Hormones and Behavior 94 (2017) 53–60

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

Male mice ultrasonic vocalizations enhance female sexual approach and hypothalamic kisspeptin neuron activity

Akari Asaba [a,1], Takuya Osakada [b], Kazushige Touhara [b], Masahiro Kato [c], Kazutaka Mogi [a], Takefumi Kikusui [a,*]

[a] Companion Animal Research, School of Veterinary Medicine, Azabu University, Kanagawa, Japan
[b] Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, and JST ERATO Touhara Chemosensory Signal Project, The University of Tokyo, Tokyo 113-8657, Japan
[c] Kato Acoustics Consulting Office, Kanagawa, Japan

ARTICLE INFO

Article history:
Received 21 February 2017
Revised 30 May 2017
Accepted 19 June 2017
Available online 29 June 2017

Keywords:
Ultrasonic vocalization
Mice
Sexual behavior
Kisspeptin neurons
Ultrasound emitter

ABSTRACT

Vocal communication in animals is important for ensuring reproductive success. Male mice emit song-like "ultrasonic vocalizations (USVs)" when they encounter female mice, and females show approach to the USVs. However, it is unclear whether USVs of male mice trigger female behavioral and endocrine responses in reproduction. In this study, we first investigated the relationship between the number of deliveries in breeding pairs for 4 months and USVs syllables emitted from those paired males during 3 min of sexual encounter with unfamiliar female mice. There was a positive correlation between these two indices, which suggests that breeding pairs in which males could emit USVs more frequently had more offspring. Further, we examined the effect of USVs of male mice on female sexual behavior. Female mice showed more approach behavior towards vocalizing males than devocalized males. Finally, to determine whether USVs of male mice could activate the neural system governing reproductive function in female mice, the activation of kisspeptin neurons, key neurons to drive gonadotropin-releasing hormone neurons in the hypothalamus, was examined using dual-label immunocytochemistry with cAMP response element-binding protein phosphorylation (pCREB). In the arcuate nucleus (Arc), the number of kisspeptin neurons expressing pCREB significantly increased after exposure to USVs of male as compared with noise exposure group. In conclusion, our results suggest that USVs of male mice promote fertility in female mice by activating both their approaching behavior and central kisspeptin neurons.

1. Introduction

The reproduction process and selection of a suitable mating partner begins with male-female communication, such as vocalization. Many reports have shown that, in various animals, male vocalizations are attractive to female mice (Asaba et al., 2014a). Further, it has been suggested that the male vocalizations could also promote female fertility. For example, hearing the conspecific male's vocalizations increase plasma estrogen levels in female túngara frogs (Lynch et al., 2006). In songbirds, the male's vocalizations presented *via* audio playback are sufficient to enhance female luteinizing hormone (LH) secretion, follicle growth, egg laying, and nest-building behavior (Brockway, 1965; Kroodsma, 1976; Hinde and Steel, 1978; Morton et al., 1985; Leboucher et al., 1998; Gibson et al., 2008). In mammals, the roars of red deer males advance the onset of seasonal ovulatory activity in female deers (McComb, 1987). Similarly, the auditory signals emitted by bucks are strong enough to stimulate the secretion of LH from the pituitary grand, ovulation, and sexual behavior in female goats (Shelton, 1980; Delgadillo et al., 2012).

In the mouse (*Mus musculus*), males emit song-like "ultrasonic vocalizations" (USVs) when they encounter female mice or female urinary pheromones. It has been shown that most USVs are emitted by the male than female when adult male mice interact with female mice (White et al., 1998; Wang et al., 2008; Scattoni et al., 2011). USVs of mice consist of several syllable types that are organized into phrases (Holy and Guo, 2005). The characteristics of USVs of male mice differ between inbred strains (Kikusui et al., 2011; Panksepp et al., 2007; Sugimoto et al., 2011), and the larger number of syllables depends on higher dominance ranking (Wang et al., 2011), level of testosterone (Dizinno and Whitney, 1977; James et al., 2006; Nunez et al., 1978), and the phase of the courtship sequence (Matsumoto and Okanoya, 2016). To demonstrate that female mice could discriminate USVs of various strains, we previously conducted playback study (Asaba et al., 2014b). Female mice could discriminate the characteristics of USVs, and preferred the USVs of mice

* Corresponding author at: Department of Animal Science and Biotechnology, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan.
E-mail address: kikusui@azabu-u.ac.jp (T. Kikusui).
[1] Current address: Department of Ultrastructure Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan.

http://dx.doi.org/10.1016/j.yhbeh.2017.06.006
0018-506X/

that were from different strains than their own. This may contribute to disassortative mating, which is an important strategy to avoid inbreeding and to facilitate the heterozygosity of offspring. On the other hand, the effect of USVs of male mice on female reproductive function in mice has yet to be explored.

Kisspeptin neurons are key hypothalamic neurons that regulate reproductive function. The major kisspeptin neuronal population in adult female mice is in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (Arc) (Clarkson et al., 2009). It has been suggested that the kisspeptin neurons in each population play different roles in modulating gonadotropin-releasing hormone (GnRH) neurons followed by luteinizing hormone (LH) release (Smith et al., 2005). Considering that the conspecific male's vocalization increase LH secretion of females in many species, we hypothesized that the USVs of male could activate these kisspeptin neurons as a central mechanism that USVs of male mice activate reproductive function in female mice.

The aim of the present study was to determine (1) whether a relationship exists between USVs of male mice and number of offspring deliveries, (2) whether USVs of male mice induce female sexual behavior, and (3) whether USVs of male mice activate kisspeptin neurons in female mice. In experiment 1, we investigated the relationship between the number of deliveries in breeding pairs and the numbers of USV syllables emitted from the males of those pairs. In experiment 2, we examined the effect of USVs of male mice on sexual behavior in female mice by comparing the effect of devocalized males and of sham-operated males. In experiment 3, we examined the activation of kisspeptin neurons in the AVPV and Arc, by using dual-label immunocytochemistry with phosphorylated cyclic AMP response element binding protein (pCREB) expression as indicator of neural activation.

2. Materials and methods

2.1. Animals and housing

We used inbred strains and closed colonies of mice (CLEA Japan), C57BL/6 (B6), BALB/c (BALB) and ICR. Food and water were supplied *ad libitum*, and animals were kept under a standard 12:12-h light-dark cycle. The environment was maintained at a constant temperature (24 ± 1 °C) and humidity (50 ± 5%). All experiments were carried out in a soundproof chamber in a soundproof room. All experimental procedures were approved by the Ethics Committee of Azabu University.

2.2. Experiment 1: the correlation between USVs of male mice and female deliveries

21 pairs of B6 mice were used in this experiment. Virgin B6 female mice were paired with virgin B6 male mice in a medium-sized Plexiglas cage (172 × 240 × 129 mm, CLEA Japan) for breeding. Female deliveries were checked every morning for 4 months. After parturition, they were allowed to care for their own litters for 3 weeks. Even if a pup died after parturition, it was counted as a delivery.

We also investigated the number of USVs syllables emitted from the paired males after the 4 months of delivery observations. Male mice were isolated from their female pairs and housed in small cages (172 × 240 × 129 mm, CLEA Japan) one week before USVs were recorded. The cages were padded with Alpha-dri bedding (Shephard Specialty Papers) to reduce rustling noises that could contaminate the recordings. During USV recordings, the cage that contained the male mouse was placed in a soundproof chamber, then an unfamiliar female was introduced. Ultrasound recordings of USVs were recorded for 3 min using a condenser microphone (CM16/CMPA, Avisoft Bioacoustics) and an A/D converter (Avisoft-UltraSoundGate116H, Avisoft Bioacoustics) as previously described (Asaba et al., 2015). The recorded sound was visualized with sound analysis software (SAS-Lab Pro, Avisoft Bioacoustics), and the USVs syllables were detected and counted.

2.3. Experiment 2: courtship behavior testing in female mice exposed to devocalized male mice

B6 female mice were used in this experiment. In order to control the estrus cycle, sexually naive female mice were ovariectomized under isoflurane anaesthesia (5%). After around 2 weeks of recovery, the ovariectomized female mice were primed with 17β-Estradiol (Cayman chemical) at a concentration of 1 μg per 0.05 mL in corn oil at 24 h and 48 h before testing.

ICR mice experienced in mating were used as stud males. It is known that ICR mice are more active than B6 mice in novel environment (Kulesskaya and Voikar, 2014). Also, outbred male mice showed stable testosterone-dependent sexual behavior (Luttge and Wallis, 1973; Luttge et al., 1974). Therefore, ICR mice are thought to be the most suitable male to examine the effect of devocalization on the courtship behavior test in experiment 2. The stud males that showed stable mounting behavior (>20 times in 1 h) were used in the following experiments. The males were devocalized by sectioning of the inferior laryngeal nerve according to previous reports (Nunez et al., 1985; White et al., 1998). The same procedure without sectioning of the inferior laryngeal nerve was used as the sham operation for the control group.

Courtship behavior tests were conducted in the medium-sized Plexiglas cages that were placed on the soundproof chamber. Female mice were transferred from their home cage and introduced into the test cage. Then, the devocalized male or sham-operated male was introduced into the cage. Their behavior and USVs were recorded for 30 min using a digital camera to allow ventral viewing, and the microphone was placed 10 cm above the cage. We measured 4 behaviors according to a previous study (Haga et al., 2010): female approaching behavior towards males (female stretching out, approaching and sniffing a male's body, including the head and anogenital area), female lordosis behavior towards mounts (female with all four paws grounded, hind the region elevated from the floor, and no evidence of attempt to escape or exhibition of a defensive upright posture), male mounting behavior (male places his forelegs on female's back).

2.4. Experiment 3: effects of USVs of male mice on activation of kisspeptin neurons

2.4.1. Preparing for USVs of male mice for playback

In experiment 3, we used the playback sounds recorded from BALB male, since our previous study revealed that B6 female showed more preference for USVs recorded from BALB male than USVs recorded from B6-male (Asaba et al., 2014b). In order to prepare the playback sounds, we recorded USVs from BALB male mouse, according to a previous study (Asaba et al., 2015). Briefly, USVs were recorded for 3 min when the male encountered a devocalized female. Playback sounds consisted of 20 s of the recorded WAV file representing the strain character of BALB (161 syllables per 20 s). Background noise of the recorded file was used as control sounds.

2.4.2. Protocol for USV playback and pheromone exposure

In a previous study, we found that the preference of female mice for USVs from a different strain was only observed when female mice were concomitantly exposed to the male pheromone, "Exocrine grand-secreting peptide 1 (ESP1)" (Asaba et al., 2014b). Hence, for experiment 3, we prepared 4 groups with consideration for the effect of male pheromone (Tris-HCL (vehicle) or ESP1) and playback sound (background noise or USVs of male mice); Tris + noise, ESP1 + noise, Tris + USVs, and ESP1 + USVs.

Another set of subject virgin female was prepared (Tris + noise, n = 8; ESP1 + noise, n = 9; Tris + USVs, n = 9; ESP1 + USVs, n = 9), to analyze the number of kisspeptin neurons and pCREB using immunohistochemistry. Mice were isolated from the familiar group, and housed in small cages over one week before playback experiments. The cages had two 6 cm-diameter holes in the sidewall which were covered