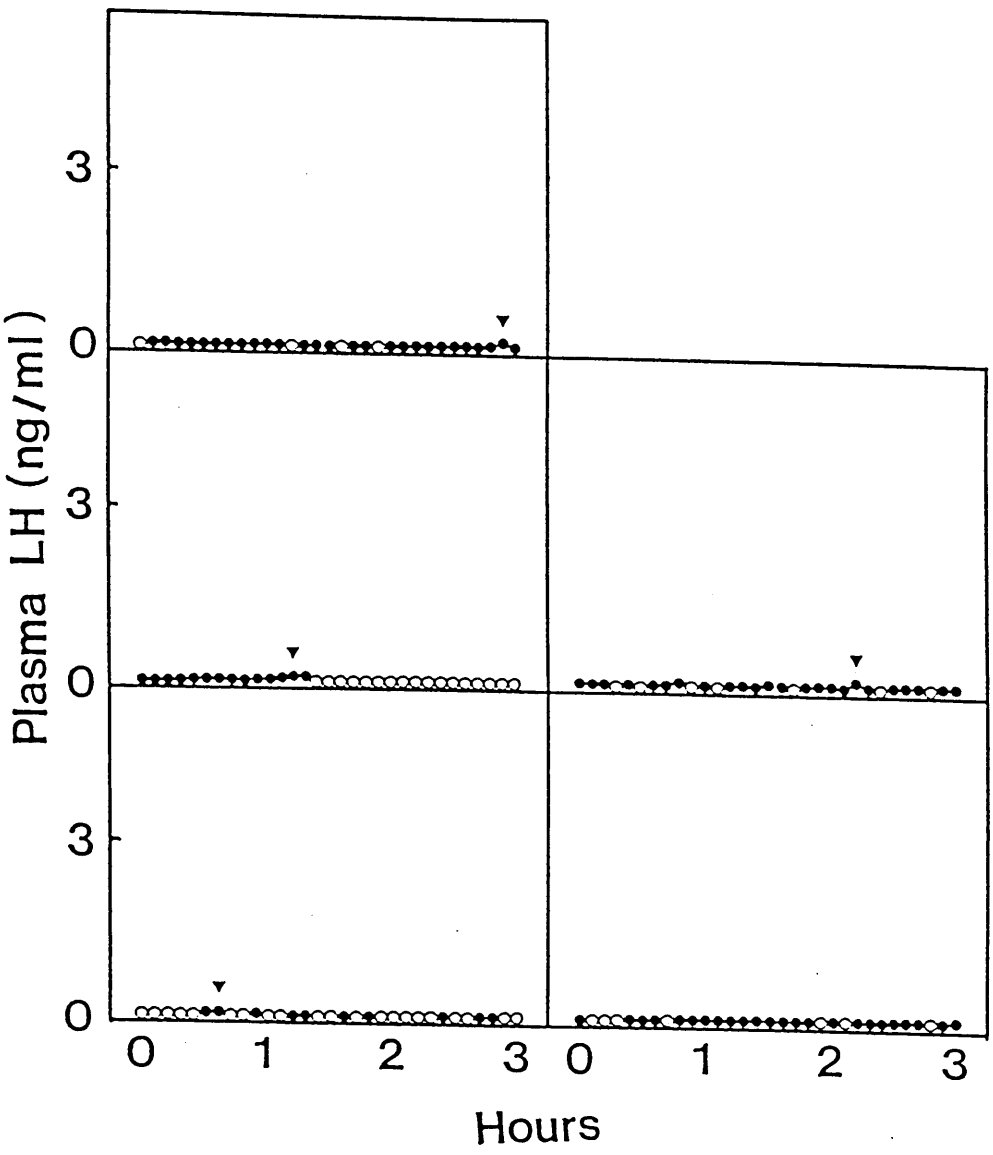


Fig. 5.3. Profiles of plasma LH concentrations in ovariectomized lactating rats injected daily with CB-154 and infused with ovine PRL. All rats were ovariectomized on day 2 and blood samples were taken on day 7 or 8 at 6-min intervals for 3 h. Open circles and arrowheads represent the values which were lower than the limit of assay (0.39 ng/ml plasma) and the peak of LH pulses identified with the PULSAR computer program, respectively.

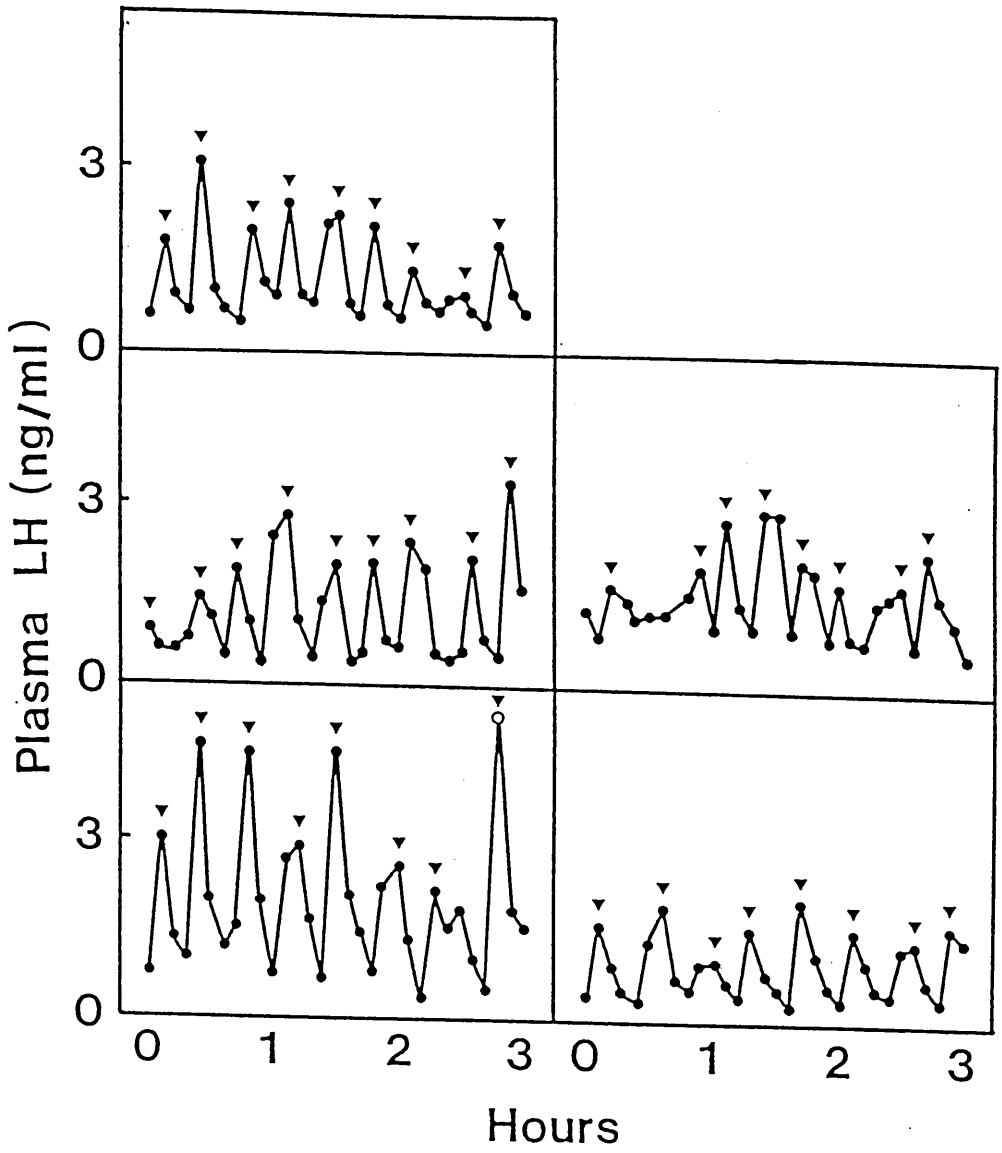


Fig. 5.4. Profiles of plasma LH concentrations in ovariectomized non-lactating rats injected daily with saline. Pups were removed from their mothers on day 2. All rats were ovariectomized on day 2 and blood samples were taken on day 7 or 8 at 6-min intervals for 3 h. Open circles and arrowheads represent the values which were lower than the limit of assay (0.39 ng/ml plasma) and the peak of LH pulses identified with the PULSAR computer program, respectively.

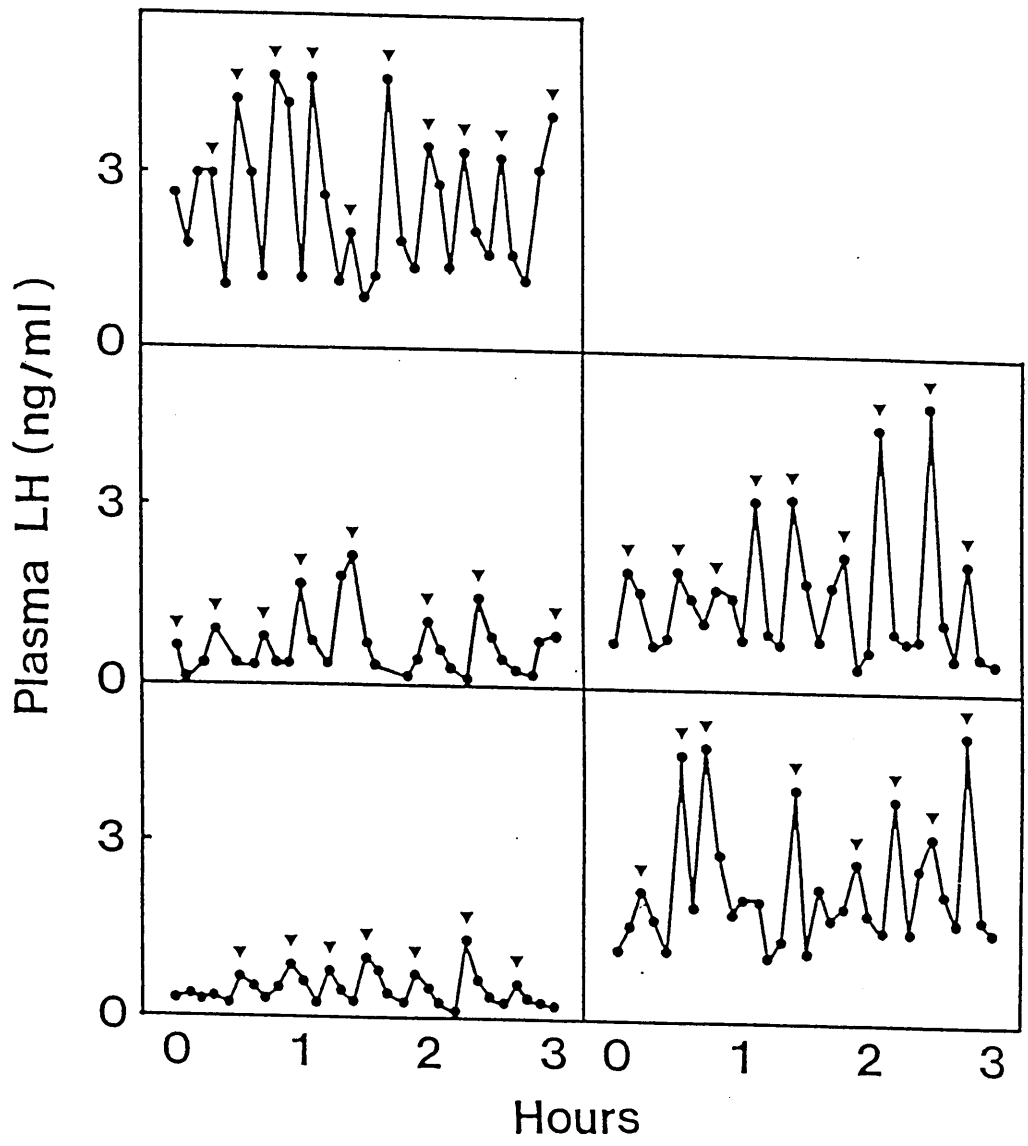


Fig. 5.5. Profiles of plasma LH concentrations in ovariectomized non-lactating rats injected daily with CB-154. Pups were removed from their mothers on day 2. All rats were ovariectomized on day 2 and blood samples were taken on day 7 or 8 at 6-min intervals for 3 h. Open circles and arrowheads represent the values which were lower than the limit of assay (0.39 ng/ml plasma) and the peak of LH pulses identified with the PULSAR computer program, respectively.

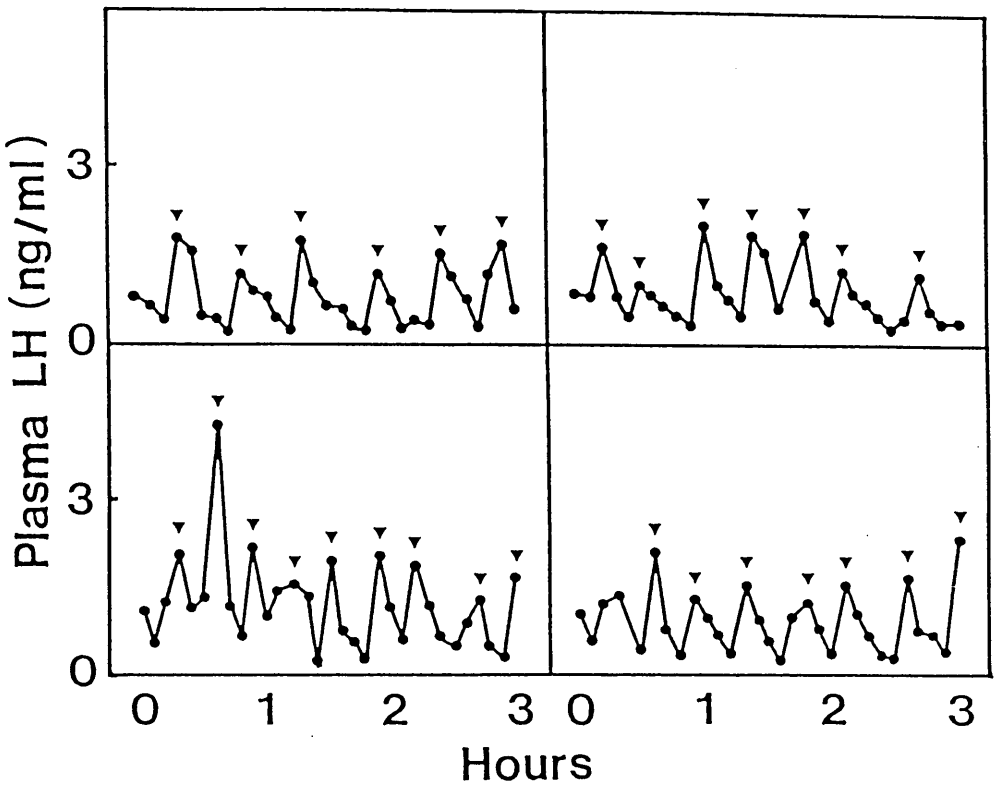


Fig. 5.6. Profiles of plasma LH concentrations in ovariectomized non-lactating rats injected daily with CB-154 and infused with ovine PRL. Pups were removed from their mothers on day 2. All rats were ovariectomized on day 2 and blood samples were taken on day 7 or 8 at 6-min intervals for 3 h. Open circles and arrowheads represent the values which were lower than the limit of assay (0.39 ng/ml plasma) and the peak of LH pulses identified with the PULSAR computer program, respectively.

CHAPTER 6

Neural pathway conveying the inhibitory signal of the suckling stimulus for pulsatile LH secretion-I

-Effects of hypothalamic deafferentation on pulsatile secretion of LH in ovariectomized lactating rats-

Introduction

The results shown in the previous chapters suggest that the suckling stimulus is directly involved in suppressing pulsatile LH secretion and that the suppressing effect is conveyed to a system or systems responsible for the release of LHRH by a neural pathway rather than by humoral factors, such as PRL and ovarian steroids.

It has been reported that the hypothalamic LHRH content in lactating rats is comparable to that of cyclic rats at dioestrus (Guller *et al.*, 1982; Jakubowska-Naziemblo *et al.*, 1985) and daily LH surges occur in ovariectomized lactating rats implanted with oestradiol (Chapter 4). These results suggest that the suckling stimulus suppresses LH release principally by inhibiting pulsatile LHRH release rather than LHRH production.

The putative pulse generator regulating pulsatile LHRH release has been thought to be located in the mediobasal hypothalamus (MBH), since pulsatile LH secretion has been observed in either cyclic or ovariectomized rats with complete deafferentation of the MBH (Blake & Sawyer, 1974; Soper & Weick, 1980), and since LHRH has been reported to be released in a pulsatile manner from the MBH *in vitro* (Bourguignon *et al.*, 1987).

Tindal (1978) has identified the central pathways which convey the suckling stimulus for oxytocin and PRL release in the rabbit. Little is known, however, about the final neural pathway in the hypothalamus for suppressing LH pulses in lactating rats. Therefore, various hypothalamic deafferentations have been made in the present chapter in ovariectomized lactating rats to determine the neural pathway which conveys the inhibitory signal of the suckling stimulus for pulsatile LH secretion.

Materials and Methods

Treatment and blood sampling

The hypothalamus was deafferentated in the morning on day 6 or 7 of lactation. All mothers were injected with oxytocin (Atonin-O, Teikoku Zoki Co., Tokyo, Japan; 1 IU/ml saline/rat, i.p.) at 14.00 and 19.00 h on the day of operation and at 10.00 h on the following day to ensure the maintenance of lactation. Each litter was weighed immediately before and one h after the injection of oxytocin. The sum of the differences in the weight of the litter following the injections in each mother was designated as the total weight gain. Twenty four h after the hypothalamic deafferentation blood samples (120 μl) were collected every 6 min for 3 h.

Hypothalamic deafferentation

The complete hypothalamic deafferentation was performed by a slightly modified method of Halasz and Pupp (1965). Two types of knives were employed (Fig. 6.1). Knife A had a bayonet shape with 1.0 mm radius and 1.8 mm vertical extent and was used for complete (CD), anterior (AD), anterolateral (ALD) and posterior deafferentation (PD). The L-shaped knife B with 1.0 mm radius was used for roof deafferentation (RD). Animals were placed in a stereotaxic instrument (Narishige, Tokyo, Japan) with the level of the bregma 1.6 mm below the lambda under ether anaesthesia. The tip of knife A was positioned 1.5 mm posterior to the bregma and lowered to the base of the skull. Operation procedures for AD, ALD and PD were identical to CD, but the lateral-posterior, posterior and anterolateral portions were not cut in AD, ALD and PD, respectively. In rats with RD, knife B was lowered into the brain at 1.5 mm posterior to and 8.0 mm below the bregma and was moved in the same way as in CD. In rats with

sham-deafferentation (SD), only the outer guide cannula of the knife was inserted into the brain and was moved in the same way as in CD. At the end of the experiment, the brain was perfused with saline followed by 10 % formalin. Cryostat sections (30 μm) of the brain were stained with cresyl violet for histological examinations.

Data analysis

Statistical differences were determined by Mann-Whitney *U*-test.

Results

The suckling behaviour of the litter and the nursing behaviour of ovariectomized lactating rats with the various hypothalamic deafferentation was similar to that of rats with SD. There was no significant difference in the total weight gain of the litter obtained after the oxytocin injections in the various groups (Mann-Whitney *U*-test; Table 6.1).

Fig. 6.2 shows a schematic illustration of the site of each of the hypothalamic deafferentations. Photomicrographs of the frontal sections of the brain in representative rats with CD or RD are shown in Fig. 6.3. The hypothalamic island in rats with CD included the arcuate nucleus, the caudal part of the suprachiasmatic nucleus and the medial anterior hypothalamic area. The medial part of the ventral and ventral part of the dorsal hypothalamic nucleus were also included. The caudal limit of the island was the rostral end of the mammillary body. The site of the cut was similar in rats with AD, ALD and PD to that in rats with CD except that the caudal-lateral-dorsal, caudal and rostral-lateral-dorsal portions were spared in rats with AD, ALD and PD, respectively. In rats with RD, the cut was almost parallel to the base of the brain from the end of the medial preoptic area to the rostral end of the mammillary body, at the level just above the ventral margin of the paraventricular nucleus (PVN).

LH secretion was markedly suppressed in rats with SD, although a few LH pulses were observed (Fig. 6.4). In contrast, LH pulses with high frequency and amplitude were observed in most of rats with CD (Fig. 6.5). The mean LH level for 3 h, and the frequency and the amplitude of LH pulses were significantly higher in rats with CD than in those with SD ($P<0.05$, Mann-Whitney *U*-test; Table 6.2). The plasma LH levels were very low and did not include any pulses in three out of four rats with AD, but LH pulses with high frequency and amplitude were observed in the remaining animal (Fig.

6.6 & Table 6.2). Pulsatile LH secretion was observed in four out of five rats with ALD; in the remaining animal the mean LH level was high but no pulses were detected by the PULSAR computer program (Fig. 6.7). The mean LH level and the amplitude of the pulses were significantly higher in rats with ALD than in those with SD ($P<0.05$, Mann-Whitney *U*-test; Table 6.2). Pulsatile LH secretion was absent in all rats with PD (Fig. 6.8) and the mean LH level in those rats was lower than that in rats with SD ($P<0.01$, Mann-Whitney *U*-test; Table 6.2). All rats with RD showed pulsatile LH secretion (Fig. 6.9). The mean LH level and the frequency and amplitude of the pulses in animals with RD were very close to those in rats with CD, but significantly higher than those in rats with SD ($P<0.05$, Mann-Whitney *U*-test; Table 6.2).

Among the various groups the only significant difference in mean plasma PRL concentrations during the 3-h sampling period consisted of a reduction in the PRL level in rats with RD in comparison to those with SD ($P<0.01$, Mann-Whitney *U*-test; Table 6.2).

Discussion

The results in the present chapter demonstrate that the signal for the inhibition of pulsatile LH release by suckling is conveyed to the hypothalamus by afferent neural fibres projecting through the dorsal part of the hypothalamus. Pulsatile LH secretion was apparent only in lactating rats in which the dorsal part of the hypothalamus had been severed (CD, ALD and RD; Figs. 6.5, 6.7 & 6.9); it did not occur in rats in which this dorsal region was left intact (SD, AD and PD; Figs. 6.4, 6.6 & 6.8). In other words, fibres entering the hypothalamus dorsally may convey a signal which originates from the teats during lactation and ultimately suppresses LHRH release and thus the LH pulses. The difference in the pattern of LH secretion between the groups examined could not be attributed to the difference in the intensity of the suckling stimulus, since the suckling behaviour of the litters in all deafferentated groups was similar to that following SD and since the weight gain of the litters induced by oxytocin injections to the mother was not different between the groups (Table 6.1). The present finding that the secretory pattern of LH was pulsatile after CD suggests that the pulse generator for pulsatile LH release is located within the hypothalamic island, i.e. the MBH. These findings agree well with those of Soper and Weick (1980). However, there are some arguments against the existence of the pulse generator in the MBH since the subchiasmatic LHRH neuronal pathway (Coen, 1987) would remain intact in rats with CD and may play a role in maintaining the pulsatile LH secretion.

The reason for the pulsatile LH secretion observed in one of the four rats with AD (Fig. 6.6) is not clear since no appreciable difference was found upon histological analysis of the deafferentation. Nevertheless, it is unlikely that the afferent neural fibres projecting to the MBH from the anterior hypothalamus (or more rostral) conduct the

suckling-induced inhibitory signal for the pulsatile LH secretion, since the LH pulses appeared in all rats with only RD in the present study.

Levels of PRL were markedly reduced by RD, while the levels in rats with CD, AD and ALD were maintained at levels similar to those in rats with SD (Table 6.2). Therefore, it is likely that dorsal inputs to the MBH inhibit the LH secretion and enhance the PRL secretion in lactating rats. Although this input enhancing PRL secretion was severed in rats with ALD or CD, plasma levels of PRL in these rats were higher than those with RD (Table 6.2). These findings imply the possibility of the presence of two kinds of neural input to the MBH; dorsal inputs which enhance PRL secretion, and anterior inputs which suppress PRL secretion. Results obtained by Weiner *et al.* (1972) in rats with AD seem to suggest the presence of fibres from the anterior hypothalamus to the MBH which inhibit PRL release. Since the connection between tuberoinfundibular dopaminergic (TIDA) neurons and the anterior pituitary must be intact in the present experiment, enhancing and suppressing inputs could regulate PRL release by controlling dopamine release into the portal vessel from the TIDA neurons.

The finding that CD did not affect the PRL secretion but reinstated pulsatile LH secretion (Table 6.2) suggests that the suckling stimulus can suppress the LHRH release in ovariectomized lactating rats irrespective of plasma PRL levels. The mechanisms that are responsible for the suppression of LH secretion and maintenance of a high level of PRL secretion may be quite different from each other at the level of the hypothalamus and the mediation by PRL may not be required for inhibition of LH secretion. This conclusion is consistent with that of Chapter 5 and the work of Smith (1978a) and Maeda *et al.* (1990) which indicate that treatment with bromocriptine to reduce PRL secretion in mid-lactation of intact and ovariectomized lactating rats did not cause an increase in LH levels.

In conclusion, afferent neural fibres conducting impulses triggered by the suckling stimulus project to the MBH through the dorsal part of the hypothalamus and thus inhibit pulsatile LH secretion, and PRL does not mediate the suppressive effect of the suckling stimulus on LH secretion at mid-lactation.

Table 6.1. Total weight gain (g) of the litters after oxytocin injection to the mother.

	Mean	Range[a]	n[b]
SD	5.0	1.0-7.5	5
CD	5.9	2.0-10.5	7
AD	5.5	4.0-8.5	4
ALD	4.7	2.0-7.0	5
PD	5.0	1.0-12.5	7
RD	2.3	-1.0-8.5	5

Mothers were injected with oxytocin (1 IU/ml saline/rat, i.p.) at 14.00 and 19.00 h on the day of deafferentation and 10.00 h on the following day. The litter was weighed together immediately before and one h after the injection, and the sum of the differences in the weight of the litter for each mother was recorded as the total weight gain. SD-sham-, CD-complete, AD-anterior, ALD-anterolateral, PD-posterior, RD-roof deafferentation. [a]The minimum and maximum values in each group. [b]Number of animals used. There were no significant differences among the groups (Mann-Whitney *U*-test).

Table 6.2. Mean plasma LH and PRL concentrations and frequency and amplitude of LH pulses (Means ± S.E.M.) in ovariectomized lactating rats bearing various hypothalamic deafferentations.

	n[a]	Mean LH (ng/ml)	LH pulse frequency (pulses/3 h)	LH pulse amplitude[b] (ng/ml)		Mean PRL (ng/ml)
SD	5	0.07±0.00	1.40±0.40	0.14±0.02	(4)[c]	63.8±17.5
CD	7	0.73±0.17**	5.71±0.81*	0.52±0.08**	(7)	74.4±11.4
AD	4	0.24±0.19	2.25±2.25	0.50	(1)	62.2±31.7
ALD	5	0.78±0.27**	4.00±1.38	0.48±0.07*	(4)	67.7±29.7
PD	7	0.04±0.00**	0*	-	(0)	64.4±18.2
RD	5	0.66±0.13**	8.00±0.63	0.44±0.07	(5)	20.1±1.0**

SD-sham-, CD-complete, AD-anterior, ALD-anterolateral, PD-posterior, RD-roof deafferentation. *P<0.05, **P<0.01 compared with SD (Mann-Whitney *U*-test). [a]Number of animals used. [b]Pulse amplitudes were calculated in animals showing LH pulses. [c]Number of animals showing LH pulses.

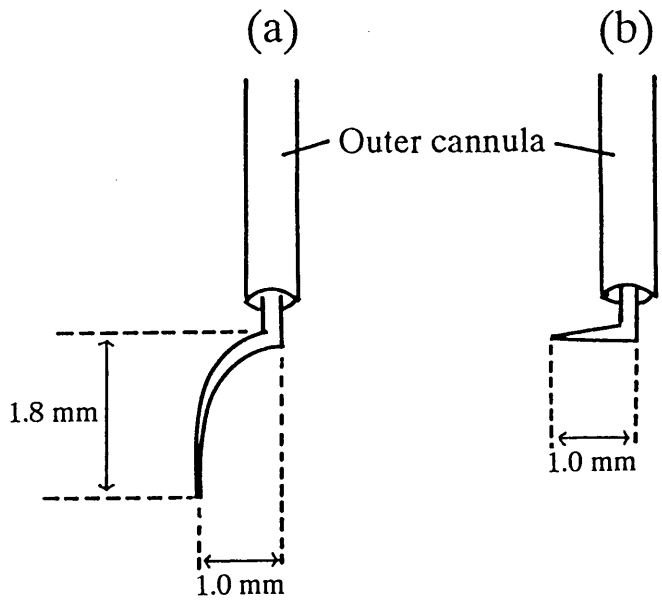


Fig. 6.1. Schematic illustration of the knife used in the present experiment.

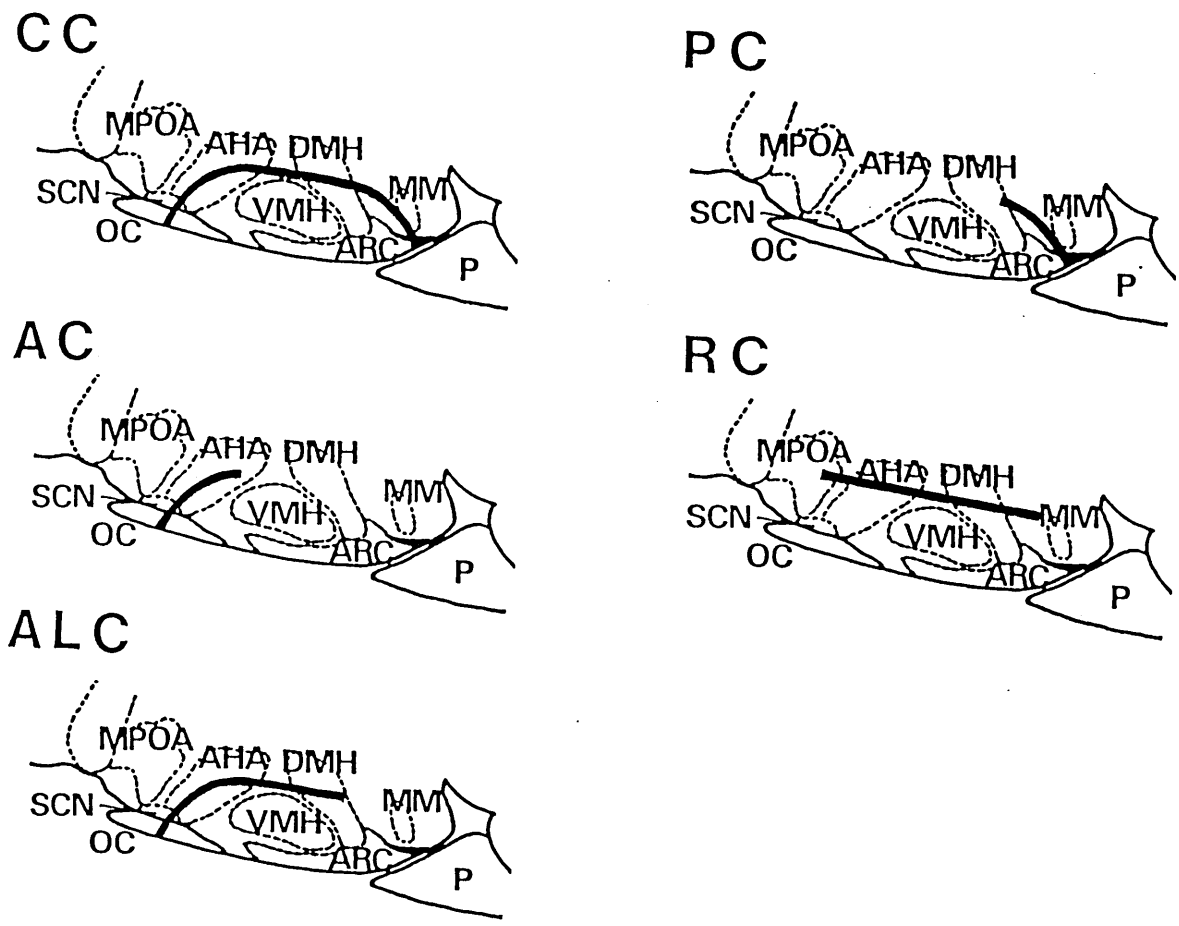
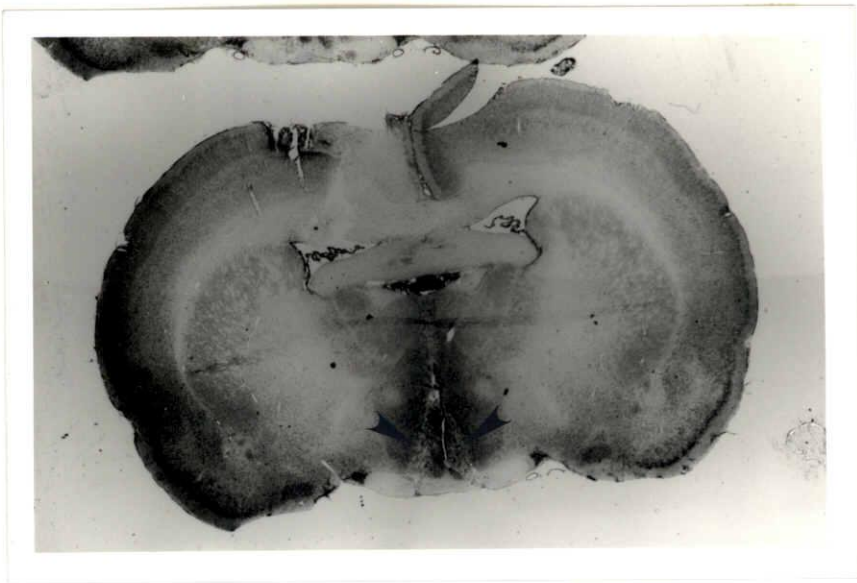


Fig. 6.2. Schematic illustration of the site of the hypothalamic deafferentation. CD-complete, AD-anterior, ALD-anterolateral, PD-posterior, RD-roof deafferentation; AHA-anterior hypothalamic area; ARC-arcuate nucleus; DMH-dorsomedial hypothalamic nucleus; MM-mammillary body; MPOA-medial preoptic area; OC-optic chiasm; P-anterior pituitary; SCN-suprachiasmatic nucleus; VMH-ventromedial hypothalamic nucleus.



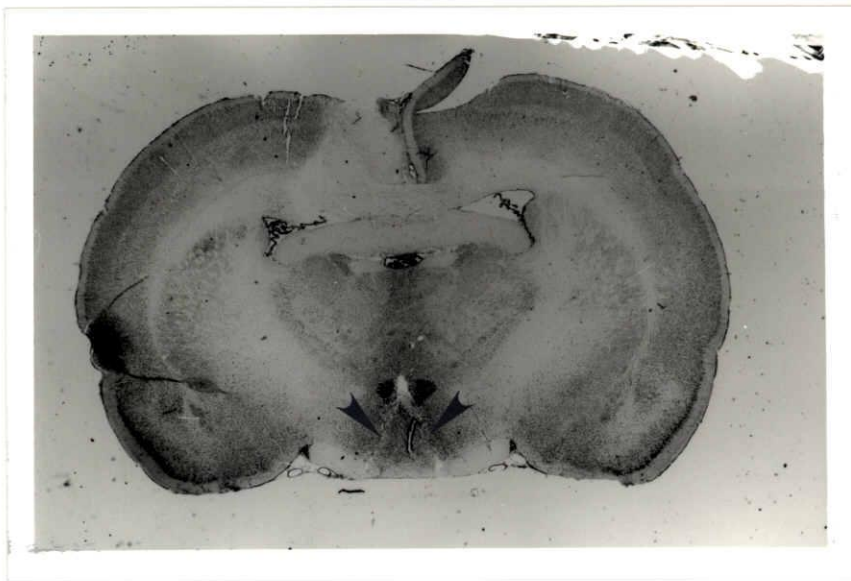
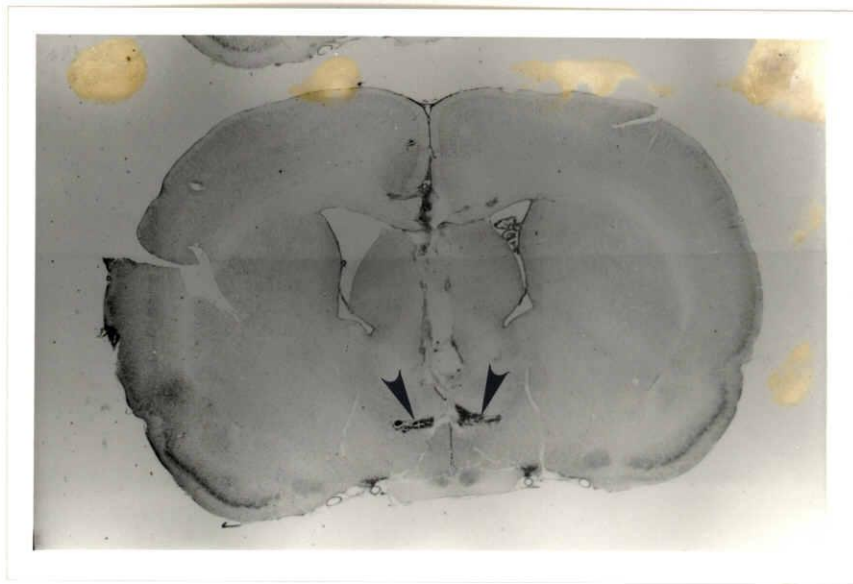
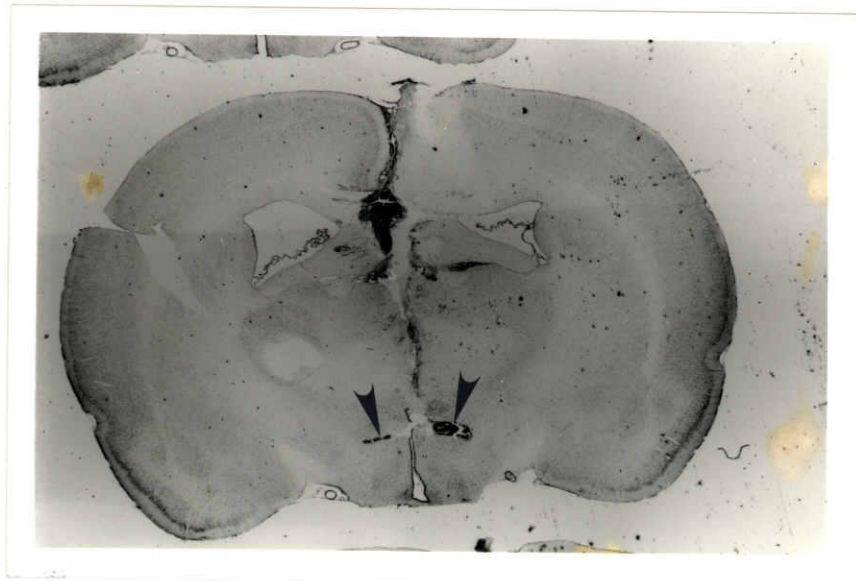


Fig. 6.3a. Photomicrographs of frontal sections of the brain of a representative rat with complete deafferentation (CD). Arrowheads indicate the cut line.





g. 6.3b. Photomicrographs of frontal sections of the brain of two representative rats ith roof deafferentation (RD). Arrowheads indicate the cut line.

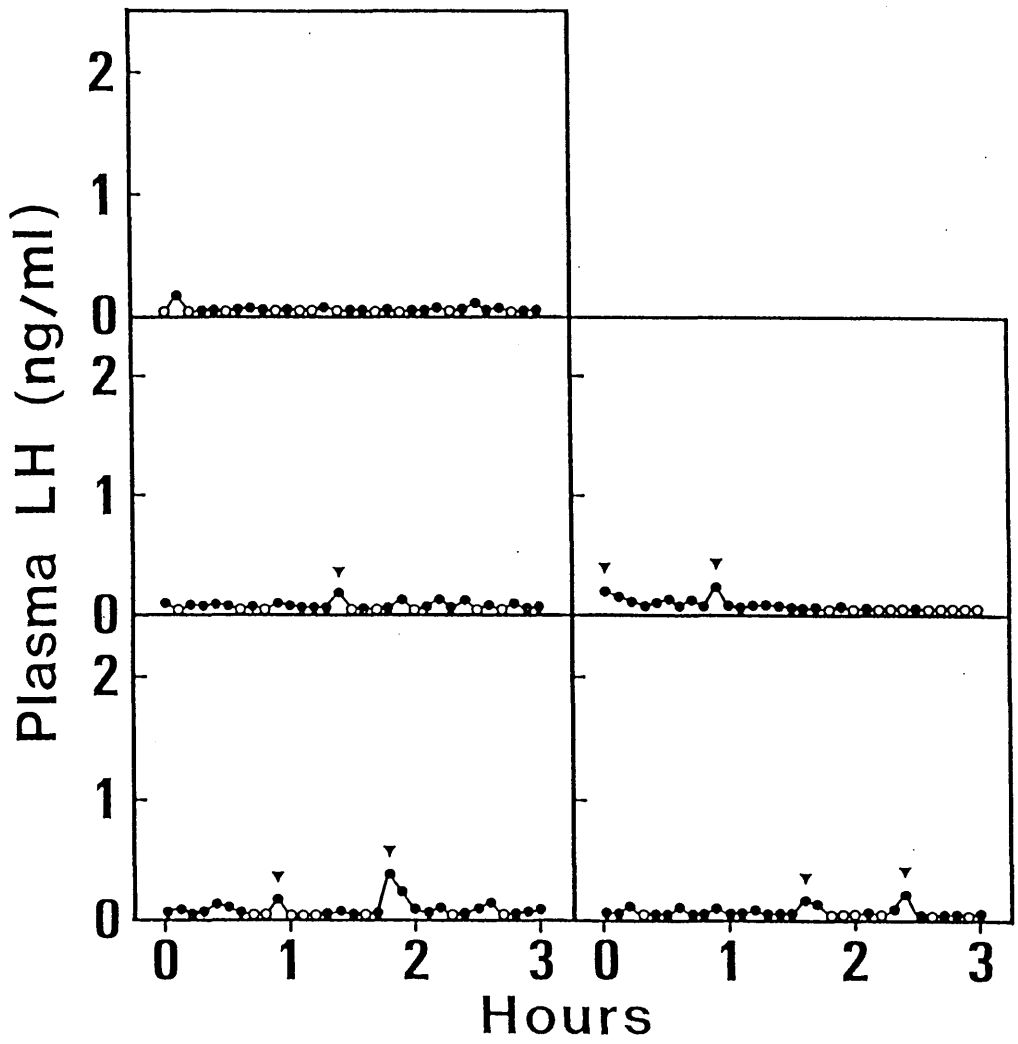


Fig. 6.4. Profiles of plasma LH concentrations in individual ovariectomized lactating rats with sham-deafferentation (SD). Ovariectomy and hypothalamic deafferentations were performed on day 2 and day 6 or 7, respectively. Blood samples were collected every 6 min for 3 h beginning 24 h after the brain surgery. Open circles and arrowheads represent the values which were lower than the limit of assay (0.039 ng/ml plasma) and the peak of LH pulses identified with the PULSAR computer program, respectively.

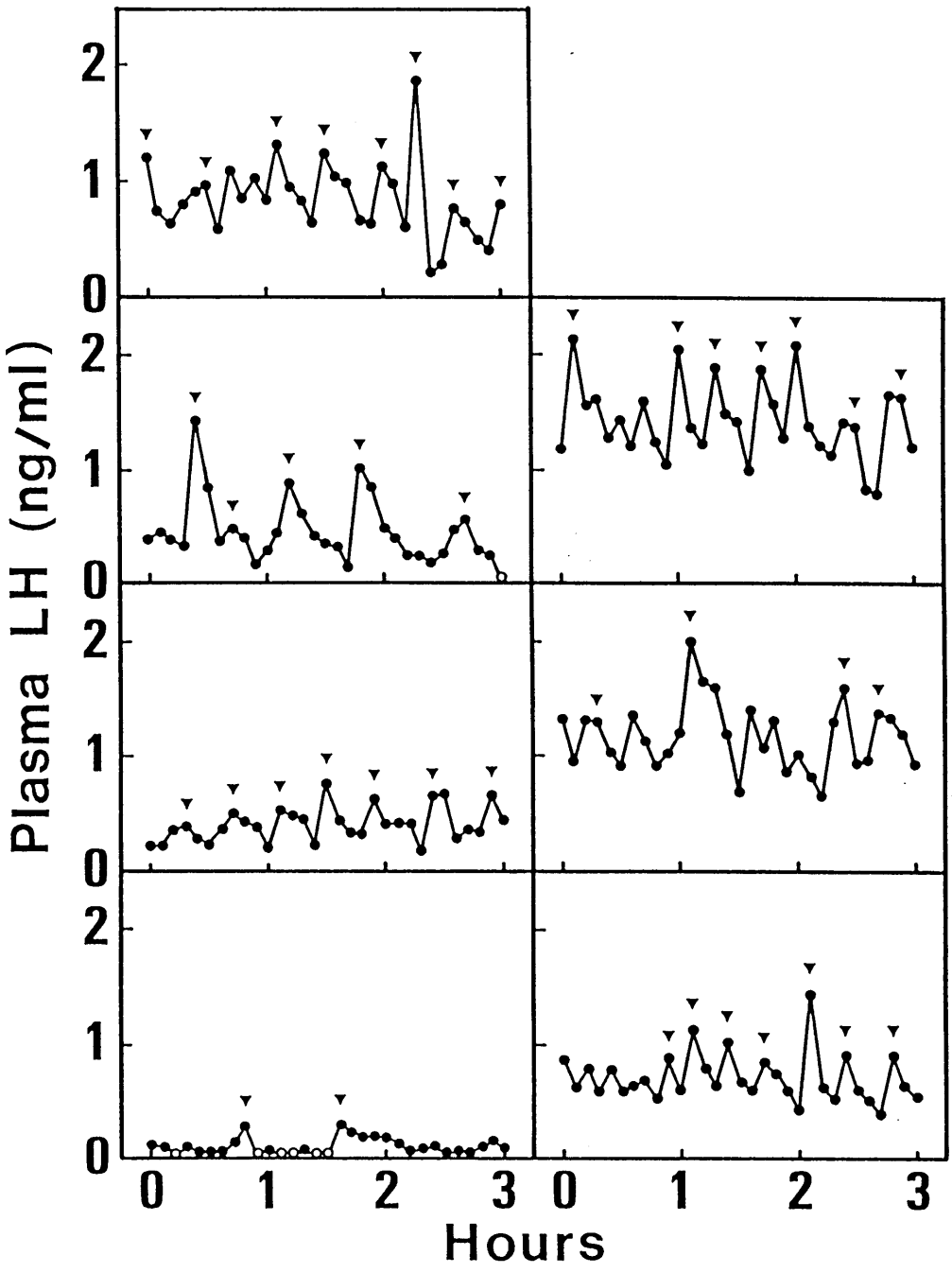


Fig. 6.5. Profiles of plasma LH concentrations in individual ovariectomized lactating rats with complete deafferentation (CD). Ovariectomy and hypothalamic deafferentations were performed on day 2 and day 6 or 7, respectively. Blood samples were collected every 6 min for 3 h beginning 24 h after the brain surgery. Open circles and arrowheads represent the values which were lower than the limit of assay (0.039 ng/ml plasma) and the peak of LH pulses identified with the PULSAR computer program, respectively.

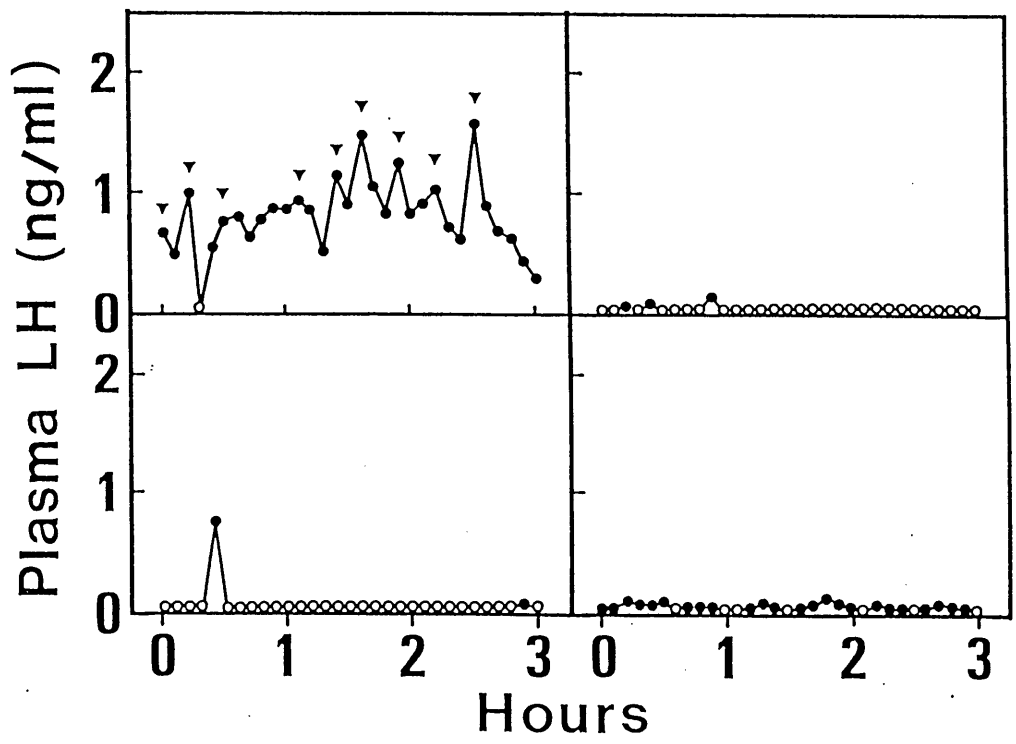


Fig. 6.6. Profiles of plasma LH concentrations in individual ovariectomized lactating rats with anterior deafferentation (AD). Ovariectomy and hypothalamic deafferentations were performed on day 2 and day 6 or 7, respectively. Blood samples were collected every 6 min for 3 h beginning 24 h after the brain surgery. Open circles and arrowheads represent the values which were lower than the limit of assay (0.039 ng/ml plasma) and the peak of LH pulses identified with the PULSAR computer program, respectively.

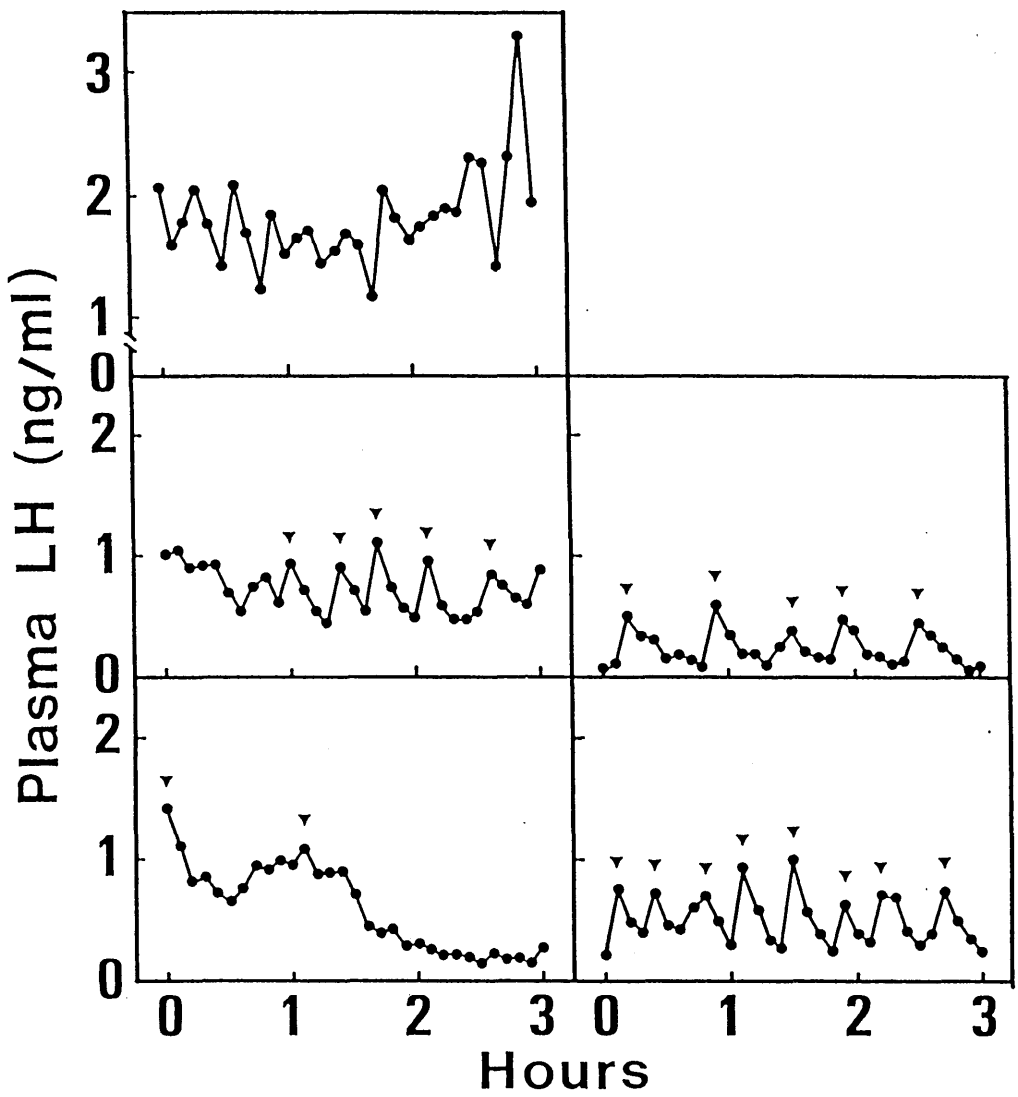


Fig. 6.7. Profiles of plasma LH concentrations in individual ovariectomized lactating rats with anterolateral deafferentation (ALD). Ovariectomy and hypothalamic deafferentations were performed on day 2 and day 6 or 7, respectively. Blood samples were collected every 6 min for 3 h beginning 24 h after the brain surgery. Arrowheads represent the peak of LH pulses identified with the PULSAR computer program.

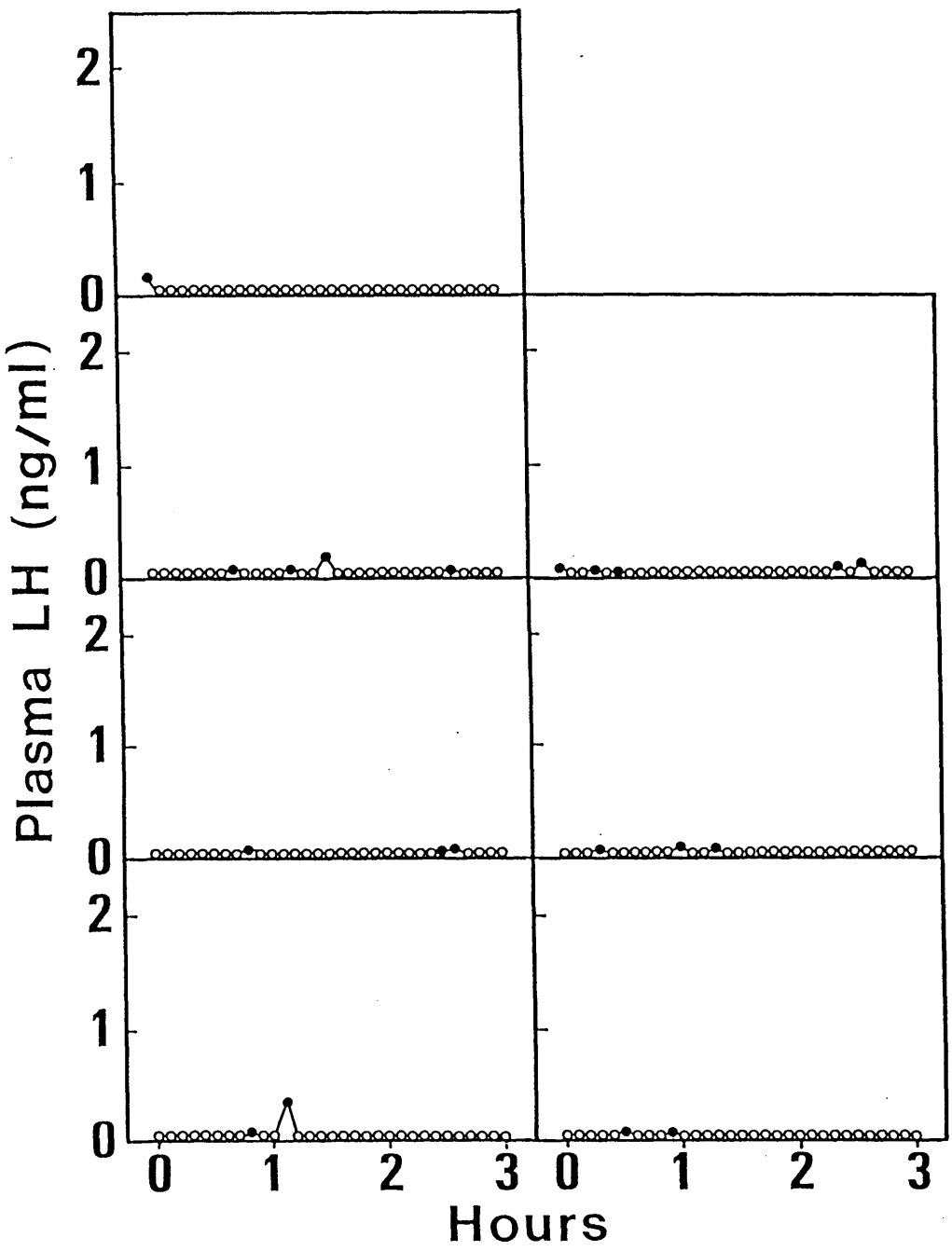


Fig. 6.8. Profiles of plasma LH concentrations in individual ovariectomized lactating rats with posterior deafferentation (PD). Ovariectomy and hypothalamic deafferentations were performed on day 2 and day 6 or 7, respectively. Blood samples were collected every 6 min for 3 h beginning 24 h after the brain surgery. Open circles and arrowheads represent the values which were lower than the limit of assay (0.039 ng/ml plasma) and the peak of LH pulses identified with the PULSAR computer program, respectively.

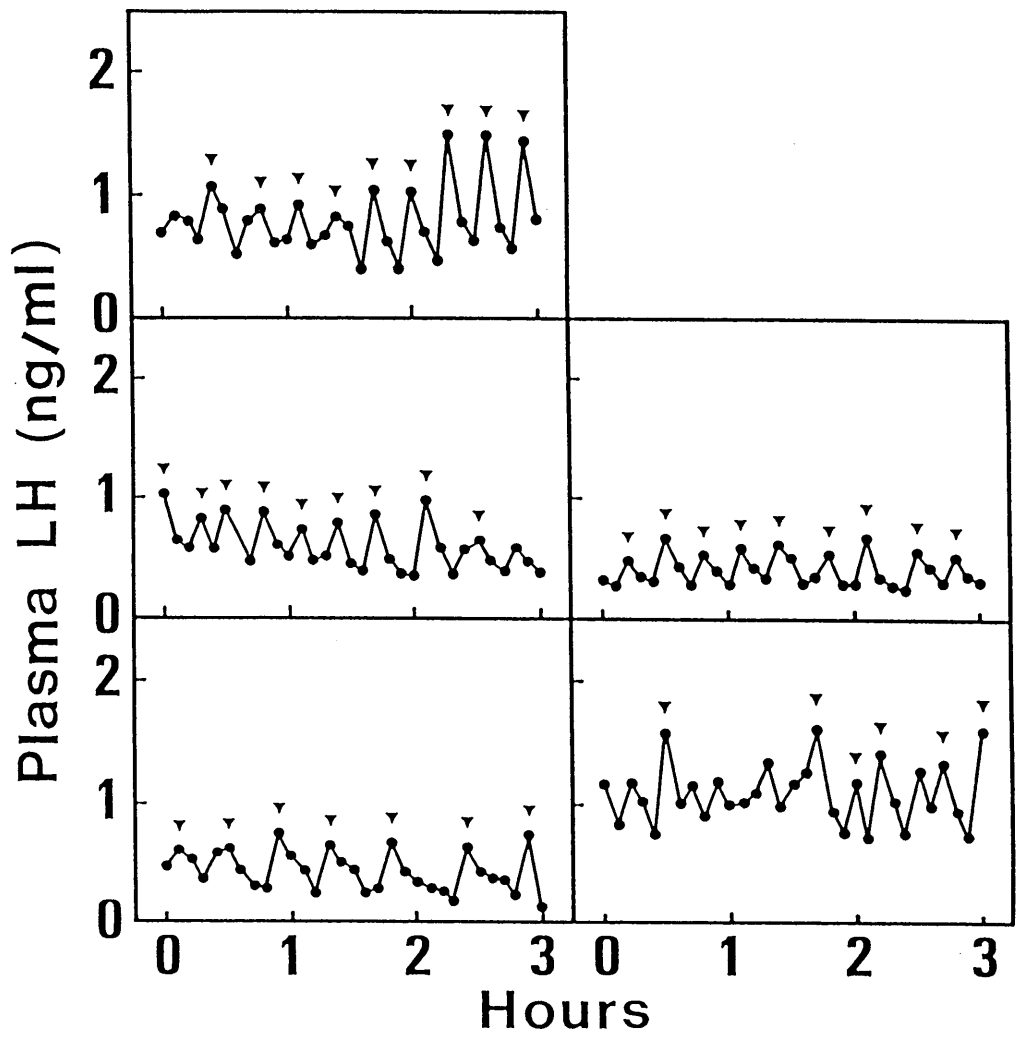


Fig. 6.9. Profiles of plasma LH concentrations in individual ovariectomized lactating rats with roof deafferentation (RD). Ovariectomy and hypothalamic deafferentations were performed on day 2 and day 6 or 7, respectively. Blood samples were collected every 6 min for 3 h, 24 h after the brain surgery. Arrowheads represent the peak of LH pulses identified with the PULSAR computer program.

CHAPTER 7

Neural pathway conveying the inhibitory signal of the suckling stimulus for pulsatile LH secretion-II

-Effects of various hypothalamic roof deafferentations or electrolytic lesions of the paraventricular nucleus on pulsatile secretion of LH in ovariectomized lactating rats-

Introduction

I have demonstrated in Chapter 6 that complete, anterior-lateral and roof deafferentation of the MBH restored LH pulses in ovariectomized rats in mid-lactation, and concluded that the neural signal responsible for the suppression of pulsatile LH secretion by suckling was conveyed through the dorsal part of the hypothalamus. In the experiments presented in this chapter, various roof deafferentations were performed to determine the more precise region of the dorsal hypothalamus through which the inhibitory neural input passes to suppress the LH pulses. In addition, electrolytic lesions of the paraventricular nucleus (PVN) were performedto investigate the significance of this nucleus in the suppression of LH secretion during lactation.

Materials and Methods

Treatment and blood sampling

The hypothalamic deafferentation and electrolytic lesion of the PVN were performed on day 7 of lactation. The litter weight gain was calculated with the same methods as used in Chapter 6. Blood samples (120 μl) were collected every 6 min for 3 h beginning 24 h after the brain surgery.

Hypothalamic deafferentation

Four types of roof deafferentation (RD) were performed according to the modified method of Halasz and Pupp (1965): 1) large anterior (LARD), 2) large posterior (LPRD), 3) small anterior (SARD), and 4) middle (MRD). The same L-shaped knife with 1.0 mm radius as described in Chapter 6 (Fig. 6.1) was used for all deafferentations. Animals were fixed in a stereotaxic instrument (Narishige, Tokyo, Japan) with the level of bregma set 1.6 mm below that of lambda. In rats with LARD or LPRD, the knife was lowered into the brain with the horizontal blade directed rostrally and the axis 1.5 or 3.5 mm posterior to the bregma; following the initial 90° rotation it was moved 3.5 or 5.0 mm posterior to the bregma, respectively, and then turned through a further 180° before being moved to its original position to complete the cut. In rats with SARD or MRD, the knife was rotated 360° in the brain with the axis 1.5 or 3.5 mm posterior to the bregma, respectively. The knife was placed at 8.0 mm depth from the bregma in LARD, LPRD or SARD groups, and 8.0 or 8.5 mm depth in MRD group.

Electrolytic lesions of the paraventricular nucleus (PVN)

The tip of the electrode was placed in the brain at 2.1 mm posterior, 0.5 mm lateral and 8.1 mm ventral to the bregma. Bilateral lesions of the PVN were made by passing a cathodal direct current of 1 mA for 40 seconds through a monopolar stainless-steel electrode (0.25 mm in diameter) insulated with epoxylite but possessing an exposed tip.

Data analysis

Statistical differences were determined by Mann-Whitney *U*-test.

Results

A schematic illustration of the site of each hypothalamic roof deafferentation is shown in Fig. 7.1. The suckling behaviour in the litters of ovariectomized lactating rats with the various hypothalamic deafferentations was similar to that of the rats with SD described in the Chapter 6. There was no significant difference in the total weight gain of the litters after the oxytocin injections in the various groups.

Rats with LARD or LPRD demonstrated LH pulses with high frequency and amplitude (Figs. 7.2 & 7.3), and the LH level in rats with SD was maintained at a very low level (Fig. 6.4). The mean LH level during the period of sampling and the mean pulse frequency and amplitude were significantly higher in rats with LARD or LPRD than those observed after SD ($P<0.05$, Mann-Whitney *U*-test, Table 7.1). LH pulses with high frequency and amplitude were found in 1 out of 5 rats with SARD, but the LH secretion in the remaining animals was profoundly suppressed and similar to that observed in the rat with SD (Fig. 7.4).

The dorsoventral level of the large cuts (LARD, LPRD) was consistently in close association with the ventral margin of the PVN. In contrast, the animals with MRD could be classified into 2 groups according to the depth of the knife cut; thus, MRD-1 designates those with the cut on or below the ventral margin of the PVN while MRD-2 represents those in which the lesion passed through the PVN (Fig. 7.5). LH secretion with small amplitude pulses was observed in MRD-1 rats (Table 7.1 & Fig. 7.6), but only the mean LH level was significantly higher than that following SD ($P<0.05$, Mann-Whitney *U*-test); in contrast, MRD-2 rats failed to show any significant difference in the parameters of pulsatile LH release compared with those following SD (Mann-Whitney *U*-test, Table 7.1 & Fig. 7.7).

Four out of 9 rats with electrolytic lesions in the region of the PVN showed LH secretion with small amplitude pulses; these animals were classified as PVN-1 (Table 7.1 & Fig. 7.8). The remaining 5 rats showed a complete suppression of the pulsatile LH release and were designated as PVN-2 (Table 7.1& Fig. 7.9). Histological assessment revealed varying degrees of damage to the periventricular nucleus of the hypothalamus in all rats in the PVN-1 group, but this area was spared in the PVN-2 rats (Figs. 7.10 & 7.11).

Discussion

The findings in Chapter 6 demonstrated that the neural signal for the inhibition of pulsatile LH release by suckling is conveyed to the MBH via the dorsal hypothalamus, since the roof deafferentation restored the frequent LH pulses(Fig. 6.9). Furthermore, pulsatile LH release with high frequency and amplitude was also apparent in the rats with LARD or LPRD in which the extent of the deafferentation was approximately half of that produced by RD in Chapter 6 (Fig. 7.1). It is important to note that the mothers in each experimental group throughout this study received a vigorous suckling stimulus, since the suckling behaviour and total weight gain of the litters nursed by deafferentated animals were indistinguishable from those by SD animals.

These initial findings suggest that the suckling stimulus may be transmitted ventrally through the region common to the LARD and LPRD. To test this possibility, the small cut identified as MRD (Fig. 7.1) in the region overlapped by the LARD and LPRD was made. The animals with MRD were classified into 2 groups according to the level of the deafferentations; when the cut was on or under the ventral margin of the PVN (MRD-1, Fig. 7.5) pulsatile LH release was observed (Fig. 7.6), but when it passed through the PVN (MRD-2, Fig. 7.5) the normal suppression of the pulses persisted (Fig. 7.7). These observations suggest that the PVN is a critical site through which the suckling stimulus projects to the MBH. Nevertheless, when the PVN was lesioned electrolytically, pulsatile LH release remained suppressed in the rats (Fig. 7.9) in which the periventricular nucleus ventral to the PVN was left intact (PVN-2, Fig. 7.10). In contrast, LH pulses were apparent in the animals (Fig. 7.8) with damage to the periventricular nucleus (PVN-1, Fig. 7.10). These results indicate that part of the inhibitory signal for pulsatile LH secretion may be conveyed through the periventricular

region rather than the PVN.

It should be noted that the cuts or lesions designated as MRD-1 or PVN-1 did not restore pulsatile LH release with the high amplitude and frequency that were observed following LARD and LPRD in this chapter (Table 7.1) and RD in Chapter 6 (Table 6.2). This suggests that a relatively large region within the dorsal hypothalamus must be damaged to block the suckling-induced suppression of LHRH release completely; the pathways associated with this inhibition may be rather diffusely distributed. The periventricular region of the hypothalamus ventral to the PVN may also be significant in this inhibitory system, since partial restoration of pulsatility was observed (Table 7.1) in animals with cuts immediately above or lesion within this region (MRD-1 and PVN-1; Figs. 7.5 & 7.10).

It has been previously reported that a small roof deafferentation posterior to the anterior commissure advances the timing of the preovulatory LH surge (Schuiling & Van Rees, 1974) and that the electrical stimulation of the hippocampus inhibits the increase in LH secretion induced by the electrical stimulation of the preoptic area (Kawakami *et al.*, 1973). These findings suggest that pathways which have the capacity to inhibit LH release enter the hypothalamus dorsally. Whether the pathways involved in the suppression of LH secretion during lactation correspond to previously described projections into the periventricular region of the hypothalamus remains to be established.

In conclusion, the results in this chapter indicate that the inhibitory neural pathway involved in the inhibition of the LH pulses during lactation enters the hypothalamus dorsally and may be quite diffuse ventral to the PVN. A critical component of this pathway may pass through the periventricular region towards the MBH.

Table 7.1. Mean plasma LH concentrations and frequency and amplitude of LH pulses (Means ± S.E.M.) in ovariectomized lactating rats with various hypothalamic roof deafferentations or electrolytic lesions of the PVN.

	n[a]	Mean LH (ng/ml)	LH pulse frequency (pulses/3 h)	LH pulse amplitude[b] (ng/ml)	
SD	5	0.07±0.00	1.4±0.40	0.14±0.02	(4)[c]
LARD	6	0.71±0.10**	5.0±0.89**	0.89±0.14**	(6)
LPRD	8	0.61±0.09**	6.6±0.60**	0.57±0.08**	(8)
SARD	5	0.36±0.24*	2.4±1.29	0.49±0.37	(3)
MRD-1	8	0.19±0.04*	2.9±0.88	0.20±0.02	(7)
MRD-2	5	0.07±0.00	1.4±0.51	0.23±0.06	(4)
PVN-1	4	0.12±0.05*	5.3±1.03*	0.27±0.04*	(4)
PVN-2	5	0.06±0.01	0.4±0.40	0.49	(1)

SD-sham-, LRD-large, LARD-large anterior, LPRD-large posterior, MRD-middle roof deafferentation, PVN-electrolytic lesion of the paraventricular nucleus. Rats with MRD or PVN lesions were classified into 2 groups (see text for details). *P<0.05, **P<0.01, compared with SD (Mann-Whitney *U*-test) [a]Number of animals used. [b]Pulse amplitude was calculated in animals showing LH pulses. [c]Number of animals showing LH pulses. The data for the rats with SD is taken from Chapter 6 (Table 6.2).

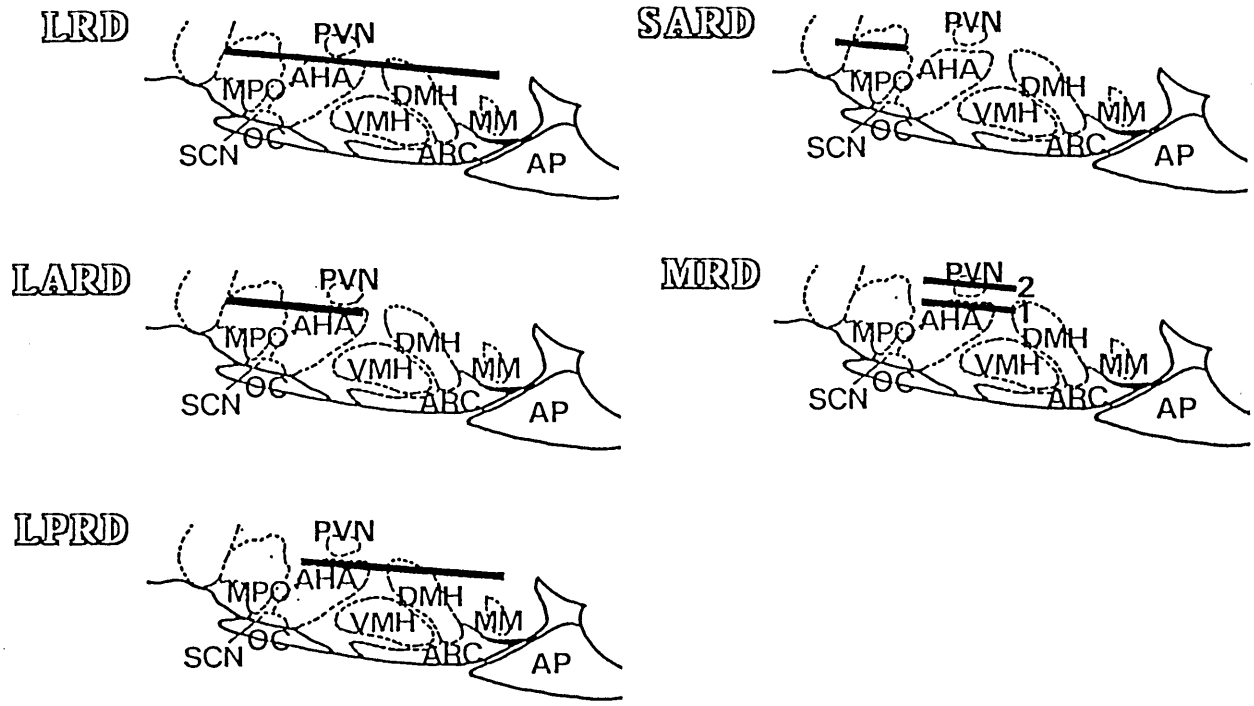


Fig. 7.1. Schematic illustration of the site of the hypothalamic roof deafferentation (RD) in the sagittal plane. LARD-large anterior, LPRD-large posterior, SARD-small anterior, MRD-middle roof deafferentation. The animals with MRD were classified into 2 groups according to the position of the cut. In MRD-1 the cut was on or below the ventral margin of the paraventricular nucleus; in MRD-2 the cut passed through this nucleus. AHA-anterior hypothalamic area; AP-anterior pituitary; ARC-arcuate nucleus, DMH-dorsomedial hypothalamic nucleus; MM-mammillary body; MPO-medial preoptic area; OC-optic chiasm; PVN-paraventricular nucleus; SCN-suprachiasmatic nucleus; VMH-ventromedial hypothalamic nucleus.

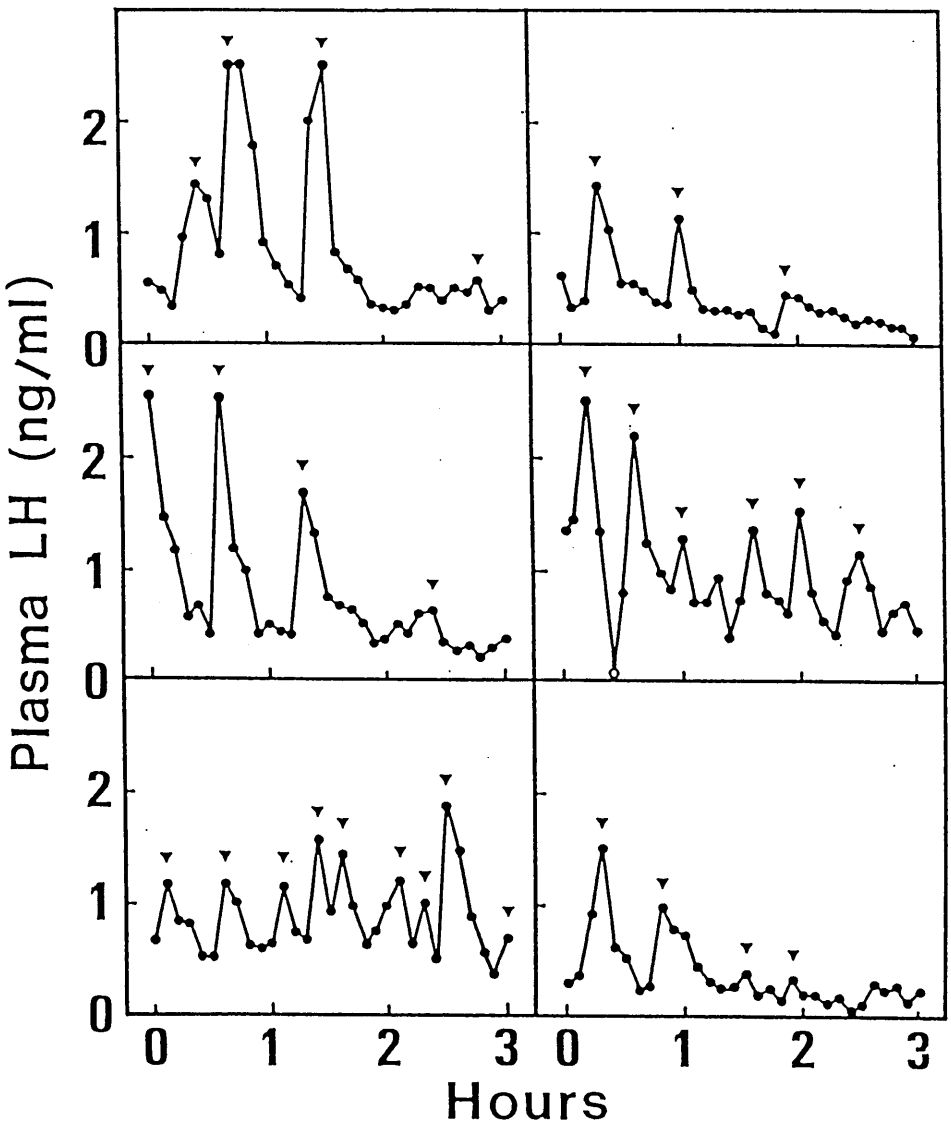


Fig. 7.2. Profiles of plasma LH concentrations in individual ovariectomized lactating rats with large anterior roof deafferentation (LARD). Ovariectomy and hypothalamic deafferentations were performed on day 2 and day 7, respectively. Blood samples were collected every 6 min for 3 h beginning 24 h after the deafferentation. Arrowheads represent the peak of LH pulses identified with the PULSAR computer program.

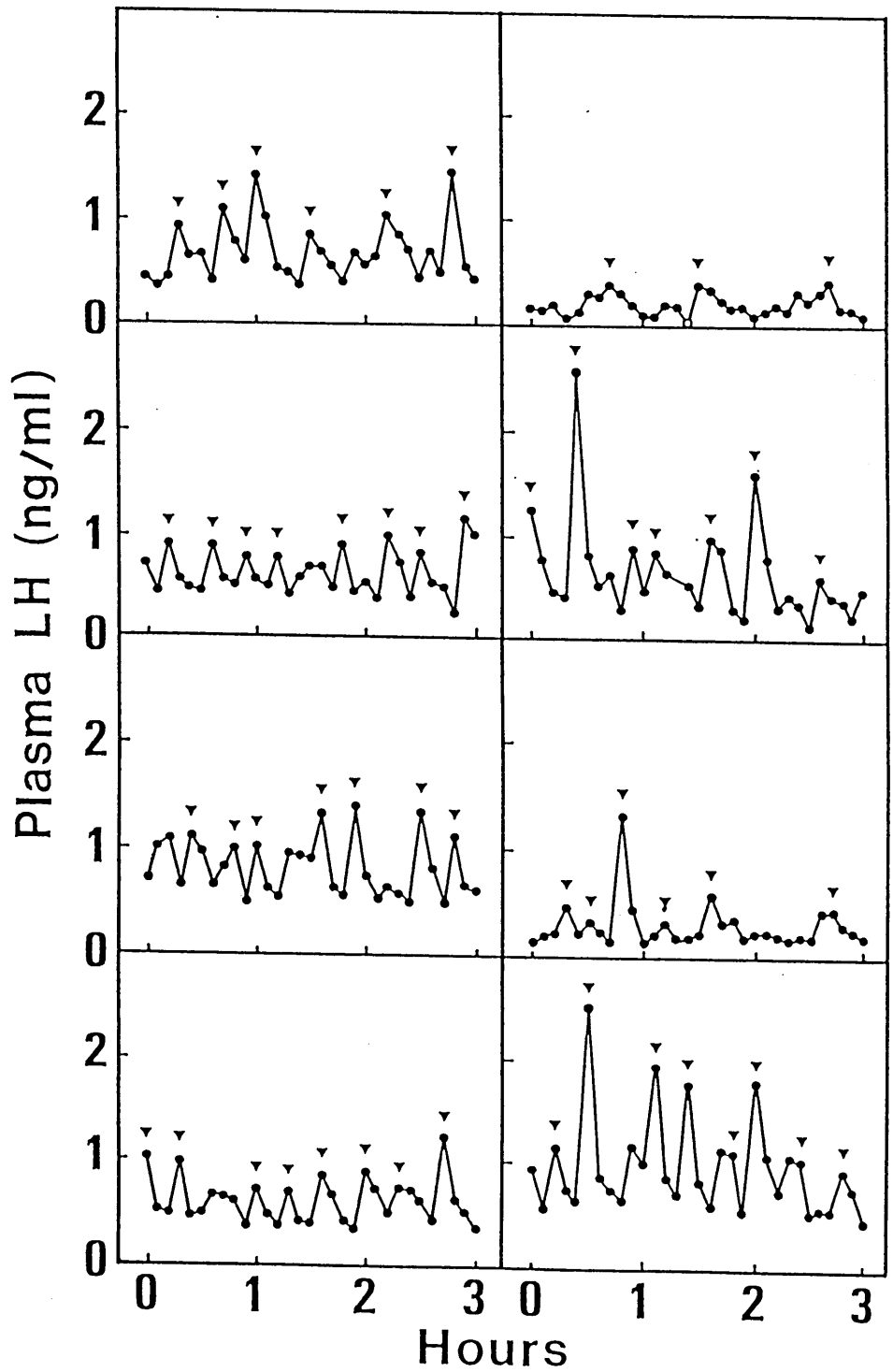


Fig. 7.3. Profiles of plasma LH concentrations in individual ovariectomized lactating rats with large posterior roof deafferentation (LPRD). Ovariectomy and hypothalamic deafferentations were performed on day 2 and day 7, respectively. Blood samples were collected every 6 min for 3 h beginning 24 h after the deafferentation. Arrowheads represent the peak of LH pulses identified with the PULSAR computer program.

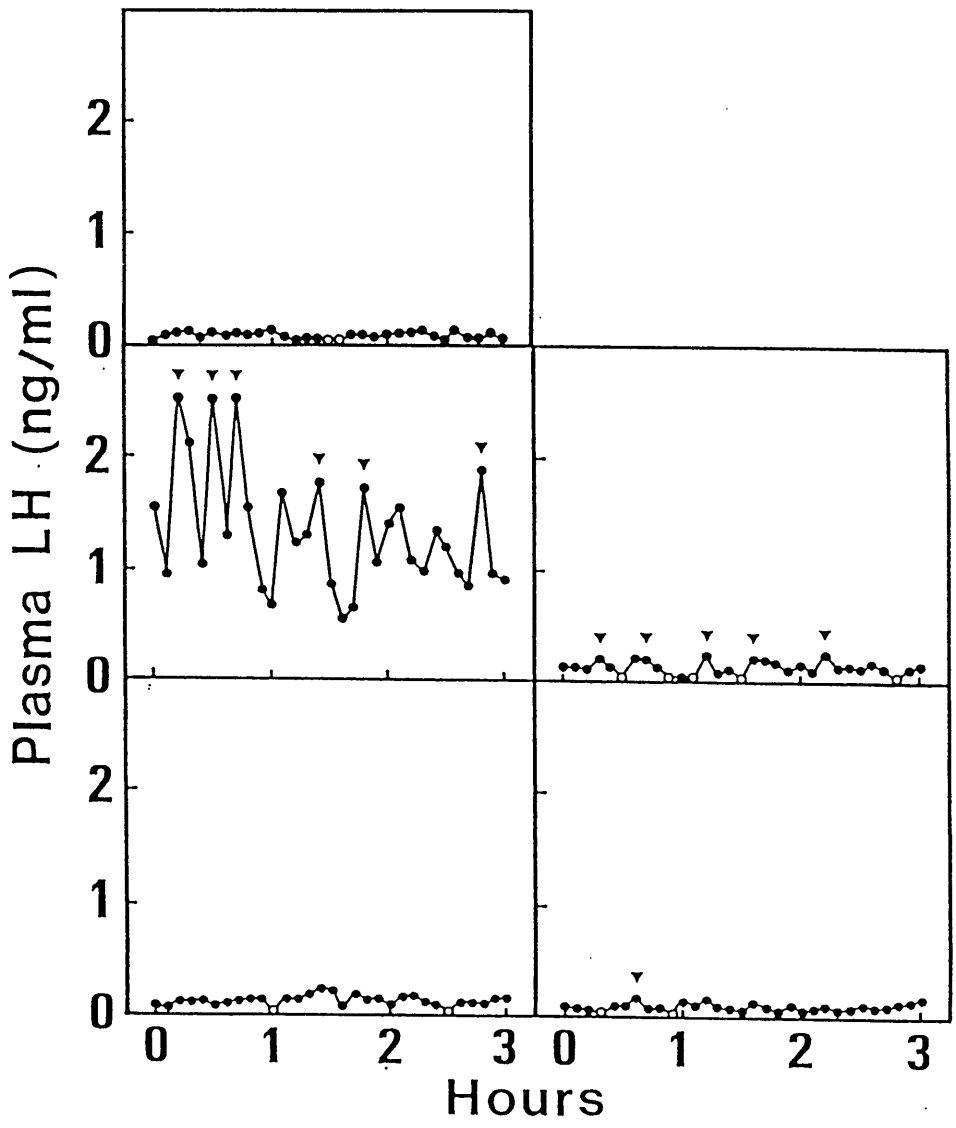


Fig. 7.4. Profiles of plasma LH concentrations in individual ovariectomized lactating rats with small anterior roof deafferentation (SARD). Ovariectomy and hypothalamic deafferentations were performed on day 2 and day 7, respectively. Blood samples were collected every 6 min for 3 h beginning 24 h after the deafferentation. Arrowheads represent the peak of LH pulses identified with the PULSAR computer program.

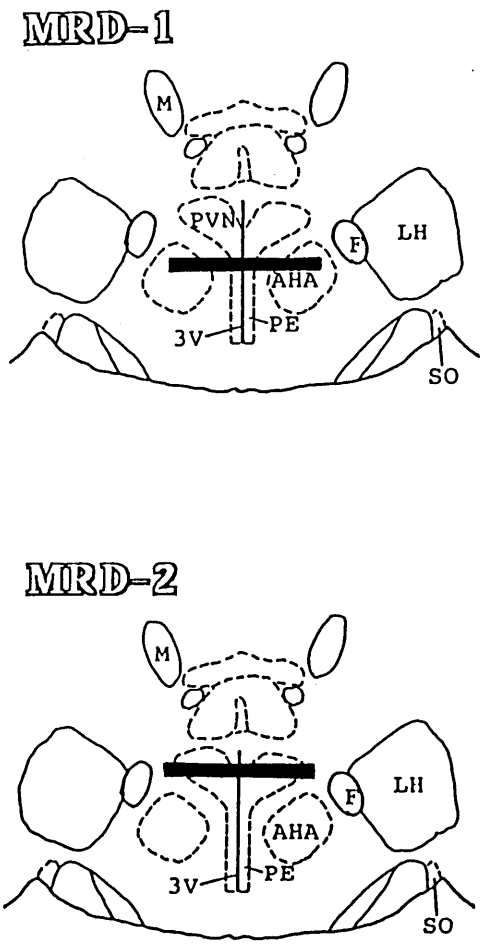


Fig. 7.5. Schematic illustration of the middle roof deafferentation (MRD) in the coronal plane. Animals were classified into 2 groups according to the position of MRD. The cut was on or below the ventral margin of the PVN in MRD-1 and passed through the PVN in MRD-2.

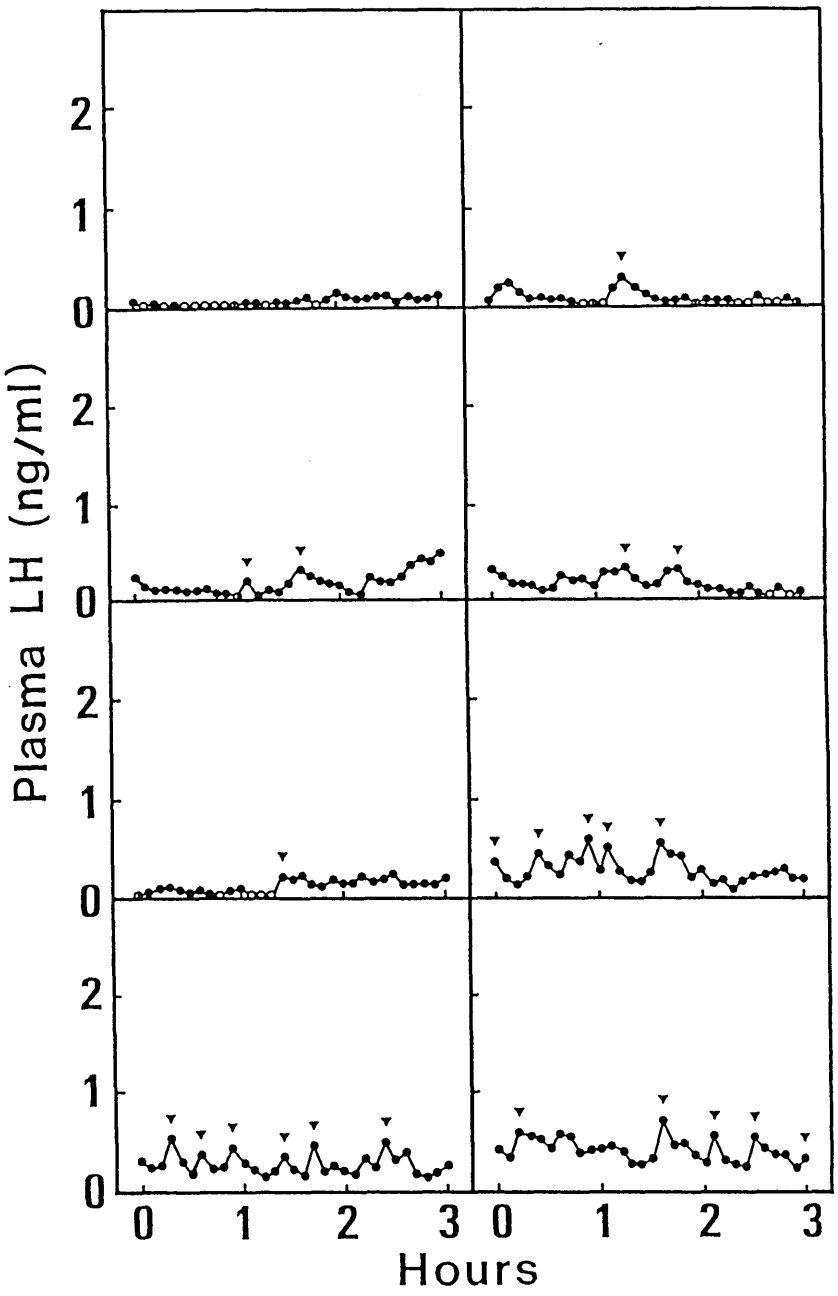


Fig. 7.6. Profiles of plasma LH concentrations in individual ovariectomized lactating rats with middle roof deafferentation (MRD-1) which was located on or under the ventral margin of the PVN. Ovariectomy and hypothalamic deafferentations were performed on day 2 and day 7, respectively. Blood samples were collected every 6 min for 3 h beginning 24 h after the deafferentation. Arrowheads represent the peak of LH pulses identified with the PULSAR computer program.

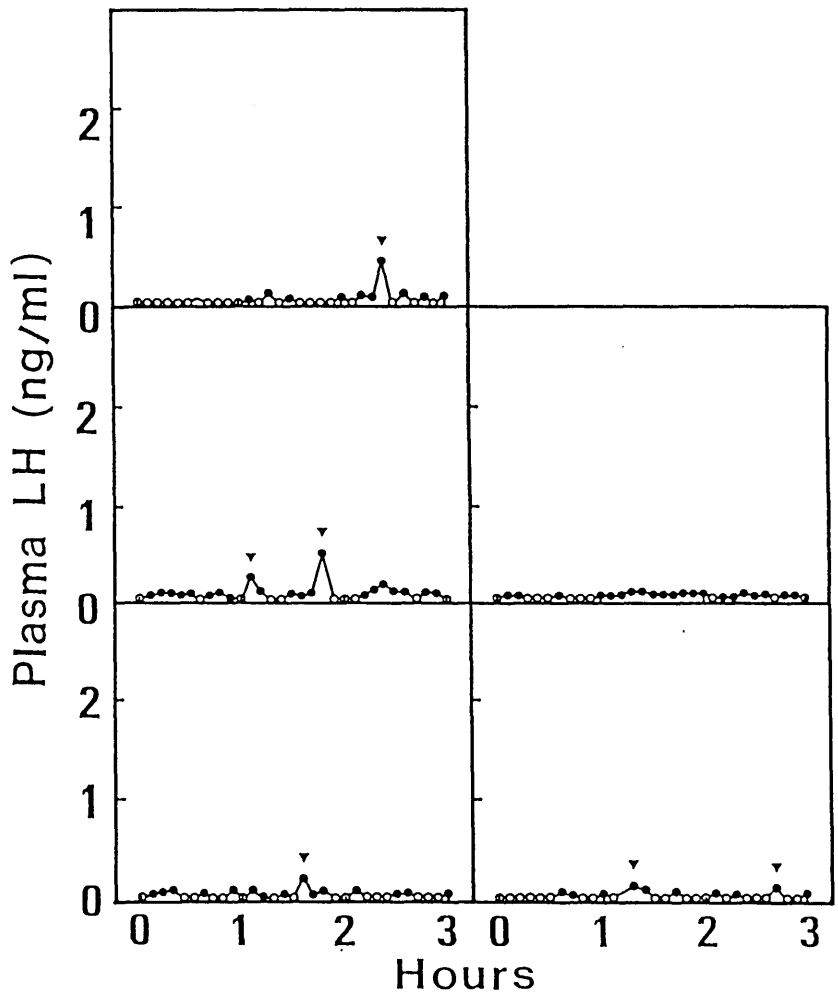


Fig. 7.7. Profiles of plasma LH concentrations in individual ovariectomized lactating rats with middle roof deafferentation (MRD-2) which was located above the ventral margin of the PVN. Ovariectomy and hypothalamic deafferentations were performed on day 2 and day 7, respectively. Blood samples were collected every 6 min for 3 h beginning 24 h after the deafferentation. Arrowheads represent the peak of LH pulses identified with the PULSAR computer program.

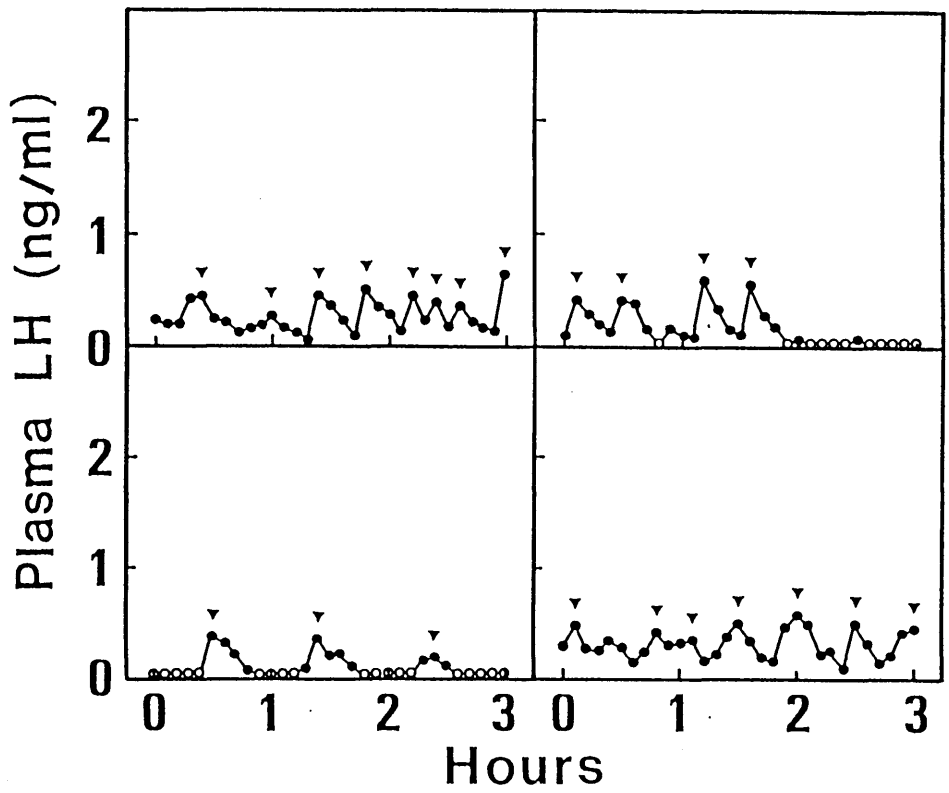


Fig. 7.8. Profiles of plasma LH concentrations in individual ovariectomized lactating rats with the electrolytic lesion of the PVN (PVN-1) showing the pulsatile LH secretion. Ovariectomy and the lesioning were performed on day 2 and day 7, respectively. Blood samples were collected every 6 min for 3 h beginning 24 h after the brain surgery. Arrowheads represent the peak of LH pulses identified with the PULSAR computer program.

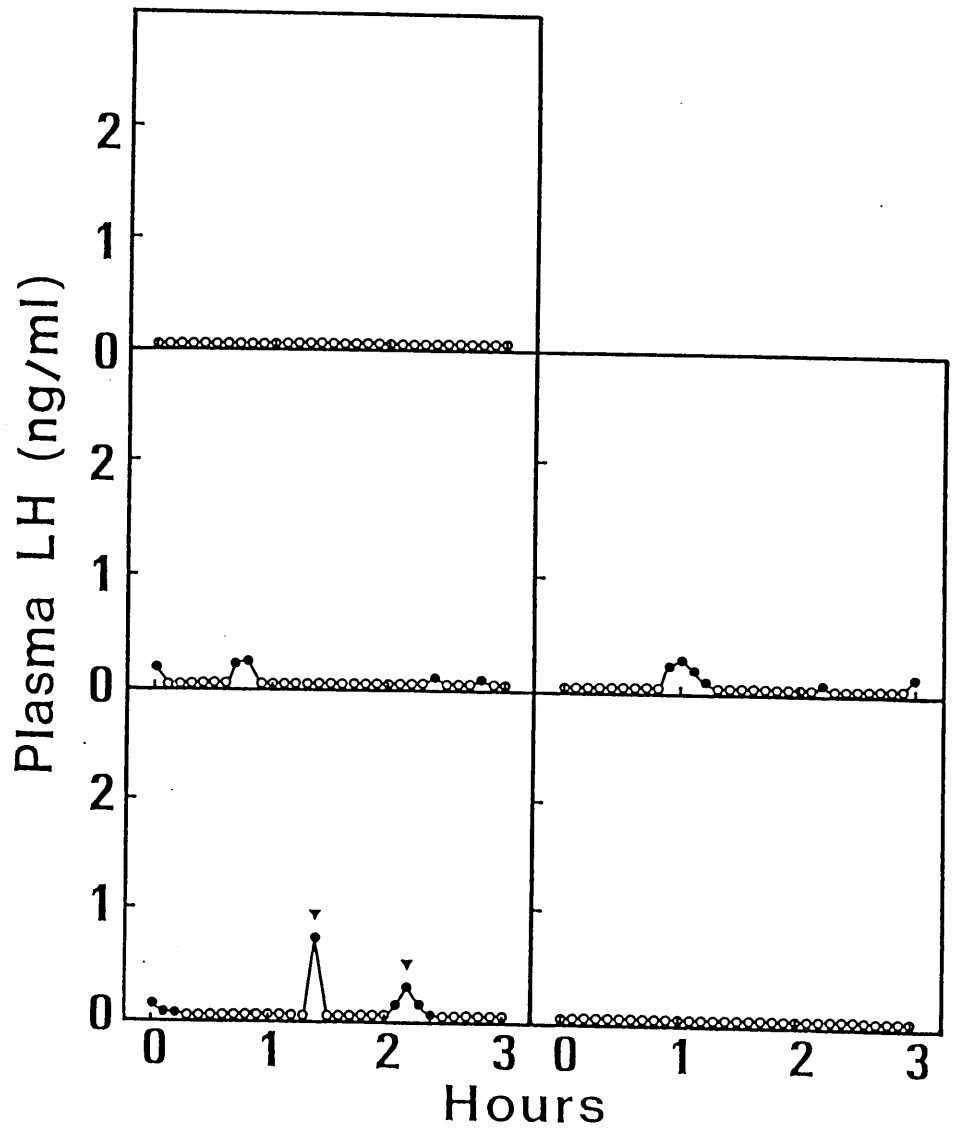


Fig. 7.9. Profiles of plasma LH concentrations in individual ovariectomized lactating rats with the electrolytic lesion of the PVN (PVN-2) showing no pulsatile LH secretion. Ovariectomy and the lesioning were performed on day 2 and day 7, respectively. Blood samples were collected every 6 min for 3 h beginning 24 h after the brain surgery. Arrowheads represent the peak of LH pulses identified with the PULSAR computer program.

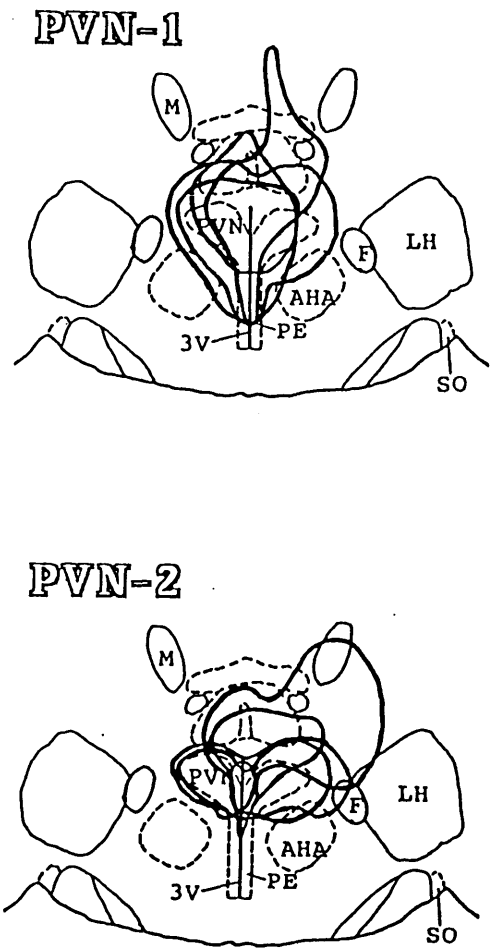


Fig. 7.10. Illustration of the PVN lesions in the coronal plane. The animals were classified into 2 groups according to the presence of LH pulses (PVN-1 group showed LH pulses and PVN-2 group did not). AHA-anterior hypothalamic area, F-fornix, LH-lateral hypothalamic area, M-mammillothalamic tract, PE-periventricular hypothalamic nucleus, PVN-paraventricular nucleus, SO-supraoptic nucleus, 3V-third ventricle.

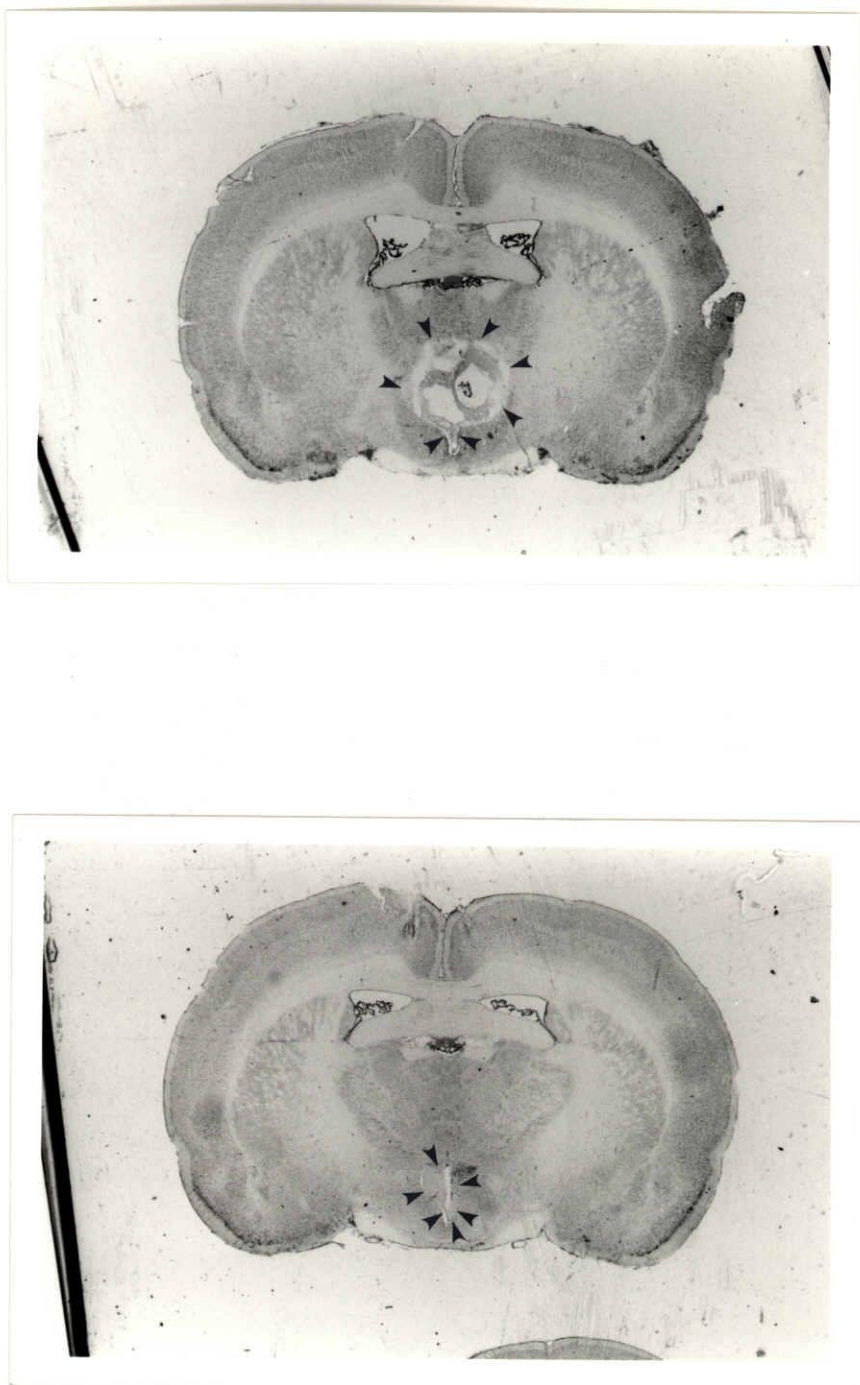


Fig. 7.11a. Photomicrographs of frontal sections of the brain of two representative rats classified as PVN-1 group. Arrowheads indicate the site of the lesion.

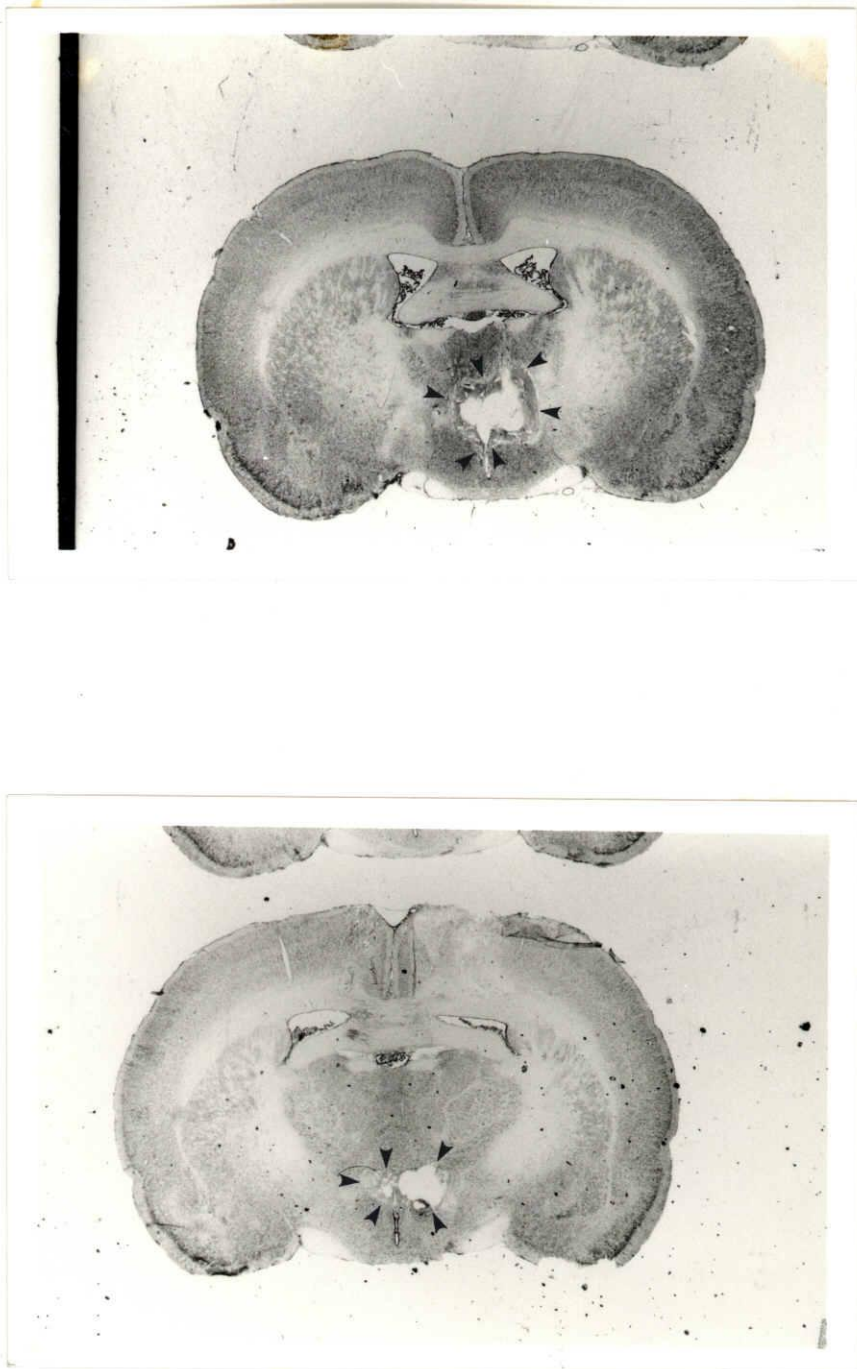


Fig. 7.11b. Photomicrographs of frontal sections of the brain of two representative rats classified as PVN-2 group. Arrowheads indicate the site of the lesion.

CHAPTER 8

General discussion

Suppression of pulsatile LH secretion by the suckling stimulus in the absence of the negative-feedback effect of ovarian steroids

Chapter 3 reveals that the suckling stimulus suppresses pulsatile LH release in the absence of the negative feedback effect of steroids. The removal of pups induced pulsatile secretion of LH and the subsequent resuckling by pups inhibit the secretion in ovariectomized lactating rats. These results suggest that the suckling stimulus is a predominant factor suppressing the pulsatile LH secretion. On the other hand, LH surges were able to be induced by the high level of circulating oestrogen in ovariectomized lactating rats even if the vigorous suckling stimulus was provided; i.e. the suckling stimulus was unable to suppress daily LH surges induced by the chronic oestrogen treatment (Chapter 4). These results indicate that the suckling stimulus suppresses the tonic LH release which is responsible for the maturation of ovarian follicles, but not LH surge which is indispensable for ovulation. Since tonic LH secretion and the surge have been reported to be controlled by the mediobasal hypothalamus (MBH) and the preoptic area (POA), respectively, the suckling stimulus seems to inhibit the activity of the MBH, but not the activity of the POA (Fig. 8.1).

Among factors that have been reported to suppress pulsatile secretion of LH, fasting and stress need the ovarian steroids to suppress the LH release completely (Cagampang *et al.*, 1990; Higuchi *et al.*, 1986). Moreover, LH secretion is depressed under long days in short-day breeders treated with ovarian steroids (Karsch *et al.*, 1984). Thus, most of these environmental factors are able to control LH secretion only in the presence of ovarian steroids. These observations have been explained by the change in the sensitivity of LH-releasing mechanism to the negative-feedback effect of ovarian steroids by the exogenous stimuli. On the contrary, the suckling stimulus is a novel stimulus

ıo ovarian steroids for inhibiting LH secretion, as demonstrated in this thesis.

lvement of humoral factors

The suckling stimulus has been known to induce the secretion of such hormones as PRL, ACTH, growth hormone (GH) and oxytocin (Tucker, 1988; Voogt *et al.*, 1969; Wakerley *et al.*, 1988). The possibility arises whether or not these hormones can play a role in suppressing LH secretion during lactation. PRL has been shown to have an antigonadal property, since hyperprolactinaemia is often accompanied by the decline of plasma LH levels and since the exogenous PRL inhibits the LH secretion (Cohen-Becker *et al.*, 1986; Smith & Bartke, 1987). However, results shown in Chapter 5 demonstrate that the blockade of PRL secretion by the administration of a dopamine agonist CB-154 did not affect the suppression of pulsatile LH release by the suckling stimulus. Moreover, complete deafferentation could reinstate pulsatile LH secretion without influencing the plasma level of PRL (Chapter 6). Considering these results together, it is concluded that the suckling stimulus can suppress directly LHRH release without the mediation of PRL in ovariectomized lactating rats. The mechanisms that are responsible for the suppression of LH secretion and maintenance of a high level of PRL secretion may be independent from each other at the level of the hypothalamus.

Since the high plasma level of ACTH induced by adrenalectomy did not prevent the suppression of LH secretion during lactation (Taya & Sasamoto, 1990), ACTH could not participate in suppressing LH secretion caused by the suckling stimulus. Whisnant *et al.* (1985) suggested that circulating cortisol was not a physiological inhibitor of LH secretion in the lactating cow. Moreover, no relationship was observed between episodic GH secretion and the actual suckling in rats (Nagy *et al.*, 1986). These results suppc the idea that the neural input originated from the suckling stimulus suppresse

pulsatile LH secretion without mediation of the humoral factors.

Neural pathway conveying suppressing effects on the activity of the LHRH pulse generator

There has been reported a considerable body of evidence suggesting that the inhibition of LH secretion during lactation is resulted from suppression of LHRH release by the suckling stimulus at the hypothalamic level. Many workers have demonstrated that LHRH administration enhanced LH secretion in lactating animals (rat: Lee *et al.*, 1989; cow: Jaeger *et al.*, 1987; goat: Knight *et al.*, 1988; ewe: Newton & Edgerton, 1989). Smith (1984) reported that the suckling stimulus reduced the number of LHRH binding sites without affecting the affinity of the receptor, and that the degree of the reduction was directly related to the intensity of the suckling stimulus. The large decrease in contents of the pituitary LHRH receptor in the presence of the strong suckling stimulus is most likely to be due to a dramatic suppression of LHRH release from the hypothalamus, because the administration of exogenous LHRH reversed the reduction of LHRH receptor caused by the suckling stimulus. Moreover, pro-oestrous surge-like LH release was induced by oestradiol administration to the ovariectomized lactating rat (Chapter 4; Copping & McCann, 1979; Smith, 1978b). Therefore, the blockade of LH secretion imposed by suckling should occur at the level of the hypothalamus, but not at the pituitary level.

Pulses of LH secretion correspond well to LHRH pulses that are thought to be generated by the putative pulse generator located in the MBH (Soper & Weick, 1980); the frequency of LH pulses seems to reflect directly the frequency of LHRH pulses and the frequency could be regulated by the LHRH pulse generator. The removal of the suckling stimulus dramatically increased the frequency of LH pulses and the subsequent

resuckling quickly reduced it as shown in Chapter 3. It is highly possible that the suckling stimulus could suppress the activity of LHRH pulse generator. This possibility was evinced by the results in Chapter 6; the LH pulses became apparent after complete deafferentation, suggesting that the LHRH pulse generator is located in the hypothalamic island, namely the MBH, made by the complete deafferentation. The suckling stimulus could input to the LHRH pulse generator located in the MBH and suppress its activity. The results of Chapters 6 and 7 reveal that the inhibitory signal for LH secretion originated from the suckling stimulus is conveyed to the MBH dorsally and this signal passes through the periventricular nucleus (Fig. 8.2). Whether pathways involved in the suppression of LH secretion during lactation correspond to the previously described projections into the periventricular region of the hypothalamus (Swanson, 1987) remains to be established.

Possible neurotransmitters

Little is known about the characteristics of the fibres conveying the signal from the teat to the MBH for suppressing the activity of the LHRH pulse generator. It has been reported that noradrenaline may perform an inhibitory role in LH release (Leung *et al.*, 1981) via neurons originating from the pons and medulla and projecting rostrally to the hypothalamus (Moore, 1979; Ungerstedt, 1971). Noradrenaline is, therefore, a candidate to be involved in the mechanism suppressing pulsatile LH secretion in lactating rats. γ-aminobutylic acidergic (GABAergic) neurons might also be associated with this inhibition, since it has been reported that GABA concentrations in the cerebrospinal fluid in lactating rats with their pups were higher than those deprived of their pups for several hours (Qureshi *et al.*, 1987) and that the suckling stimulus increased the concentration of the enzyme of GABA biosynthesis in the MBH (Racagni, *et al.*, 1984). In addition,

inhibitory effects of GABA have been demonstrated on pulsatile LH secretion in ovariectomized lactating rats (Donoso & Banzan, 1984). Opioidergic neurons might also be a candidate mediating the suppression of LH secretion during lactation. Plasma concentrations of β-endorphin in peripheral (Riskind *et al.*, 1984) and portal blood (Gordon *et al.*, 1987) in lactating animals receiving the suckling stimulus were higher than those without the suckling stimulus, and the opioid peptide suppressed the pulsatile LH release in ovariectomized rats (Babu *et al.*,1988). Many workers have suggested the involvement of endogenous opioids in suppression of LH release during lactation in various animals (rat: Sirinathsinghji & Martini, 1984; cow: Whisnant *et al.*, 1986; Myers *et al.*, 1989; Rund *et al.*, 1989; sow: Barb *et al.*, 1986; Mattioli *et al.*, 1986; Armstrong *et al.*, 1988; ewe: Malven & Hudgens, 1987). However, my recent work reveals that the endogenous opioid transmitted signals involved in the negative feedback of the ovarian steroid for LH secretion but not signals of the suckling stimulus (unpublished data).

Involvement of corticotrophin releasing factor (CRF) has been suggested in an inhibition of pulsatile LH release by stress (Rivier & Vale, 1984; Rivier *et al.*, 1986; Petraglia *et al.*, 1986; Gindoff & Ferin, 1987), indicating the possibility that CRF could mediate the inhibitory signal of the suckling stimulus on pulsatile LH secretion. As shown in Chapter 7, the electrolytic lesion of the PVN consisting of a large number of cell bodies containing CRF failed to recover the pulsatile secretion of LH. Moreover, the intracerebroventricular injection of α-helical CRF, an antagonist of CRF, in our preliminary experiment could not remove the suppression of LH release in ovariectomized rats. These results suggest that CRF does not participate the suppression of LH release by the suckling stimulus.

Our preliminary experiments using antagonists to α and β-adrenergic, $GABA_A$,

$GABA_B$ and glycine receptors, failed to prevent the suppression of LH release by the suckling stimulus. Further studies are required to identify the nerves which convey the inhibitory signal of the suckling stimulus to the putative pulse generator for LH secretion located in the MBH.

Conclusion

The present dissertation describes the results of experiments demonstrating the neuroendocrine mechanism by which the suckling stimulus suppresses pulsatile LH secretion in lactating rats used as a model for another animals and women.

The results in this thesis demonstrate the neuroendocrine mechanism by which the suckling stimulus suppresses LH secretion during lactation; 1) the suckling stimulus suppresses pulsatile LH release in the absence of the negative feedback effect of ovarian steroids, but not daily LH surges induced by the chronic treatment of oestradiol. 2) PRL does not mediate the suppressing effect of the suckling stimulus on LH pulses. 3) the inhibitory signal emanating from the mother's teat is conveyed dorsally to the MBH through the periventricular nucleus and suppresses pulsatile LH secretion.

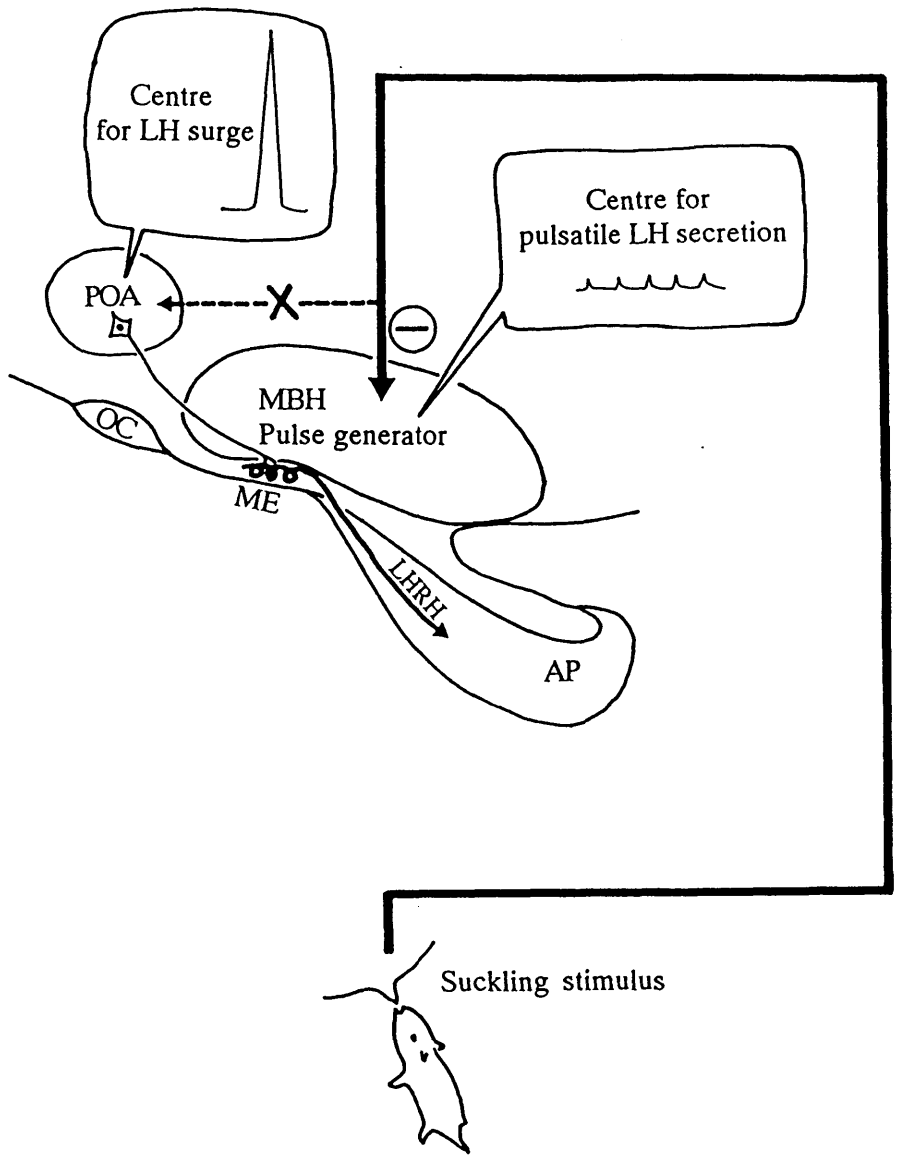


Fig. 8.1. Schematic illustration of the neural input of suckling stimulus to the hypothalamus. AP-anterior pituitary, MBH-mediobasal hypothalamus, ME-median eminence, OC-optic chiasm, POA-preoptic area.

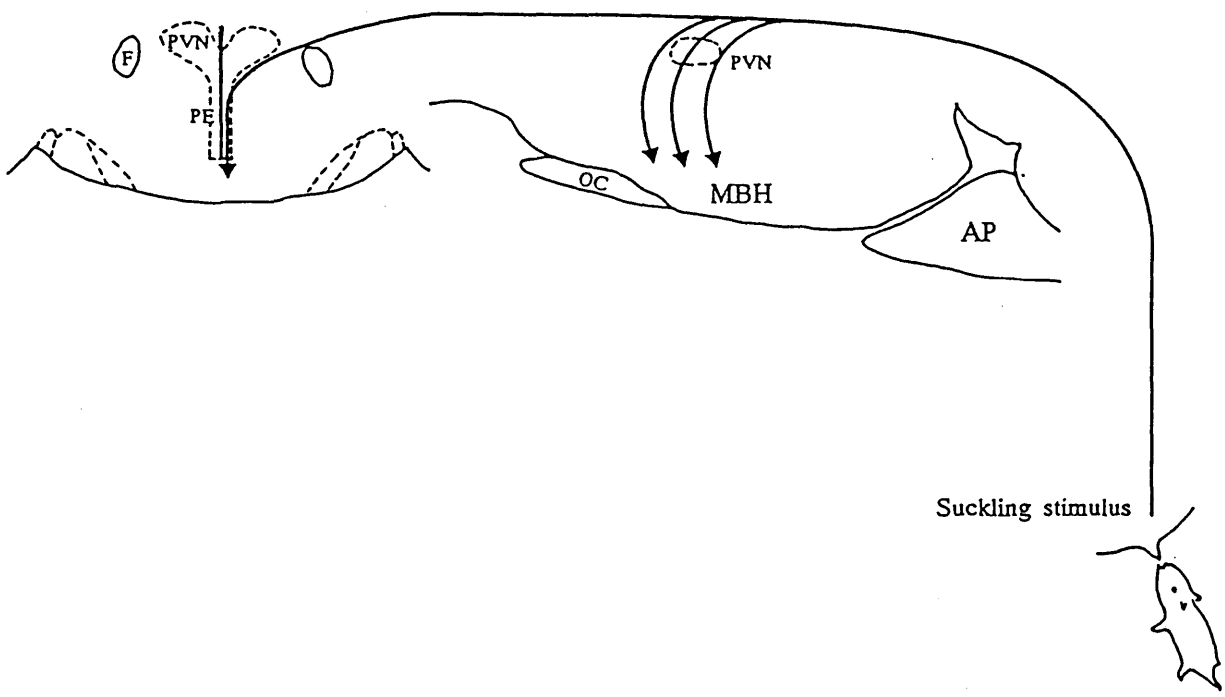


Fig. 8.2. Schematic illustration of the neural pathway of the suckling stimulus within the hypothalamus. AP-anterior pituitary, F-fornix, MBH-mediobasal hypothalamus, OC-optic chiasm, PE-periventricular hypothalamic nucleus, PVN-paraventricular nucleus.

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LIST OF PUBLICATIONS CONCERNING THIS DISSERTATION

1. Tsukamura, H., Maeda, K.-I. & Yokoyama, A. (1988). Effect of the suckling stimulus on daily LH surges induced by chronic oestrogen treatment in ovariectomized lactating rats. *Journal of Endocrinology* 118, 311-316.
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