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Electrophysiological study on the neural control mechanism of the gonadotropin-releasing hormone release in the female goat

（シバヤギ性腺刺激ホルモン放出ホルモン分泌調節機構に関する
電気生理学的研究）

1994

The United Graduate School of Veterinary Sciences,
Gifu University

(Tokyo University of Agriculture and Technology)

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Abbreviations

FSH : follicle-stimulating hormone

GnRH : gonadotropin-releasing hormone

IC : integrated circuit

LH : luteinizing hormone

MBH : medial basal hypothalamus

MUA : multiple unit activity

OVLT : organum vasculosum of the lamina terminalis

PBST : phosphate buffered saline containing Triton X-100

PGF2α : prostaglandin F2α

POA : preoptic area

SCN : suprachiasmatic nucleus

SD : standard deviation

SEM : standard error of mean

Chapter 1

General Introduction

Brain control of reproduction

It is now widely accepted that the reproductive process in mammals is governed by the brain. This concept was first proposed by Geoffrey W. Harris in 1950's. He found that disconnecting of the pituitary stalk resulted in an atrophy in the gonad, the thyroid, and adrenal glands, and then predicted the existence of the hypothalamic humoral factors regulating the pituitary hormone secretion [35]. His prediction broke the dawn of the reproductive neuroendocrinology and first put the brain in the highest position in a network regulating the reproductive system in mammals.

Many external and/or internal environmental changes modulate the reproductive activities through the brain mechanism. For example, the photoperiod, namely an annual changes in daylength, is the principal external cue for determining the period of annual reproductive cyclicity in most mammalian species [43,64,77,129]. Under short-day condition, the short-day breeders such as sheep, goat, and some species of primate continue the ovulatory cycle, whereas the long-day breeder such as horse, ferret and hamster become anovulatory [43,65,77]. Physiological cessation of ovarian cyclicity during lactation is caused by the suckling stimulus by pups or babies [37,66,70,122,123]. The ability of stress including infectious diseases, psychiatric disorders, and surgical trauma to interfere with human reproductive functions has long been recognized by clinicians [110]. In addition, pheromonal cues influence the ovarian function; the most striking example of this is the induction of ovulation in anestrus ewes by exposure to rams, i.e. "ram effect" [18,27,109,117].

The information emanating from the environmental factors is accepted by the various sensory system and conveyed to the brain through the neural pathway. The brain then integrates these sensory inputs and transduces them to humoral signals such as hypothalamic hormones, which in turn regulate the gonadal function by facilitating or

suppressing the secretion of gonadotropins or other hormones from the anterior pituitary gland.

Rhythmic nature of GnRH release

Gonadotropin-releasing hormone (GnRH, also called LHRH for luteinizing hormone-releasing hormone) was first defined from porcine and ovine brain by laboratories headed by Guillemin and Schally in 1971 [2,69,115]. This neurohormone is synthesized in the precursor form in the cell bodies located in the hypothalamus [1], and released as a decapeptide molecule from neurovascular terminals within the median eminence into hypophysial portal veins. After diffusion from the secondary portal plexus into the extracellular spaces of the anterior lobe of the pituitary, GnRH molecule binds to specific membrane receptors on gonadotrophs to activate one or more second messenger systems, and induces secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [23]. While 23 years since discovery of GnRH, another gonadotropin releasing humoral factor has not been identified yet in mammals, and GnRH is still considered to be the sole neuropeptide mediating secretion of gonadotropins.

It has long been predicted that the secretory profile of GnRH is pulsatile, because 1) the pulsatile pattern of gonadotropin secretion was found in many species of both sexes, 2) the blockade of the GnRH actions by immunoneutralizing the endogenous decapeptide [16,21,26,36] or antagonizing its receptor [30] arrested the pulsatile gonadotropin secretion. Recent technological advances of the method for neurophysiology and radioimmunoassay have made it possible to analyze directly the release pattern of GnRH *in vivo*, and this hypothesis was verified in the ewe [12] by clearly showing the pulsatile pattern of GnRH release into the pituitary portal circulation that was exclusively synchronized with LH pulses in peripheral circulation. Until now, temporal relationships

between GnRH and LH pulses have been documented in the monkey [62,99,131,133], sheep [9,12,15,74], rabbit [100], rat [60,61,130] and goat [145]. Therefore, there is now general agreement that GnRH is released into the portal circulation in the pulsatile manner and then regulates the pulsatile gonadotropin release from the pituitary.

The physiological necessity of pulsatility of GnRH release for the hormonal interaction of the hypothalamo-pituitary-gonadal axis has been demonstrated. In the ovariectomized rhesus monkey bearing radiofrequency lesions of the medial basal hypothalamus (MBH) that depleted endogenous GnRH release, the attempt was made to resume gonadotropin secretion by constant infusion of exogenous GnRH, but it only induced a transitory release of LH and FSH regardless of the rate of decapeptide infusion [103]. On the contrary, when this GnRH replacement regimen was changed to an intermittent mode with a physiological frequency of administration as seen in ovariectomized monkeys with intact hypothalami, the gonadotropin secretion was reestablished [4,85]. Moreover, the ovulatory menstrual cycles were maintained by mimicking the pulsatile GnRH release in intact rhesus monkey that had been MBH-lesioned [53]. A poor responsiveness of adenohypophysial gonadotrophs to continuous GnRH stimulus has been extensively demonstrated in many species, suggesting that constant exposure to GnRH leads to desensitization or down-regulation of the anterior pituitary [53]. These results indicate that the intermittent (pulsatile), but not continuous, mode of GnRH release is intrinsically essential to maintain the secretory activity of the adenohypophysial gonadotrophs and thereby the gonadal activity.

Furthermore recent studies have demonstrated that the pattern of pulsatile GnRH release, especially its frequency transition, plays an important role in controlling the activity of the reproductive function. For example, Vallel et al. [3] found that the increment of GnRH pulse frequency in the portal blood was found during the artificial luteolysis

induced by programmed administration of progesterone in ewes during the breeding season, but not in the anestrous season, and suggested that absence of the increment of GnRH pulse frequency following luteolysis would induce the switch from the breeding to anestrous season. In sexually immature rhesus monkey, by using the push-pull perfusion technique, it was shown that the frequency of pulsatile GnRH release in the median eminence was increased from the prepubertal to early pubertal period [134]. The data suggest that a rise in GnRH pulse frequency is also involved in the induction of sexual maturation.

The pulsatility of GnRH release is governed by the yet to be identified neural construct, so-called the "GnRH pulse generator", that is considered to reside in the hypothalamus. Thus, many internal and external environmental changes finally modulate the neural operation of the GnRH pulse generator in the brain; this, in turn, gives rise to the pattern of pulsatile GnRH release and then LH secretion, which eventually dictates the gonadal function [24,27,53,63,77]. Therefore, the GnRH pulse generator has been recognized as a key determinant of all influence on the reproduction conveyed through the central nervous system in mammals.

Estrous cycle and GnRH secretion

In female animal, patterns of reproductive activity are practically dominated by the estrous cycle. The estrous cycle is controlled by a complex interaction between cues from the external environment and internal hormonal signals. External cues determine whether or not the estrous cycle occurs as described above, but are not directly involved in the sequences of the estrous cycle. Instead, these are governed by the endocrine communication among hypothalamus, pituitary and gonads.

Using the pattern of LH pulses as an index of the GnRH pulse, it has long been

suspected that pulsatile GnRH release varies during the estrous cycle. For instance, during the follicular phase of the estrous cycle, LH is secreted as a series of high frequency and low amplitude pulses occurring approximately once every hour in the ewe [27]. In contrast, the luteal phase is characterized by the pattern of low frequency, high amplitude, and irregular pulses often with extremely long interval among them [27]. Direct evidence for this was first presented by Clarke and Cummins [17] and then by Moenter et al. [76]. In these studies the pituitary portal-blood sampling technique was adopted, and they demonstrated GnRH pulses of low frequency and high amplitude during the luteal phase, whereas those of high frequency and low amplitude during the follicular phase [17,76]. The concept drawn from these data is that the GnRH pulse generator, which drives pulsatile GnRH release, is subjected to the feedback modulation by gonadal steroids.

Karsch et al. [46] demonstrated that the increment of the LH concentration induced by the surgical removal of corpus luteum during the luteal phase was accompanied by a sustained rise in circulating estradiol. Closed correlation was shown between the pattern of endogenous LH pulses and the amount of estradiol secretion during the follicular phase in the ewe [113,128]. McNeilly et al. [71] reported that the inhibition of high frequency LH pulses by the immunoneutralization of GnRH during the follicular phase resulted in an immediate cessation of the ovarian estradiol secretion. These findings suggest that increased pulse frequency of GnRH neurosecretion that occurs following the luteolysis plays a key role for the preovulatory follicular development as well as for the estradiol rise in the systemic circulation.

The preovulatory rise of estradiol then induces a large amount of LH secretion (LH surge) that causes ovulation and initiates luteinization. Sarker et al. [112] were the first to show that a surge of GnRH was noted at the time of the preovulatory LH surge in the rat. Similarly, in rhesus monkeys the LH surge induced by exogenous estradiol was

shown to be associated with elevated concentrations of stalk blood GnRH [87]. Subsequently, the effect of administration of high dose estradiol on the GnRH secretion was reported in ovariectomized ewes by Clarke and Cummins [13] and Schillo et al. [116]. Although in these earlier studies the pattern of GnRH secretion during the LH surge was not consistent among individual animals, recent studies have clearly demonstrated that the surge release of GnRH into the portal circulation occurred coincident with both estradiol-induced [11,75] and preovulatory [76] LH surges. On the other hand, it was reported that administration of antiserum against GnRH [16,25,36], or transection of the pituitary stalk [14], completely blocked estrogen-induced LH surge in the ewe. Taken together, it is evident that a transient increase of GnRH secretion from the hypothalamus (GnRH surge) is the primary cue for the onset of the LH surge and thereby ovulation.

Thus, it can be summarized at this stage that the estrous cycle in the female animal is governed by the endocrine system consisting of the hypothalamo-pituitary-gonadal axis, and that the neural mechanism dictating the shift from the pulsatile to the surge mode secretion of GnRH may be of central importance for the institution of the estrous cyclicity.

Neural mechanism underlying pulsatile and surge mode secretion of GnRH

Halasz and Pupp [33] first demonstrated that female rats bearing complete hypothalamic deafferentation showed either persistent estrus or persistent diestrus, and suggested that the center for the tonic LH secretion was located inside the hypothalamic island which is isolated by the complete hypothalamic deafferentation from the rest of the brain. Subsequently, Blake & Sawyer [5] and Krey et al. [55] reported that the pulsatile LH and FSH secretion were still apparent in both rats and rhesus monkeys after complete disconnection of the MBH from the remainder of brain. In contrast, discrete radiofrequency lesions encompassing the region of the arcuate nuclei completely disrupted

tonic LH release in the rhesus monkey [103]. These observations are suggesting that the central neural mechanism governing the tonic GnRH release, namely the GnRH pulse generator, is located within the MBH.

However, the questions of what neural elements are involved in GnRH pulse generation or what mechanism controls the generation of a quantum of GnRH release are yet to be answered. Escalera et al. [22] and Wetsel et al. [138] have reported the establishment of clonal GnRH-producing neuronal cell lines, which are immortalized by genetically directed tumorigenesis in transgenic mice, secreting GnRH with a rhythmic pattern to the perifused medium *in vitro*. In the superfusion medium of primary cultures of fetal rat hypothalamic neurons, as well as the GnRH neuronal cell line, episodic GnRH release was spontaneously exhibited; the frequency of which was comparable to that observed in perifused adult or fetal hypothalamic tissues [56]. These results indicate that there is an endogenous pulse-generating mechanism within the GnRH neuron. On the contrary, several researchers hypothesize that endogenous pulse generation of the GnRH neuron is controlled by the input of neurotransmitters and/or neuromodulators. For instance, Terasawa et al. [124] reported that administration of α-adrenergic blockers suppressed GnRH release from the median eminence, and that direct infusion of methoxamine, an α_2-adrenergic stimulant, or norepinephrine into the median eminence facilitated GnRH release in the rhesus monkey. Furthermore, they investigated that norepinephrine release from the median eminence was pulsatile and was synchronous with GnRH pulsatile release, suggesting that this neurotransmitter may be involved in GnRH pulse generation [124].

On the other hand, the GnRH pulse generator activity during the GnRH/LH surge is controversial. Does estradiol induce the GnRH surge by accelerating or heightening the GnRH pulse generator activity? In the rhesus monkey, some have reported high-frequency

pulses during the spontaneous [92] or estradiol-induced LH surge [62]. Clarke and Cummins [13] and Caraty et al. [10] found an increase in the GnRH pulse frequency in the pituitary portal circulation during the LH surge in ovariectomized ewe injected with estradiol. In contrast, a dramatic decline in the frequency of the hypothalamic pulse generator activity was observed in estradiol-induced [51] and preovulatory [94] LH surge in the rhesus monkey by using the electrophysiological recording technique. Moenter et al. [73] recently reported that the secretory profile of GnRH into the portal blood collected every 30 sec during the surge was not the pulsatile but continuous mode in ovariectomized ewe given estradiol.

Thus, although the recent technological advances have made it possible to analyze directly the pattern of the GnRH release, the neural mechanism generating pulsatile and surge mode release of the GnRH is yet poorly understood.

Electrophysiological manifestation of the GnRH pulse generator

An attempt to electrophysiologically monitor the hypothalamic GnRH pulse generator activity was initiated in the late 1970s on the basis of the concept that the neural component periodically fires with a high-frequency burst of action potentials which culminate in a neurosecretion of GnRH. Knobil [54] first described characteristic increases in the neural activity coincided with the initiation of the LH pulse in the MBH of the anesthetized ovariectomized rhesus monkey utilizing acutely placed electrodes to record hypothalamic multiple unit activity (MUA). His group further developed a decade ago the methodology for high clarity recording in both anesthetized and conscious rhesus monkey with multiple recording electrodes chronically placed in the MBH [140]. Successful recording of this specific MUA was then reported in unrestrained ovariectomized rats by Kimura et al. [52] and Nishihara et al. [88].

Recently, in my laboratory, characteristic increases in the frequency of the electrical activity (MUA volley), each of which is synchronized with the initiation of pulsatile LH secretion, have also been successfully recorded in the MBH of conscious ovariectomized goats by means of the MUA recording technique [79,82,89]. The unitary relationship between the MUA volley and the LH pulse is invariably observed under a variety of experimental conditions [38,79], providing evidence that this specific electrical signal refects the hypothalamic GnRH pulse generator activity governing release of GnRH. Using the MUA as an index, it is now possible to access directly to the hypothalamic controlling system of pulsatile secretion of GnRH.

Objectives

The purpose of studies described in this dissertation is by utilizing the new experimental procedure, namely the MUA recording technique, to analyze the neural mechanism underlying pulsatile and surge mode secretion of GnRH, and to examine the interaction between its activity and endocrine events during the estrous cycle in the female goat. The 'Shiba' goat was employed as an experimental model throughout the study. The GnRH pulse generator activity was assessed based on the changes in the characteristic bursts of the multiple unit activity which were exclusively associated with LH pulses (MUA volleys).

In chapter 3, the location of the tip of electrodes, from which MUA volleys were recorded, was histologically examined, and its correlation to the distribution of GnRH immunoreactive neurons was studied in order to obtain information on the location and component of the GnRH pulse generator. In chapter 4, the role of the GnRH pulse generator in the induction of the LH surge was examined in ovariectomized goat infused with estradiol. Then, in chapter 5, changes in the GnRH pulse generator activity were examined during the programmed administration of ovarian steroids which mimicked

luteal and follicular phase steroidal milieu in ovariectomized animals. Finally, in chapter 6, GnRH pulse generator activity was monitored throughout the estrous cycle in ovary-intact females.

Chapter 2

General Materials and Methods

Animals

Adult female Shiba goats weighing about 25 kg were used. They are from a closed colony of University of Tokyo, and the non-seasonal breeder which continued to cycle throughout a year under the natural daylight conditions in Japan as described previously [42,65]. Animals were kept in the Faraday cages where the daylight were maintained at 12 h light and 12 h dark during the experiment. They were fed pelleted foodstuff (Ace Mare 10 g/kg/day ; Nihon Nosan Kogyo Co., Yokohama, Japan) twice daily and allowed free access to water.

In chapters 3-5, female goats were bilaterally ovariectomized under anesthesia with halothane inhalation at least 3 months prior to experiments to eliminate effects of endogenous steroids.

MUA recording procedures

Electrode construction

The electrode assembly is shown in Fig.2.1. Multiple electrode array consists of six 113 μm (0.0045 inch) teflon-insulated platinum (90 %) - iridium (10 %) wires (#7770, A-M Systems, Evertt, WA, USA) which are encased in a stainless-steel guide tube (22-gauge) with outer guide tube (18-gauge) and a shaft length of 40-50 mm. To implant the electrodes into the bilateral MBH, two guide tubes were paired by covering with dental acrylic after connecting one end of the wires to an integrated circuit (IC) socket (10×18 mm) having 14 pins with solder. The distance between the guide tubes was 4 mm. The other end was cut to extend about 3 mm beyoned the tip of the guide tube and the insulation was removed from the electrode only at the end of each electrode tip. The wires

were slightly bended to splay out. The each guide tube was also connected to the pins of the IC socket with a fine copper wire and used as ground. During the implantation, these recording wires were retracted into the outer guide tube and spacer was inserted between the electrode connector block and the head of outer guide tube (Fig.2.1).

Surgical preparation

Electrode implantation was conducted by the method of stereotaxic surgery for the Shiba goat with radiographic monitoring [79,81]. Under halothane anesthesia, the head of the goat was mounted in the stereotaxic apparatus, and median incision was made on the scalp. After calvarium was exposed, a window (20×10 mm) was drilled open and a spinal needle (21-guage 70 mm; Terumo Co., Tokyo, Japan) attached to a micromanipulator was lowered at one of the rostral corners until the tip reached the lateral ventricle. A 0.5 ml of radio-opaque material, Iopamidol (Iopamiron 370; Schering Co., Berlin, Germany) was injected slowly through the spinal needle and a lateral radiography was taken 30 sec later. According to the radiography in which the infundibular recess of the third ventricle was visualized, the stereotaxic coordinates were calculated so that the tip of the electrodes was placed in the MBH. The electrode assembly was lowered until the tip of the outer guide tube reached 3 mm above the target, and was fixed to the calvaria with dental acrylic. Then the spacer was removed and the electrodes were extruded to splay out at the target region. Fig.2.2 is a radiography showing the final position of the electrodes. The whole assembly was secured to the calvaria using anchor screws and dental acrylic.

MUA recording and Data analysis

The schematic illustration of the MUA recording system is shown in Fig.2.3. After 1-2 weeks of recovery, the goat was transferred to the recording room and tied

loosely to the stanchion where it could feed and rest even during the recording period. At recording, a buffer amplifier IC (TL084; Texas Instruments Co., Dallas, TX, USA) which formed a high input-impedance and low output-impedance voltage follower circuit was directly plugged to the electrode assembly to reduce the noise level along the signal path. To reject the artifact of the common input such as electromyogram of moving animal, differential input signals between two electrodes were further amplified by means of a high gain amplifier (VC11; Nihon Kohden Co., Tokyo, Japan) with low and high cut off frequencies of 500 Hz and 3 kHz, respectively. Since the electrode assembly consisted of twelve electrodes, the MUA by different combinations of electrodes were screened and one that showed the most stable electrical activity was selected. After amplitude discrimination with a window discriminator (Dia Medical System, DPC-1310-PI, Tokyo, Japan), MUA signals were stored as the activity rate in a personal computer (PC-9801 vm; NEC, Tokyo, Japan) for data storage and processing.

Electrophysiological correlates of the hypothalamic GnRH pulse generator activity is manifested as the characteristics increase in the frequency of the MUA associated with the pulsatile LH secretion (MUA volley) as shown in Fig.2.4. MUA volleys are characterized by the initial brief over-shoot followed by a gradual decline to the base line, and always precede the LH pulses even when the pulse frequency changes under a variety of experimental circumstances [38,79]. In this dissertation, the changes in the GnRH pulse generator activity were assessed by measuring the frequency (or the time of inter-volley interval) and duration of MUA volleys.

Blood sampling

The indwelling vinyl chroride catheter (18-gauge, 95 cm; Japan Sharwood, Tokyo, Japan) was fitted caudally into the latelal jugular vein a few days before the blood sampling. The catheter was filled with physiological saline containing heparin sodium (10 unit/ml; Wako Chemical Industries, Ltd., Osaka, Japan) to prevent blood coagulation.

Blood samples (1-5 ml) were collected within a heparin-included (1-10 unit) syringe. Plasma was separated immediately by centrifugation at 4°C for 20 min and stored at -20°C until assayed for hormone concentrations by radioimmunoassays.

Radioimmunoassays

Luteinizing hormone (LH)

Plasma concentrations of LH were measured in triplicate aliquots of 50 μl of plasma samples by a double-antibody radioimmunoassay for ovine LH using ^{125}I-ovineLH (NIADDK-ovineLH-I-3) as a tracer and NIH-LH-S-19 as a standard as previous described [78]. The rabbit antiserum to ovine LH, YM #18, and caprine anti-rabbit gamma globulin antiserum for second antibody were provided by Dr. Y. Mori of the University of Tokyo and Dr. Y. Kanai of Tsukuba University, respectively. The average sensitivity was 0.019 ng/tube. The intra- and inter-assay coefficients of variation were 8.6 % and 13.7 %, respectively.

Ovarian steroids

Plasma concentrations of progesterone and estradiol were determined by a double-antibody radioimmunoassay as reported previously [78]. The rabbit anti-progesterone antiserum was prepared in the Laboratory of Veterinary Physiology, the University of Tokyo, and the rabbit anti-estradiol antiserum was supplied by Dr. Y. Kanai of Tsukuba University. ^{3}H-progesterone (NEW-TRK641) and ^{3}H-estradiol (NEW-TR587K), and progesterone (Teikoku Zoki Pharmaceutical Co., Kanagawa, Japan) and estradiol (Sigma Chemical Co., St. Louis, MO., USA) were used as tracers and standards, respectively. Plasma samples (200 μl) were extracted with 2 ml diethyl ether (Wako Chemical Industries, Ltd., Osaka, Japan) and assayed for progesterone. For estradiol, 2 ml plasma samples were extracted with 6 ml diethyl ether, and the extract was redissolved in 50 % methanol (Wako Chemical Industries, Ltd., Osaka, Japan) and distilled water, and were further deffated with hexane (Wako Chemical Industries, Ltd., Osaka, Japan) before assayed. The sensitivity for progesterone and estradiol were 10 pg/tube and 6.4 pg/tube, respectively. The intra- and inter-assay coefficients of variation were 10.4 % and 15.1 % for progesterone, and 8.5 % and 16.9 % for estradiol, respectively.

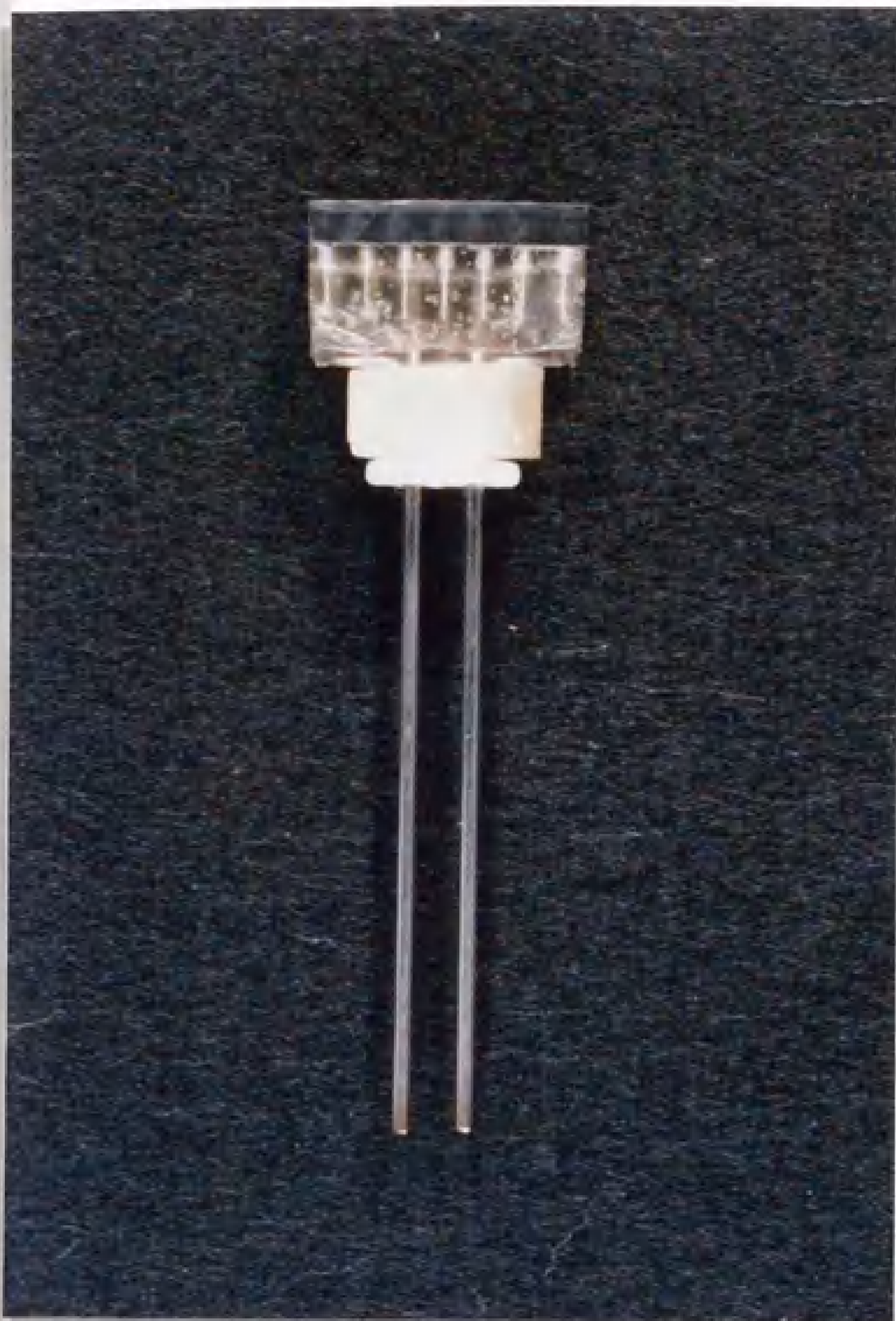


Fig. 2.1. Electrode assembly. At implantation, electrode wires are retracted into the outer tube by inserting the spacer (left). Then the spacer is removed and the electrodes are extruded to splay out at the target region (right).

Fig. 2.2. A fluoroventriculogram of the goat head mounted in the stereotaxic instrument. Radio-opaque material injected into the lateral ventricle is diffused into the third ventricle. The tip of the electrodes are extruded from the guide tube to splay out within the MBH.

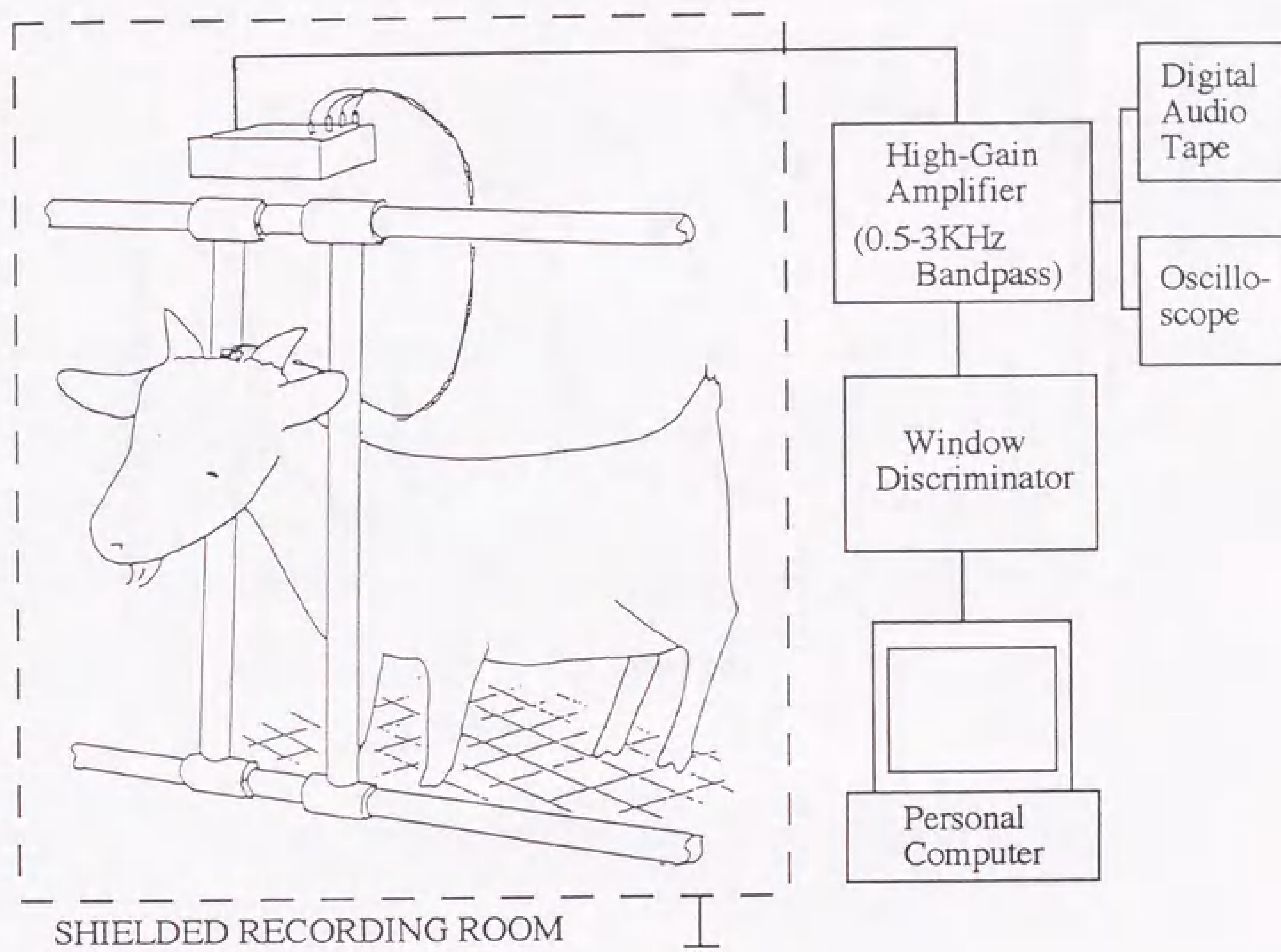


Fig. 2.3. Schematic illustration of MUA recording system. A buffer amplifier is directly plugged onto the electrode assembly chronically implanted in the MBH. Electrical signals are further amplified by means of a high gain amplifier with low and high cutoff frequency of 500 Hz and 3.0 kHz, respectively. After amplitude discrimination with a window discriminator, MUA signals are stored as the activity rate in a personal computer.

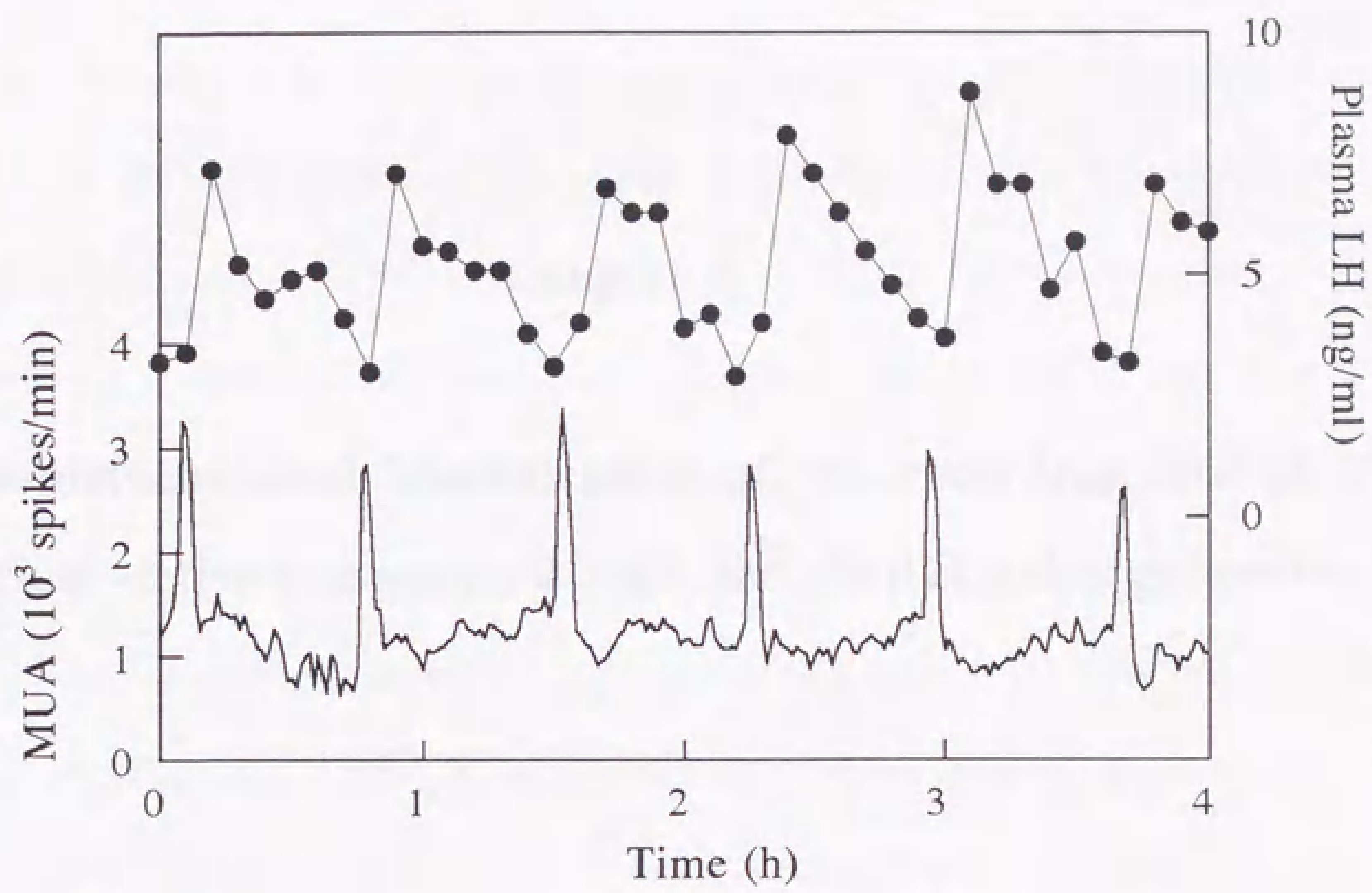


Fig.2.4. Characteristic increase in the hypothalamic MUA (MUA volley) and luteinizing hormone (LH) pulse in peripheral circulation. Each MUA volley always preceds the LH pulse.

Chapter 3

Immunohistochemical identification of the recording site of the electrical activity associated with the GnRH pulse generator

Introduction

Two mutually exclusive hypotheses for the mechanism of GnRH pulse generator have been proposed: 1) there is an endogenous pulse-generating component within GnRH neurons, and 2) pulsatile GnRH neurosecretion is controlled by the synaptic input of neurotransmitter and/or neuromodulator. In my laboratory, characteristic increases of the hypothalamic MUA that are synchronized consistently with LH pulses (MUA volleys) have been successfully recorded through the electorodes implanted in the MBH (see Fig.2.4) [79,82]. However, it is still not certain as to whether this electrical signal reflect the activity of GnRH neurons or not. Identification of the neuronal component from which the MUA volley is recorded is extremely important to understand the mechanism underlying GnRH pulse generation. The present study was, therefore, attempted to lacalize the recording site of the electrophysiological manifestation of the hypothalamic GnRH pulse generator activity (MUA volley) and to examine their topographical correlation to the structure of the GnRH immunoreactive neurons.

Materials and Methods

Seven ovariectomized goats were implanted with the electrodes array into the MBH. MUA was recorded from these animals, and active electrodes (n=4) from which specific MUA volleys were recorded, and inactive electrodes (n=3) from which no specific MUA was recorded, were selected and their positions were examined as described below.

The head of goat was mounted in the stereotaxic apparatus under halothane anesthesia, and direct current (200 μA for 20 sec) was passed through the selected electrodes to localize the recording site. The electrode array was then removed carefully, and a cannula (19-guage) was implanted into the lateral ventricle and fixed with dental acrylic to the calvarium. About a week later, colchicine (Sigma Chemical Co., St Louis, USA) dissolved in the physiological saline (200 μl, 0.1mg colchicine per kg of body weight) was intracerebroventricularly administered through the cannula, and the animal was sacrified on the next day with sodium pentbarbital (Nembutal; Abbott laboratories, North Chicago, IL, USA) at the dose of 30 mg/kg body weight following systemic heparinization (5,000 IU heparin per animal). The head was perfused bilaterally through the carotid arteries with 2.5 l of saline (the first 1 l containing the heparin at the dose of 10 IU/ml), and then with 2.5 l of Zamboni's fixative (4% parafolmaldehide, 7% saturated picric acid in 0.1 M phosphate buffer, pH 7.4). Following perfusion, the brain was removed from the skull and trimmed down to a block. The brain block was then stored in the fixative containing 15 % sucrose at 4°C for 36-48 h.

The brain blocks were cut coronally at 40 μm intervals on a freezing microtome. Every fourth section was mounted on slides and stained with the hematoxylin-eosin to localize the lesion site, and its adjacent sections were processed for GnRH immunohistochemistry. In the present study, a modified avidin-biotinylated peroxidase

complex procedure [34] was used to visualize GnRH immunoreactive neurons. Free-floating sections were washed in 0.1 M phosphate buffered saline containing 0.05 % Triton X-100 (PBST) and then incubated in PBST containing 1 % bovine serum albumin (BSA) and 2 % normal goat serum at 4°C over-night to minimize nonspecific staining. Sections were exposed to the primary anti-GnRH antiserum (UCB-Bioproducts, SA, USA) at a dilution of 1:30,000 in 0.1 M PBST containing 1 % bovine serum albumin for 72 h at 4°C. They were then treated with a biotinylated goat anti-rabbit IgG at room temperature for 2 h and then an avidin-biotin-horseradish peroxidase (Vectastain; Vector Laboratories, Inc., CA, USA) for 1 h. The horseradish peroxidase was visualized using 0.05 % 3,3-diaminobenzidine tetrahydrochloride as the chromogen. Sections were mounted onto slides, dried, dehydrated, cleared and coverslipped. All sections were examined under a light microscope with a bright field, and the location of the lesion placement was mapped out in the goat stereotaxic atlas (Shimada et al. unpublished).

Results

Electrical lesion was made by passing direct current through the electrode tip. The sites of lesion were found as circle area of about 400 μm-diameter easily distinguishable from surrounding normal tissue as shown in Fig.3.1. The localization of such electrodes that was estimated to be at the center of lesion in each case is shown in Fig.3.2. The tips of active electrodes from which the MUA volleys were recorded were located in the median eminence on the border of the third ventricle (3 electrodes) and in the ventral region of the mammillary recess at the level of the caudal aspect of the median eminence (1 electrode). On the other hand, the tips of inactive electrodes were located in the periventricular region dorsal to the arcuate nucleus, in the lateral region of the mammillary recess, and in the anterior lobe outside the median eminence.

Representative photomicrographs taking the relation of active electrodes to GnRH immunoreactive fibers are presented in Fig.3.3. All the tips of active electrodes were found to be embedded in the mass of GnRH-immunoreactive fibers in the median eminence, whereas no immunostaining was found in the vicinity of the tips of inactive electrodes.

Discussion

The results described in this chapter demonstrated that the electrical activity derived from the GnRH pulse generator was recorded from the median eminence and its adjacent regions in the MBH. Similarly, in ovariectomized rat, the tip of the active electrode, from which MUA volley was recorded, was located in the arcuate nucleus-median eminence region, mostly in the median eminence [52]. In the rhesus monkey, the majority of active electrodes were also found in the MBH including the region of the arcuate nucleus, the retrochiasmatic zone and the dorsal aspect of the median eminence [121]. These histological studies in three different species have unambiguously shown that the GnRH pulse generator is located at least in part in the MBH around the median eminence.

The vast majority of GnRH containing perikarya are immunohistochemically identified in the medial preoptic area, and axons arising from these cell bodies terminate primarily in either the organum vasculosum of the lamina terminalis or the median eminence in the goat [34,147]. The distribution pattern of GnRH neurons is similar to that those reported in sheep [59,143] and rat [120], but different from the one in rhesus monkey where the major GnRH cell group resides in the periventricular, as well as in the tuberal regions [119]. Despite the apparent interspecies differences in the distribution of cell bodies of GnRH neurons among monkey, rat and goat, the MUA volley associated with the LH pulse has been recorded consistently from the same region of the hypothalamus, i.e. MBH. Moreover, in the present study tips of all active electrodes were always embedded in the mass of GnRH immunoreactive fibers in the median eminence, whereas no GnRH immunoreactive fiber was found in the vicinity of the tip of inactive electrodes. Similarly, in the rhesus monkey the recording site of MUA volleys was

localized in moderate to heavy GnRH immunoreactive fiber bundles in the majority of instances [121]. Therefore, the MUA volley may be recorded from the axonal tract or the terminal of GnRH neurons as a product of the coordinated firing of GnRH neurons during the pulsatile neurosecretion. However, because both the size of recording electrodes and the lesions used to mark their tips are too large to pinpoint the location at the cellular level, and because there are numerous neuronal inputs such as monoamines, opioid peptides, excitatory amino acids in the MBH, it is still premature to conclude that the MUA volley is recorded specifically from the GnRH neuron. In fact, it was reported that inactive electrodes appeared equally close to GnRH elements as the active electrodes in the rhesus monkey [121], and so one can not discount the possibility that the MUA volley reflects the activity of other neural elements regulating the neurosecretion of GnRH from nerve terminals.

In conclusion, present results suggest that at least a part of the neural component governing GnRH pulse generation resides in the MBH around the median eminence. However, further study is required to determine as to whether MUA volley is recorded from the GnRH nerve terminal or from non-GnRH neurons.

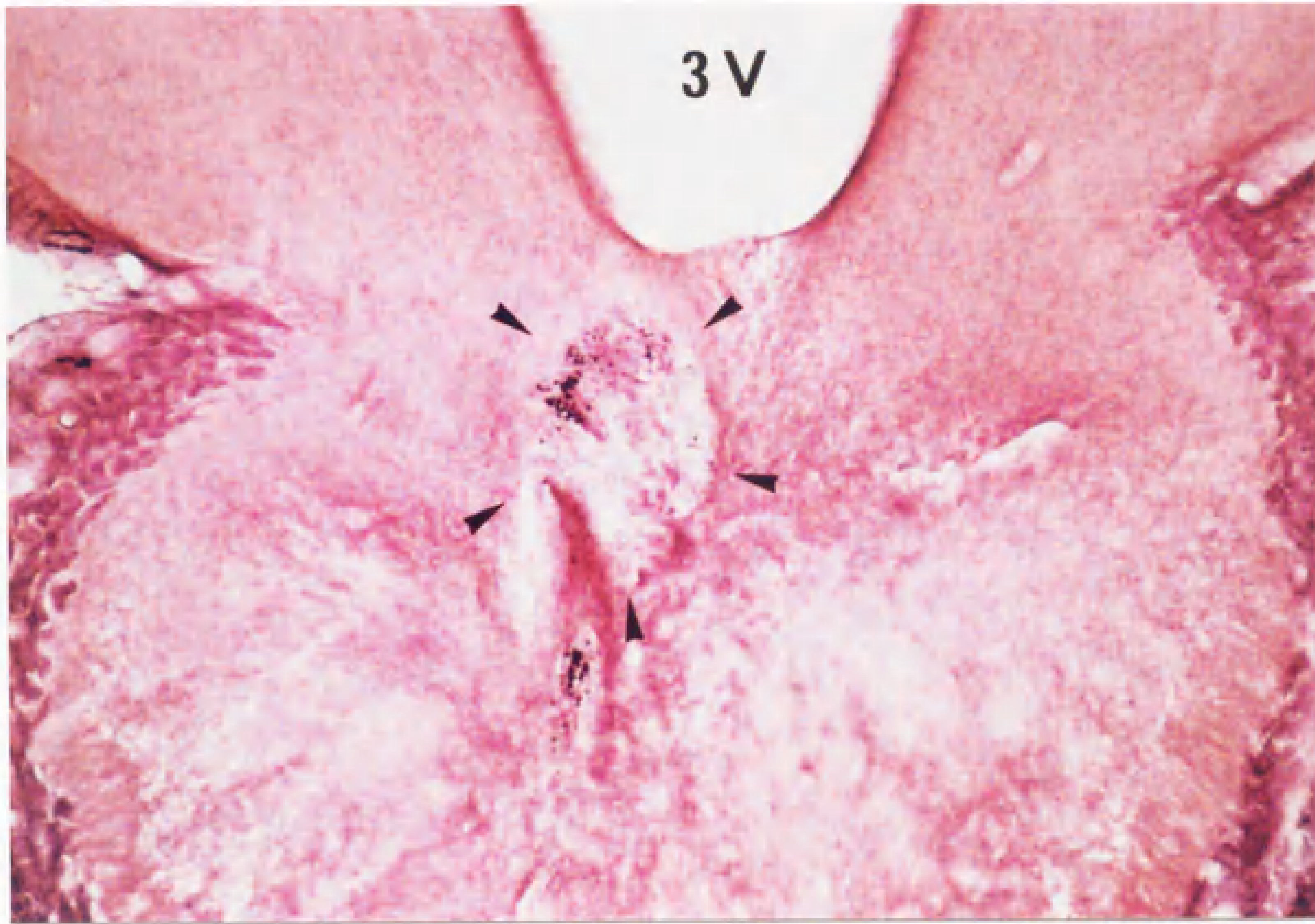


Fig.3.1. A photomicrographs showing a lesion of an active electrode centered in the median eminence. Arrowheads indicate the area damaged by lesion. 3V: third ventricle × 50

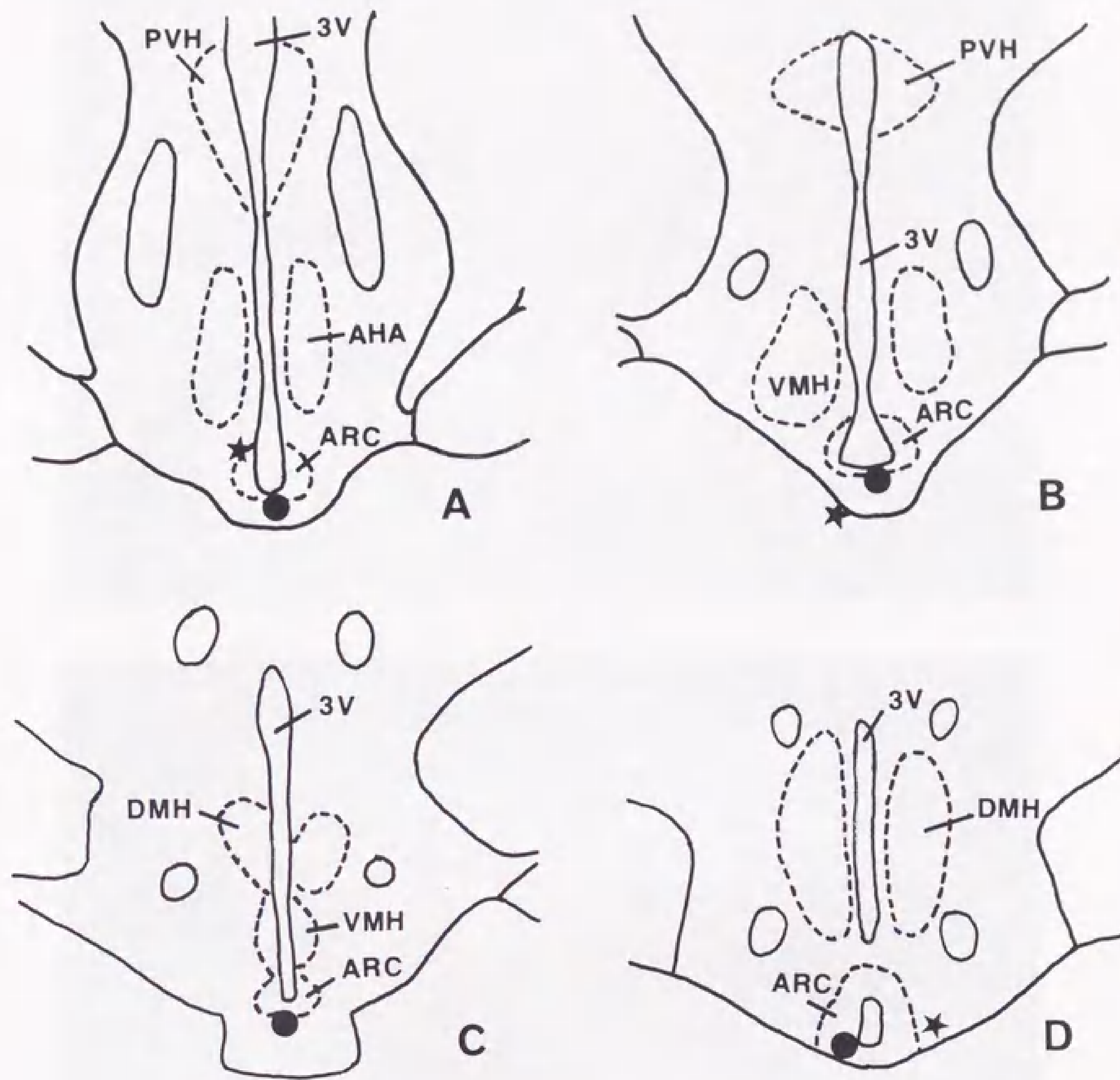
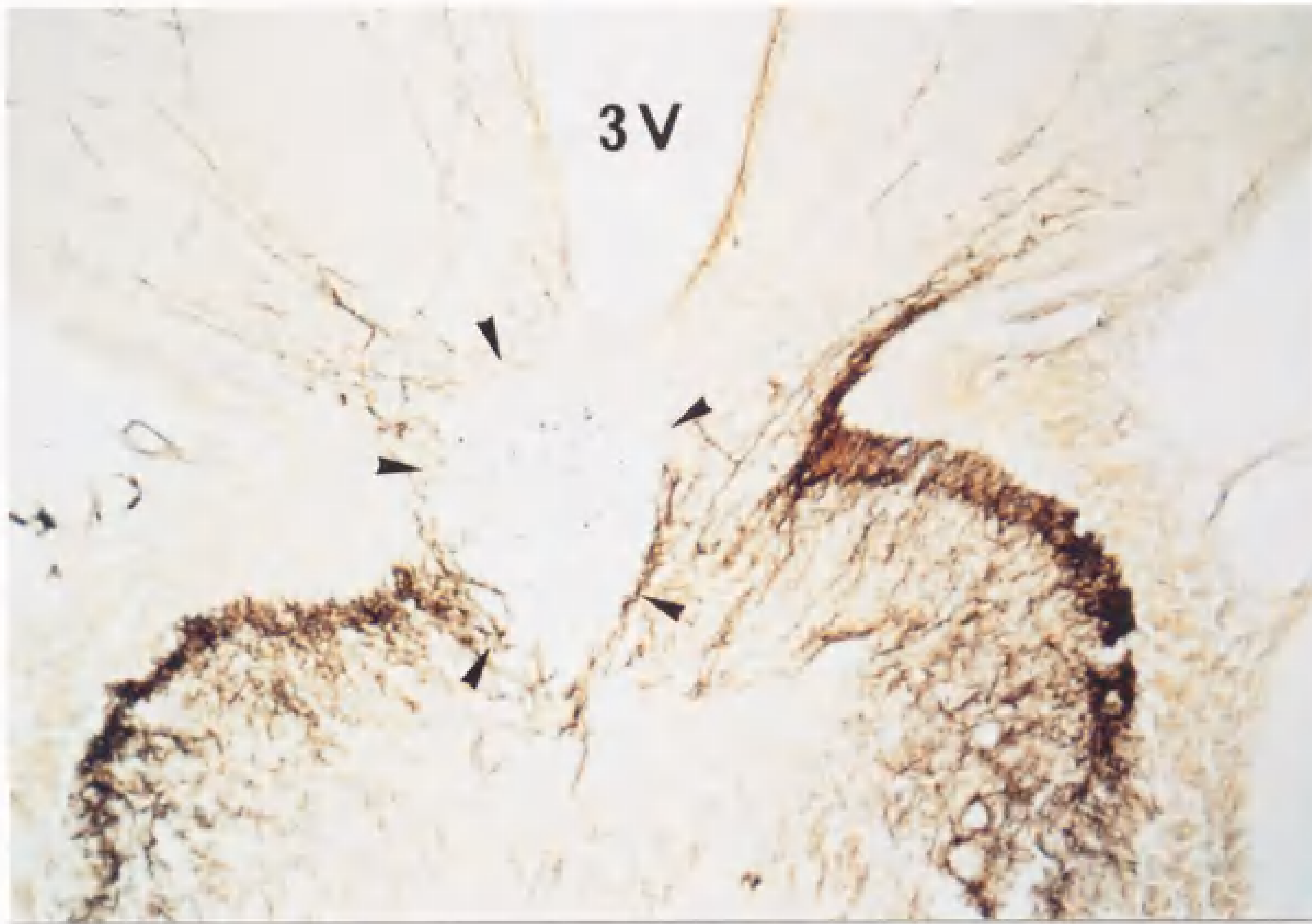


Fig.3.2. Diagrammatic representation of lesions placed in the hypothalamus to mark site of recording electrodes. The solid circles represent active electrodes, the stars, inactive electrodes. PVH: paraventricular hypothalamic nucleus, AHA: anterior hypothalamic area, ARC: arcuate nucleus, VMH: ventro-medial hypothalamic nucleus, 3V: third ventricle, DMH: dorso-medial hypothalamic nucleus.



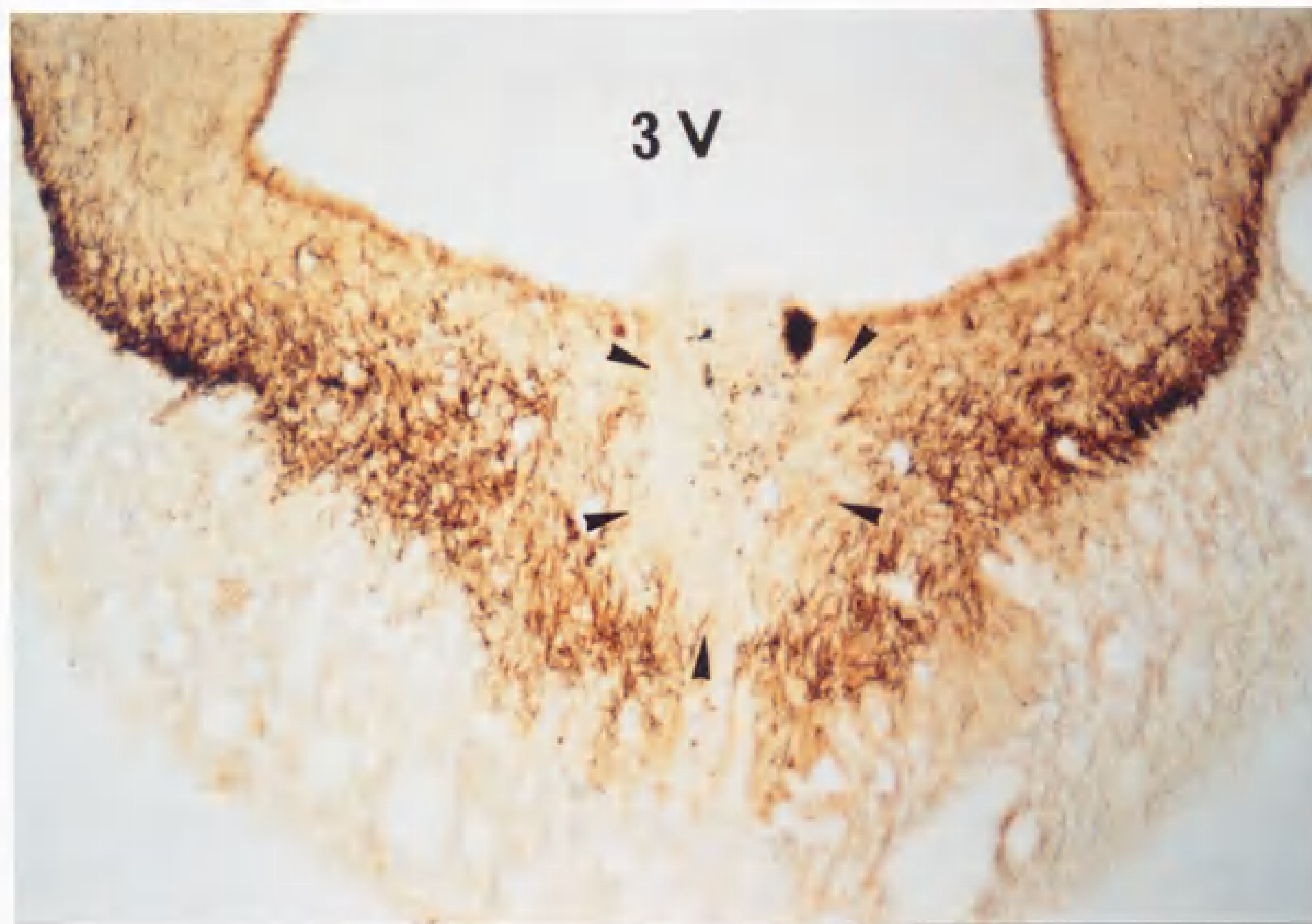


Fig.3.3. Representative photomicrographs (#21, #26) showing correlation between lesion site through active electrodes and GnRH immunoreactive fibers in the median eminence. Electrodes are surrounded by a dense plexus of GnRH fibers. Arrowheads indicate the area damaged by lesion. 3V: third ventricle × 50

Chapter 4

GnRH pulse generator activity during the estradiol-induced LH surge

Introduction

Exogenously administered estradiol can elicit preovulatory-like LH surges in ovariectomized female animals of various species including the goat [83]. While estradiol is known to act directly on the pituitary gland to enhance the responsiveness to GnRH by increasing the number of GnRH receptors [31,84], the importance of hypothalamic input has well been demonstrated in experiments using neurophysiological techniques [31,40] and immunization against GnRH [36] in ovariectomized ewes given estradiol. Recent studies have demonstrated clearly that GnRH secretion into the pituitary portal circulation is substantially increased during the LH surge in estradiol-treated ovariectomized ewes [75] and also during the preovulatory surge in intact ewes [76]. Similarly, in ovariectomized goats given estradiol, a large increase in GnRH concentration in microdialysis perfusates of the median eminence was observed during the LH surge [67]. Taken together, the increased GnRH secretion from the hypothalamus appears to be the primary cue for the onset of the LH surge.

However, the role of the hypothalamic GnRH pulse generator in the induction of the LH surge is poorly understood. Using the technique for sequential collection of pituitary-portal blood and the algorithms for endocrine pulse detection [72], Clarke and Cummins [13] and Caraty et al. [10] found an increase in GnRH pulse frequencies during the LH surge in ovariectomized ewes given estradiol. In contrast, a dramatic decline in the frequency of the hypothalamic pulse generator activity was observed in estradiol-induced LH surge in ovariectomized rhesus monkeys using the electrophysiological recording [51] and during the preovulatory LH surge in cycling monkeys [94]. It is therefore still not certain as to whether the LH surge is associated with acceleration of the GnRH pulse generator activity that regulates basal gonadotropin secretion. The experiment described in

this chapter was undertaken to examine, by recording the specific MUA, the changes in the GnRH pulse generator activity during the estradiol-induced LH surge in ovariectomized goats.

Materials and Methods

Ovariectomized goats (n=3), from which MUA volley were recorded, were used for the following experiments.

Each goat was infused intravenously with estradiol at a rate of 3 μg/h for 16 h via one of the catheters fitted bilaterally to the jugular veins a few days prior to the experiment. Crystalline estradiol (Sigma Chemical Co., St Louis, U.S.A) was dissolved in ethanol (100 μg/ml), diluted with sterilized physiological saline to a concentration of 0.3 μg/ml (0.3 % ethanol solution) and infused 10 ml hourly with a microtubing pump (SJ-1211 Atto, Tokyo, Japan). In the preliminary examination, the infusion of vehicle solution (0.3 % ethanol saline) was confirmed to have no effect by itself on the frequency of LH pulses in ovariectomized goats [80].

Blood samples (1 ml) were collected via the jugular catheter every 6 min for 4 h on the day before estradiol infusion to confirm the synchrony between MUA volleys and LH pulses, and then every 30 min for a further 24 h starting from 4 h prior to the initiation of estradiol infusion to the time of the onset of the LH surge. In one goat (#902) the same experimental procedure was repeated a month later to examine the reproducibility.

Effects of estradiol on the GnRH pulse generator activity were assessed by measuring the inter-volley interval and the duration of MUA volleys during the course of estradiol treatment. The 24 h period of blood sampling was divided into 3 sections as follows: Phase 1, pretreatment control period; Phase 2, the period from the start of estradiol infusion to the onset of the LH surge; Phase 3, from the onset of the LH surge to the end of blood sampling. The onset of the LH surge was defined as the time when the sustained rise in plasma LH exceeded twice the average baseline level during the pretreatment control period. The data were expressed as the mean±SEM and Student's *t*-

test, Cochran-Cox test or paired *t*-test was adopted to detect statistical differences between mean values.

Results

Characteristic patterns of hypothalamic MUA associated with LH pulses in the plasma were recorded in all the 3 goats examined during the pretreatment control period as shown in Fig.4.1. The initiation of each LH pulse was always preceded by an abrupt rise in MUA (MUA volley) and thus regarded as an electrophysiological manifestation of the GnRH pulse generator activity. Fig.4.2 shows representative patterns of MUA and plasma LH during the 24 h period in an ovariectomized goat infused with estradiol for 16 h. The onset of the LH surge averaged 10.8±0.6 h (range 10.0-11.5 h) after the start of estradiol infusion, and the recurrence of MUA volleys was observed throughout the 24 h including the period of the LH surge in all 4 cases as shown Fig.4.3. Virtually identical results were obtained in two experiments repeated at a one month interval in goat #902. As shown in Table 4.1 the interval between MUA volleys was longer after the onset of the LH surge than in the pretreatment control period. On the other hand, the duration of the MUA volley was shortened after the onset of the LH surge as indicated in Table 4.2.

Discussion

It has previously been reported that a large amount of GnRH is released into the pituitary portal circulation synchronized with the onset of the LH surge in ovariectomized ewes treated with estradiol [75]. Manabe et al. have observed a similar sudden increase in the GnRH concentration at the onset of the LH surge in the microdialysis perfusate from the median eminence in ovariectomized goats given estradiol [67]. These results suggest that an abrupt rise in GnRH secretion into the pituitary portal circulation is a primary requisite for induction of the LH surge in ovariectomized ewes and goats given estradiol, while increased pituitary responsiveness to GnRH may also be necessary to achieve full expression of the LH surge. In support of this view hypothalamo-pituitary disconnection [40] and immunoneutralization against GnRH [36] have been shown to abolish or largely reduce the LH surge induced by estradiol in ovariectomized ewes. Taking these and the present findings into consideration it appears unlikely that an increased pulse frequency is a cause of estradiol-induced GnRH surge. If this is the case, what role has the GnRH pulse generator in the initiation of the GnRH surge? Two mutually exclusive interpretations can be drawn from these results; 1) each MUA volley may generate a large amount of GnRH secretion which is enough to induce a GnRH surge even with decreased pulse frequency, or 2) the onset of the GnRH surge is under the control of a neuronal mechanism that is independent of the GnRH pulse generator. The former explanation seems unlikely, however, considering a rapid turnover of GnRH released into the portal circulation [74], a relatively long interval between the MUA volleys, and the shape of the GnRH surge that is reported to be non-pulsatile [73,75]. It appears that previous observations tend to favor the latter explanation. For example, the hypothalamic deafferentation with a Halasz knife [33] placed between the arcuate nucleus and the

suprachiasmatic nuclei did not alter the pulsatile pattern of LH secretion but blocked the LH surge induced by estradiol benzoate [40]. Robinson et al [111] reported that the release of the inhibitory amino-acid gamma-aminobutyric acid in the preoptic/septal area decreased during the preovulatory LH surge in the ewe. These data support the view that the neuronal system governing the surge release of GnRH resides in a different brain area from that for pulsatile GnRH release.

The decreased frequency of GnRH pulse generator activity during the LH surge observed in the present study is inconsistent with previous findings; Clarke & Cummins [13] and Caraty et al. [10] reported an increase in the pulse frequency of GnRH secretion during the LH surge in ovariectomized ewes given estradiol. The reason for the discrepancy between these and the present results is unclear, though it may be ascribed to differences in either the species (ewes vs. goats), ways of estradiol administration (injection vs. infusion), or parameters (GnRH release into the portal circulation vs. MUA volleys). In previous studies using ewes as experimental animals, the GnRH pulse generator activity was assessed by detecting a pulse with aid of statistical methods from sequential patterns of GnRH concentrations in the pituitary portal blood. Although these computing methods are useful for examining the pulsatility of basal hormone secretion, they seem to have limitations when applied to the period of enhanced secretion, e.g. the LH/GnRH surge due to the ever-changing base-line levels.

In conclusion, the present study has demonstrated that the electrophysiological manifestation of the hypothalamic GnRH pulse generator activity becomes less frequent during the LH surge induced by estradiol in ovariectomized goats. It is therefore suggested that the GnRH/LH surge is induced by the neural mechanism that is intrinsically different from the GnRH pulse generator dictating basal gonadotropin secretion.

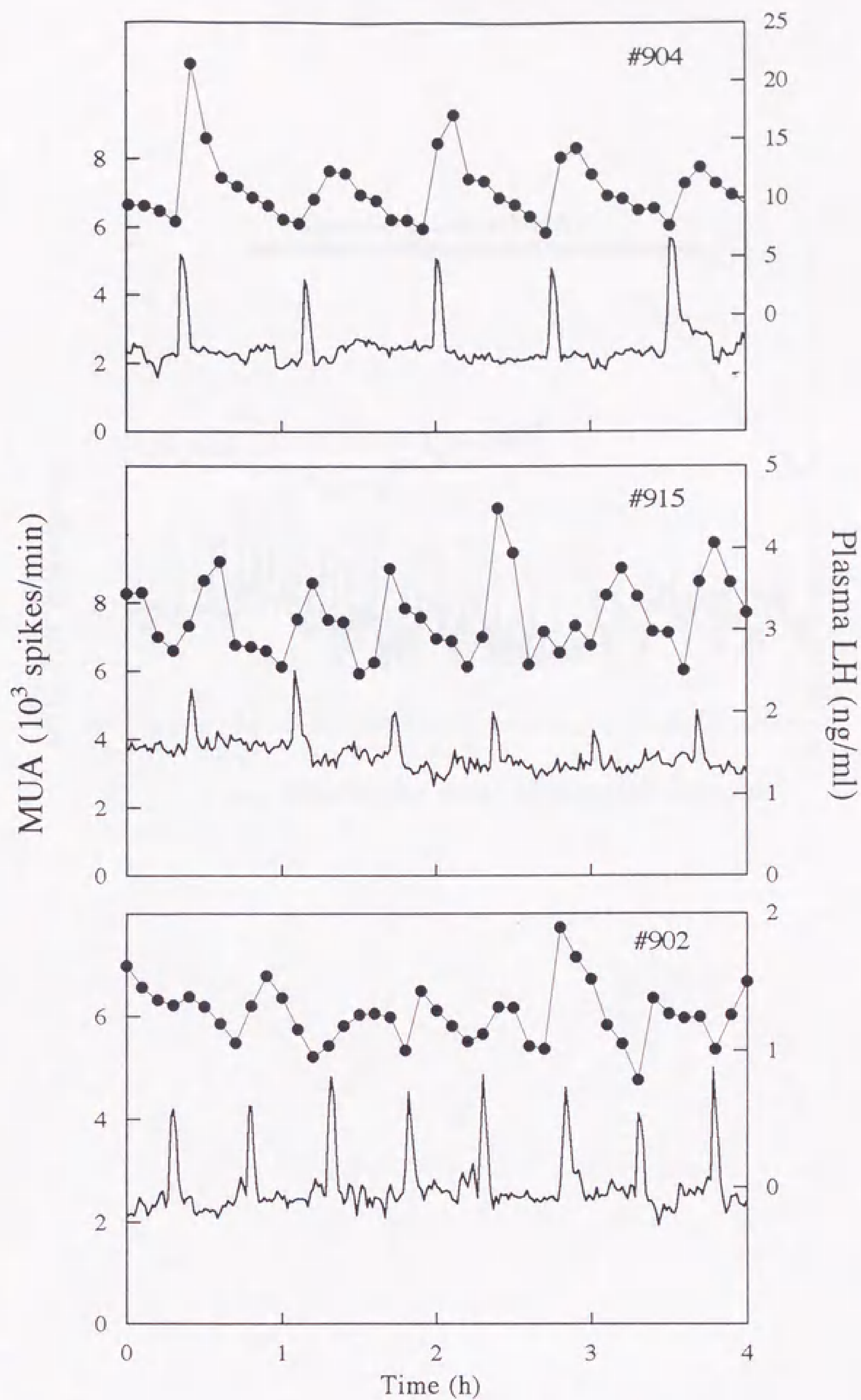


Fig.4.1. Correlation between the MUA volleys and LH pulses in 3 ovariectomized goats during the pretreatment control period.

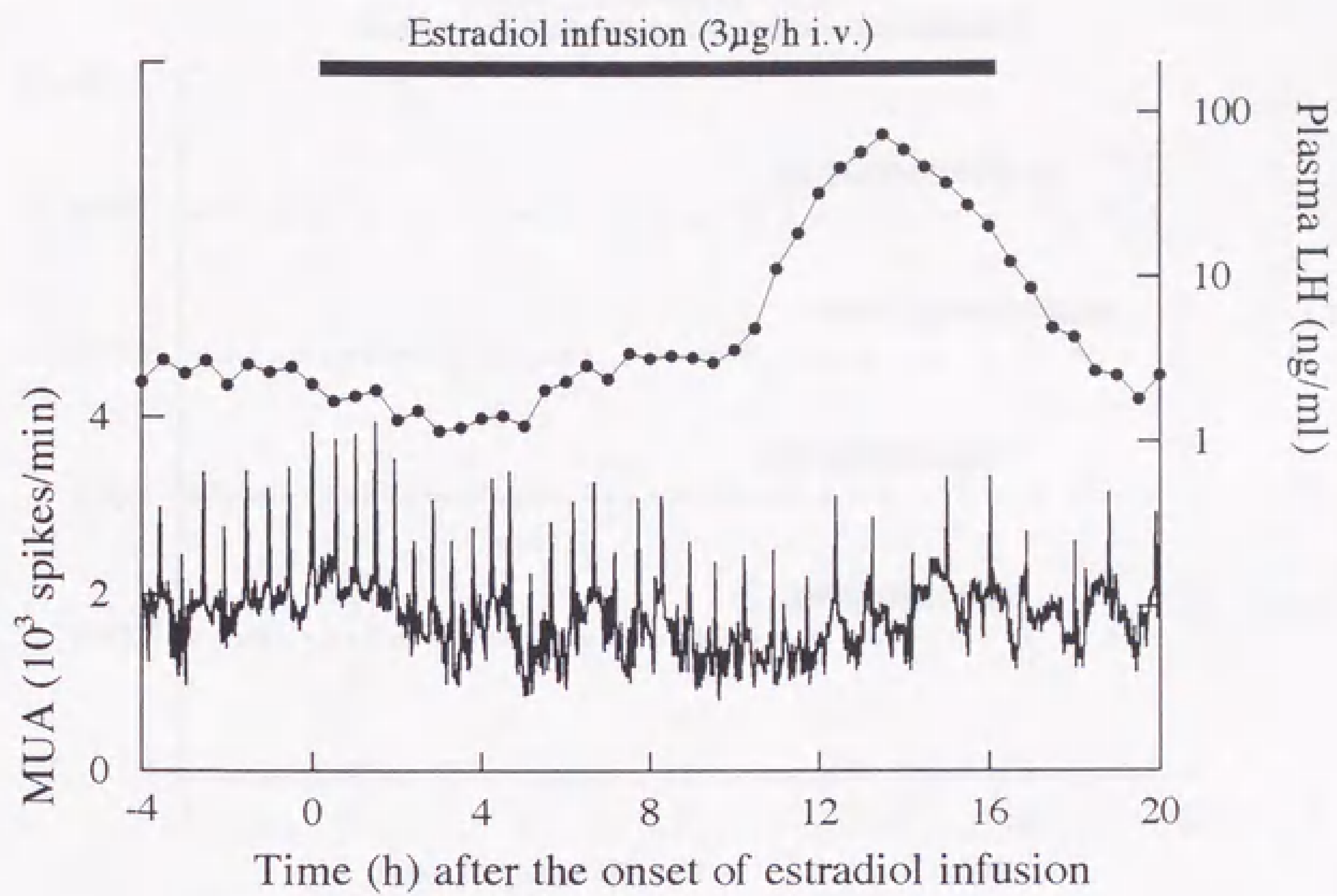


Fig.4.2 A representative pattern of the GnRH pulse generator activity, as exemplified by MUA volley (Fig.4.1), during the estradiol-induced LH surge in an ovariectomized goat (#902). The inter-volley intervals became longer after the onset of the LH surge as compared with the pretreatment control period.

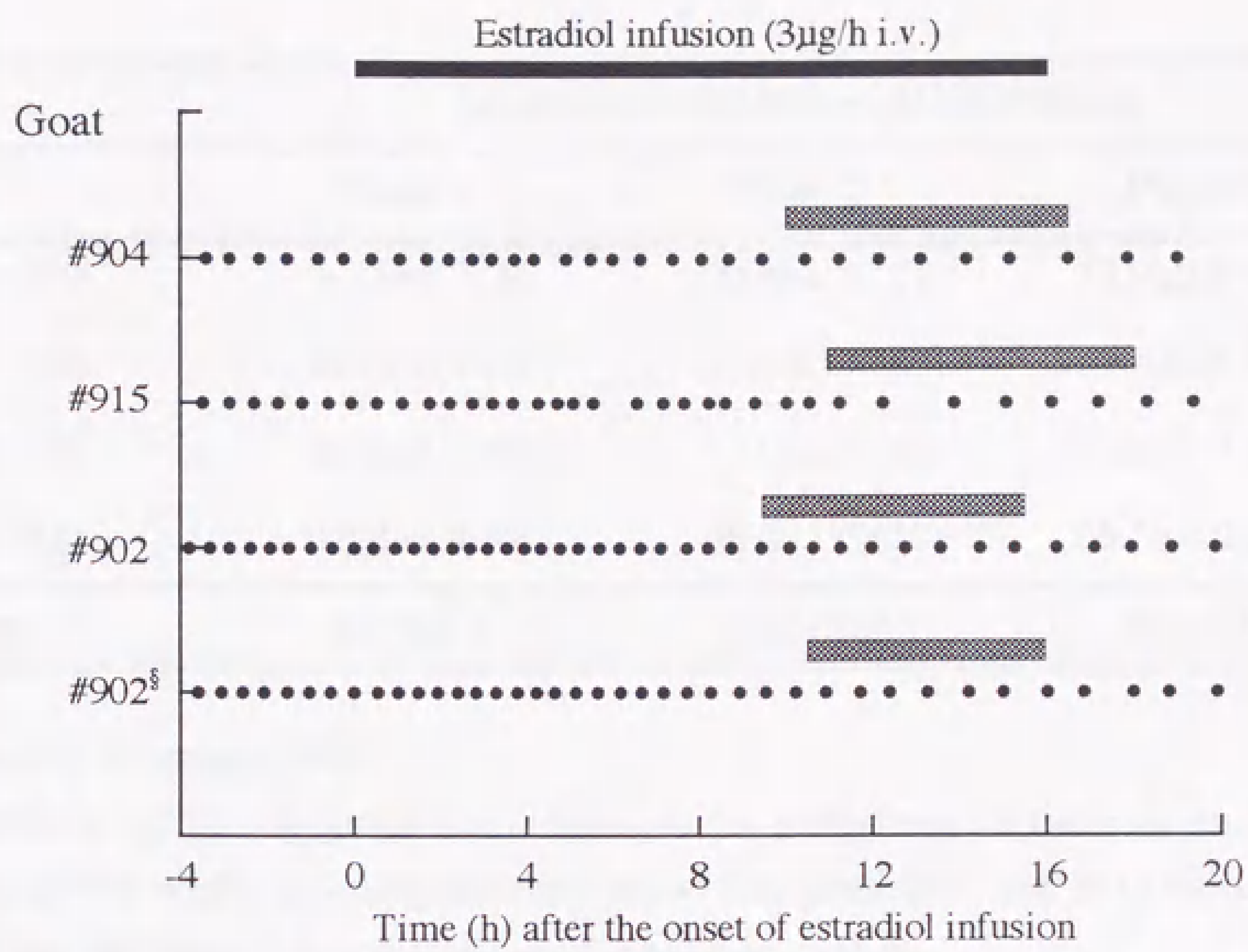


Fig.4.3 Recurrence of MUA volleys (•) in ovariectomized goats given estradiol (see Fig.4.2). Decreased frequency of the pulse generator activity was observed after the onset of the LH surge as shown by the shadow bars. The same experimental procedure was repeated a month later in one goat (#902).

Goat	Intervals (min) between MUA volleys		
	Phase 1	Phase 2	Phase 3
#902 (39)	30.7±0.5 (8)	30.4±1.0 (21)	53.9±2.8 (10)**
#902[1] (43)	28.5±0.5 (9)	27.3±0.7 (20)	49.4±2.3 (14)**
#915 (35)	34.3±1.1 (7)	34.0±1.9 (20)	71.5±4.7 (8)**
#904 (32)	38.2±1.9 (6)	36.4±1.6 (17)	65.3±4.0 (9)**
Average	32.9±2.1	32.0±2.0	60.0±5.1*

Values are the mean±SEM.

Numbers of MUA volleys for each phase and for each shown in parentheses.

*p<0.01, **p<0.001 as compared with phase 1 by Student's *t* test or Cochran-Cox test for individual data, and by paired *t* test for averaged values.

[1] The same experimental procedure was repeated a month later in one goat (#902).

Table 4.1 Intervals between MUA volleys during the pretreatment control period (phase 1), during the period from the initiation of estradiol infusion to the onset of the LH surge (phase 2) and from the onset of the LH surge to the end of blood sampling (phase 3) in ovariectomized goats infused with estradiol (3μg/h, i.v.) for 16 h.

	Duration (min) between MUA volleys		
Goat	Phase 1	Phase 2	Phase 3
#902 (39)	3.0±0.3 (8)	2.9±0.2 (21)	2.0±0.2 (10)*
#902[1] (43)	2.8±0.1 (9)	2.8±0.1 (20)	2.1±0.1 (14)**
#915 (35)	2.9±0.1 (7)	2.3±0.2 (20)*	1.9±0.4 (8)*
#904 (32)	3.5±0.2 (6)	3.2±0.2 (17)	2.2±0.2 (9)**
Average	3.1±0.2	2.8±0.2	2.1±0.1**

Values are the mean±SEM.

Numbers of MUA volleys for each phase and for each shown in parentheses.

*$p<0.05$, **$p<0.01$ as compared with phase 1 by Student's *t* test or Cochran-Cox test for individual data, and by paired *t* test for averaged values.

[1] The same experimental procedure was repeated a month later in one goat (#902).

Table 4.2 Duration of MUA volleys during the pretreatment control period (phase 1), during the period from the initiation of estradiol infusion to the onset of the LH surge (phase 2) and from the onset of the LH surge to the end of blood sampling (phase 3) in ovariectomized goats infused with estradiol (3μg/h i.v.) for 16 h.

Chapter 5

Steroidal modulation of the GnRH pulse generator activity

Introduction

It is well known that the progesterone and estradiol exerted a tonic negative feedback effect on gonadotropin secretion during the luteal phase of the estrous cycle. Using ovariectomized ewes, Goodman and Karsch [28] demonstrated differential effects of these two steroids; progesterone suppressed the LH pulse frequency whereas estradiol pulse amplitude. Karsch et al. [44] further showed that treatment with either estradiol or progesterone greatly diminished or abolished detectable pulsatile secretion of GnRH and LH in ovariectomized ewe. On the contrary, Martin et al. [68] reported that a treatment with progesterone alone did not influence the LH pulse frequency, but in combination with estradiol reduced markedly it in ewes. Thus, it is still equivocal whether the suppression of the GnRH pulse generator activity during the luteal phase is regulated by progesterone alone or in combination with estradiol.

In the present chapter, changes in the GnRH pulse generator activity was examined by monitoring MUA volleys in ovariectomized goats during a programmed steroidal treatment, which mimicked endocrine environments during the luteal and follicular phases. The purposes of this chapter were 1) to examine whether the progesterone with luteal phase levels could suppress the GnRH pulse generator activity to the extent as seen in the luteal phase of cycling goats [41], and 2) to confirm the previous finding in chapter 4 that the pulse generator is not accelerated prior to the onset of the LH surge by using more physiological model in terms of steroidal profiles in the systemic circulation.

Materials and Methods

Three ovariectomized Shiba goats, from which MUA volleys were recorded consistently, were used for the following experiments.

Ovarian steroids were administered by means of subcutaneous implantation as described previously [80]. Animals were first implanted with progesterone capsules made of silastic sheet (50×75 mm; Dow Corning Co., MI, USA) for 3 days, then implanted additionally with an estradiol capsule (3.35 mm i.d., 4.65 mm o.d., 40 mm long, Dow Corning) for another 3 days, which together reproduced the steroidal milieu during the luteal phase. To mimic luteolysis and the preovulatory increase in estradiol secretion, the progesterone capsules were removed and then estradiol solution (0.54 μg/ml in 0.54 % ethanol saline) was intravenously infused for 36 h at a gradually increasing infusion rate from 0.5 μg/h (0 h) to 3.0 μg/h (36 h). The recording of MUA was commenced about 12 h before the progesterone implantation and continued for about 10 days except for 1 min interruptions every 24 h to change computer files.

Blood samples (5 ml) were collected every 24 h during the period of progesterone treatment, and every 2 h for 48 h from 2 h before the removal of the progesterone packets. Additionally, 1 ml blood samples were collected every 6 min for 4 h on 3 occasions to examine the pulsatility of LH secretion in different steroidal environments.

Data are expressed as the mean values±SEM, and the difference between mean values for the control and the steroid-treated periods was examined by paired t-test.

Results

Constant release of ovarian steroids from the subcutaneous implants maintained plasma concentrations of progesterone and estradiol at 4.5±0.6 ng/ml and 5.5±0.9 pg/ml, respectively, which were within the physiological ranges for these steroids during the luteal phase in intact goats [78].

The relation between the hypothalamic MUA and LH pulses in the peripheral circulation that was seen in the control period was maintained during the periods of treatment with progesterone alone or in combination with estradiol as shown in Fig.5.1. The initiation of each LH pulse was always preceded by the MUA volley regardless of interpulse intervals.

The MUA volley frequency during the period of treatment with progesterone alone was not changed significantly ($p>0.05$) as compared to the control period (21.7±0.3 MUA volleys/12h), but it declined dramatically ($p<0.05$) to 10.7±0.9 MUA volleys/12h at 36 h after the additional implantation of the estradiol capsule as shown in Fig.5.2.

Changes in the MUA volley frequency during the mimicked follicular phase in two goats are shown in relation to the endocrine changes (Fig.5.3). Plasma progesterone declined to the basal level (<1 ng/ml) within 6 h of progesterone withdrawal. The estradiol level, on the other hand, increased gradually and the LH surge was induced 30 (#04)-32 (#24,#27) h after the start of estradiol infusion. The MUA volley frequency increased from 10.0±2.9 volleys/12h (P+E2 3day) to 13.7±1.9 volleys/12h after the removal of progesterone, but then decreased gradually in parallel with an increase in the plasma estradiol concentration (Fig.5.2, Fig.5.3). For the remaining goat, data recorded during the LH surge are missing due to a recording problem. Recurrence of MUA volleys was observed in the presurge period and this was maintained during the LH surge as well.

Discussion

Present results demonstrated that the MUA volley frequency was not suppressed by progesterone alone of the luteal phase level, but synergetic action of progesterone and estradiol was necessary to suppress the frequency. In ovariectomized ewes, however, the frequency of pulsatile LH release was shown to be suppressed by progesterone alone [28], and Karsch et al. [44] reported that this is caused by the reduced frequency of pulsatile GnRH release into the portal circulation. The reason for this inconsistency with the present results is unclear, though it may be ascribed to differences in the species, the duration of the progesterone treatment or the parameters used to monitor the GnRH pulse generator activity. In previous studies [28,44] short-term ovariectomized animals were used and progesterone treatment was commenced only 10 days after ovariectomy, whereas I used long-term (>3 months) ovariectomized animals. Therefore, in the former experimental model there might have been some carry-over effects of endogenous estradiol considering the fact that the MUA volley frequency was abruptly suppressed after additional treatment with estradiol of the luteal phase level. This idea is also supported by the previous observation that the pulse frequency of LH secretion was little suppressed by progesterone alone in the long-term ovariectomized ewe [68]. In addition, the frequency of the MUA volley increased after the progesterone withdrawal, indicating that the GnRH pulse generator activity is also not fully suppressed by low-level estradiol alone. Taken together, the results suggest that progesterone plays a predominant role to hold the activity of the GnRH pulse generator with a synergetic action of basal levels of estradiol which appears to play a rather permissive role during the luteal phase in cycling goats.

The acceleration of MUA volley frequency after the progesterone removal is consistent with the previous observation that the LH pulse frequency increased following

luteolysis in cycling goats [78]. In the present study, however, this increase in MUA volley frequency was found to fall off gradually in parallel with the increase in the plasma estradiol level, although plasma estradiol reached supraphysiological peak levels which were several times higher than normal preovulatory peaks. It has been reported that estradiol treatment greatly diminished the pulsatile release of GnRH in the portal circulation of the ewe [44], and the frequency of MUA volleys was shown to decline dramatically following the injection of estradiol in the ovariectomized rhesus monkey [32,51] and during the follicular phase being the rise in the estradiol levels in cycling monkey [94]. The present results thus support the concept that high levels of estradiol act on the brain to suppress the GnRH pulse generator activity.

In the present study, plasma estradiol level was raised gradually to simulate the preovulatory estradiol profile in the follicular phase, which resulted in the induction of the preovulatory-like LH surge with similar latency after the progesterone decline to that seen in cycling females [78]. Similar to the previous observation in chapter 4, namely in the case of constant estradiol infusion, the recurrence of the MUA volleys continued during the LH surge and there was no acceleration of the MUA volley frequency at the transition from the tonic to the surge mode secretion of LH. This result strengthens the previous hypothesis that the GnRH pulse generator may not be directly involved in the formation of the preovulatory LH surge.

In conclusion, 1) the GnRH pulse generator activity is suppressed by the synergetic action of progesterone and estradiol at luteal phase levels, 2) GnRH pulse generator is also suppressed by high levels of estradiol, and 3) there is no acceleration of the GnRH pulse generator activity during the preovulatory-like LH surge in the artificial follicular phase in accordance with previous observations in chapter 4.

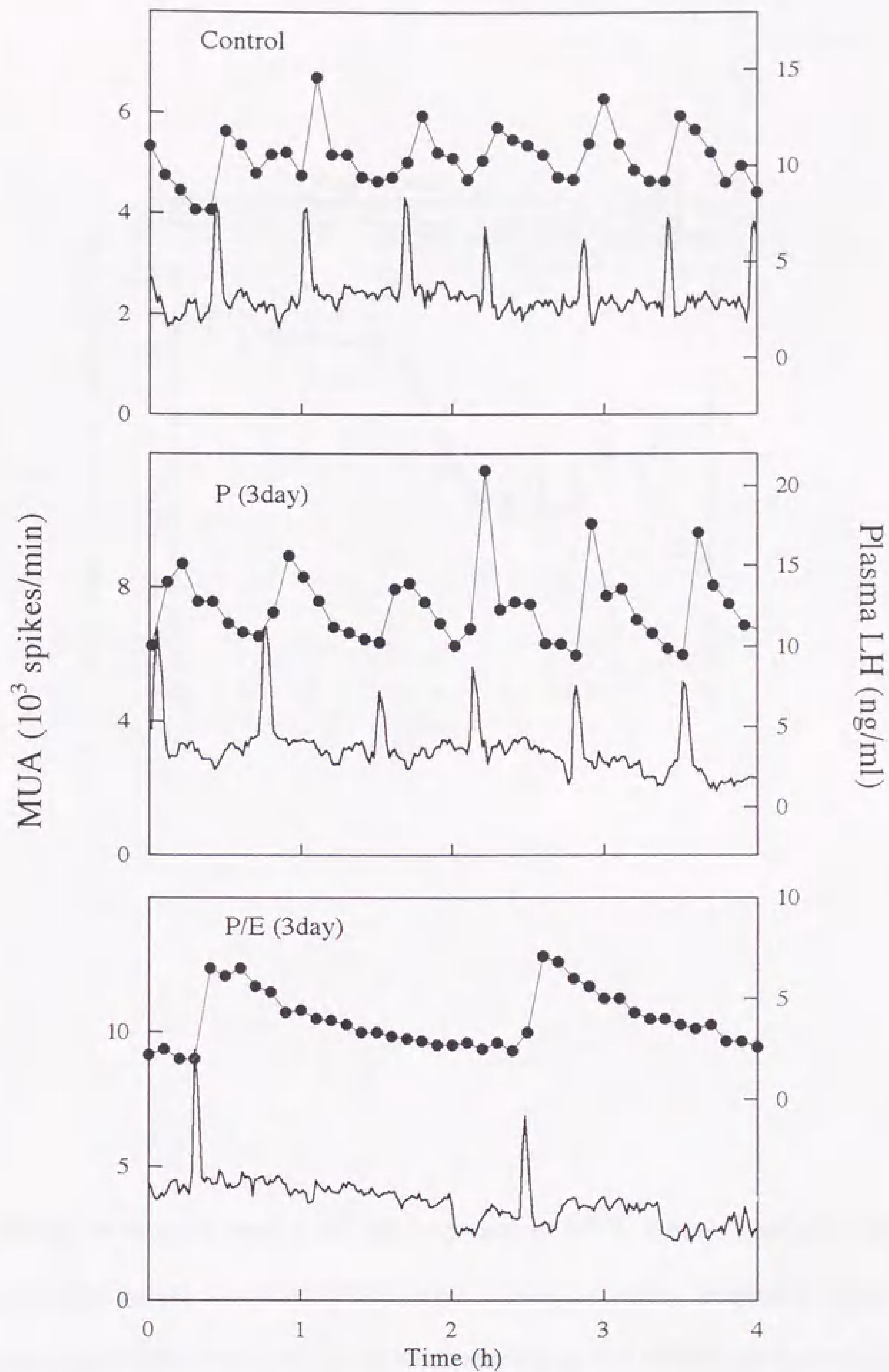


Fig.5.1. The relation between MUA volleys and LH pulses in a representative ovariectomized goat treated with luteal-phase levels of ovarian steroids. Upper, control period; Middle, treatment piriod with porgesterone alone (3rd day); Bottom, combined treatment period with progesterone and estradiol (3rd day).

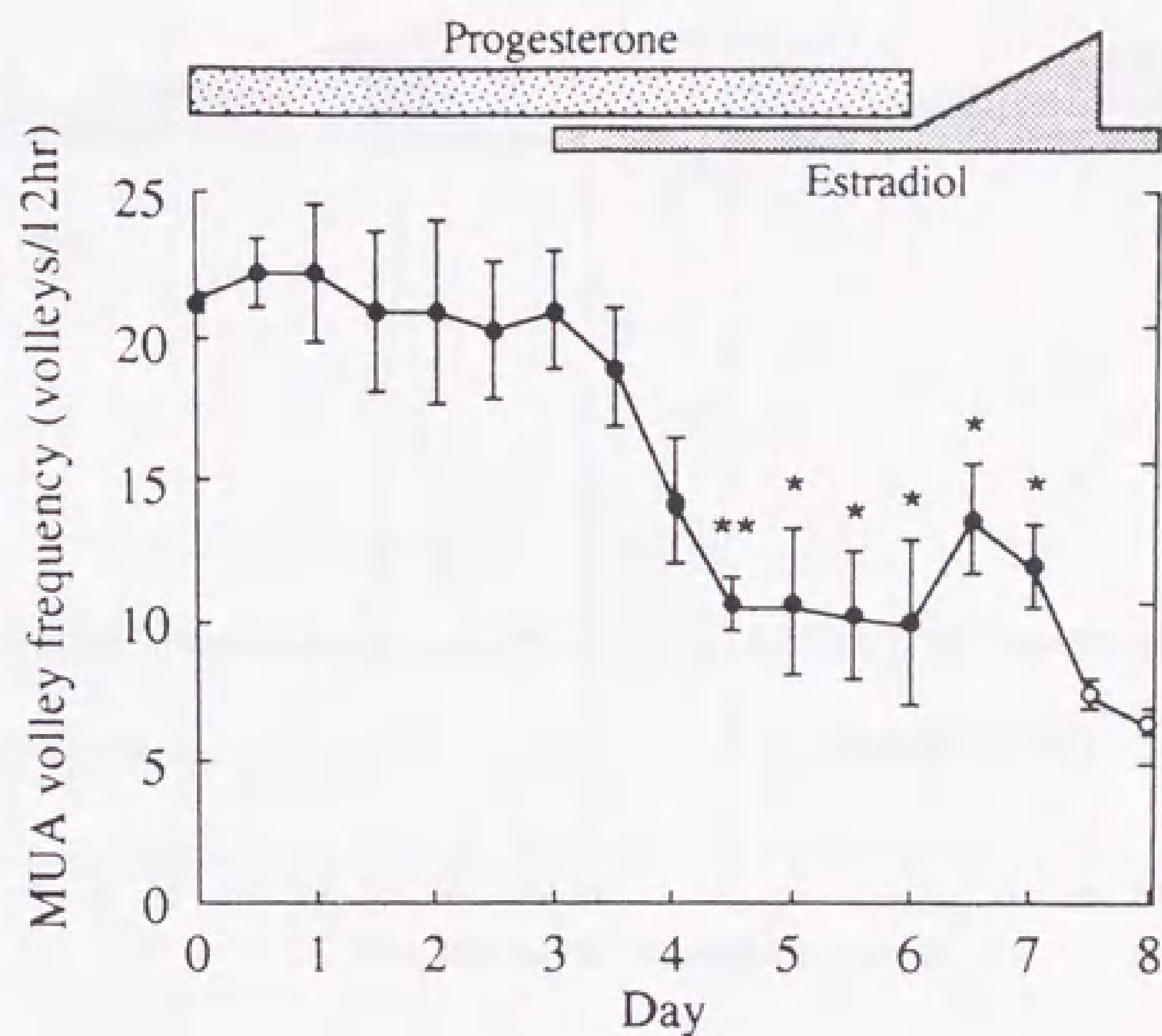


Fig.5.2. The effects of ovarian steroids on the frequency of MUA volleys throughout the experimental period in ovariectomized goats. The MUA volley frequency was not changed significantly ($p>0.05$) during the treatment period with progesterone alone as compared to the control period, but it declined (*$p<0.05$, **$p<0.01$) after the additional treatment with estradiol. Data are expressed the mean±SEM for 3 (solid circle) or 2 (open circle) ovariectomized goats. Data obtained from 2 animals were not subjected to the statistical test.

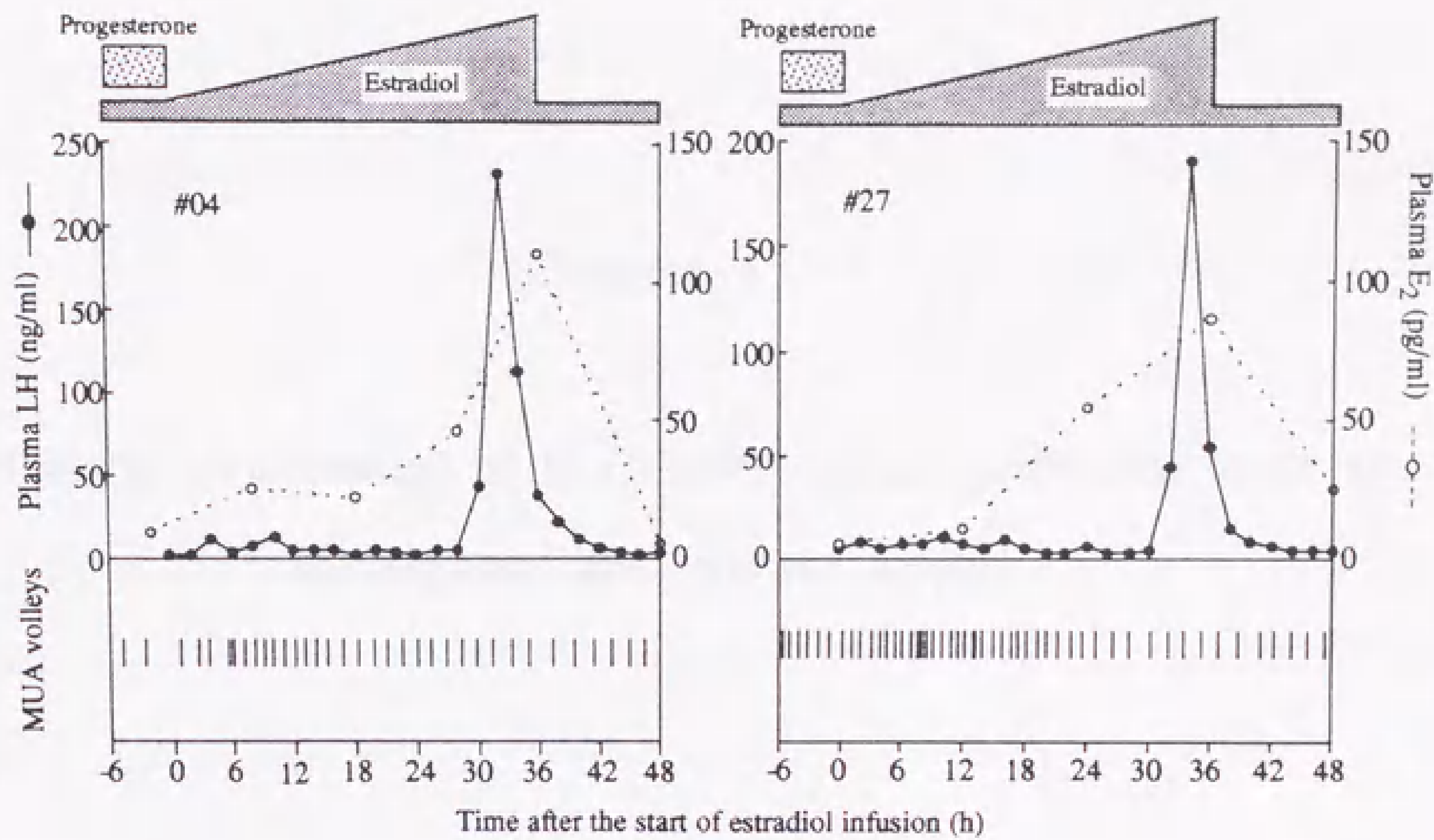


Fig.5.3. The appearence of the MUA volley (|) and the endocrine changes during the mimicked follicular phase in two ovariectomized goats. The plasma estradiol (E2) level was raised gradually by increasing the rate of infusion of estradiol, which resulted in the induction of the preovulatory-like LH surge. The frequency of the MUA volley increased after progesterone removal but then decreased again in parallel with the increase in estradiol.

Chapter 6

Continuous monitoring of the GnRH pulse generator activity throughout the estrous cycle

Introduction

In cycling animals, the GnRH pulse generator activity changes dramatically during the estrous cycle with generally high frequency of pulsatility in the follicular phase and low in the luteal phase [27]. This phenomenon became clear in various species by detection of LH pulses in the peripheral circulation or by direct measurement of GnRH release into the pituitary portal blood or the extracellular fluid at the median eminence [47,60]. However, in the light of technical difficulties, the attempt to continuously monitor the process of dramatic changes in the GnRH pulse generator activity throughout the estrous cycle has been unpractical.

In the previous chapter, it was shown that the steroidal treatment which mimicked the luteal phase endocrine environments suppressed the GnRH pulse generator activity profoundly, and that the onset of the LH surge induced by estradiol was not accompanied by the increased pulse frequency suggesting the involvement of the surge producing mechanism independent from the GnRH pulse generator. With respect to the ovulatory cycle in intact animals, however, the changes in MUA volley throughout the estous cycle have been reported so far only in rhesus monkey [94] and no imformation is available for other species.

In the present chapter, I have therefore attempted to record the MUA volleys in intact female goats to examine the profile of the cyclical changes of the hypothalamic GnRH pulse generator activity throughout the estrous cycle in relation to the hormonal profiles in the peripheral circulation.

Materials and Methods

Three cycling female goats with normal histories, from which specific MUA volleys had been recorded, were used in the following experiment.

The MUA volleys were recorded continuously throughout the estrous cycle (about 3 weeks) except for daily interruptions within 1 min to change computer files. Blood samples (5 ml) for progesterone and estradiol assays were taken daily through the jugular catheter to monitor ovarian cyclicity. At the mid-luteal phase of the succeeding estrous cycle, prostaglandin F2α (PGF2α; dinoprost 2 mg) was administered i.m. to induce luteolysis, and the change in the MUA volley was investigated in detail with respect to the endocrine changes at the transition from the luteal to the follicular phase. Blood samples were obtained every 2 h from 4 h before PGF2α injection to 8 or 14 h after the end of the LH surge, which was assessed by the rapid insensitive radioimmunoassay with shortened incubation periods.

Changes in the GnRH pulse generator activity throughout the estrous cycle were expressed in terms of mean frequency and duration of MUA volleys. The duration of MUA volly was designated as the time when sustained rise in the number of MUA spikes exceeded mean±5SD of baseline levels during 5 min prior to the onset of each volley.

Results

The characteristic increases in the frequency of hypothalamic MUA (MUA volley) were recorded continuously throughout the estrous cycle of 3 intact female goats. As shown in ovariectomized goats [38,79], the initiation of each LH pulse was always preceded by the MUA volley (Fig.6.1).

The frequency and duration of the MUA volley changed dramatically throughout the estrous cycle. Representative MUA volleys during the course of luteolysis, in the follicular phase and the luteal phase are shown in Fig.6.2. The frequency of MUA volleys was low during the luteal phase, whereas increased during the follular phase. On the other hand, the volley duration was prolonged in the luteal phase as compared with in the follicular phase as shown in Fig.6.3. The contours of MUA volley characterized by initial brief over-shoot and following decline during the follicular phase were changed to have much blunter initial elevation during the luteal phase.

Changes in the frequency and the duration of MUA volleys and in plasma concentrations of gonadal steroids for 4 estrous cycles in 3 animals are shown in Fig.6.4. The data for 3 days of goat #14 and 5 days of goat #18 (second cycle) during the luteal phase were missing due to recording problems. The frequency of MUA volley showed a reciprocal relationship with the plasma progesterone profile; the frequency of MUA volley abruptly increased at the end of the luteal phase as progesterone levels declined and continued to accelerate during the follicular phase. On the other hand, the volley duration was gradually prolonged accompanying the rise in the concentration of plasma progesterone from the end of follicular phase, and altered in parallel with the plasma progesterone levels. There was no apparent relationship between the frequency as well as the duration of MUA volleys and the plasma estradiol concentration throughout the estrous

cycle. At day 0 of the estrous cycle when progesterone level was minimum and estradiol level maximum, high frequency of MUA volley during the follicular phase was slightly decreased.

A representative pattern of MUA volley, plasma estradiol and LH levels in one goat given PGF2α during the luteal phase is shown in Fig.6.5. Plasma progesterone declined rapidly after PGF2α injection, and plasma estradiol increased gradually until the occurrence of the preovulatoty LH surge and then decreased rapidly. Changes in the MUA volley in terms of frequency and duration during the PGF2α induced follicular phase in 3 animals examined are shown in Fig.6.6. The MUA volley frequency began to increase abruptly from 4 h after the PGF2α injection, whereas the MUA volley duration became shorter gradually, and the high frequency of the MUA volley was maintained throughout the follicular phase except for the period around the LH surge, when the volley frequency was transiently lowered.

Discussion

In this chapter, an attempt was made to monitor continuously the GnRH pulse generator activity throughout the entire estrous cycle of the intact female goat and to relate it to the secretory profile of reproductive hormones. The characteristic rise of MUA (MUA volley) specifically associated with the LH pulse in the peripheral circulation was recorded throughout the estrous cycle in ovary-intact goats. The synchrony between MUA volleys and LH pulses was similar to that observed in the ovariectomized as shown in chapters 4-5, providing further evidence that each MUA volley reflects the electrical activity directly related to the periodical activation of the GnRH pulse generator.

It has been reported that the frequency of pulsatile LH secretion reduces during the luteal phase in a number of species including goat [41]. Recently, it was clearly shown in the ewe that this reduction was a result of decreased frequency of pulsatile GnRH release into the pituitary portal circulation [17,47,76]. In the present study, it was also shown that the GnRH pulse generator activity in terms of frequency reduced dramatically during the luteal phase of the estrous cycle in female goats. In addition, there was a reciprocal relationship between the MUA volley frequency and plasma progesterone profile, suggesting that progesterone was suppressive on the GnRH pulse generator activity. However, because it was demonstrated in chapter 5 that progesterone treatment in combination with estradiol but not porgesterone alone was capable of suppressing the MUA volley frequency to the extent observed in the luteal phase, it seems likely that the suppression of GnRH pulse generator activity during the luteal phase can be ascribed to synergetic action of progesterone and estradiol.

The duration of each MUA volley has been reported rather stable in ovariectomized rat [52], monkey [140] and goat [79]. On the other hand, in intact female

goats, the volley duration changed considerably throughout the estrous cycle showing apparent prolongation during the luteal phase. Although the physiological role of this alteration is yet to be clarified, present results demonstrated for the first time that the duration of each activation of GnRH pulse generator changes according to the stage of the estrous cylcle. In the rhesus monkey, the MUA volley duration prolonged gradually after ovariectomy [93] and was shortened by the estradiol injection of physiological levels [32,51]. Similarly, in ovariectomized goat, the estradiol infusion induced a reduction of the MUA volley duration (see Chapter 4). Moreover, in the present study, volley duration was gradually shortened accompanying the preovulatory rise in plasma estradiol levels during the PGF2α induced follicular phase. Therefore it is suggested that estradiol is an inhibitory factor on the GnRH pulse generator activity, which shortens the duration of each pulse during the estrous cycle. In the present study, the duration of the MUA volley changed in parallel with plasma progesterone profile throughout the estrous cycle, and it, in fact, became shorter following the luteolysis induced by PGF2α administration. In intact female rhesus monkey, O'byrne et al. [94] showed that there was a slight prolongation in the duration of MUA volley during the luteal phase as compared with the follicular phase. Therefore, it seems plausible that the prolongation of the volley duration during the luteal phase is caused by an increase in progesterone secretion.

Present results also demonstrated that the MUA volley frequency was transiently lowered around the time of the preovulatory LH surge. This is in accordance with the previous obsevation that the occurrence of MUA volleys continued with decreased frequency during the estradiol-induced LH surge in ovariectomized goats (chapter 4,5). It was also shown that the MUA volley frequency was decreased concurrently with the initiation of the preovulatory [94] and estradiol-induced [51] LH surge in the rhesus monkey. O'byrne et al. [94] suggested that this phenomenon was the consequence of a

preovulatory rise in plasma estrogen concentrations. Similar observation was made in chapter 5 that the MUA volley frequency decreased gradually in parallel with an increase in plasma estradiol in ovariectomized goats infused with estradiol at a gradually increasing infusion-rate. Therefore, the suppression of the MUA volley frequency during the period around the LH surge may be caused by elevated estradiol levels. Alternatively, either the short-loop or ultrashort-loop feedback system may be involved in the modulation of the GnRH pulse generator activity, because reduced frequency of MUA volleys was only seen around both the preovulatory LH surge in intact females (present study) and estradiol-induced LH surge in ovariectomized animals (chapter 4). In fact, it has been shown in ovariectomized ewes that an injection of GnRH into the third cerebral ventricle resulted in the reduction of the frequency of pulsatile LH secretion [86]. In the rhesus monkey, however, it was reported that the GnRH analog-induced elevation of LH secretion had no effect on the GnRH pulse generator activity by using the MUA recording technique [50]. Similary, Yamada et al. [144] in my laboratory could not find any change in the frequency of MUA volleys following either peripheral or intracerebroventricular administration of GnRH in ovariectomized goats.

In conclusion, it has been demonstrated in this chapter that a dramatic change in the GnRH pulse generator activity during the estrous cycle is related to plasma levels of gonadal hormones in the female goat. The results can be summarized as follows, 1) the frequency of GnRH pulse generator activity shows a reciprocal relationship with the plasma progesterone profile, 2) the duration of GnRH pulse generator activity changes in parallel with the plasma progesterone levels with considerable prolongation in the luteal phase 3) in the follicular phase, the frequency of GnRH pulse generator activity is lowered during the preovulatory LH surge.

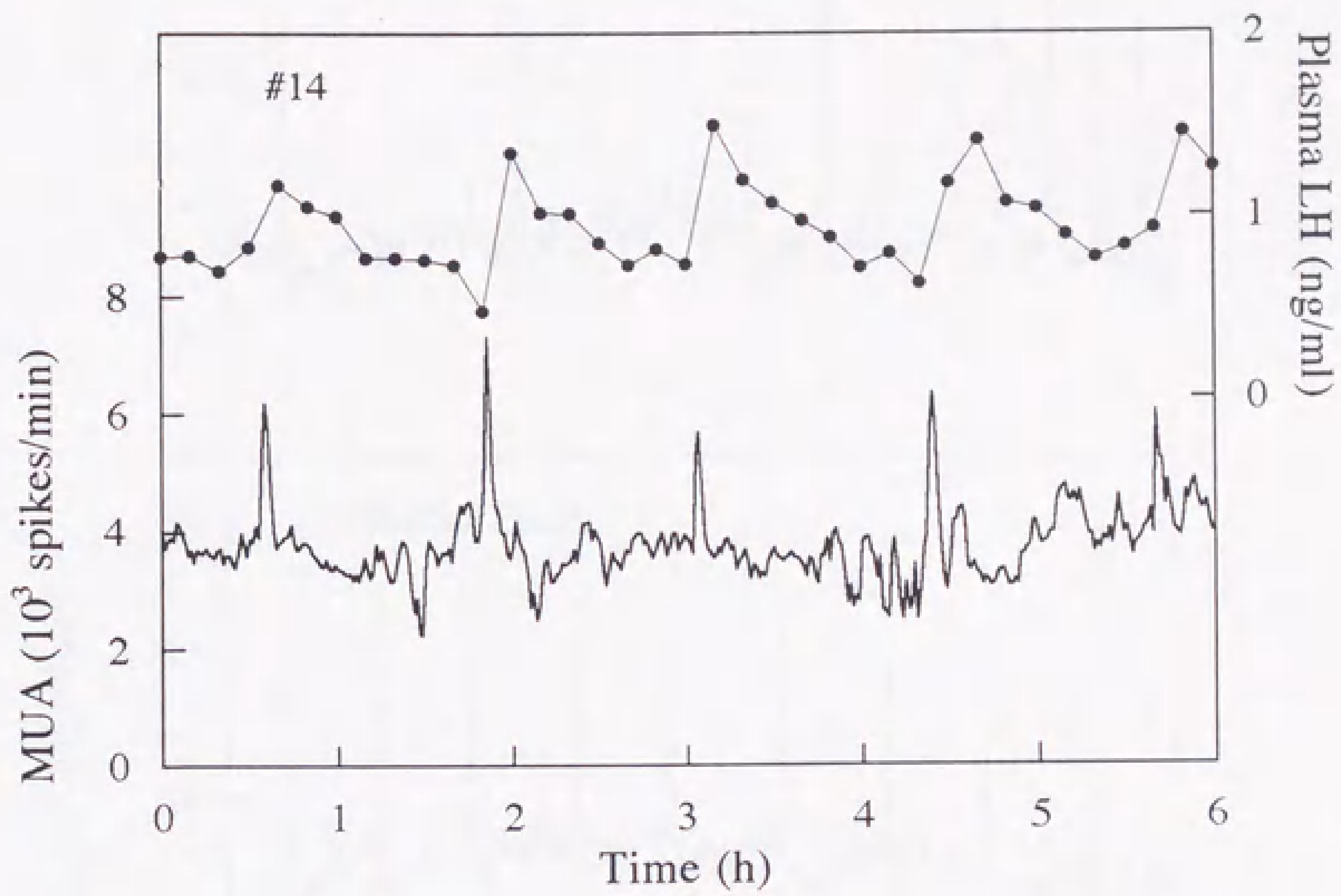


Fig.6.1. The correlation between MUA volleys and LH pulses in an ovary-intact goat (#14). The initiation of each LH pulse was always preceded by an abrupt rise in MUA (MUA volley).

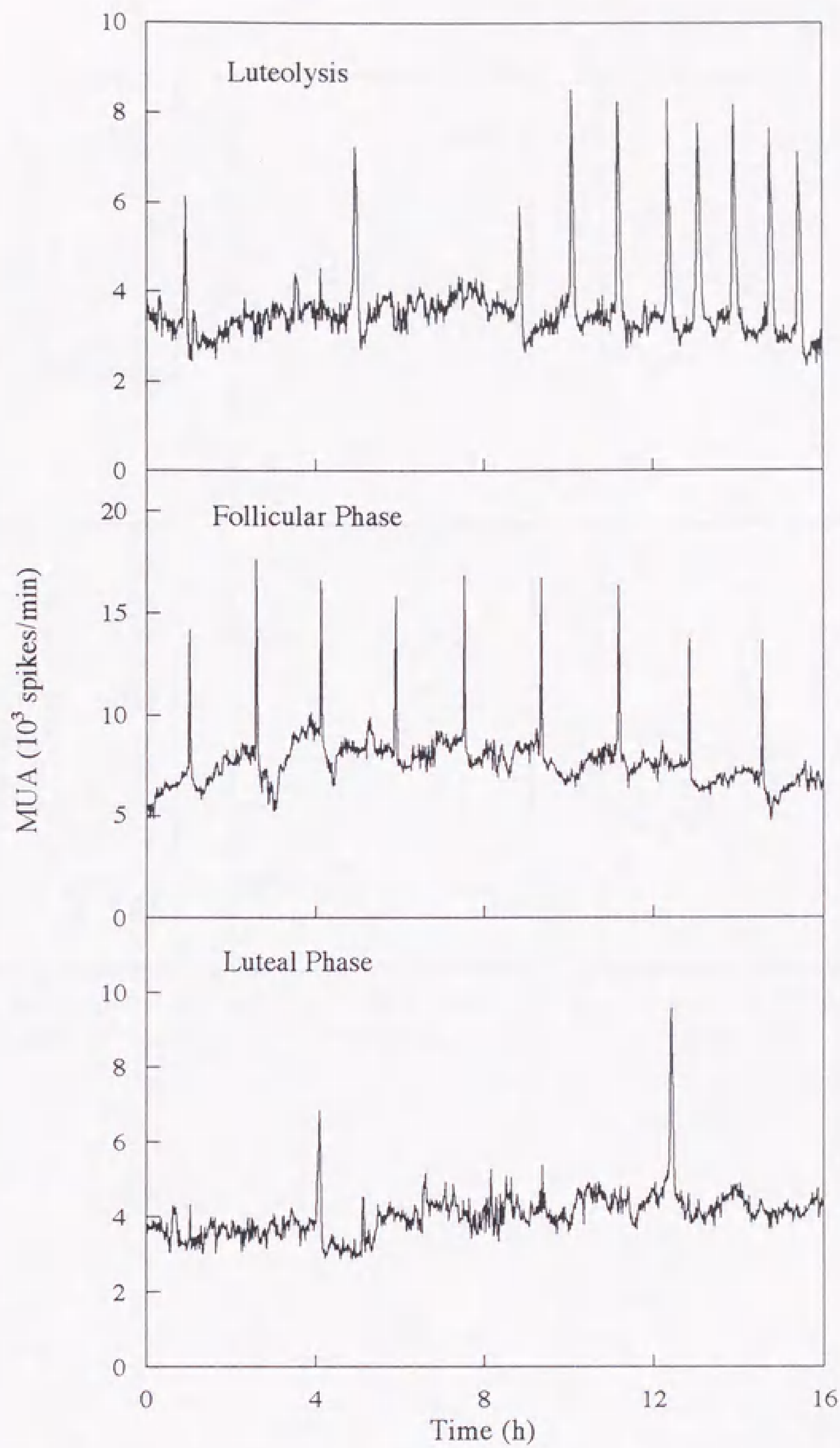


Fig.6.2. Representative changes in the MUA volleys during the luteolysis (top), follicular phase (middle) and luteal phase (bottom) in a cycling female goat. The volley frequency was high in the follicular phase whereas low in the luteal phase, and an abrupt increase was seen during the course of luteolysis.

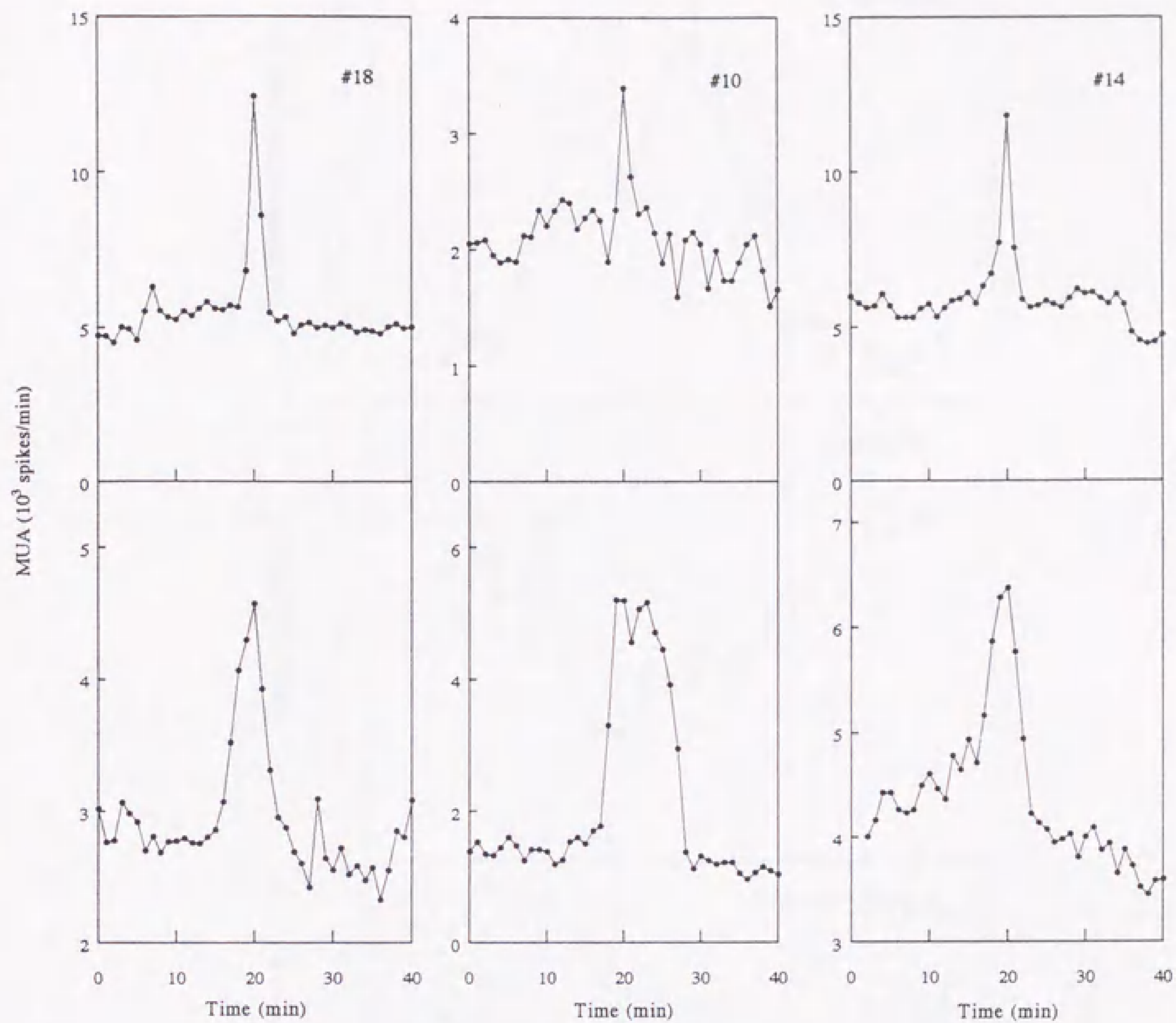


Fig.6.3 Representative MUA volleys in 3 individual goats during the follicular phase (upper) and the luteal phase (bottom). The duration of MUA volley was prolonged during the luteal phase.

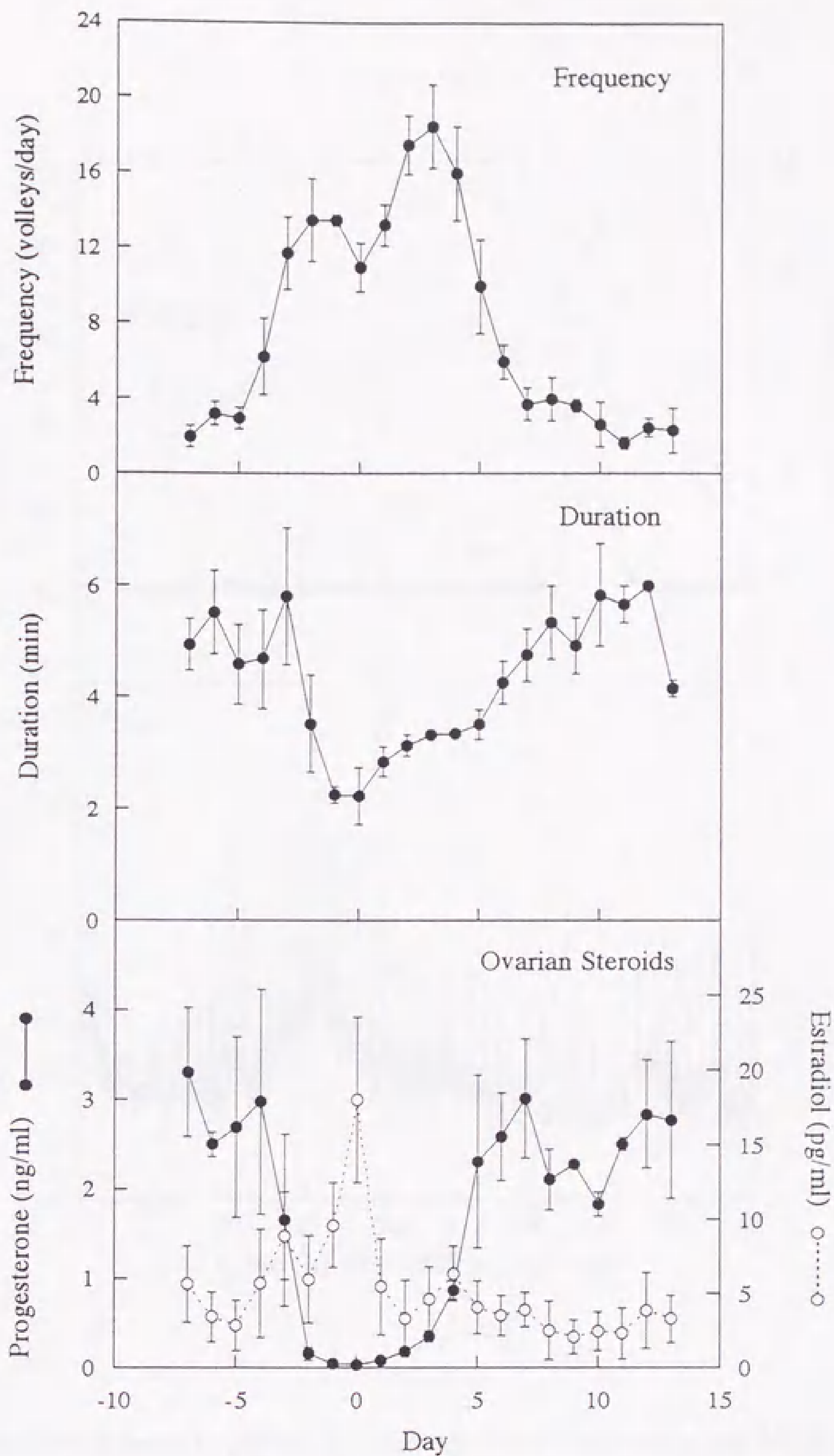


Fig.6.4. Changes in frequency and duration of the MUA volleys in relation to plasma profile of ovarian steroids throughout the estrous cycle in female goats. Each point represents the mean±SEM for 3-4 observations (4 cycles in 3 goats). Day 0 indicates the day of minimum progesterone concentration during the estrous cycle.

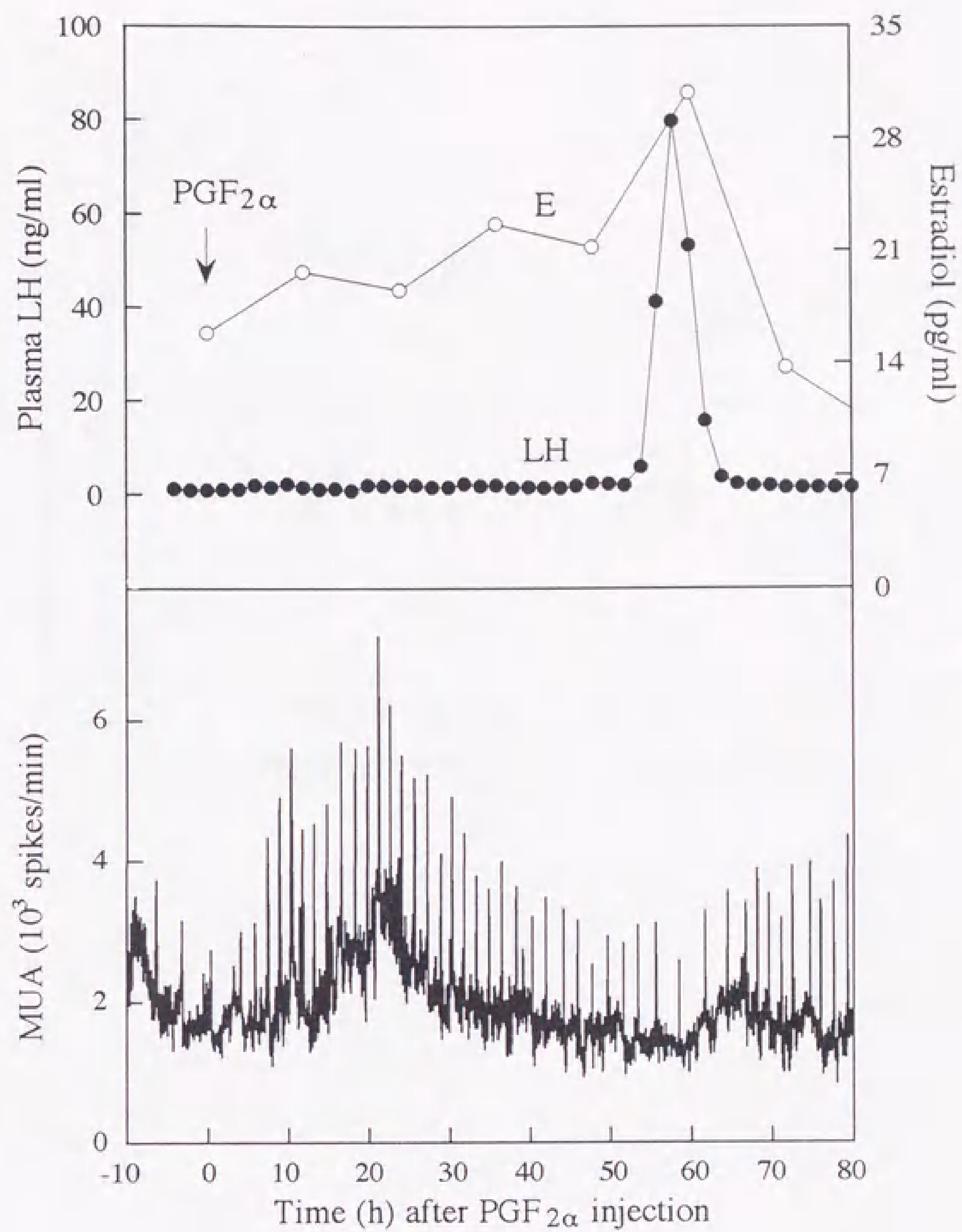


Fig.6.5. Representative changes in plasma LH and estradiol (top) and in the MUA volley (bottom) throughout the follicular phase following the luteolysis induced by PGF2α administration. Plasma estradiol (E) increased gradually and the preovulatory LH surge was induced after 60 h of PGF2α injection. Reccurence of the MUA volley was observed to continue during even at the time of preovulatory LH surge.

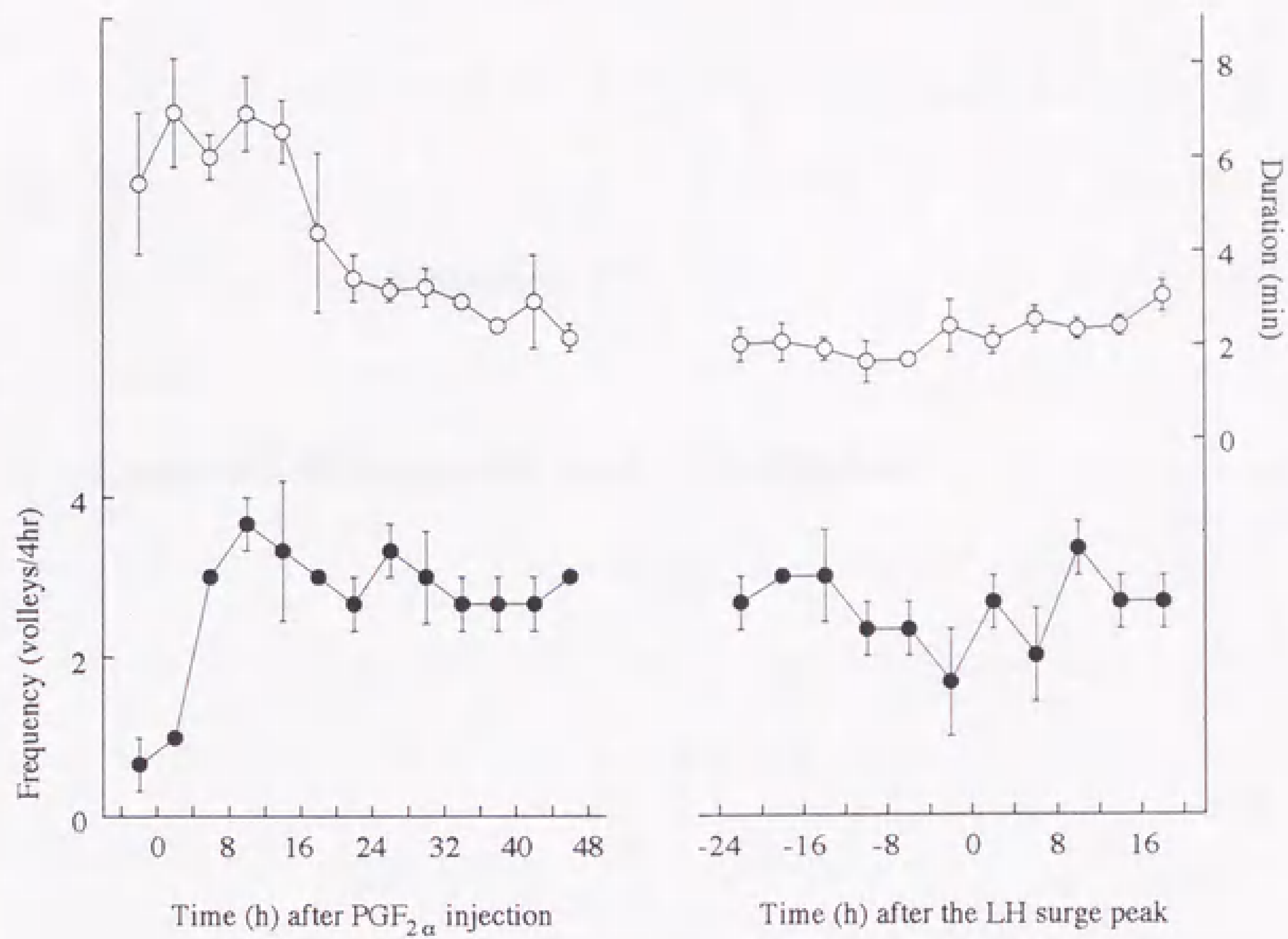


Fig.6.6 Changes in the frequency (solid circle) and duration (open circle) of MUA volleys during the periods around the PGF2α induced luteolysis and the LH surge in 3 female goats. The data are normalized to either the time of PGF2α injection (left) or the peak time of the LH surge (right). Each point represents the mean±SEM for 3 animals. The volley frequency increased abruptly after PGF2α injection, and then the duration became shorter. The volley frequency was lowered slightly around the LH surge.

Chapter 7

General Discussion and Conclusion

Neural mechanisms governing the GnRH release

In the present dissertation, temporal relationship between the MUA volley and the following LH pulse was maintained under all experimental conditions including the situation where the pulse frequency was altered by the administration of exogenous steroids (chapter 5). Using microdialysis technique, it was shown in ovariectomized goats that LH pulses in the systemic circulation were preceded by episodic increases of GnRH neurosecretion at the median eminence [145] with the interval being much the same as between the MUA volley and the LH pulse. Moreover, Moenter et al. [74] found a similar relationship when they investigated the pattern of GnRH release into the portal circulation in ovariectomized ewes; the shape of most GnRH pulses approximated a square wave with an abrupt ascent (within 1 min of pulse onset), a release period averaging 5.5 min, and a precipitous descent to the pre-pulse baseline level within 2-3 min. This release pattern of GnRH in the sheep resembles the contour of the MUA volley in the goat. By considering all these information, MUA volley associated with LH pulse was regarded as the specific electrical signal which reflects the hypothalamic GnRH pulse generator activity governing the release of GnRH.

Recent studies in the ewe have demonstrated clearly that GnRH secretion into the portal circulation is substantially increased during both the estradiol-induced [75] and preovulatory [76] LH surges. Similarly, in the ovariectomized goat given constant intravenous infusion of estradiol, the GnRH surge was shown to take place at the same time as the LH surge [67] using the intracerebral microdialytic perfusion technique [145]. In chapter 4, I used the same experimental model, namely the ovariectomized goats receiving constant estradiol infusion, to test the change in MUA volley around the time of the LH surge and to determine the role of the GnRH pulse generator in induction of the GnRH surge. As a result recurrence of MUA volleys with slightly decreased pulse

frequency around the LH surge was observed. In accordance with this finding that the MUA volley continues to appear throughout the artificial (chapter 5) and natural follicular phase (chapter 6) with a slight slow-down around the LH surge, there was no acceleration of the MUA volley frequency at the transition from the basal to the surge mode of LH secretion. In this connection, it was shown that the GnRH pulse generator activity, as assessed by recording the specific MUA, was strongly suppressed during the LH surge in both estradiol-treated ovariectomized [51] and intact [94] rhesus monkeys, and ovariectmized rats [90]. Moenter et al. [73] recently reported that the pattern of GnRH in the portal circulation was not strictly episodic during the GnRH surge in ovariectomized ewes, suggesting that one important action of estradiol in inducing the GnRH surge may be to switch the pattern of GnRH secretion from episodic to continuous. These previous and present findings support the hypothesis as described in chapter 4 that the positive feedback actions of estradiol on LH/GnRH secretion which is essential for ovulation is exerted through a neuronal mechanism that is intrinsically differnt from the pulse generator. Consequently, it is suggested that there are two independent neural components governing the GnRH release in the hypothalamus; one is the "GnRH pulse generator" regulating the pulsatile GnRH release and the other is the "GnRH surge generator" which is activated by the exposure to estradiol and induces a surge mode secretion of GnRH.

Blake and Sawyer [5] reported that rats bearing complete deafferentation of the MBH showed pulsatile LH secretion after ovariectomy. Ohkura et al. [95] also reported that LH pulses appeared in rats bearing anterior, anterolateral or complete hypothalamic deafferentation and that these types of deafferentation did not affect the LH pulse frequency. Similarly, in the ovariectomized ewe, neither anterior deafferentation at the level of the suprachiasmatic nucleus (SCN) [20,40,98,106,107] nor posterior deafferentation at the level of the mamillary bodies [40,126] altered pulsatile LH secretion,

whereas similar cuts in the MBH suppressed or abolished pulsatile LH secretion. In the present study the tip of the electrode, from which specific electrical activity synchronized with the pulsatile LH secretion (MUA volley) was recorded, was located in the MBH around the median eminence. Likewise, MUA volley was always recorded from the MBH in rats [52] and rhesus monkeys [121] regardless of interspecies difference of the distribution pattern of immunoreactive GnRH neurons. It is therefore most likely that the GnRH pulse generator is located in the hypothalamus and most probably within the MBH around the median eminence.

Then, where is the GnRH surge generator located? In the rat, the isolation between the medial basal and anterior structures of the hypothalamus by a knife-cut resulted in the cessation of estrous cyclicity, but basal [33] and pulsatile [5] gonadotropin secretion was maintained. In contrast, cuts across more anterior planes, leaving intact paths linking the MBH and septo-preoptic region, did not perturb the ovarian cycles. The lesions restricted to the medial preoptic area (POA) induced anovulatory persistent estrus [48,104,136,137] and the annulment of LH surges [137]. On the other hand, in ewes, knife cuts [20,40,105,126] or lesions[107] just posterior to the SCN or POA blocked the ovulation and estradiol-induced LH surge, whereas cuts anterior to the SCN did not [20,40,105]. Moreover, although there is no available information as to the hypothalamic localization of estrogen receptor in goats, in other domestic ruminants such as ewes which share many similarities in the brain structure with goats, dense collections of cells containing estrogen receptors have been found in both POA and MBH [57,58]. The present observation that the baseline of MUA between volleys recorded from the MBH did not change during the LH surge suggests that the estrogen-responsive neuronal system mainly involved in generating the LH suge is located in the POA, but not in the MBH, while that in the MBH mainly participates to the regulation of GnRH pulse generator

activity.

In addition, almost complete absence of immunoreactive estrogen receptor in preoptic and hypothalamic GnRH neurons have been shown in various species such as ewe [58], rat [118] and guinea pig [135], suggesting that estrogen actions on GnRH neurons are not direct, but are mediated via estrogen-responsive neurons in the hypothalamus. However, exact information about the neural mechanism mediating the LH surge is still lacking. Recently, Robison et al. [111] found a decline in gamma-aminobutyric acid and an increase in norepinephrine in the POA associated with the LH surge in ovariectomized ewes suggesting the involvement of these neurotransmitters, although it is known that adrenergic antagonists do not consistently block the LH surge [39].

In this dissertation I could not determine the neuronal component responsible for the GnRH pulse generating mechanism. In chapter 3, I postulated that MUA volleys reflect the neuronal activity of axons or terminals of GnRH neurons activating, because positive electrodes, from which specific MUA are recorded, are always found in the vicinity of condensed GnRH immunoreactive fibers in the median eminence region. This leads me to hypothetize that the GnRH pulse generator consists of only GnRH neurons, in other words, the GnRH neuron system has a pulse generating mechanism within itself. However, this idea is incompatible with the fact that during the LH surge, probably when the excitability of GnRH neuron is markedly increased for acceleration of GnRH neurosecretion [67], MUA volleys continued to recur without any acceleration of pulse frequency and actually there was no appreciable increase in the baseline of MUA as demonstrated in chapters 4-6.

Although the reason for this discrepancy is unknown, I hypothesize two mutually exclusive ideas as follows. One is that the MUA volley is not recorded from GnRH

neurons but from non-GnRH neurons through electrodes located in the GnRH concentrating region. In other words, the GnRH pulse generator consists of neuronal components other than GnRH neurons residing in the MBH which periodically activate GnRH neurons during the pulse. This idea is supported by numerous studies which revealed that various neurotransmitters as well as neuromodulators are involved in the control of GnRH neurosecretion. For instance, administration of prazosin, an α_1-adrenegic blocker, resulted in smaller GnRH pulses, whereas the stimulation by α-adrenergic input to the GnRH neurons appeared to enhance the amount of the GnRH pulse [124]. Similary, an infusion of neuropeptide-Y to the stalk-median eminence stimulated *in vivo* GnRH release in a dose dependent manner [141]. Moreover, these neurotransmitters were released from the stalk median eminence in a pulsatile fashion which was synchronized with the pulsatile GnRH release [124,141,142], providing a rationale to consider that these substances are involved in the pulse generation.

The other possibility is that the surge mode GnRH neurosecretion occurs at a part of the brain away from the region where the electrodes are located, namely, there are at least two subpopulations of GnRH neurons; one is residing in the MBH and responsible for the pulsatile secretion, whereas the other residing presumably in the POA including the organum vasculosum of the lamina terminalis (OVLT) and responsible for surge mode secretion. In fact, the axons of up to 50 % of GnRH cells terminate in the OVLT, and the remainder terminates in the median eminence [125]. Lehmen et al. [57] reported that one of the regions of densest accumulation of estrogen receptor immunoreactive cells was seen in the medial POA at the level of the OVLT. In support of this notion, Norman et al. [91] suggested that induction of the LH surge in the rhesus monkey involves activation of two groups of GnRH neurons; one group is normally involved in the tonic (pulsatile) secretion of GnRH and the other in secretion of GnRH with continuous stream during the estradiol-

induced LH surge.

However, the latter hypothesis seems unlikely, because the immunohistochemical studies revealed that the most neuronal cell bodies of GnRH neurons are scattered over the region anterior hypothalamus, i.e. the medial POA, diagonal band of Broca, supraoptic nuclei, and there are few cell bodies of GnRH neurons in the MBH of the goat [34,147]. This distribution pattern is different from those in the monkey in which the major hypothalamic GnRH cell groups reside in the MBH [119]. Moreover, a large increase in GnRH concentration in microdialysis perfusates of the 'median eminence' is shown during the LH surge in ovariectomized goat infused with estradiol [67]. Jackson et al. [40] reported that the cuts which disrupted the anterior portion of the arcuate nucleus without affecting the anterior region of hypothalamus blocked estrogen-induced LH surges in ovariectomized ewes. Therefore, the former hypothesis seems a plausible explanation on the discrepancy between the immunohistochemical and the electrophysiological studies.

Fig.7.1 gives a schematic model of the possible mechanism governing the GnRH release in the female goat. The GnRH pulse generator resides within the MBH around the median eminence and consists of a group of neurons other than the GnRH neurons. Rhythmic activity of the GnRH pulse generator stimulates the GnRH neuronal axon or terminal in the median eminence and causes pulsatile release of GnRH. On the other hand, GnRH surge generator, which consists of the neuronal group containing the estrogen receptors other than GnRH neurons, is activated by the exposure to estradiol, and induces the surge mode secretion of GnRH.

Neural control of the estrous cycle

In chapter 6, sequential change in the GnRH pulse generator activity throughout the estrous cycle was first demonstrated in ruminant species by recording specific MUA volleys, and it was revealed that the frequency and the duration of MUA volleys were alterd dramatically.

Clarke et al. [17] and Moenter et al. [76] reported that the GnRH pulse frequency was low during the luteal phase, whereas high during the follicular phase of the estrous cycle in the ewe. The results obtained in chapter 6 also demonstrated that the frequency of GnRH pulse generator, which was assessed by the MUA volley, decreased markedly during the luteal phase but increased during the follicular phase showing a reciporcal relationship with the plasma progesterone profile throughout the estrous cycle. Regarding this regulatory mechanism it was shown in chapter 5 that exogenously administered progesterone exerted the negative feedback effect on the GnRH pulse generation when combined with estradiol treatment which mimicked the steroidal milieu of the luteal phase in long-term ovariectomized goats, but not by progesterone alone. Therefore, the reciprocal relation between the pulse generator frequency and plasma progesterone level observed throughout the estrous cycle suggests that the progesterone level in peripheral circulation would be the predominant determinant of the GnRH pulse generator activity in the goat estrous cycle but the basal level of estradiol is necessary presumably as a permissive factor.

On the other hand, the duration of MUA volley also changed in parallel with the plasma progesterone levels showing considerable prolongation in the luteal phase of the estrous cycle. Cardenas et al. [8] recently demonstrated that the fluctuations in the MUA volley duration was not due to the sequential activation of different units but due to fluctuation in the bursts of individual neurons contributing to the MUA volleys in the

rhesus monkey, although the physiological role of the duration of each MUA volley, e.g. whether or not these changes are related to modification of the pattern of GnRH release, is still unknown. In the rhesus monkey it was reported that the duration of each MUA volley was not correlated with the amount of LH release [139]. However, further investigation would be necessary to address this question, since it has been reported that the amplitude as well as the frequency of GnRH pulses changes throughout the estrous cycle with higher amplitude in the luteal than in the follicular phase in ewes [3,17,45], and that the pulse amplitude of GnRH released in the stalk median eminence increased after progesterone treatment in ovariectomized rhesus monkeys [142].

Then, what are the endogenous mediater(s) responsible for these actions of gonadal steroids on the GnRH pulse generator activity? It has been shown that the suppressive effects of gonadal steroids during the luteal phase are canceled partially, but not completely, by the administration of naloxone, an endogenous opioid peptide antagonist in rhesus monkey [49,96,132], rat [19,101,102], and sheep [6,7,146], indicating the involvement of endogenous opioid peptides in the steroidal suppression of the frequency of GnRH pulse generator activity during the luteal phase. Similarly, the inter-MUA volley interval was shorter during the period of naloxone treatment than the control period in ovariectomized goat treated with progesterone and estradiol that mimicked a steroidal milieu during the luteal phase [38]. Therefore, it seems likely that endogenous opioid peptides are one of the main neural factors, but not the sole one, responsible for mediating the steroidal negative feedback on the frequency of the GnRH pulse generator activity. On the other hand, the neural factor mediating the duration of MUA volley is unkown. Because the naloxone treatment prolonged the duration of the MUA volley in ovariectomized goat treated with ovarian steroids [38], it is suggested that neural mediator(s) other than opioid peptides is involved in the prolongation of MUA

volley duration.

It was demonstrated in the ewe that LH concentrations increased accompanying the rise in the concentration of plasma estradiol in the follicular phase [29,97]. Furthermore, there is a close correlation between endogenous LH pulse and estradiol [113,128] with each LH pulse being followed by an estradiol secretion [127] in the follicular phase following luteolysis. On the othe hand, suppression of endogenous LH secretion by treatments with progesterone [46], pentobarbital [108], or dopamine [114] prevented the preovulatory estradiol rise, whereas the pulsatile injection of LH to the anestrus ewes induced an increase in the secretion of estradiol which culminated in the ovulation [46]. In addition, blokade of the pulsatile secretion of LH by immunoneutralization against GnRH during the follicular phase without affecting basal LH and FSH concentrations in the peripheral circulation, strikingly suppressed the estradiol secretion [71]. These observations indicate that an increase in pulsatile LH secretion drives the preovulatory estradiol rise during the follicular phase. In chapter 6, it was also observed that the MUA volley frequency was abruptly increased accompanying with the reduction of progesterone concentration after PGF2α administration, which then was followed by the estradiol rise. Therefore, it is suggested that the abrupt rise in the frequency of the GnRH pulse generator activity following the luteolysis is playing an important role for the increase of estradiol secretion from developing follicles. Both in intact and ovariectomized goats, further acceleration of MUA volley frequency did not take place during the period of preovulatory LH surge, when the frequency was transiently lowered instead. These results suggest that an increment in the GnRH pulse generator frequency caused by the decline of the progesterone concentration following luteolysis is indispensable for follicular development and the estradiol rise in the systemic circulation, which eventually induces the preovulatory LH surge via the GnRH surge generator.

Conclusion

The results presented in this dissertation have shown that the neurosecretion of GnRH from the hypothalamus is governed by two functionally independent systems; namely, the pulse generator and the surge generator. The GnRH pulse generator, which appears to reside in the MBH around the median eminence, controls the pulsatile pattern of GnRH and then gonadotropin secretion from the pituitary gland, which mediates various external and internal environmental factors to regulate gonadal activities such as follicular development and steroid secretion. On the other hand, the GnRH surge generator, which may reside in the preopic area, is activated by the rise in systemic estradiol levels and is responsible for a massive discharge of GnRH into the portal circulation (GnRH surge) and thereby inducing the gonadotropin surge, which is essential for ovulation.

The ovarian cyclicity in female animals is maintained by the hormonal interactions between hypothalamic GnRH which receives dual modulation by the pulse generator and the surge generator, pituitary gonadotropin, and ovarian steroids. During the luteal phase, the frequency of the GnRH pulse generator activity is suppressed by synergetic action of progesterone and estradiol, whereas the duration of that is facilitated by progesterone. Subsequently, the fall in progesterone associated with the luteolysis removes an inhibitory tone on the frequency of GnRH pulse generator, allowing the GnRH and LH pulse frequency to increase, which then stimulates estradiol secretion from the ovarian follicles. The sustained elevation in circulating estradiol provokes the GnRH surge generator, which then induces preovulatory GnRH and LH surge. The LH surge, in turn, induces ovulation and luteinization so that next cycle begins.

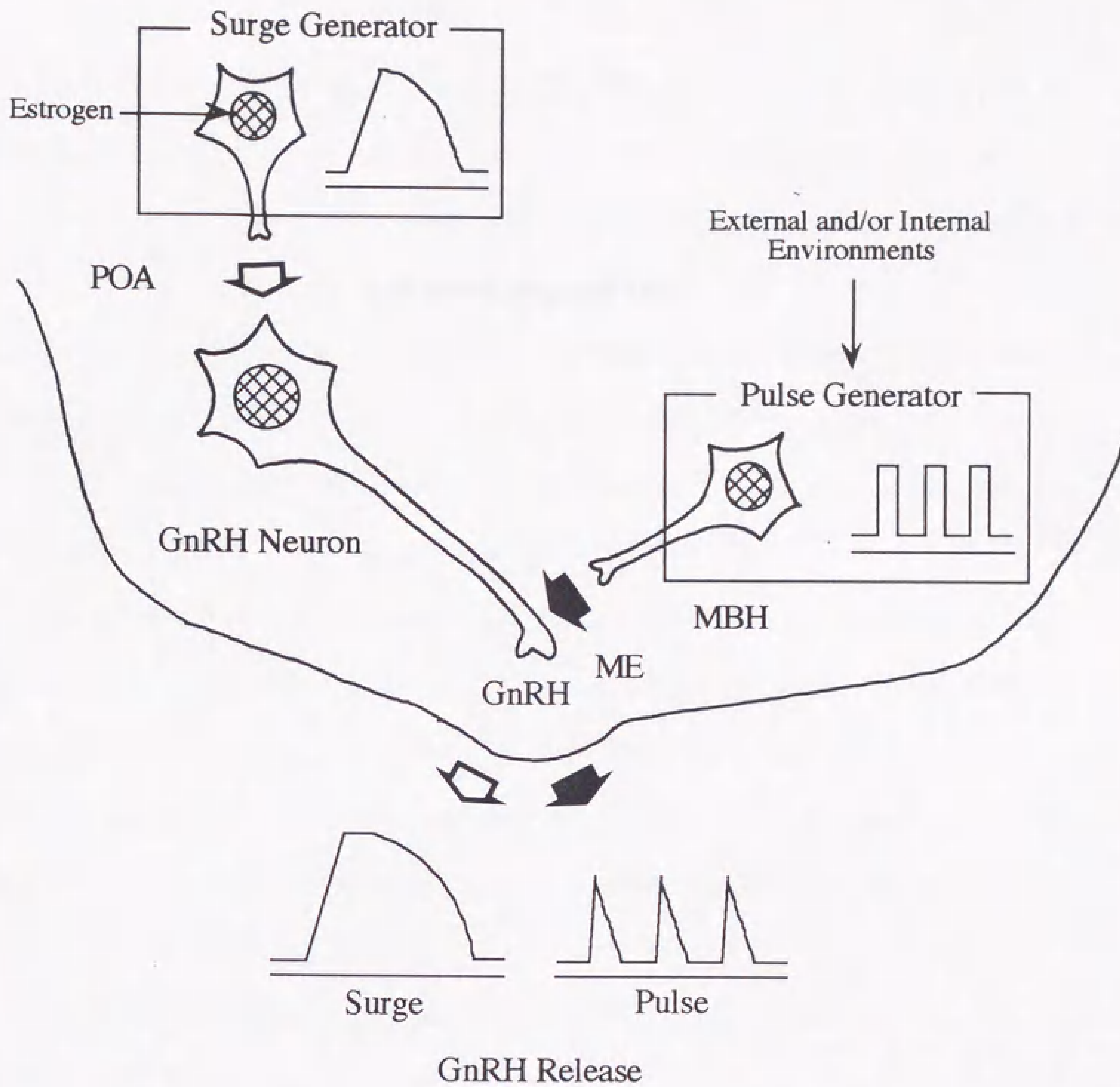


Fig.7.1. Schematic illustration of the possible neural mechanism governing the GnRH release in the female goat. MBH: medial basal hypothalamus, ME: median eminence, POA: preoptic area

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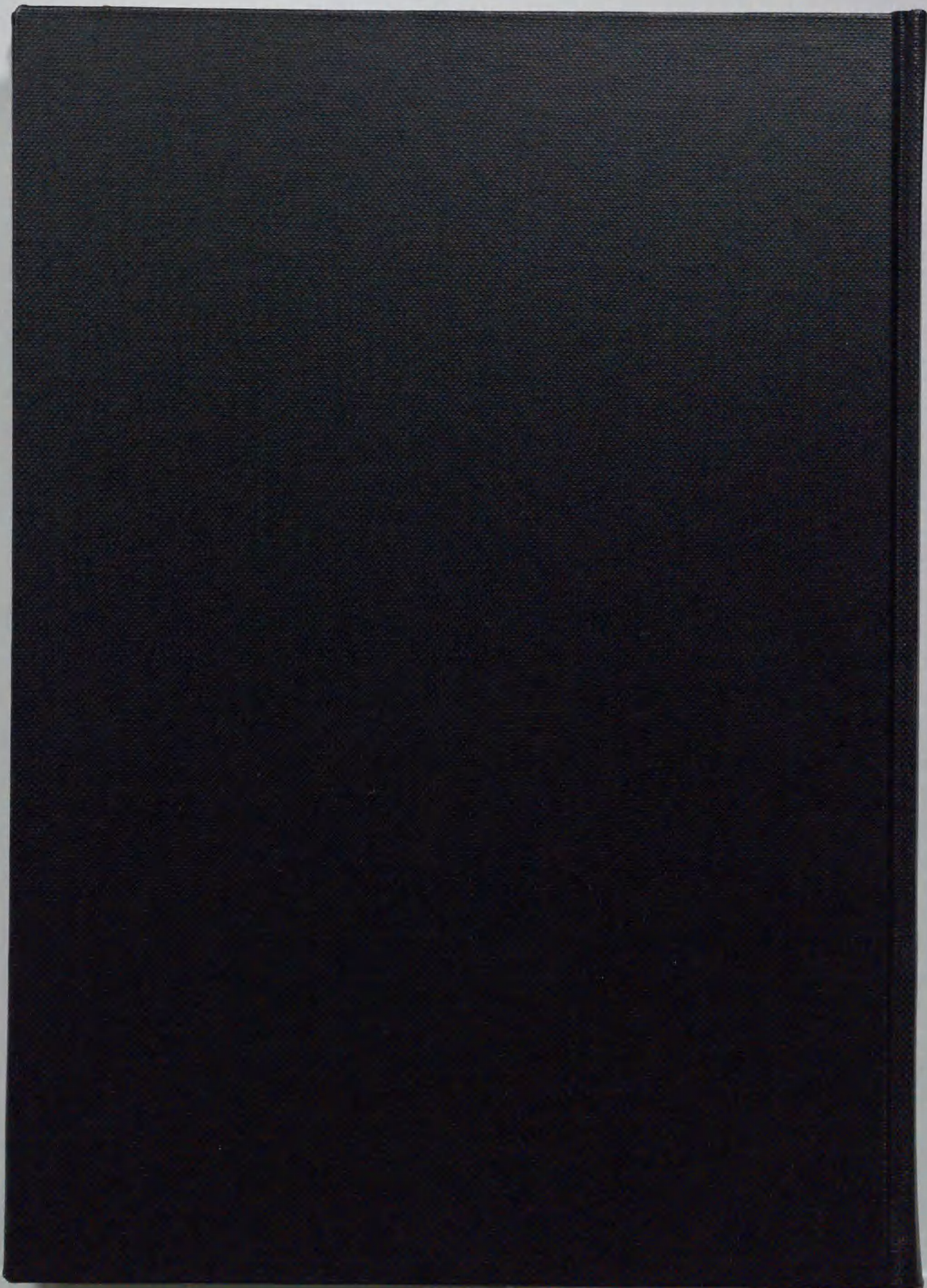

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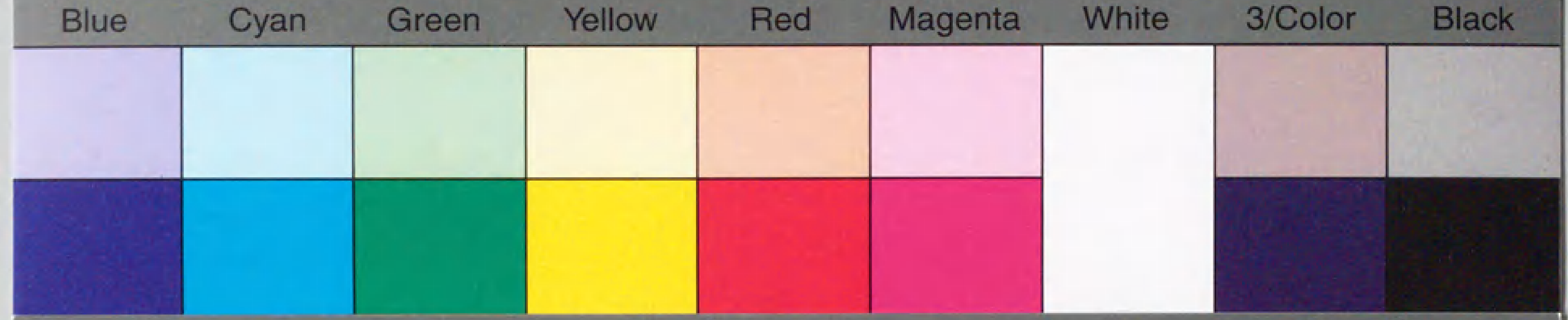
Blue Cyan Green Yellow Red Magenta White 3/Color Black



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A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19