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—Original Article—

Mating-induced increase in *Kiss1* mRNA expression in the anteroventral periventricular nucleus prior to an increase in LH and testosterone release in male rats

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Abstract. Kisspeptin has an indispensable role in gonadotropin-releasing hormone/gonadotropin secretion in mammals. In rodents, kisspeptin neurons are located in distinct brain regions, namely the anteroventral periventricular nucleus-periventricular nucleus continuum (AVPV/PeN), arcuate nucleus (ARC), and medial amygdala (MeA). Among them, the physiological role of AVPV/PeN kisspeptin neurons in males has not been clarified yet. The present study aims to investigate the acute effects of the olfactory and/or mating stimulus with a female rat on hypothalamic and MeA *Kiss1* mRNA expression, plasma luteinizing hormone (LH) and testosterone levels in male rats. Intact male rats were exposed to the following stimuli: exposure to clean bedding; exposure to female-soiled bedding as a female-olfactory stimulus; exposure to female-soiled bedding and mating stimulus with a female rat. The mating stimulus significantly increased the number of the AVPV/PeN *Kiss1* mRNA-expressing cells in males within 5 minutes after the exposure, and significantly increased LH and testosterone levels, followed by an increase in male sexual behavior. Whereas, the males exposed to female-soiled bedding showed a moderate increase in LH levels and no significant change in testosterone levels and the number of the AVPV/PeN *Kiss1* mRNA-expressing cells. Importantly, none of the stimuli affected the number of *Kiss1* mRNA-expressing cells in the ARC and MeA. These results suggest that the mating-induced increase in AVPV/PeN *Kiss1* mRNA expression may be, at least partly, involved in stimulating LH and testosterone release, and might consequently ensure male mating behavior. This study would be the first report suggesting that the AVPV/PeN kisspeptin neurons in males may play a physiological role in ensuring male reproductive performance.

Key words: Kisspeptin, Gonadotropin-releasing hormone, Luteinizing hormone, Male sexual behavior, Testosterone

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Accumulating evidence suggests that kisspeptin neurons play an essential role in mammalian reproductive function [1, 2]. Kisspeptin neurons are indispensable for gonadotropin-releasing hormone (GnRH) and consequent luteinizing hormone (LH) and follicle-stimulating hormone (FSH) release in mammals including rodents [3–5], ruminants [6–8] and primates [9, 10]. Indeed, plasma LH and FSH levels are undetectable in male and female *Kiss1* (kisspeptin gene) knockout (KO) rats [3]. Cell bodies of kisspeptin neurons are located in distinct brain regions, such as the anteroventral periventricular nucleus (AVPV)/periventricular nucleus (PeN)/preoptic area (POA), arcuate nucleus (ARC) and medial part of the amygdala (MeA) [4, 11–13] in the brain of mammalian species including rodents [14–17], ruminants [7, 18–20], primates [21–23]. Since the discovery of kisspeptin neurons, the role of kisspeptin neurons in reproduction has been intensively studied mainly in females.

In male mammals, it is postulated that the ARC kisspeptin neurons play a role in the regulation of tonic GnRH/gonadotropin release [3, 24–26] similar to females. The ARC kisspeptin neurons are suggested to serve as a GnRH pulse generator, which governs pulsatile gonadotropin release to control various reproductive events, such as puberty onset, spermatogenesis/folliculogenesis and sex steroid synthesis in both sexes [3, 25, 27]. On the other hand, a number of evidence suggests that the AVPV/PeN/POA kisspeptin neurons mediate the estrogen-positive feedback to induce GnRH/LH surge that leads to ovulation in female mice [28], rats [29], sheep [30], goats [31], cattle [32], musk shrews [33], pigs [34] and monkeys [35]. Moreover, our previous study suggests that the AVPV/PeN kisspeptin neurons integrate the external information, such as a male-olfactory stimulus, to enhance GnRH/LH surge in female rats [36]. Whereas, the number of AVPV/PeN/POA kisspeptin neurons in males is lower than in females in rodents [37] and monkeys [35]. Thus, the physiological importance of the AVPV/PeN/POA kisspeptin neurons in males has not been clarified yet. The AVPV/PeN *Kiss1* mRNA expression in male mice is augmented by exogenous

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androgen [38] and the POA kisspeptin neurons in male monkeys [35, 39] and goats [31] are activated by exogenous estrogen followed by a surge-like increase in plasma LH levels as shown in females. These studies suggest that the AVPV/PeN/POA kisspeptin neurons may also function to control reproduction in males.

The sex-related olfactory stimulus and/or mating stimulus are suggested to be involved in a rapid induction of GnRH release, and the GnRH may directly or indirectly (via LH and then testosterone increase) enhance mating behavior in male animals [40–42]: Previous studies reported that plasma LH levels increase after female urine exposure within 15 min in male mice [43]; plasma LH and testosterone levels increase after copulation within 10 min and 60 min, respectively, in male rats [44]; rapid LH or testosterone increase after copulation has been reported in other mammals including rabbits (testosterone: within 45 min) [45], cows (LH: within 60 min) [46] and pigs (LH: within 20 min) [47]. Further, a central administration of GnRH facilitated male-sexual behavior in gonad-intact male rats within 15 min [40] and testosterone-implanted castrated male rats within 30 min [41], suggesting that the GnRH may rapidly and positively affect the brain circuit involved in the male sexual behavior. These results suggest that the olfactory stimulus and/or mating stimulus may rapidly stimulate kisspeptin neurons, consequently enhances GnRH release and then LH and testosterone release in circulation in male mammals. Further, the MeA kisspeptin neurons are suggested to mediate pheromonal cues to increase LH release in male mice [43, 48]. Thus, we hypothesized that a rapid increase in *Kiss1* mRNA expression in the brain of male rats may mediate the signals originated from the olfactory and/or mating stimuli from/with female rats to enhance GnRH/LH and then testosterone release; eventually, the information(s) augment male sexual behavior.

The present study, thus, aims to investigate the acute effects of the olfactory stimulus derived from female rats and/or mating stimulus with a female rat on *Kiss1* mRNA expression in the brain, LH and testosterone release in male rats. To address this issue, we examined the effects of three-stimulus, such as exposure to clean bedding, exposure to female-soiled bedding as a female-olfactory stimulus, and exposure to female-soiled bedding and mating stimulus with a female rat on the number of the *Kiss1* mRNA-expressing cells in the AVPV/PeN, ARC, and MeA, and plasma LH and testosterone levels in intact male rats. *Kiss1* mRNA expression in the nuclei was investigated within 5 min after the stimuli, because plasma LH levels increased within 12 min after the stimuli in the present study. Sexual behavior was also analyzed in the males mated with a female rat.

Materials and Methods

Animals

Adult males (ages 10–13 wk; 300–400 g body weight) and females (ages 10–12 wk; 250–300 g body weight) Wistar-Imamichi strain rats were maintained under a controlled environment (14 h light and 10 h darkness, lights on at 0500 h; 23 ± 3°C) and allowed free access to standard laboratory rat chow (CE2; Clea, Tokyo, Japan) and water. All male and female rats were sexually experienced before the experiments. Sixteen male rats were subjected to the analysis for plasma LH and testosterone levels, and the other fifteen males were subjected to the histological analysis for *Kiss1* mRNA expression in the brain after exposure to clean or female-soiled bedding, or exposure to female-soiled bedding and mating with a female rat. The surgical procedures for all animals were performed under ketamine (27.0 mg kg^{-1})/xylazine (5.3 mg kg^{-1}) mixture and inhalant isoflurane (1–3%) anesthesia, if not otherwise specified. Seven days before blood or brain tissue sampling from the males, the females for bedding/mating stimulus were bilaterally ovariectomized (OVX) and subcutaneously implanted with Silastic tubing (1.0-mm inner diameter; 1.5-mm outer diameter; 20 mm in length; Dow Corning, Midland, MI, USA) containing crystalline estradiol-17β (E_2) (Sigma, St Louis, MO, USA) to mimic a proestrous level of plasma E_2 to ensure the olfactory stimulus from the female and acceptance of copulation by a male rat: The OVX + E_2 females were confirmed to show robust lordosis behavior [49], and the plasma E_2 levels in OVX rats with the same E_2 treatment were 1.86 ± 0.34 nmol/l as determined in our previous study [50]. All animal experiments were conducted in accordance with the Guidelines of the Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University (Accession number 2018031358).

Olfactory or mating stimulation for male rats

Intact male rats were divided into three groups: exposure to clean bedding, exposure to female-soiled bedding, and placed with a female for mating with female-soiled bedding (Fig. 1A). The female-soiled bedding was prepared by housing an OVX + E_2 female rat in a clean acrylic cage covered with bedding for seven days. On the day of blood or brain sampling, the testing cages (60 cm long × 45 cm wide × 45 cm high, the front side was transparent, and the other sides were opaque) were covered with clean (fresh) or female-soiled bedding 30 min before introducing a male. The OVX + E_2 female rat was placed in the testing cage covered with their own female-soiled bedding at the same time. A male rat was then transferred from his home cage to the testing cage at 1730 h and kept there for 60 min for blood sampling (Fig. 1B) or 5 min for brain sampling (Fig. 1C).

Blood sampling for analyzing plasma LH and testosterone levels and monitoring for male sexual behavior

A silicon cannula (inner diameter 0.5 mm; outer diameter 1.0 mm; Shin-Etsu Polymer, Tokyo, Japan) was inserted into the right atrium through the jugular vein on a day before the blood sampling in male rats (n = 4–6 in each group). To determine the effect of olfactory and/or mating stimuli on the plasma LH and testosterone levels, blood samples (150 µl) were obtained from free-moving conscious intact male rats (n = 4–6 in each group) for 84 min at 6 min intervals: Male rats were subjected to the blood sampling in their home cage (for the first 24 min) and then in the testing cage (for the last 60 min). Plasma samples were obtained by immediate centrifugation and stored at –20°C until assayed for LH and testosterone. The sexual behavior of the male rats placed in the testing cage with a female rat was recorded on a video camera and the total number of mounting, intromission, and ejaculation of the males was counted every 6 minutes according to previous studies [51, 52].

LH and testosterone assays

Plasma LH concentrations in 50-µl plasma samples were determined by a double-antibody radioimmunoassay (RIA) with a rat LH RIA kit

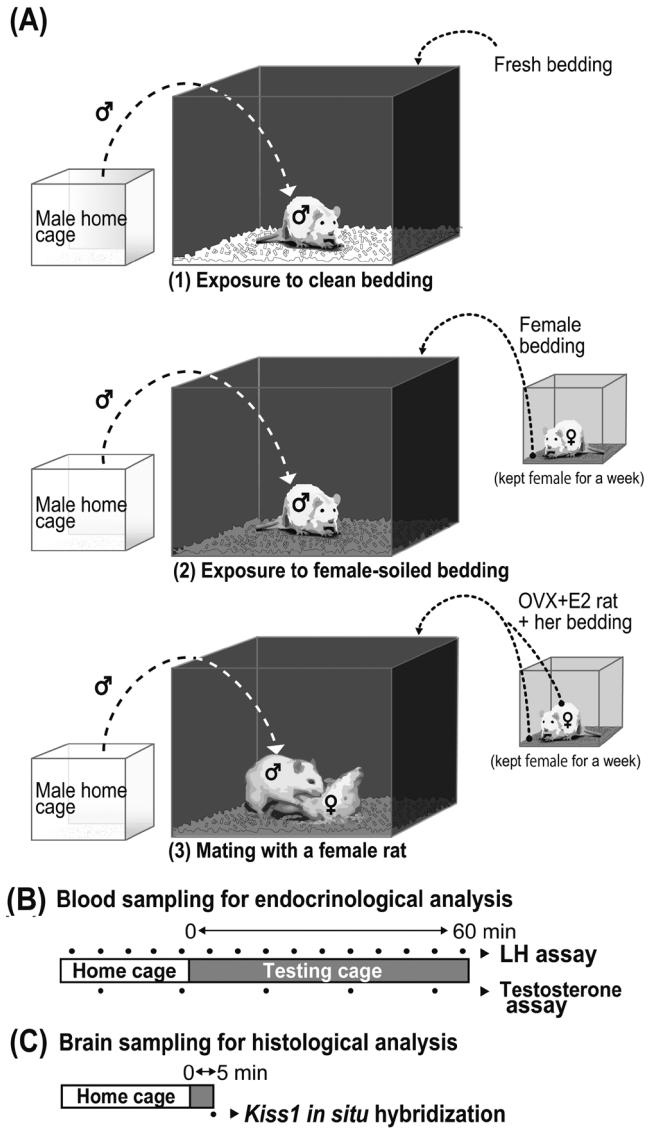

Fig. 1. Experimental scheme of olfactory or mating stimulation. Intact male rats were divided into three groups: exposure to clean bedding, exposure to female-soiled bedding, and placed with a female for mating with female-soiled bedding. The males were kept in their home cages and then introduced to each testing cage containing fresh bedding or female-soiled bedding without/with an ovariectomized (OVX) + estradiol-17β (E_2) female rat. The fresh bedding or the female-soiled bedding taken from the cage, where an OVX + E_2 female rat had been kept for a week, was placed into a testing cage without/with an OVX + E_2 rat 30 min before introducing a male rat into the testing cage (A). Male rats were subjected to the blood sampling in their home cage (for the first 24 min) and then in the testing cage (for the last 60 min) at 6 min intervals, and the plasma samples were used for luteinizing hormone (LH) and testosterone assays (B). Five minutes after the onset of the exposure to each stimulus in the testing cage, the male brain samples were taken for histological analysis for *Kiss1* mRNA expression (C).

provided by the National Hormone and Peptide Program (Baltimore, MD, USA), and were expressed in terms of the NIDDK rat LH RP-3. The least detectable level of LH assay was 156 pg/ml, and the intra- and inter-assay coefficients of variation were 6.75 and 2.65% at 0.98 ng/ml, respectively.

Plasma testosterone levels were determined by an enzyme-linked sorbent immunoassay using a testosterone ELISA kit (Cat No. 582701, Cayman Chemicals, Ann Arbor, MI, USA, RRID: AB_328059) according to the manufacturer instructions. Testosterone extracted from 40-μl plasma samples with a mixture of hexane and ether (3:2) was dissolved in 0.1% gelatin-0.05 M phosphate solution and then quantified. The least detectable level of testosterone was 6 pg/ml, and the intra- and inter-assay of coefficients of variation were 2.61 and 11.06% at 1.40 ng/ml, respectively.

Brain sampling

Five minutes after the transfer to the testing cages, male rats (n = 5 in each group) were deeply anesthetized with sodium pentobarbital and perfused with 0.05 M PBS followed by 4% paraformaldehyde in 0.05 M PB to obtain the brain sample for analysis of *Kiss1* mRNA expression. The timing of the brain sampling for *Kiss1* mRNA analysis was chosen, because the plasma LH levels in male rats were significantly increased 12 minutes after mating stimulation and thereafter with kisspeptin being a dominant stimulator for GnRH/LH release in male rats [3]. Note that all male rats in the group for mating stimulus immediately showed mating behavior. Brains were immediately removed from the skull, postfixed with the same fixative at 4°C overnight, and then immersed in 30% sucrose in 0.05 M PB at 4°C for 2–3 days until they sank. Serial 50-μm coronal sections containing the AVPV/PeN, ARC, and MeA were obtained.

In situ hybridization of Kiss1 mRNA

In situ hybridization for *Kiss1* was performed in the brain sections taken from male rats in each group as previously described [36, 37]. The brain sections were hybridized with 1 μg/ml DIG-labeled anti-sense *Kiss1* cRNA probe (position 33-348; GenBank accession no. AY196983) overnight at 60°C. The sections were washed with 2 × SSC containing 50% formamide for 15 min at 60°C twice, then treated with 20 μg/ml RNase A for 30 min at 37°C and alkaline phosphatase-conjugated anti-DIG antibody (sheep IgG, dilution 1:1000; Roche Diagnostics, Indianapolis, IN, USA, RRID: AB_514497) 2 h at 37°C, and then treated with a chromogen solution (337 μg/ml 4-nitroblue tetrazolium chloride, 175 μg/ml 5-Bromo-4-Chloro-3-indoyl-phosphate) for 1.5 h. The number of *Kiss1* mRNA-expressing cells on each brain section was bilaterally counted twice by a blind investigator under a microscope, and the average per section was calculated in each group. The number of *Kiss1* mRNA-expressing cells was counted every second section through the AVPV/PeN (from 0.48 mm anterior to 0.48 mm posterior to the bregma; eleven sections in total) or every fourth section through the ARC (rostral division, from 1.80 to 2.60 mm; middle division, from 2.60 to 3.40 mm; caudal division, from 3.40 to 4.20 mm posterior to the bregma; three sections for each division of the ARC and nine sections in total) and MeA (from 2.40 to 3.60 mm posterior to the bregma; four sections in total) according to the rat brain atlas [53]. No positive signal for *Kiss1* mRNA was detected in the brain

sections hybridized with the corresponding sense probe as described previously [36].

Data and statistical analysis

Statistical differences in the plasma LH or testosterone concentrations were determined by two-way (stimulus and time as main effects) ANOVA, followed by the Bonferroni test using js-STAR software (http://www.kisnet.or.jp/nappa/software/star/). Statistical differences in the number of mounts, intromission or ejaculation in male rats were determined by one-way repeated measures ANOVA, followed by the Bonferroni test using js-STAR software. Statistical differences in the number of *Kiss1* mRNA-expressing cells per section in the AVPV/PeN, ARC (rostral, middle, caudal divisions) or MeA were determined by one-way factorial ANOVA, followed by the Bonferroni test using js-STAR software.

Results

The mating stimulus with a female rat increased plasma LH and testosterone levels in intact male rats

The profiles of changes in mean plasma LH concentrations in intact male rats in each group were shown in Fig. 2A. The LH level showed an immediate increase in intact male rats after the mating with a female rat, while the LH level gradually increased after the exposure to female-soiled bedding. The LH level was stable when the male rats were exposed to the clean bedding. Two-way ANOVA analysis (main effects, stimuli and time) revealed significant effects of stimuli ($F_{2,13} = 6.37$, $P < 0.05$), time ($F_{14,182} = 2.72$, $P < 0.05$) and interaction between stimuli and time ($F_{28,182} = 2.57$, $P < 0.05$) on the plasma LH levels (Fig. 2A). Specifically, the plasma LH levels in the male rat at 18, 24, and 54 minutes after the mating with a female rat were significantly higher (†, $P < 0.05$, analyzed by the Bonferroni test) than those in clean-bedding- and female-soiled-bedding-exposed male rats (Fig. 2A). The plasma LH levels in the male rat at 12, 30, and 36 minutes after the mating were significantly higher (*, $P < 0.05$, analyzed by the Bonferroni test) than those in clean-bedding-exposed male rats (Fig. 2A). The plasma LH levels in the male rat at 12 and 30 min after the exposure to female bedding were significantly higher (*, $P < 0.05$, analyzed by the Bonferroni test) than those in clean-bedding-exposed male rats (Fig. 2A).

The mean plasma testosterone concentrations in the male rats mated with a female gradually increased, while testosterone levels were stable in the males exposed to the clean or female-soiled bedding during the sampling period (Fig. 2B). Two-way ANOVA analysis (main effects, stimuli and time) revealed significant effects of time ($F_{4,52} = 4.94$, $P < 0.05$) and interaction between stimuli and time ($F_{8,52} = 2.99$, $P < 0.05$), but no significant effects of stimuli ($F_{2,13} = 0.98$, $P > 0.05$), on the plasma testosterone levels (Fig. 2B). Specifically, the plasma testosterone levels in the male rat at 54 minutes after the mating were significantly higher ($P < 0.05$, analyzed by the Bonferroni test) than those of male rats exposed to clean bedding (Fig. 2B).

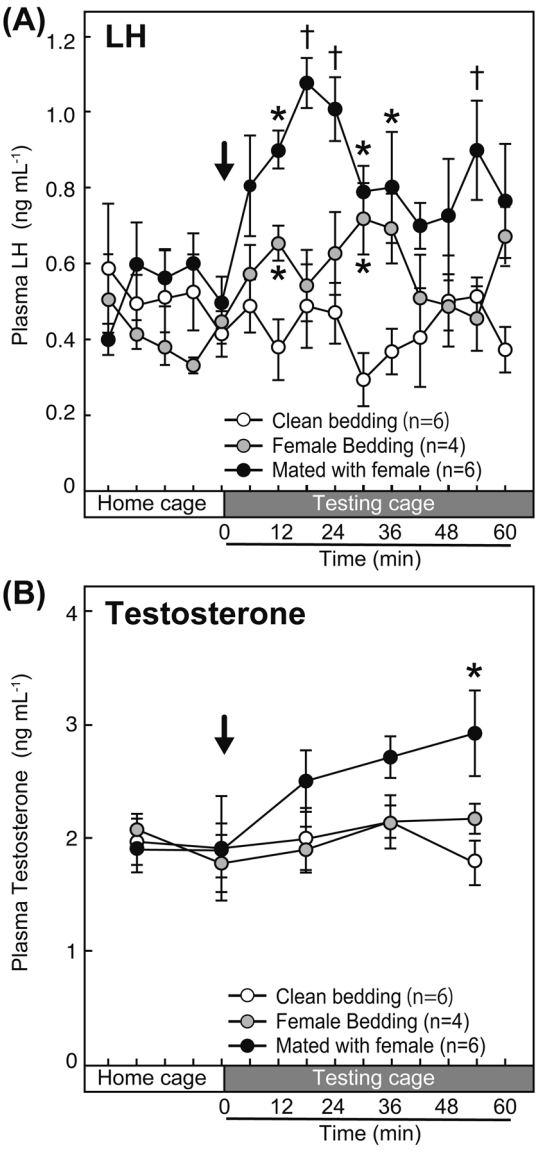

Fig. 2. Effects of olfactory and/or mating stimulation on plasma luteinizing hormone (LH) and testosterone levels in intact male rats. The changes in the mean plasma LH levels of intact male rats in each group that were exposed to the clean bedding, the female-soiled bedding, or female-soiled bedding and mated with an ovariectomized (OVX) + estradiol-17β (E_2) rat (A). The changes in the mean plasma testosterone levels in each group (B). Arrows indicate the onset of each bedding exposure and/or mating with a female. †, values indicating significant difference compared with the value in the males exposed to clean bedding and female-soiled bedding ($P < 0.05$, two-way ANOVA). *, values indicating significant difference compared with the value in the males exposed to clean bedding ($P < 0.05$, two-way ANOVA).

Temporal analysis of the number of male-type sexual behavior

Temporal changes in the number of mounting, intromission, and ejaculation, and the total number of these sexual behavior in male rats for 60 min after the cohabitation with a female rat are shown in Fig. 3. All male rats showed sexual behaviors during the 60 min blood sampling/behavior test period. The mounting and intromission behavior seemed to show a biphasic increase during the period, but no significant difference was found in the number of mounting

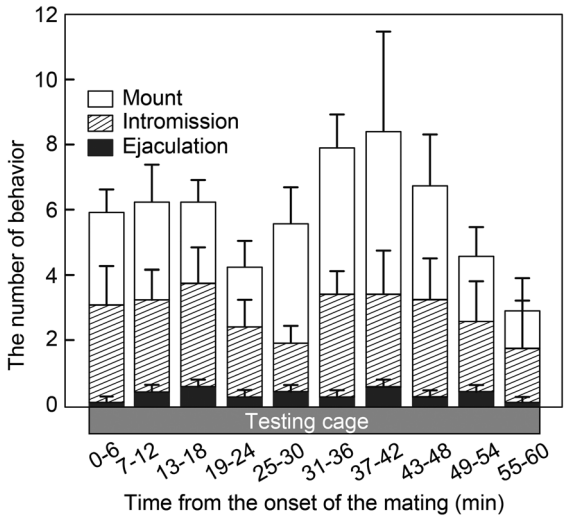

Fig. 3. Temporal changes in the number of male-type sexual behaviors in intact male rats after the introduction to the female-soiled bedding and a female rat. The number of mounting, intromission, and ejaculation were measured every 6 min for 60 min in intact male rats exposed to female-soiled bedding and mated with an ovariectomized (OVX) + estradiol-17β (E_2) rats. Values are the mean ± SEM. There was no significant difference between each time point (one-way ANOVA).

($F_{9,45} = 0.78$, $P > 0.05$), intromission ($F_{9,45} = 0.33$, $P > 0.05$), ejaculation ($F_{9,45} = 0.72$, $P > 0.05$) or total ($F_{9,45} = 0.56$, $P > 0.05$) in male rats (Fig. 3).

The mating stimulus with a female rat increased Kiss1 mRNA expression in the AVPV/PeN, but not in the ARC and MeA in intact male rats

Kiss1 mRNA-expressing cells in the AVPV/PeN, ARC and MeA in representative intact male rats are shown in Fig. 4. *Kiss1* mRNA-expressing cells were found in the AVPV/PeN (Fig. 4A), ARC (Fig. 4B) and MeA (Fig. 4C) of all male rats exposed to clean or female-soiled bedding, and mated with a female rat.

The mating stimulus significantly increased the number of *Kiss1* mRNA-expressing cells in the AVPV/PeN, but not in the ARC and MeA of intact male rats (Fig. 4D). Specifically, one-way ANOVA analysis showed a significant effect of the stimulation on the number of *Kiss1* mRNA-expressing cells in the AVPV/PeN in the males ($F_{2,12} = 7.23$, $P < 0.05$) (Fig. 4D), and the number in the AVPV/PeN in male rats mated with a female rat was significantly higher than the rats exposed to clean bedding or female-soiled bedding ($P < 0.05$, analyzed by the Bonferroni test) (Fig. 4D). No significant difference was found in the number of *Kiss1* mRNA-expressing cells in the rostral ARC ($F_{2,12} = 0.18$, $P > 0.05$, Fig. 4D), middle ARC ($F_{2,12} = 4.74$, $P > 0.05$, Fig. 4D), caudal ARC ($F_{2,12} = 0.57$, $P > 0.05$, Fig. 4D) or MeA ($F_{2,12} = 0.54$, $P > 0.05$, Fig. 4D) in male rats between groups.

Discussion

The present study demonstrated that the AVPV/PeN kisspeptin neurons are, at least partly, involved in ensuring the mating behavior in male rodents, because the mating stimulus with a female rat rapidly (within 5 min) increased the number of AVPV/PeN *Kiss1* mRNA-expressing cells and then LH release followed by testosterone release in male rats. Further, our previous study showed that kisspeptin neurons are indispensable for male-type behavior: *Kiss1* KO male rats failed to show the ejaculation, even if the KO rats were supplemented with exogenous testosterone [52]. The central GnRH has been suggested to be involved in enhancement of male sexual behavior in a previous study [41]: The central GnRH administration restored mounting behavior in hyperprolactinemic male rats showing deficits of the behavior. Taken together, the present findings suggest that the mating stimulus induces *Kiss1* mRNA expression in the AVPV/PeN to enhance GnRH/LH and then testosterone release, consequently strengthening male sexual behavior. To our knowledge, this is the first report suggesting a physiological role of AVPV/PeN kisspeptin neurons in males.

Interestingly, solely female-olfactory stimulus failed to increase AVPV/PeN *Kiss1* mRNA expression and plasma testosterone levels in male rats, suggesting that physical stimulus caused by mating with a female but not female-derived olfactory stimulus is mainly involved in the induction of the AVPV/PeN *Kiss1* mRNA expression to activate the hypothalamic-pituitary-gonadal (HPG) axis. We have previously demonstrated that the male-derived olfactory stimulus increased c-Fos expression in the AVPV/PeN kisspeptin neurons and LH release in estrogen-primed-female rats [36], suggesting that the male derived-olfactory stimulus activated AVPV/PeN kisspeptin neurons in females and that the olfactory stimulus derived from the mates is less important in male rats than females for the activation of AVPV/PeN kisspeptin neurons. The notion is consistent with a previous study, showing that exposure to a mate's bedding increased the number of AVPV/PeN kisspeptin neurons in female mice, but not in males [54]. Thus, these results suggest a sexual differentiation of neural responses to sex-related olfactory cues to affect kisspeptin neurons and then GnRH and gonadotropin release.

The present results concerning the number of *Kiss1* mRNA-expressing cells suggest that the ARC and MeA kisspeptin neurons in male rats would be less important than AVPV/PeN kisspeptin neurons for the induction of the LH release after copulation. On the other hand, a recent study showed that exposure to female urine (including pheromonal signals) for 30 min increased the number of kisspeptin neurons co-expressing c-Fos in the MeA in intact male mice, suggesting that activation of the MeA kisspeptin neurons may be involved in an increase in LH release in intact male mice [43]. Moreover, it is suggested that the MeA kisspeptin neurons were positively regulated by sex steroids in male mice [55]. These studies suggest that there might be species difference in the central mechanism regulating kisspeptin neurons and sexual behavior in males. Further studies are required to clarify whether the MeA and/or ARC kisspeptin neurons are also involved in the enhancement of LH and testosterone release by the olfactory/mating stimulus in the male rodents and other mammalian species.

Interestingly, the number of *Kiss1* mRNA-expressing cells in the

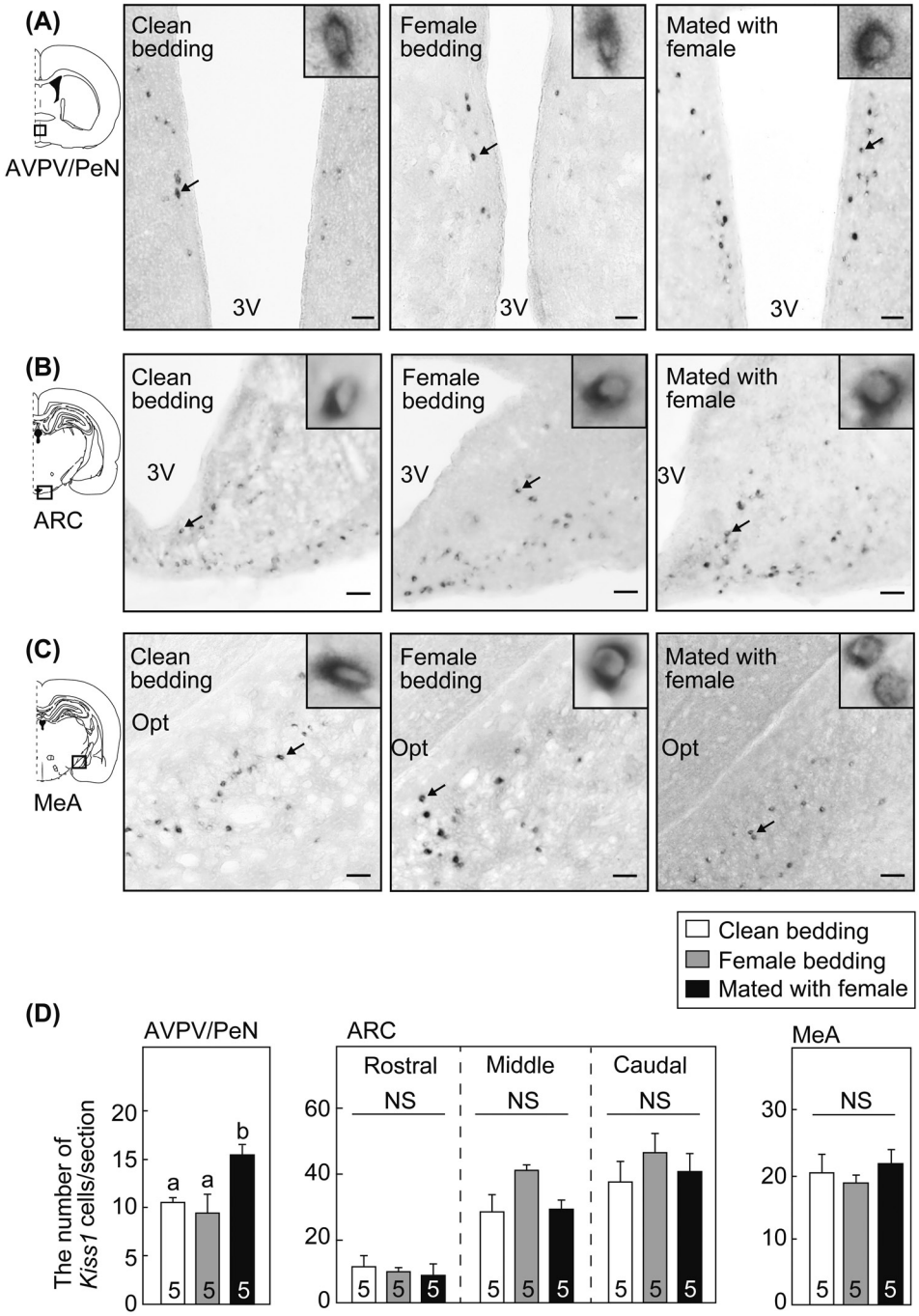

Fig. 4. Effects of olfactory and/or mating stimulation on *Kiss1* mRNA expression in the anteroventral periventricular nucleus-periventricular nucleus continuum (AVPV/PeN), arcuate nucleus (ARC) and medial amygdala (MeA) in intact male rats. *Kiss1* mRNA expression determined by *in situ* hybridization in the AVPV/PeN (A), ARC (B) and MeA (C) in representative intact male rats exposed to clean bedding, female-soiled bedding, or female-soiled bedding with a female rat. The boxed area in the schematic illustration of the coronal section of the rat brain shows the location of the AVPV/PeN, ARC, or MeA examined. Insets show *Kiss1* mRNA-expressing cells at higher-magnification in each brain area indicated by arrows in the photomicrographs. Scale bars, 100 μm; 3 V, the third ventricle; Opt, optic tract. The number of *Kiss1* mRNA-expressing cells per section in the AVPV/PeN, ARC (the rostral, middle, and caudal divisions), and MeA of intact male rats exposed to clean bedding, female-soiled bedding, or female-soiled bedding with a female rat (D). The value with different characters shows a significant difference within the three groups ($P < 0.05$, one-way ANOVA followed by Bonferroni test). There was no significant difference (NS) between the groups (one-way ANOVA) in the number of the *Kiss1* mRNA-expressing cells per section in the ARC (the rostral, middle, and caudal divisions) and MeA. Values are the means ± SEM. The number in each column indicates the number of animals used.

AVPV/PeN increased rapidly after the mating stimulus. This rapid increase in *Kiss1* mRNA expression is consistent with previous studies, suggesting that mRNA expression of some peptides increased within 15 min after the stimuli: corticotropin-releasing hormone mRNA expression in the hypothalamic paraventricular nucleus was induced by isotonic saline injection as a mild stressor [56]; brain natriuretic peptide mRNA expression in the cultured ventricular cardiocytes was induced by endothelin-1 stimulation [57]. These results suggest that some peptide mRNA could be rapidly expressed after the stimuli as immediate early genes do. Generally, the amount of mRNA in the cell depends on both the rates of mRNA transcription in the nucleus and mRNA degradation in the cytoplasm [58]. Thus, the rapid increase in the AVPV/PeN *Kiss1* mRNA expression in the current study would be the results of an increase in transcription and/or decrease in degradation of the *Kiss1* mRNA. Future studies are required to clarify if the rapid increase in the AVPV/PeN *Kiss1* mRNA expression would be associated with an activation of the AVPV/PeN kisspeptin neurons.

In conclusion, the present results suggest that the increase in the number of *Kiss1* mRNA-expressing cells in the male AVPV/PeN induced by the mating stimulus may be involved in stimulating the HPG axis, and might consequently assure the mating behavior in male rats. The current study suggests that the AVPV/PeN kisspeptin neurons in males may play a physiological role in ensuring male reproductive performance.

Conflict of Interest: The authors have nothing to disclose.

Acknowledgments

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