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Characterization of the Primer Pheromone Molecules Responsible for the 'Male Effect' in Ruminant Species

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Introduction

Pheromones are chemical signals used among consepcifics for social interactions such as sex attraction, mate selection, modulation of neuroendocrine function, and individual identification (Vandenbergh, 1994; Singh, 2001; Halpern and Martínez-Macros, 2003). The male effect is a representative example of such pheromonal actions in goats and sheep, in which exposure to the male pheromone accelerates the pulsatile gonadotropin-releasing hormone (GnRH)/luteinizing hormone release and thereby the ovarian activity in the female (Schinkel, 1954; Martin *et al.*, 1986).

Several attempts have been made to isolate pheromone molecule(s) responsible for the male effect using the induction of ovulation (Knight and Lynch, 1980) or the stimulation of LH release (Claus *et al.*, 1990; Cohen-Tannoudji *et al.*, 1994) as indices for the pheromone activity. These studies found that the male hair but not urine contained the pheromone activity. Therefore, chemical constituents in the male hairs were extracted and processed for further fractionation and analysis. Although the pheromone activity was observed in the fractions of initial steps, it eventually disappeared as the fractionation proceeded. Thus, the chemical identity of the primer pheromone has not yet been determined so far.

A major problem of those studies appears to reside in the bioassay systems they employed, which required several time consuming steps to determine whether the sample in question contained the pheromone or not. To overcome this matter, we have established a bioassay system to assess the pheromone activity in a real-time manner with high sensitivity. Utilizing this system, we have been conducting a series of experiments aiming at elucidation of production mechanism and the isolation of chemical molecule (s) of pheromone responsible for the male effect in the Shiba goat, a Japanese indigenous breed.

GnRH pulse generator and monitoring method of its activity

The GnRH pulse generator is the neural substrate that governs intermittent GnRH discharges into the pituitary portal circulation and thereby modulates the pulsatile LH release from the pituitary (Knobil, 1980).

To monitor the neural activity of GnRH pulse generator, female goats were stereotaxically implanted with an array of recording electrode in the median eminence/arcuate region under halothane anesthesia. After recovery, neural signals were recorded as the multiple-unit activity (MUA) in conscious goats, and analyzed on a personal computer in a real-time manner. It was shown under a variety of experimental conditions that the temporal relation between the periodic increases in the MUA (MUA volleys) and LH pulses in the circulation were exclusively maintained. Thus, that the MUA observed in this method actually reflects the neural activity of GnRH pulse generator has been proved (Mori *et al.*, 1991; Mori and Tanaka, 1995).

Then, we examined the effects of the pheromone on the GnRH pulse generator. Exposure to male hair resulted in a rapid increase in the MUA (Hamada *et al.*, 1996). When male hair was continuously presented, the repeated occurrence of MUA with shorter inter-volley intervals than the control was observed (Ichimaru *et al.*, 1999). These results demonstrated for the first time a direct evidence for the intimate association of the primer pheromone action with the hypothalamic GnRH pulse generator activity. Furthermore, the validity of the MUA monitoring method as the bioassay system of the pheromone activity was also provided.

Production of the primer pheromone

Utilizing the bioassay system, we examined the pheromone activity in skin samples taken from various regions of male goat in the presence or absence of androgens to understand mechanism of the primer pheromone production.

The treatment with testosterone of castrated male goats for 4 weeks induced the pheromone activity in the skins of head, neck and shoulder regions but not in those of flank, back or rump region. The pheromone activity disappeared in 2 weeks after the removal of testosterone. These results indicated that the pheromone responsible for the male effect was produced testosterone-dependently in the skins of specific regions of the body (Iwata *et al.*, 2000). Immunohistochemical observation revealed that this regional difference of pheromone production correlated the expression pattern of 5α-reductase converting testosterone to dihydrotestosterone (DHT) (Wakabayashi *et al.*, 2000).

Therefore, we then examined effects of DHT on the pheromone production. Interestingly, the pheromone activity was induced in the skins not only of head but also rump regions after four weeks of the DHT treatment in the castrated male (Iwata *et al.*, 2001), indicating a prerequisite role of DHT in the primer pheromone production.

Taken together, it seems likely that testosterone is converted to DHT in the skins of 5α-reductase rich regions, such as the head and neck of male goats and that it is DHT that is actually responsible for the induction of pheromone production. Because the hairs obtained from those regions of male goat always possess relatively high pheromone activities, the pheromone produced in the skins might be released on the skin and accumulated on the hair surface.

Isolation of the pheromone molecule(s)

Based on the above results, we first collected 200 g of hairs from the head and neck regions of sexually matured male goats. Then, lipid components in the hair sample were extracted by the supercritical CO_2 fluid extraction (SFE) system. After 20 runs of the SFE, a total amount of 600 mg of lipid components were obtained. They were pooled, dissolved in diethylether, divided into small aliquots and stored at –80°C. The presence of pheromone activity in the extract was confirmed by the bioassay.

The SFE sample (0.25 mg) was loaded to the gas chromatography (GC) equipped with a fused silica capillary column and eluted in 60 min. The eluted sample was separated into several fractions according to the retention time, and they were collected through the preparative fraction collector (PFC), which was directly attached to the GC. A small portion of the fraction was also loaded to the mass spectrometer (MS) and chemical components were analyzed with the reference of the mass spectra database.

Each fraction was assessed for the pheromone activity by the bioassay. Once the pheromone activity was confirmed in a single fraction, the SFE sample was fractionated by the GC/PFC with the same manner as described above except that only the sample eluted around the retention time for the positive fraction was separated into smaller fractions in this step.

By repeating these processes, the GC/PFC fraction containing the pheromone activity has been downsized step by step to about one minute fraction, which still contains a few substances as revealed by the GC/MS analysis.

Conclusions

We established a method to electrophysiologically monitor the activity of GnRH pulse generator in conscious goats. Using this method as the bioassay, we have been attempting to identify the pheromone molecule responsible for the male effect. Through several analytical steps, the pheromone activity was separated into a small fraction comprised of a few compounds. Although further fractionation is needed, we hope that the identification of the pheromone molecule will be soon. Because the action of this primer pheromone is intimately associated with the hypothalamic GnRH pulse generator, the pheromone molecule will become a powerful tool to investigate the control mechanisms of the reproductive neuroendocrine system.

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