Abstract: Among the intact male rats, a subpopulation has been found to show little or no sexual behavior, even after experiencing several mating sessions. This study investigated whether sexually sluggish (SS) males show behavioral differences from normal copulatory (NC) males, other than those concerning sexual behavior. The olfactory preference of males was measured through the time spent displaying nose-poking behavior directed at sexually active males and estrous females for odor exploration in a three-chamber apparatus. Both the NC and SS males showed a significant preference for the odor of estrous females compared with that of male odors. However, SS males spent significantly less time nose-poking estrous females than NC males. The food-finding test was performed after overnight fasting. Our findings showed that all the NC males found the buried pellet within 5 min, whereas over 60% of the SS males failed to find it. The males were also tested for their ability to find a buried bag containing soiled bedding from estrous female cages. The bag was found by 80% of NC males, but only by 20% of SS males. Our results suggest that SS and NC male rats differ not only in sexual behavior but also in other functions such as olfaction.

Key words: estrous odor, food-finding, olfactory preference, rats, sexual behavior

Introduction

While screening the sexual activity of male rats, researchers became aware of the existence, among sexually inexperienced rats, of a subpopulation with little or no sexual behavior, even after completing several mating sessions [16]. In this study, such rats were designated as sexually sluggish (SS) males, as opposed to normal copulatory (NC) males, which typically achieve ejaculation. However, little is known about the differences between NC and SS males as the latter appear to resemble NC males with regard to behavior and physiology.

Similar to NC males, SS males can discriminate between neutral odors (e.g., amyl acetate and mint) as well as sociosexual odors, intact male urines, and estrous and anestrous females [5]. Moreover, no difference has been observed in sexually active males in terms of preference for physical interaction with either receptive females or the soiled bedding of estrous versus anestrous females [13]. NC and SS males are also similar in terms of non-social, anxiety-like behavior, as measured in elevated-plus maze trials [11]. Additionally, no physiological differences were found between NC and SS males regarding the serum levels of the sexual steroids testosterone and estradiol [13]. Supplemental testosterone treatment of SS males did not promote sexual behavior, whereas supplemental estradiol treatment suppressed ejaculation [2]. Neural activation of the vomeronasal system via exposure to estrous soiled bedding was also comparable between SS and NC males [13].

As shown above, the underlying physiological difference responsible for the sexual inactivity of SS males is yet to be identified. Therefore, we investigated whether SS males suffer from olfactory dysfunction, which could attribute to their low sexual activity. It has been previously reported that, although SS males clearly prefer soiled bedding from estrous females to that of other
males, they spend a significantly shorter time investigating female soiled bedding than NC males [13]. In this study, three mating tests were performed to select NC males with a normal level of sexual behavior and SS males with distinctly low sexual activity that could not achieve ejaculation. Both male groups were then assessed with the aim of investigating their olfactory function using the following behavioral tests: (1) a test concerning their olfactory preference for estrous airborne odor, (2) a test concerning their ability to find a food pellet after overnight fasting, and (3) a test concerning their ability to find a buried bag containing soiled bedding from estrous females.

Materials and Methods

Animals

Eight-week-old male and female Long–Evans rats were acquired from the Institute for Animal Reproduction (Ibaraki, Japan). All the animals were separated by gender, and they housed in groups of two or three per cage, under temperature-controlled (23 ± 2°C) and light/dark cycle (lights off from 20:00 to 8:00) conditions, with free access to food and water. All experimental and animal housing procedures followed the Guidelines for the Care and Use of Laboratory Animals of Teikyo University of Sciences and were approved by our institutional Committee for Experimental Animal Ethics.

Females were ovariectomized under isoflurane anesthesia and primed with estradiol benzoate (5 µg/0.1 ml sesame oil) 48 h before and with progesterone (500 µg/0.1 ml sesame oil) 3–6 h before behavioral tests. The three mating tests, which are described below, were performed weekly. Males were divided into two groups according to their sexual activity: NC males, which achieved ejaculation in the third test, and SS males, which displayed low sexual activity and failed to achieve ejaculation in any of the three mating tests. In total, we identified 20 NC males and 24 SS males for the olfactory preference test (see Tables 1 and 2). All the NC males and 15 SS males were subjected to the receptive female odor preference tests, and 10 NC males and the remaining 9 SS males were used in the food-finding tests.

Olfactory preference test

The apparatus utilized for the olfactory preference test has been previously described by Xiao et al. [18]. Briefly, it has three compartments (Fig. 1, 110 cm in total length (L) × 12 cm width (W) × 30 cm height (H)) partitioned by triplicate opaque plates with 3-cm-diameter holes at different levels. A ventilation fan, connected to the top of the middle compartment, transported airflow from the two side compartments to the middle one. A 2-cm-deep transparent tube was attached to the air inlets in the middle compartment (2 cm above the floor).

Before each test, the apparatus was cleaned with 70% ethanol (v/v) and bedded with fresh paper chips (Alpha-dri, Shepherd Specialty Paper, Watertown, TN, USA). Each experimental male was allowed to acclimate to the apparatus for 5 min. Unattended behavioral observations were recorded for 5 min with a video camera. The time spent nose-poking the left and right air inlets was calculated using an event recorder software.

The olfactory preference tests were performed three times, once a week. Each test was followed by the mating test described below.

Mating test

Each male was placed in a transparent observation cage (50 (L) × 30 (W) × 40 (H) cm), which is bedded with wood shavings, and was allowed to acclimate for 5 min. Behavioral tests began with the introduction of estrous females and terminated either after the first ejaculation or when 60 min had elapsed. The total amounts and latencies of mounts and intromissions and the latency of ejaculation were recorded. The mating tests were replicated three times, once a week.

Food-finding test

The test cage (50 (L) × 30 (W) × 40 (H) cm) was

Table 1. Incidences and median latencies (s) of mount, intromission and ejaculation in each sexual behavior test

| MOUNT          | INTROMISSION | EJACULATION |
|----------------|--------------|-------------|
| Tests          | 1  | 2  | 3  | 1  | 2  | 3  | 1  | 2  | 3  |
| Incidencea)    |    |    |    |    |    |    |    |    |    |
| NC males       | 10/20 | 18/20 | 20/20 | 7/20 | 17/20 | 20/20 | 6/20 | 17/20 | 20/20 |
| SS males       | 1/24 | 5/24 | 5/24 | 0/24 | 0/24 | 2/24 | 0/24 | 0/24 | 0/24 |
| Median Latencyb) |    |    |    |    |    |    |    |    |    |
| NC males       | 3,088.50 | 130.5 | 10  | 3,600 | 210  | 52.5 | 3,600 | 854.5 | 496.5 |
| SS males       | 3,600 | 3,600 | 3,600 | 3,600 | 3,600 | 3,600 | 3,600 | 3,600 | 3,600 |

a) number of rats who behaved at least once (numerator) / total number of rats (denominator). b) the latency of rats showing no response was analyzed as the maximum observation time (3,600 s).
bedded with a 5 cm layer of wood shavings, where a food pellet was buried at the bottom of the cage. Experimental males fasted overnight for 15 h, and they were then placed in the center of the cage to start the behavioral observation. The test was terminated either after the male discovered the pellet or after 5 min had elapsed.

Female soiled bedding finding test
The test cage was the same as in the food-finding test, but a cloth bag (3 × 3 cm) containing soiled bedding collected from the cages of estrous females was buried. Nonfasted experimental males were placed in the cage, and the time taken to reach the bag was recorded.

Statistics
For each olfactory preference test, the nose-poking time was analyzed by two-way analysis of variance (ANOVA) with repeated measures (2 groups × 2 stimulus rats). The time required for the food and soiled bedding finding tests was analyzed by survival analysis using the log-rank test.

Results
Sexual behavior
Tables 1 and 2 summarize the parameters of rat sexual behavior on which the group assignment (NC and SS) in this study was based. Mount and intromission latency were time from the introduction of stimulus estrous females to the first expression of these behaviors, and ejaculation latency (EL) was time from the first intromission to the first ejaculation. Through the accumulating sexual experience of three mating sessions, all NC males displayed mounts, intromissions, and ejaculations, whereas a significantly lower number of SS males expressed those sexual behaviors (Table 1). Table 2 displays the number of mounts per 5 min (mounts/5min), interintromission interval (III, EL/number of intromissions), and intromission ratio (IR, number of intromissions/total number of mounts and intromissions), which indicate sexual behavior efficiency. The values of mount/5min of SS males were found to be extremely low, and it was impossible to calculate III and IR in SS males.

Olfactory preference
Figure 2 shows the results of the weekly olfactory preference tests, which were followed by the mating tests. The rats were sexually naive in the first test, experienced mating once in the second test, and experienced mating twice in the third test.

The mean percentages of time spent nose-poking toward estrous females to total nose-poking time were 65.5 ± 6.59% in NC males and 59.5 ± 7.00% in SS males in

![Fig. 1. The apparatus used for the olfactory preference test for conspecific odors. Experimental subjects were placed in the middle compartment, and two types of stimulus rats were positioned in the left and right compartments, respectively (in this picture, a gonadally intact male was in the left compartment, and an estrous female primed with sex hormones was in the right compartment). A ventilation fan, which was connected to the ceiling of the middle compartment, produced an airflow conveying stimulus rat odors to the middle section through the transparent tubes (as indicated by arrows). Experimental males displayed nose-poking behavior into the tubes to investigate the odors of stimulus rats.](image)

![Table 2. Mean number of mounts per 5 min, interintromission interval (III, s), and intromission ratio (IR, number of intromissions/total number of mounts that include intromissions) in each sexual behavior test](table)
the first test; two SS males were excluded from the analysis as they were never observed performing nose-poking behavior. The mean percentages of time spent nose-poking toward estrous females to total nose-poking time were 84.9 ± 3.93% in NC males and 67.4 ± 5.52% in SS males in the second test (Table 3). The two-way ANOVA (2 groups × 2 stimuli) for nose-poking time indicated that only NC males showed a significant olfactory preference (test 1: \( F_{1,33} = 11.08, P < 0.01 \); test 2: \( F_{1,33} = 64.48, P < 0.001 \)). A significant difference was also observed between the NC and SS males with respect to the time spent nose-poking toward estrous females (test 1: \( F_{1,33} = 4.86, P < 0.05 \); test 2: \( F_{1,33} = 30.86, P < 0.001 \)).

After two mating experiences, the mean percentages of time spent nose-poking toward estrous females to total nose-poking time were found to be at 80.8 ± 4.86% in NC males and 77.4 ± 4.64% in SS males; they were observed to both correspond to typical masculine olfactory preference patterns (Table 3). The difference between the time spent nose-poking toward males and estrous females was also significant in both NC males (\( F_{1,33} = 32.96, P < 0.001 \)) and SS males (\( F_{1,33} = 7.73, P < 0.01 \)). However, SS males exhibited significantly less nose-poking time toward estrous females than NC males (\( F_{1,66} = 4.71, P < 0.05 \)).

### Discussion

In this study, SS rats were selected based on prescreening sexual behavioral tests that had been performed in other experiments in our lab over recent years. These SS males, which were excluded from those previous experiments as inadequate subjects, had a normal, healthy appearance. When subjected to sexual behavioral tests,
they were initially found to be interested in the stimulus females and could be observed approaching them, examining their odor, and occasionally exhibiting failed mounting. However, thereafter, several of them ceased active interaction with the stimulus females, failing to maintain their sexual motivation throughout the mating session.

Since destruction of the olfactory epithelium decreases sexual activities in male rats [3, 7, 9, 15], we hypothesized that SS males might have an impaired reception of signals derived from estrous females, which prevented them from maintaining their sexual motivation. Therefore, these experiments have focused on the olfactory system. We investigated the difference in olfactory preference for the airborne signals of estrous females, which indicated that NC males show a clear preference for estrous odor compared with male odor in the three tests; SS males, however, showed no preference in tests 1 and 2 and a significant preference for estrous odor only in test 3 (Fig. 1, Table 3). Although olfactory preference is known to depend on previous sexual behavior experience [10], our results show that olfactory preference is sufficiently manifested not only by sexual behavior experience but also by exposure to and/or short interactions with estrous females. That is, SS males with little or no sexual behavior per se still displayed olfactory preference after two mating sessions in the third olfactory preference test. However, in all the preference tests, SS males spent a significantly shorter time exploring estrous female odor than NC males. These results suggest that SS males can recognize estrous female odors, showing a weak, but favorable preference for them.

A decrease in the time spent exploring estrous female odor in the olfactory preference test could result from either olfactory function deterioration or declined sexual motivation. We then had the rats perform food-finding tests after 15 h of fasting; this is to examine olfactory function without the influence of sexual motivation. We found that, in contrast to NC males, only one-third of SS males reached the buried food pellet within 5 min. Both NC and SS males were subjected to the same drive control and had similar body weight (NC males 378.3 ± 32.1 g, SS males 388.7 ± 29.3 g), which indicates that their feeding motivation (hunger drive) was equivalent. Therefore, the inability of the SS males to locate the food pellet might have been caused by impaired olfactory function. Moreover, using the same paradigm, we investigated the ability of rats to find a buried bag containing estrous female bedding. Consistently, 80% of SS males failed to reach the incentives. SS males have been reported to be able to identify sexually relevant odor (e.g., estrous female urine, anestrous female urine, and male urine) as well as sexually irrelevant odor, such as amyl acetate and mint [5]. In addition, as mentioned above, SS males showed a significant preference for estrous female odor in the third olfactory preference test. Taken together, our results indicate that SS males may have hyposensitive olfaction compared to NC males, that is, SS males can discriminate between sexually relevant odors but have low sensitivity to odor stimuli, which may lead to sluggish sexual behavior. In fact, male sexual behavior is suppressed by the infusion of zinc sulfate into the rat olfactory epithelium, which prevents olfactory inputs [3, 7, 9, 15]. Although we did not inves-
tigate whether the vomeronasal function of SS males had decreased, the disturbance of those chemosensory inputs must play a part in impairing sexual behavior in male rats [8, 14].

Efforts to elucidate the physiology of SS males are still ongoing, and our knowledge about them remains to be extremely limited. Because sexual behavior is dependent on sex hormones, most studies have focused on how the neuroendocrine characteristics of SS males differ from those of NC males. There is only one report describing the characteristics in the SS male olfactory system, [1] which includes an increased expression of androgen receptor (AR) and a decreased expression of estrogen receptor-α (ERα) in the olfactory bulb compared with NC males.

On the other hand, the medial amygdala (MeA) and the medial preoptic area (MPOA) have been known to play a critical role in the regulation of male sexual behavior; this is why a number of studies investigated the difference in these regions between NC and SS males. According to these studies, the MeA in SS males was significantly higher in terms of expressing AR, ERα, and aromatase (an enzyme that converts testosterone to estradiol) than that in NC males [1, 12]. The MPOA in SS males was also found to be higher in terms of expressing AR and aromatase, but lower in ERα, compared to that of the NC males [12]. However, while these neuroendocrine differences may partly contribute to the level of sexual activity, it may be insufficient to explain the difference between NC and SS males not only in sexual behavior but also in terms of olfactory sensitivity for foods as well as sex hormone sensitivity in the neural circuitry.

Our SS males showed not only declined sexual activity but hyposensitivity to food odors independent of sex hormones. Although we did not evaluate the circulating hormone levels or their receptor expression in SS males during this study, we assume that the declined sexual activity in SS males may not be caused by a neuroendocrine aberration. A previous study reported that NC and SS males differ remarkably in terms of sympathetic sensitivity, and the number of neurons with N-methyl-D-aspartate (NMDA) receptors in the hypothalamic periventricular nucleus is significantly decreased in SS males [17]. Furthermore, schizophrenia, with which patients sometimes complain of decreased libido and erectile dysfunction, also includes an anosmic symptom [4]. Thus, the marked differences in the brains of SS males might be caused by factors other than neuroendocrine distortion, which emerge as a particular symptom, such as declined sexual activity and hyposensitive olfaction.

Similar to SS rats, most men with impaired sexual behavior appear to be perfectly healthy. A recent study reported that, of a total of 574 patients with erectile dysfunction (ED), 115 patients (20.03%) had rhinologic diseases (RD), while 201 ears, nose, and throat patients with nasal congestion and nasal discharge included 29 (14.43%) that complained of ED [6]. Furthermore, the report also demonstrated that the olfactory sensitivity of patients with ED was lower than that of healthy male adults, and patients with both ED and RD had the worst olfactory sensitivity [6]. Therefore, SS male rats may constitute an excellent animal model for such symptoms and disorders in humans. Our results strongly support the need to investigate whether declined human sexual function has physiological causes, other than those related to the reproductive function, such as the endocrinology of sex steroids.

**Conflicts of Interest**

None

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