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Electrophysiological approach to the hypothalamic GnRH pulse generator

Y. Mori[1] and T. Tanaka[2]

[1]*Laboratory of Veterinary Ethology, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan; and* [2]*Laboratory of Veterinary Reproduction, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183, Japan*

The hypothalamic GnRH pulse generator that regulates intermittent GnRH discharge into the pituitary portal circulation and thereby modulates pulsatile secretion of LH has been recognized as a key determinant of the reproductive function in mammals. Thus, various internal, as well as external, factors first modify the electrical activity of the GnRH pulse generator, which then alters the pulsatile pattern of gonadotrophin secretion and eventually influences reproductive function. Here, we describe a procedure that has permitted long-term recording of electrophysiological manifestation of the GnRH pulse generator activity and its application to our research which uses ovariectomized and cyclic female goats as experimental models. We have successfully recorded characteristic increases in neuronal activity associated with pulsatile LH secretion from conscious goats by means of a multiple unit activity (MUA) recording technique, which is an adaptation of that developed originally for use in rhesus monkeys. The unitary relationship between periodical increases in MUA (MUA volleys) and LH pulses is well maintained under a variety of experimental conditions, providing evidence that these MUA volleys are the consequence of GnRH pulse generator activity.

Introduction

Rhythmic release of GnRH from the median eminence into the portal circulation is driven by a yet to be identified neural component, i.e. the GnRH pulse generator, which is considered to reside in the hypothalamus (Knobil, 1980; Lincoln *et al.*, 1985; Levine *et al.*, 1991). Many internal and external environmental changes, such as nutrition, puberty, stress, lactation, photoperiod and pheromones, are thought to modulate the neural operation of the GnRH pulse generator; this, in turn, gives rise to the pattern of pulsatile GnRH release into the portal circulation which dictates the pattern of LH secretion from the pituitary, and eventually gonadal function (Rivier and Rivest, 1991; Mori, 1992; Karsch *et al.*, 1993; Foster, 1994; Goodman, 1994). Therefore, the GnRH pulse generator is thought to be the most important final common mediator of all influences on reproduction conveyed through the central nervous system.

An attempt to monitor hypothalamic GnRH pulse generator activity electrophysiologically 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 (1981) first reported that characteristic increases in neural activity coincided with the initiation of the LH pulse in the medial basal hypothalamus (MBH) of anaesthetized ovariectomized rhesus monkeys using acutely placed electrodes to record hypothalamic multiple unit activity (MUA). His group had further developed the methodology for high clarity recording in both anaesthetized and conscious rhesus monkeys with multiple recording electrodes chronically placed in the MBH (Wilson *et al.*, 1984).

Successful recording of this specific MUA was then reported in unrestrained ovariectomized rats by Kimura *et al.* (1991) and Nishihara *et al.* (1991). Although similar attempts were made in ruminants, such as sheep (Rasmussen *et al.*, 1981; Thiery and Pelletier, 1981), conclusive results were not obtained until recently when we adopted the MUA recording technique to the miniature 'Shiba' goat (Mori *et al.*, 1991). The Shiba goat is a Japanese indigenous goat, of which a closed colony has been maintained for research purposes, particularly as an experimental model for domestic ruminants in some institutes including the University of Tokyo (Kano *et al.*, 1977). Some aspects of their reproductive characteristics have been described by Mori and Kano (1984) and Mori *et al.* (1987), and a stereotaxic procedure for accurate placement of electrodes or other devices in the brain has been developed (Mori *et al.*, 1990) together with construction of the brain atlas with stereotaxic coordinates (G. O. Zuccolilli, S. Hayashi and Y. Mori, unpublished). The data presented in this paper were all obtained from goats. We first outline the MUA recording procedure, and then discuss the possible application of this technique to the study of reproductive neuroendocrinology in domestic ruminants with special reference to practical advantages.

Long-term Recording of the Electrophysiological Manifestation of the GnRH Pulse Generator Activity

Recording of multiple unit activity

The methods for stereotaxic implantation of recording electrodes into the mediobasal hypothalamus (MBH) and for recording the electrical activity of GnRH pulse generator have been described in detail by Nishihara *et al.* (1994). In brief, we use a multiple electrode array consisting of 4–6 Teflon-insulated platinum–iridium wires (75 μm in diameter). They are encased together in a stainless steel tube which is used as the outer guide tube for chronic implantation in the brain. These electrodes are implanted stereotaxically with radioventriculographic monitoring (Mori *et al.*, 1990), and the tip of the electrode is targeted at the MBH including the arcuate–median eminence region. After 1–2 weeks of recovery, the goat is transferred to the recording room and tied loosely to the stanchion where it can feed and rest during the recording period (Fig. 1). At the time of recording, a buffer amplifier integrated circuit (IC), which forms a high input–impedance and low output–impedance voltage follower circuit, is directly plugged into the electrode assembly to reduce the noise level along the signal path and to reject the artefact of the common input such as electromyogram of the moving animal. Differential input signals between two electrodes are further amplified by means of a high gain amplifier with low and high cut-off frequencies of 500 Hz and 3 kHz, respectively. MUA signals are then fed into the amplitude discriminator and processed as the activity rate in a personal computer and simultaneously stored on a digital audio tape for further off-line analysis of MUA.

Correlation between MUA volleys and LH pulses

Specific increases in MUA (the MUA volley) were first recorded in the ovariectomized goat as shown in Fig. 1. These MUA volleys are characterized by the initial brief overshoot reaching the peak within 30 s followed by a gradual decline to baseline. The duration of the MUA volley is 3–4 min and the interval between them in ovariectomized goats is 30–40 min, and both of these are stable. The firing pattern with relatively short duration resembles that recorded in rats (Kimura *et al.*, 1991), rather than that in monkeys (Wilson *et al.*, 1984). The success rate of recording, in terms of proportion of animals from which specific MUA volleys are obtained, has been greater than 80% in ovariectomized goats.

Correlation between the MUA volleys and pulsatile LH secretion is shown in Fig. 2. Each LH pulse was invariably preceded by a MUA volley, and this temporal association between the two was consistent, even when the pulse frequency was changed by steroid treatments (Mori *et al.*, 1991; Tanaka *et al.*, 1994). Using the microdialysis technique, we determined that LH pulses in the systemic circulation

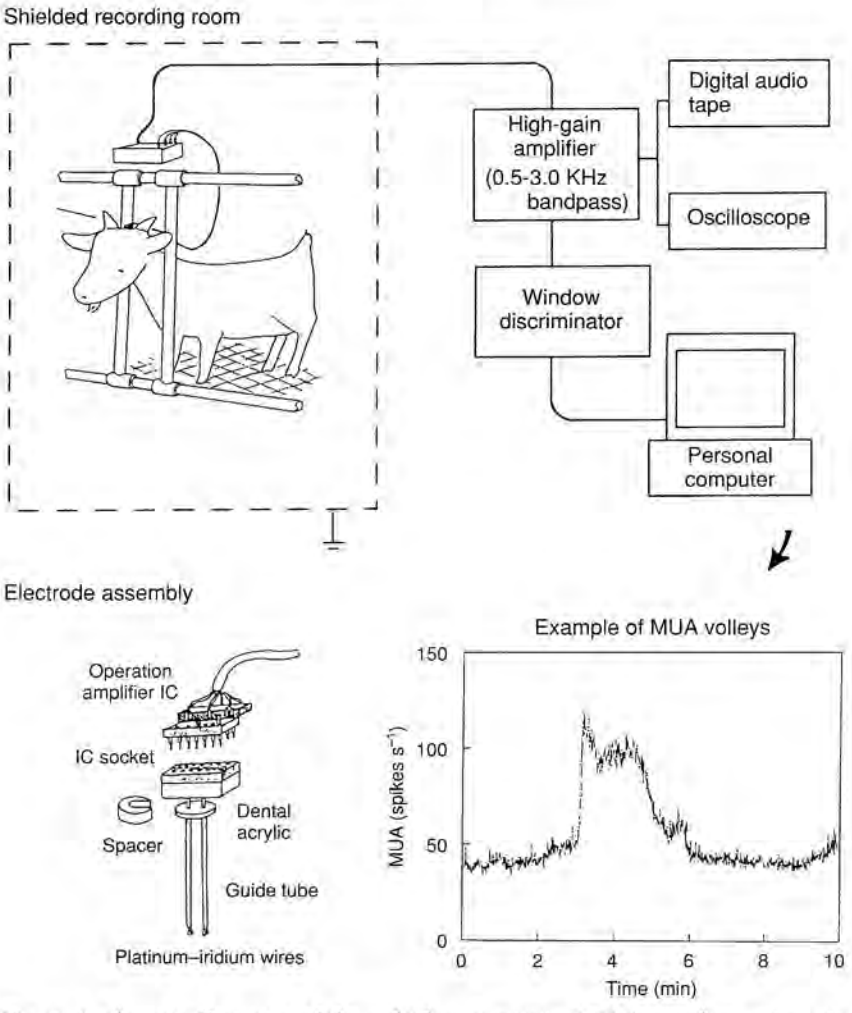

Fig. 1. A schematic illustration of the multiple unit activity (MUA) recording system. At recording, a buffer amplifier is plugged directly onto the electrode assembly permanently implanted in the medial basal hypothalamus. MUA is amplified with a high-gain amplifier, discriminated with amplitude discriminator and thus selected signals are stored in a personal computer as an activity rate. Representative MUA volley expressed in terms of spikes per second is shown in the insert (redrawn with modification from Nishihara *et al.*, 1994).

of ovariectomized goats were preceded with similar intervals by episodic increases of GnRH neurosecretion at the median eminence (Yamaguchi *et al.*, 1992), and the interval was much the same as between the MUA volley and the LH pulse. Moreover, Moenter *et al.* (1992b) 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 value within 2–3 min. This release pattern of GnRH in sheep resembles the contour of the MUA volley in goats. Considering all of the above factors, we believe that the MUA volley associated with the LH pulse is the specific electrical signal reflecting the hypothalamic GnRH pulse generator activity governing the release of GnRH.

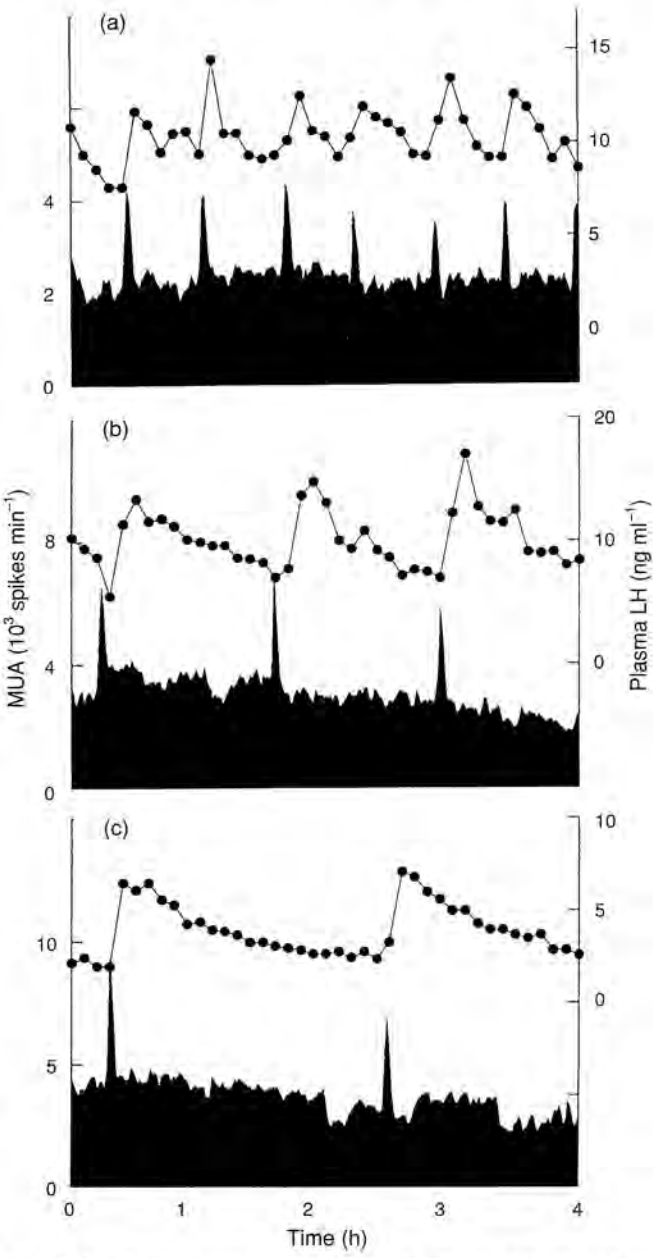

Fig. 2. Synchrony between multiple unit activity (MUA) volleys expressed in terms of spikes per minute and LH pulses in (a) an ovariectomized goat. This goat was then implanted subcutaneously with capsules containing progesterone and oestradiol for (b) 2 days and (c) 4 days to mimic the steroidal milieu in the luteal phase. The administration of steroids slowed down the pulse frequency, but the temporal relationship between the MUA volleys and LH pulses was unchanged (Modified from Mori *et al.*, 1991).

Recording site of MUA volley

At present the origin of signals underlying the operation of the pulse generator is unclear, and it is yet to be determined whether the electrical activity is recorded from GnRH neurones themselves or from other neuronal elements that stimulate neurosecretion of GnRH periodically. The vast majority of perikarya that contain GnRH are immunohistochemically identified in the medial preoptic area in goats (Hamada *et al.*, 1992; Zuccolilli *et al.*, 1994). The distribution pattern of GnRH neurones is similar to that reported in sheep (Lehman *et al.*, 1986; Wood *et al.*, 1992) and rats (Silverman *et al.*, 1994), but different from that of rhesus monkeys in which the major hypothalamic GnRH cell group resides in the periventricular, as well as in the tuberal, regions (Silverman *et al.*, 1982). Despite the apparent interspecies differences in distribution of cell bodies of GnRH neurones among monkeys, rats and goats, the MUA volley associated with the LH pulse has been recorded consistently from the same region of the hypothalamus, i.e. MBH. Recently, we searched for the site of electrodes recording specific MUA activity and found that the tips of the successful recording electrodes were always embedded in the mass of GnRH immunoreactive fibres in the median eminence (Fig. 3). In contrast, no GnRH immunoreactive fibres were found in the vicinity of the tips of inactive electrodes (T. Tanaka, H. Okamura and Y. Mori, unpublished). Similarly, in rhesus monkeys the recording sites of MUA volleys were localized in moderate to heavy GnRH immunoreactive fibre bundles in most instances (Silverman *et al.*, 1986). Thus, the MUA volley may be recorded from the axonal tract or the terminals of GnRH neurones as a product of the coordinated firing of GnRH neurones. However, because the diameter of the electrode tip is too large to pinpoint the location at the cellular level, and because there are numerous neuronal inputs such as catecholamines, opioid peptides and excitatory amino acids in the MBH, it is still premature to conclude that the MUA volley is recorded specifically from the GnRH neurones, and the possibility that the MUA volley reflects the activity of other neural elements regulating the neurosecretion of GnRH from nerve terminals cannot be discounted.

Application of the MUA Recording Technique to the Study of the Reproductive Neuroendocrine System in Ruminants

The MUA recording technique has certain advantages for the study of the central regulatory mechanism of reproductive function. For instance, a real-time analysis of the GnRH pulse generator activity is possible under conscious and unrestricted conditions for a considerably long period, up to several months if necessary. Moreover, the effect of various internal or external factors such as hormones, nutrition, suckling stimuli, stressors, environmental cues and pheromones could theoretically be assessed at the hypothalamus and pituitary simultaneously. The following are examples of the application of this technique.

Assessment of chronic changes in GnRH pulse generator activity throughout the ovulatory cycle: advantage of long-term continuous monitoring

The Shiba goat is a non-seasonal breeder, and the female is cyclic throughout the year under natural daylight conditions in Japan (Mori and Kano, 1984; Mori *et al.*, 1987; Maeda *et al.*, 1988). The duration of the ovulatory cycle is 3 weeks, and hormonal profiles during the cycle have been described by Mori and Kano (1984) and Mori (1992).

One of the important advantages of this technique is that it makes possible the long-term continuous recording of the GnRH pulse generator activity with minimum interference to the physiological condition of animals. Changes in the frequency of MUA volleys throughout the ovulatory cycle in a female goat are shown in Fig. 4 (T. Tanaka, T. Ozawa, K. Hoshino and Y. Mori, unpublished observation). There is a reciprocal relationship between the MUA volley frequency and plasma progesterone profiles. The data suggest a suppressive effect of progesterone on the GnRH pulse generator activity. However, when we examined effects of progesterone in the presence and absence of oestradiol in ovariectomized goats, progesterone treatment in combination with oestradiol could suppress the MUA volley frequency to the extent of that found in the luteal phase (Tanaka *et al.*, 1994);

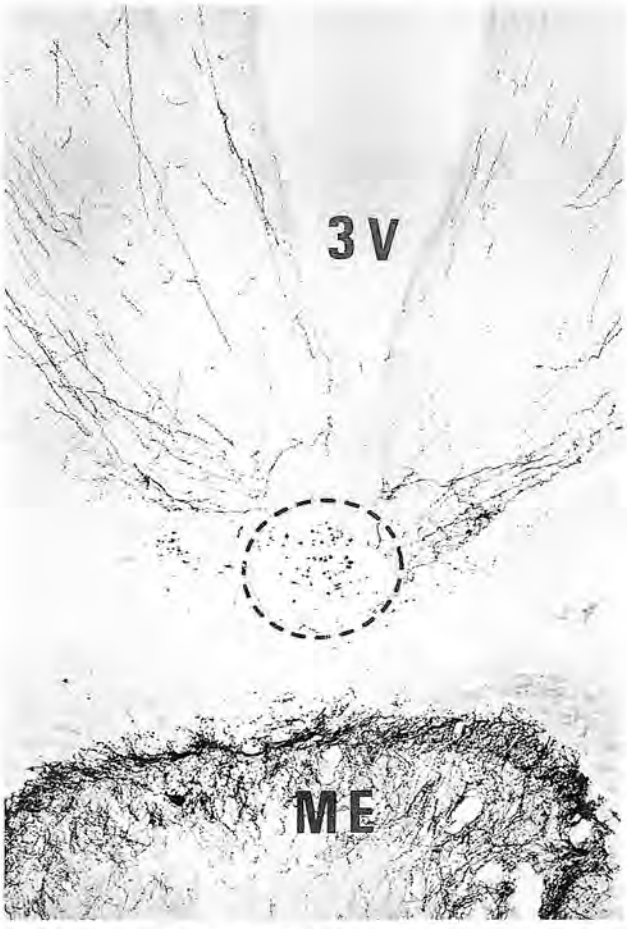

Fig. 3. The recording site of the multiple unit activity (MUA) volley in relation to the GnRH immunoreactive fibres in an ovariectomized goat. The electrical lesion made at the tip of the active electrode (broken circle) was located in the vicinity of GnRH immunoreactive fibres (dark staining) in the median eminence (ME) underneath the third ventricle (3V) (T. Tanaka, H. Okamura and Y. Mori, unpublished).

progesterone alone was ineffective in this regard. It appears, therefore, that progesterone is playing a dominant role, while oestradiol plays a rather permissive role in exerting negative feedback effects on the GnRH pulse generator activity during the luteal phase.

Figure 5 shows marked changes in the hypothalamic MUA patterns at three stages of the oestrous cycle in one female goat (T. Tanaka, T. Ozawa, K. Hoshino and Y. Mori, unpublished observation). At the luteolysis that occurred spontaneously, the volley frequency increased abruptly (Fig. 5a), and it rapidly attained the high level that was maintained during the follicular phase (Fig. 5b). This high frequency of the MUA volley declined slightly around the time of the preovulatory LH surge (this phenomenon will be discussed in detail in the next section). During the luteal phase, the volley frequency was low, and basal levels of MUA between volleys tended to fluctuate more than those in the follicular phase (Fig. 5c). It was noted that the volley frequency became extremely high during the transition from the luteal to the follicular phase. This may be responsible for a transient rise of circulating LH concentrations following luteolysis (Mori and Kano, 1984).

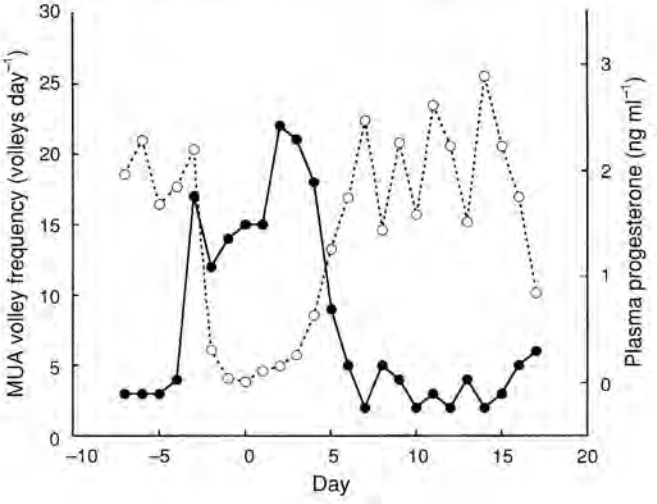

Fig. 4. The relationship between the multiple unit activity (MUA) volley frequency (●) and plasma progesterone concentration (○) during the oestrous cycle of a female goat. The volley frequency showed a reciprocal relationship to the plasma progesterone profile. Day 0 indicates the day of minimum progesterone concentration (T. Tanaka, T. Ozawa, K. Hoshino and Y. Mori, unpublished).

Analysis of GnRH pulse generator activity during the LH surge: advantage of discriminating hypothalamic and pituitary responses

In contrast to the negative feedback action, a preovulatory rise of oestradiol induces a large amount of LH secretion (LH surge). The effect of administration of a high dose of oestradiol on GnRH secretion was first evaluated in ovariectomized ewes by Clarke and Cummins (1985) and Schillo *et al.* (1985). Although in these earlier studies the pattern of GnRH secretion during the LH surge was not consistent among individual animals, recent studies have clearly determined the surge release of GnRH into the pituitary portal circulation coincident with the LH surge (Clarke, 1987; Caraty *et al.*, 1989; Moenter *et al.*, 1990; Karsch *et al.*, 1992). As shown in Fig. 6, a large increase in GnRH concentration was observed in microdialysis perfusates of the median eminence during the LH surge in ovariectomized goats infused with oestradiol (Manabe *et al.*, 1993).

However, information about the activity of the GnRH pulse generator during the GnRH and LH surges is not available from patterns of hormones owing mainly to technical limitations of detecting unambiguous pulses from the ever-changing baseline values of GnRH or LH during the period of the surge. The question arises as to whether the acceleration of GnRH pulse generator activity is necessary for induction of the preovulatory GnRH surge. To answer this, we recorded MUA during an oestradiol infusion that mimicked the preovulatory rise of oestradiol and which induced an LH surge in ovariectomized goats (Tanaka *et al.*, 1992). Representative patterns of the hypothalamic MUA and plasma LH are illustrated (Fig. 7a). MUA volleys were observed throughout the experiment including the time of the LH surge. The timing of MUA volleys during the LH surge is also shown for individuals (Fig. 7b). There was a slight decrease in frequency of the MUA volley during the pre-surge period, and this became more pronounced after the onset of the LH surge.

The finding that the GnRH pulse generator activity continued, but with decreased frequency, during the oestradiol-induced LH surge in ovariectomized goats, as monitored by MUA, has raised several questions. First, is the GnRH pulse generator directly involved in the induction of the GnRH surge? Moenter *et al.* (1992a) found that the pattern of GnRH release into the portal circulation is continuous, but not exclusively pulsatile during the surge. There is a rapid turnover of GnRH in the portal blood circulation (Caraty *et al.*, 1989; Moenter *et al.*, 1990), so the surge release of GnRH cannot be maintained

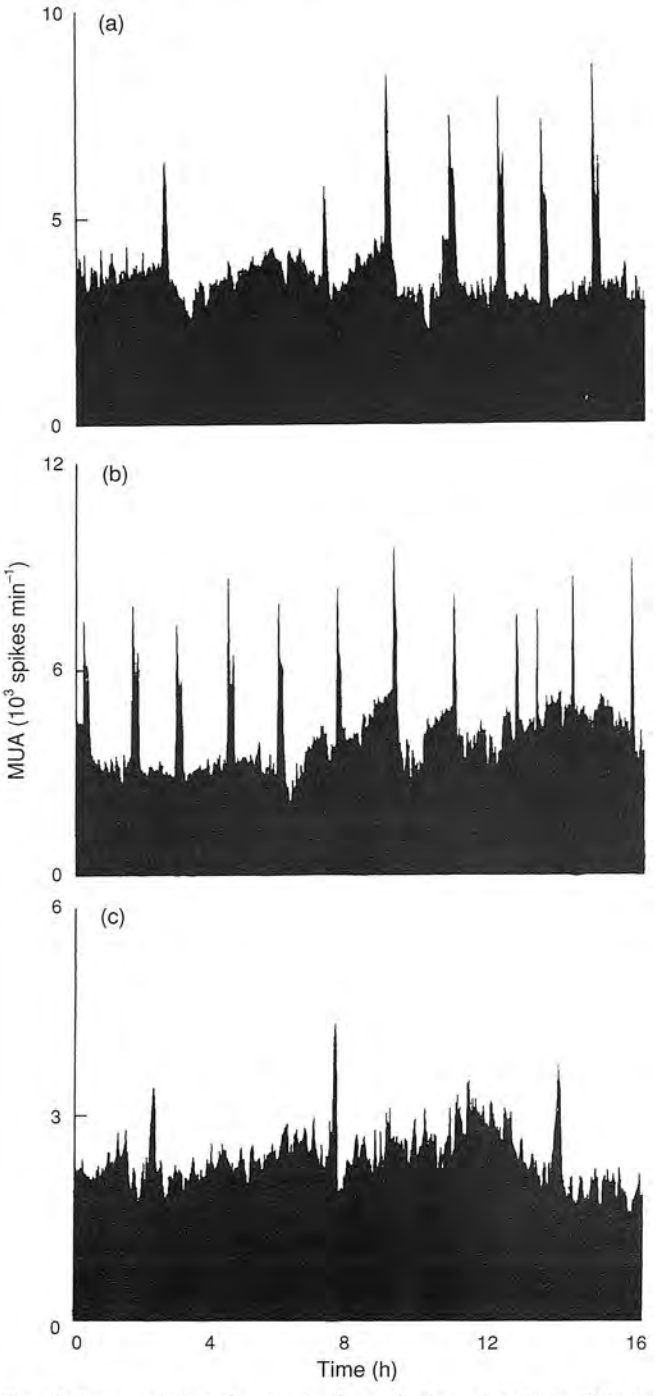

Fig. 5. Representative changes in the multiple unit activity (MUA) volleys during (a) luteolysis, (b) follicular phase and (c) luteal phase in a cyclic female goat. The volley frequency was high in the follicular phase but low in the luteal phase, and an abrupt increase was seen during luteolysis (T. Tanaka, T. Ozawa, K. Hoshino and Y. Mori, unpublished).

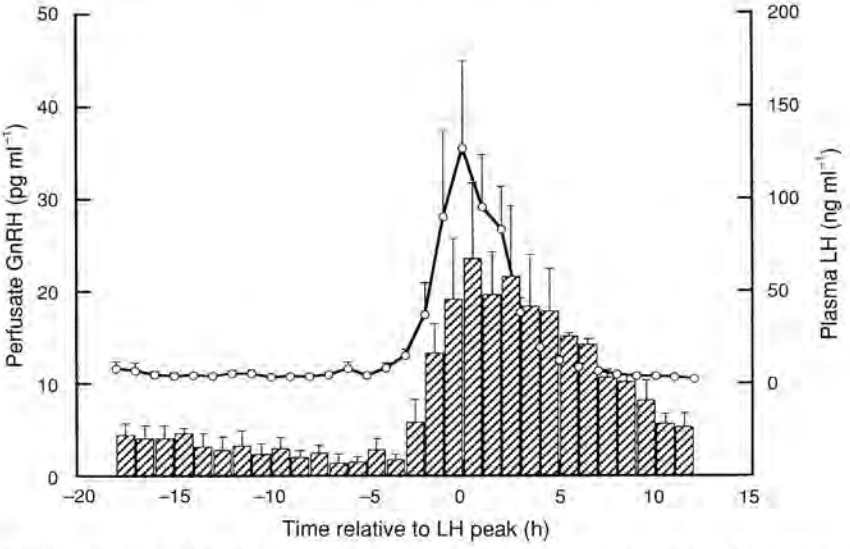

Fig. 6. Increase in GnRH concentration in the microdialysis perfusate at the median eminence (▨) synchronized with the LH surge (○) in ovariectomized goats given oestradiol. Each point represents mean ± SEM for three animals, and the data are normalized to the peak of LH surge. (Reproduced, with permission, from Manabe *et al.*, 1993.)

by pulses of GnRH at intervals of about 1 h. It therefore appears more reasonable to consider that the GnRH surge is induced by the neuronal mechanism that is intrinsically different from the GnRH pulse generator (this will be discussed later).

Second, why is the frequency of GnRH pulse generator activity suppressed during the period of the surge? In ovariectomized goats infused with oestradiol, reduction in the MUA volley frequency was associated with an increase in oestradiol in the circulation (Tanaka *et al.*, 1994). The most obvious explanation is that a rise in oestradiol reduces GnRH pulse generator activity. An alternative explanation is that, as the decline of MUA volley frequency coincided with the onset of the GnRH and LH surge, either the short-loop (LH inhibition of GnRH) or ultrashort-loop (GnRH inhibition of GnRH) feedback system may be involved in the modulation of the pulse generator activity. This is supported by the finding that in ovariectomized ewes an injection of GnRH into the third ventricle results in a significant reduction in the frequency of pulsatile LH secretion (Naylor *et al.*, 1989). However, the results of other studies do not support this hypothesis. In rhesus monkeys, it was shown by using the MUA recording technique that the LH secretion induced by the GnRH analogue had no effect on the GnRH pulse generator activity (Kesner *et al.*, 1986). Similarly, we could not find any change in the frequency of MUA volleys after either peripheral or intracerebroventricular administration of GnRH in ovariectomized goats (A. Yamada, T. Tanaka, H. Kamomae and Y. Mori, unpublished).

Third, why is there no change in the baseline of the MUA during accelerated GnRH neurosecretion, i.e. the GnRH surge? This is intriguing in view of the fact that active electrodes for MUA are always found in the vicinity of condensed GnRH fibres in the median eminence region as mentioned above.

In considering the above findings which prompted our questions, we believe that the following must be considered: (1) the GnRH pulse generator consists of neuronal components other than GnRH neurones, and in our studies the MUA volley is being recorded from neurones that do not contain GnRH through an electrode located in the GnRH concentrating region; (2) the surge mode of GnRH neurosecretion occurs at a part of the brain away from the region where the recording electrodes are located. According to this hypothesis, there are at least two subpopulations of GnRH neurones involved in the regulation of GnRH secretion during the ovulatory cycle. One subpopulation resides in the MBH

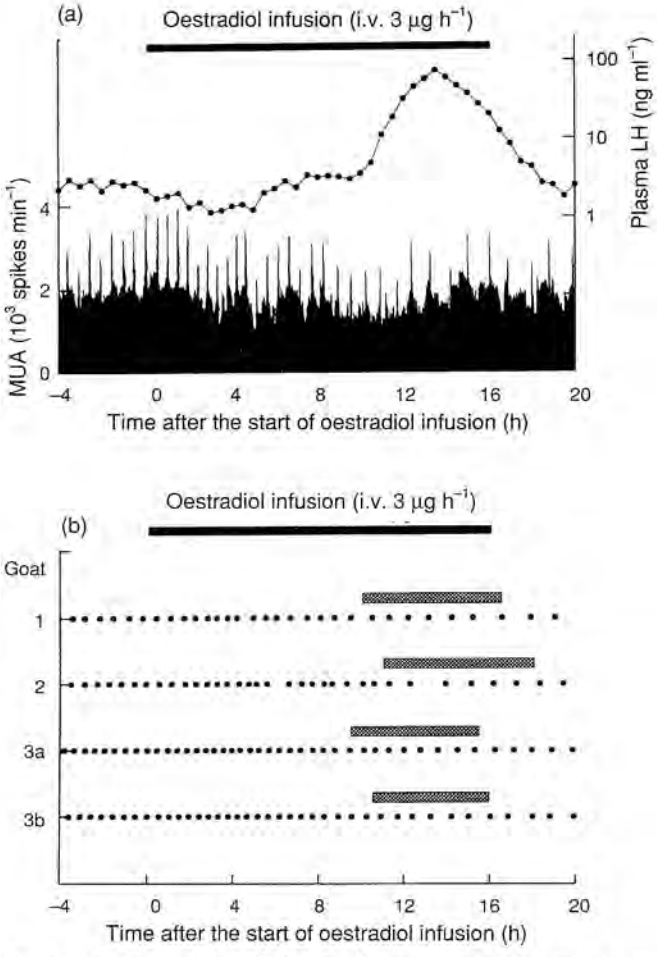

Fig. 7. (a) The pattern of the GnRH pulse generator activity as exemplified by multiple unit activity (MUA) volleys during the oestradiol-induced LH surge in an ovariectomized goat. MUA volleys were observed throughout the experiment. (b) Occurrence of MUA volleys (•) in three individual animals. In one goat (3), the same experimental procedure was repeated a month later to examine the reproducibility (3a, 3b). Decreased frequency of the pulse generator activity was observed after the onset of the LH surge (▩) (Modified from Tanaka *et al.*, 1992).

and is responsible for the pulsatile secretion which changes the frequency of LH release which drives follicular development; the other subpopulation resides presumably in the preoptic area (POA) and is responsible for the preovulatory GnRH surge.

Examining the effects of internal and external substances on the GnRH pulse generator activity: advantage of real-time analysis

Another important advantage of this technique of recording electrical activity from the hypothalamus is that bioactive substances, such as hormones, neurotransmitters and neurochemicals, can be administered at a fixed time relative to each MUA volley. Moreover, their effects on the GnRH pulse generator can be analysed on the real-time basis.

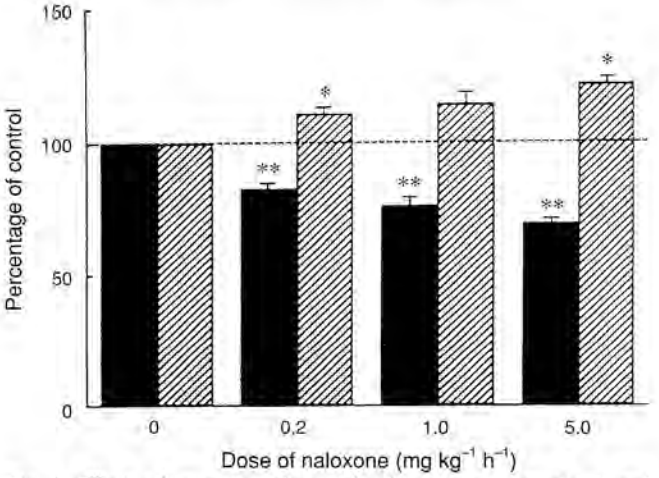

Fig. 8. Effects of increasing doses of naloxone on interval (■) and duration (▨) of multiple unit activity (MUA) volleys in ovariectomized goats. Values are means ± SEM for 3–4 animals. *, ** Significantly different from the pretreatment control period ($P < 0.05$; $P < 0.01$, respectively). (Reproduced, with permission, from Ito *et al.*, 1993.)

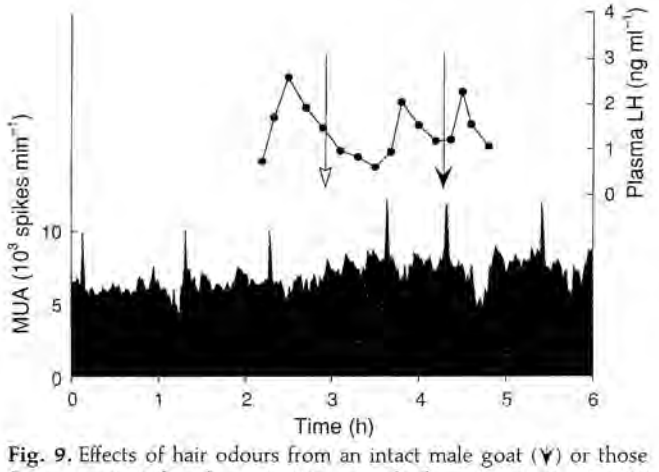

Fig. 9. Effects of hair odours from an intact male goat (▼) or those from a castrated male goat (▽) on multiple unit activity (MUA) volleys and LH pulses in an ovariectomized female goat with an oestradiol implant (T. Hamada, M. Nakajima, Y. Takeuchi and Y. Mori, unpublished).

An example of this kind of approach is the acceleration of MUA volley frequency by timed administration of naloxone, an opioid receptor antagonist. In this study, conducted in ovariectomized goats, naloxone was given 10 min after the previous MUA volley. Since the timing of naloxone administration was controlled accurately in relation to the GnRH pulse generator activity by monitoring the recurrence of MUA volleys, it was shown that the interval to the first volley was more dramatically decreased than any later intervals during the naloxone administration (Ito *et al.*, 1993). Dose-dependent effects of naloxone on the duration of, and on the interval between, MUA volleys in ovariectomized goats are shown in Fig. 8.

Another example of timed administration of bioactive materials is presented in Fig. 9 (T. Hamada, M. Nakajima, Y. Takeuchi and Y. Mori, unpublished). In this case, hair odours from intact or castrated male goats were presented 40 min after an MUA volley to an ovariectomized goat carrying an oestradiol implant and kept under 16 h light:8 h dark to increase inter-volley intervals to 60 min or longer. The exposure to hair odour of male goats, but not to that of castrated goats, induced an MUA volley within a few minutes of application. We are currently using this experimental model to develop a novel bioassay system for isolation of androgen-dependent pheromones produced in male goats, which are considered to be responsible for the 'male effect' in this species (Claus *et al.*, 1990).

Conclusion

We have described the technical procedure and the application of an electrophysiological approach to the hypothalamic GnRH pulse generator in goats. Ovariectomized goats were used in this study because the screening of the active electrode was easier owing to high frequency of MUA volleys. We then extended the application to cyclic female goats. Specific MUA volleys have also been recorded in castrated male goats (Kanai *et al.*, in press). For the next phase of our research, we are developing a telemetry system to monitor GnRH pulse generator activity in freely behaving animals. Although the MUA recording technique has certain advantages as described herein, there are limitations with this approach, because we do not yet know the identity of the neurones from which specific MUA is recorded. Extended neurophysiological, as well as neuroanatomical, approaches are required both for elucidating the brain clock generating hourly pulses, and for facilitating potential application of this technique to the study of reproduction in ruminants.

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