Stimulation of the GnRH Pulse Generator Activity by Continuous Exposure to the Male Pheromones in the Female Goat

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Abstract. Male pheromones in ruminant species such as sheep and goats are known to reactivate quiescent ovarian function in seasonally anestrus females. We have previously shown that only a few minutes exposure to the male pheromone can accelerate the GnRH pulse generator activity immediately in the female goat. In this study the effect of 4 h continuous exposure to the male pheromone on the GnRH pulse generator activity was examined by recording the specific multiple unit activity (MUA) from the medial basal hypothalamus. Pooled buck hair was used as the source of male pheromone. Six ovariectomized estradiol-primed does were fitted with a nose-mask for 8 h, and during the latter 4 h period 2.0 g of pooled male hair was set in the chamber at the rostral end of the mask. As compared with the period when only an empty mask was put on, the exposure to the male hair resulted in a shortening ($p < 0.05$) of the interval between the MUA volleys, namely the electrophysiological manifestation of the pulsatile GnRH neurosecretion. This stimulatory effect was observed throughout the time when animals were exposed to the male pheromone. This result demonstrates that the hypothalamic GnRH pulse generator of the recipient female is accelerated as long as the exposure to the male pheromone continues.

Key words: Pheromone, Hypothalamus, GnRH pulse generator, Goat.

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Pheromones are chemical signals used to communicate among conspecifics. Primer pheromones are designated to influence recipients' neuroendocrine system, and they subsequently modify various aspects of reproductive function as well as of behavior. One well-known example of such pheromone-induced gonadal stimulations is the "male effect" [1–3] in which seasonally anovulatory female sheep and goats resume their ovarian cyclicity after being exposed to the male or pheromones that are produced androgen dependently in sexually active males [4, 5].

The male effect is mediated by an acceleration of the hypothalamic gonadotropin releasing hor-

mone (GnRH) pulse generator activity, which then results in increased gonadotropin secretion and consequent follicular development in the quiescent ovary of anestrus females. Previously we investigated the effect of a single application to the male pheromone by means of recording the multiple unit activity (MUA) volleys (characteristic increases of the multiple-unit activity) from the medial basal hypothalamus of conscious ovariectomized goats [4]. We showed that the application of the male pheromone, which had been accurately timed to the midway between GnRH pulses, actually shortened the inter-pulse interval, i.e., the MUA volley appeared within a few minutes after the pheromone application, which was much earlier than expected.

Using this electrophysiological technique, we could establish a real-time bioassay system for the

primer pheromone activity with sufficient sensitivity, specificity and reproducibility in the goat [4]. Because the purpose of that study was to examine whether the sample under investigation had pheromonal activity or not, testing materials were simply presented in front of the goat's nostril for a relatively short period (< 5 min). Therefore it is not yet known how the GnRH pulse generator would respond if the female is exposed to the male pheromone for a longer period, which appears a more likely situation occurring in the natural mating scenes.

In the present study, we therefore extended the duration of pheromone application to 4 h to test the hypothesis that the increased activity of hypothalamic GnRH pulse generator would be maintained in the female goat when it was subjected to continuous stimulation of the primer pheromone.

Materials and Methods

Animals and multiple unit activity recording

Six female Shiba goats were obtained from the closed colony at the experimental station of the University of Tokyo [6]. They were ovariectomized and then implanted with an array of bilateral recording electrodes in the medial basal hypothalamus according to the stereotaxic procedure developed for this goat [7–9]. The method for recording the specific MUA which reflects the function of the hypothalamic GnRH pulse generator has been described in detail in our previous reports [10, 11]. The MUA showing abrupt increases, which had regular intervals and were exclusively associated with luteinizing hormone (LH) pulses in the peripheral circulation, was considered to be the electrophysiological manifestation of the hypothalamic GnRH pulse generator [11]. During the recording period of MUA, the animals were loosely restrained to the stanchion in the Faraday cage, where they were fed twice a day and provided water *ad libitum* except for the period when the nose-mask was fitted. The lighting was controlled to be 12L12D or 16L8D (for the long-day treatment), and the temperature and relative humidity were kept at 23 (± 2) °C and 40 (± 5)%, respectively, throughout the experiment.

Preparation of the pheromonal substance

As the source of male pheromone(s), hair sample was collected from the head, cervix and shoulder regions, where the male specific odor was most prominent, of one sexually mature male Shiba goat. The collected hair sample was pooled and stored at -20 °C. For each continuous 4 h application 2.0 g of hair was used.

The procedure for continuous application was based on the method adopted in previous studies [3, 12–14], namely the goat was fitted with a nose-mask which was equipped with a chamber to set test substances. For habituation the females were put on empty masks for 4–8 h for several successive days before they were subjected to the pheromone application.

Experimental design

In order to mimic the internal and/or external environments in the anestrus season, the ovariectomized goats were subcutaneously implanted with Silastic capsules (i.d. 3 mm, o.d. 5 mm, length 20 mm; Dow Corning, Midland MI., USA) containing crystalline estradiol (Sigma Chemical Co., St Louis MO., USA), and the light condition was changed from 12L12D to 16L8D [15, 16]. The GnRH pulse generator activity was sufficiently suppressed by these treatments, i.e., the interval between MUA volleys was prolonged (over 150% of the initial baseline levels) and reached a plateau within 3 or 4 days. After that the application of pheromone was conducted.

On Day 0, an empty mask was fitted to the nose of the animal and left for 8 h from 0900 to 1700. On the next day (Day 1), the animal was again fitted with the empty mask for the first 4 h from 0900 to 1300, and then the mask was replaced with the one containing 2.0 g male goat hair at 1300, and left for another 4 h until 1700. To minimize the residual pheromone effects, the air around the upper half of the body was ventilated individually whenever the mask was put on. On Days 2 and 3 the animals were not exposed to the pheromone and the change of MUA volley interval was observed.

The specific MUA was recorded throughout the experiment, and in one representative goat blood samples (1 ml) were collected every 5–10 min via a jugular catheter from 1100 to 1730 on Days 0 and 1 to confirm the synchrony between the MUA vol-

leys and plasma LH pulses, which had been demonstrated in our previous studies [4, 10, 18].

Radioimmunoassay

Plasma concentrations of LH were measured by the double-antibody radioimmunoassay with anti-ovine LH rabbit serum (YM#18) and ^{125}I -ovine LH (NIDDK-oLH-I-3) as described elsewhere [17].

The minimal detectable concentration was 0.38 ng/ml and the intra-assay coefficient of variation was 6.8%.

Statistical analysis

The mean interval between MUA volleys during the exposure to the pheromone (or control period) was calculated for every 2 h and normalized to the value at the pre-exposure 2 h period in each of the six individual goats. These standardized data of individual goats were then averaged, and expressed as group means ($\pm$ SEM). The effect of pheromone exposure on the interval was assessed by detecting significant decrease from the baseline level with the paired *t* test.

Results

A representative profiles of the MUA volleys and LH pulses at Days 0 and 1 are shown in Fig. 1. The activity of the GnRH pulse generator was accelerated by the male pheromone, and the synchrony between MUA volleys and LH pulses was maintained even during the continuous 4 h exposure to the male goat hair.

Figure 2 shows successive changes of mean MUA volley intervals of each 2 h period, which were normalized to the baseline level at 0500–0700 period. When animals were fitted with the empty mask, no significant change was observed in the MUA volley interval. On the other hand, the mean interval between MUA volleys was significantly shortened by exposing the female to the male goat hair, and then it returned to the baseline level within a few hours after the cessation of pheromone exposure.

As shown in Fig. 3, the male pheromone significantly shortened the MUA volley interval throughout the exposure period of Day 1: the mean interval during pheromone exposure (1300–1700) declined to $90.2 \pm 3.7\%$ of the Day 0 value. No remaining effect was observed on subsequent days.

Fig. 1. Synchrony between MUA volleys recorded from the medial basal hypothalamus and LH pulses in the systemic circulation at Day 0 (a) and Day 1 (b) in a representative ovariectomized estradiol-primed goat. The hatched and open bars are the periods during which the goat was put on the nose-mask with and without male goat hair (as the source of the male pheromone), respectively. The MUA volleys were always followed by LH pulses even when the pulse frequency was altered as a result of the exposure to the pheromone.

Discussion

In this study, the effect of continuous 4 h exposure to the pheromone on the activity of the hypothalamic GnRH pulse generator, the center of the reproductive neuroendocrine system, was investigated by monitoring the change of MUA volley intervals. Following the habituation process, the goats showed no signs of distress when fitted with the nose-mask for the continuous pheromone application. The synchrony between MUA volleys and LH pulses was maintained during the experimental period as seen in our previous studies [10, 18–21], even when the pulse frequency was increased by the exposure to the pheromones. No change was observed either in the amplitude or in the basal levels of LH pulses. These findings support the view that, at least as far as the “male effect” is concerned, the pheromone accelerates the hypothalamic GnRH pulse generator activity and then

Fig. 2. Sequential changes of MUA volley intervals at Day 0 (a) and Day 1 (b). Mean ($\pm$ SEM, $n=6$) intervals of MUA volleys for 2 h were normalized to the baseline value at 0500–0700 of each day, and then the data were averaged for the 6 goats. The asterisks show the differences ($p<0.05$, paired t test) as compared with the baseline value. The open bar indicates the period when the goat was fitted with the nose-mask (0900–1700) and the hatched bar the period when the male goat hair was contained in the mask (1300–1700).

indirectly stimulates the gonadotropin secretion from the pituitary gland [3, 4].

During the continuous exposure to the male pheromone, the interval between MUA volleys decreased significantly, indicating the pheromone-induced acceleration of the hypothalamic GnRH pulse generator activity. Phenomena that the male pheromone stimulates pulsatile LH secretion were reported in previous studies [3, 4]. However, it had not been clarified in those reports whether the persistent pheromonal stimulation was necessary to maintain the high frequency of LH pulses. An alternative explanation for this might be that a short term pheromonal stimulation triggered the consequential change of the reproductive neuroendocrine system, and thus increased frequency of LH pulses continue. The latter seems unlikely, however, based on the present observation. In this study the MUA volley interval, which was shortened throughout the pheromone exposure, returned to the former prolonged level soon after the cessation of application. Pheromones are presumed to be accepted by receptors in the vomeronasal organ in mammals [22]. It therefore seems that the central action of the pheromone on the GnRH pulse gen-

Fig. 3. Mean intervals of MUA volleys observed during the period corresponding to the continuous 4 h application of male goat hair (1300–1700) at each day between Day 0 and Day 3. Data of each animal were normalized to the Day 0 value (as 100%), and then averaged for the 6 animals. The asterisk shows the difference ($p<0.05$, paired t test) as compared with the value of Day 0. The hatched column indicates the day of the continuous 4 h pheromone application.

erator is exerted only during the period while the pheromone stimulates its receptor systems.

Our previous study with the single application [4] has shown that the pheromonal stimuli instantly induce the MUA volley. As a further extent, the present results have shown that the continuous pheromonal stimulation accelerates the oscillator activity of the hypothalamic GnRH pulse generator as long as the stimuli exists. However, the extent of shortening of the MUA volley interval during the continuous application was not comparable to those seen with the single application. Although the reason is unclear, one possible explanation is that only volatile molecules could pass through the mesh of the mask and therefore the stimulation might have been insufficient as compared with the single application where the goat was allowed to not only smell but also taste the male goat hair. Mammalian pheromones were suggested to exhibit their effects more strongly if plural molecules could act cooperatively rather than as a single molecule [23–26]. Pheromones which induce the male effect in sheep, for example, are speculated to be a combination of two or more substances [27]. Although the chemical components of the male goat pheromone are yet to be identified, it appears likely that multiple molecules

are involved in the biological action of the male pheromone on the GnRH pulse generator.

In conclusion, we have verified the hypothesis that the primer pheromone contained in the male goat hair can stimulate the hypothalamic GnRH pulse generator in the recipient female for relatively long period of time such as 4 h used in this study.

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