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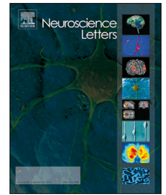
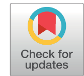


Research article

Conspecific odor exposure predominantly activates non-kisspeptin cells in the medial nucleus of the amygdala



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ABSTRACT

A small neuronal subpopulation in the medial nucleus of the amygdala (MeA), expressing the *Kiss1* gene, is now considered an important mediator that integrates socio-sexual behavior and odor information in order to modulate the Hypothalamic-Pituitary-Gonadal (HPG) axis. Previous studies demonstrated that exogenous kisspeptin administration or selective activation of *Kiss1*-expressing neurons in the MeA modulates the onset of puberty, LH secretion and sexual behavior. These functions are supported by the known MeA neuronal connections. In the MeA, as well as in the hypothalamus, *Kiss1* mRNA expression mostly depends on sex steroids levels. However, the percentage of *Kiss1*-expressing cells that co-express estrogen receptor α (ERα) in the MeA is currently unknown. Additionally, whether MeA kisspeptin neurons show Fos expression due to pheromone exposure is still undisclosed. In the present study, we used adult male and female mice that express a reporter protein under the *Kiss1* promoters to determine the percentage of *Kiss1*-expressing neurons that co-express the ERα in the MeA and, whether those cells are activated by olfactory cues. We found a high percentage of *Kiss1*-expressing neurons in the MeA co-expressing the ERα. The proportion of co-expression was similar between male and female mice in diestrus. Interestingly, a low percentage of *Kiss1*-expressing neurons in the MeA co-express Fos after conspecific odor exposure, despite a significant increase of Fos positive cells in the MeA. Additionally, odor exposition leads to a sexually dimorphic change in *Kiss1* expression in the posterior subdivision of the MeA. Our findings suggest that olfactory signals predominantly activate non-kisspeptin cells in the MeA to modulate responses to pheromones and therefore the HPG axis.

1. Introduction

The medial nucleus of the amygdala (MeA) is a sexually dimorphic area composed of different chemically defined neuronal populations, including some neurons that express the gene *Kiss1* [9,13,19,32]. The MeA expresses high levels of steroid receptors [26], and *Kiss1* mRNA expression mostly depends on sex steroid levels in this area [19,29]. *Kiss1* mRNA levels in the MeA increase as puberty approaches and sex steroid levels rises [29]. In adult rodents, MeA *Kiss1* expression is higher in males than in females at diestrus [19]. However, *Kiss1* mRNA levels increase during proestrus and *Kiss1* mRNA or kisspeptin protein content in the MeA is similar between adult male and female when estradiol (E2) levels are comparable [19,31]. In addition, the expression of the *Kiss1* gene in the MeA is specifically modulated by estrogen receptors (ERs) but not by androgen receptors [19]. Studies using ERs knockout mice further demonstrated that kisspeptin cells in the MeA are primarily regulated by estrogen receptor α (ERα), similar to what has been described for hypothalamic kisspeptin neurons [13,29]. However, although it is well known that virtually all hypothalamic kisspeptin neurons co-express the ERα [9,27], the percentage of *Kiss1*-expressing neurons in the MeA that contain ERα remains unexplored.

Recent studies have begun to uncover possible functions of kisspeptin in the amygdala. Exogenous kisspeptin administration into the MeA or activation of MeA *Kiss1* neurons modulates the onset of puberty, the secretion of luteinizing hormone (LH), and sexual behaviors [1,2,6,7,14,20]. In male rodents, MeA kisspeptin neurons maintain reciprocal connections with the accessory olfactory bulb, and with a subset (15%) of gonadotropin-releasing hormone (GnRH) neurons. This suggests that extrahypothalamic kisspeptin neurons contribute to the integration of socio-sexual behavior and odor information in order to modulate the HPG axis [1,2,22,32]. However, whether MeA kisspeptin neurons are activated by conspecific odor signals has not been

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investigated yet. In the present study, we used adult male and female mice that express a reporter protein under the *Kiss1* promoters to determine the percentage of *Kiss1*-expressing neurons that co-express the ERα in the MeA. We also investigated whether these MeA *Kiss1*-expressing neurons respond to olfactory/pheromone cues.

2. Materials and methods

2.1. Animals

The Kiss1-hrGFP strain (C57BL/6-Tg(Kiss1-hrGFP)KG26Cfe/J, Jackson Laboratories) was used for experiments. This mouse model allows the visualization of *Kiss1*-expressing cells through the hrGFP protein, whose expression is directly regulated by *Kiss1* promoters [8,9]. The mice were weaned at 3 weeks of age and genotyped via PCR using DNA extracted from the tail tip of the mice (REDExtract-N-Amp™ Tissue PCR Kit, Sigma). The Kiss1/hrGFP mice were housed in the animal care facility of the Department of Anatomy, Institute of Biomedical Sciences, University of São Paulo in an environment with controlled light (12 h on/12 h off; lights on at 6:00 am) and temperature (23 ± 2 °C). All experiments and procedures were performed in accordance with the guidelines established by the National Institute of Health's Guide for the Care and Use of Laboratory Animals and were approved by the institutional Animals Ethics Committee of the Institute of Biomedical Sciences, University of São Paulo.

2.2. ERα co-expression in hrGFP cells

Adult Kiss1-hrGFP male and female mice (14–16 weeks of age, n = 4/5 per group) were deeply anesthetized (14:00–15:00 p.m.) and perfused transcardially with saline followed by a 10% buffered formalin solution, pH 7.4. Tissue processing was performed as previous described previously [25]. Four series were collected and stored at −20 °C in cryoprotectant until processing for immunohistochemistry to detect ERα-immunoreactivity (ERα-ir) and anti-hrGFP immunoreactivity.

2.3. Pheromone exposure test

In order to investigate whether MeA *Kiss1* neurons express Fos-immunoreactivity (Fos-ir) in response to male or female odors, mice were separated into four groups (n = 4/5 per group): males exposed to fresh bedding; males exposed to female odor; females exposed to fresh bedding and females exposed to male odor. Adult female mice were selected after daily observation of vaginal smears and confirmation of a regular estrous cycle. Females were selected for experiments at the diestrus phase. To avoid interference of behavioral experience in our experiments [11,18,21], we used adult sexually naïve mice (14–16 weeks of age). Male or female mice were moved to a clean-bedding cage daily, between 12:00 and 13:00 p.m, for 90 min. This routine was repeated for 15 days. On day 16, males exposed to female odor were moved to a soiled-bedding cage where a normally cycling female had been housed individually for 15 days. On day 16 or until next diestrus phase, females exposed to male odor were moved to a soiled-bedding cage where a male had been housed individually for 15 days. Males and females exposed to fresh bedding were again moved to a clean-bedding cage before perfusion. Ninety minutes after exposure or non-exposure to odors, animals were deeply anesthetized and perfused transcardially with saline followed by a 10% buffered formalin solution, pH 7.4. Tissue processing was performed as described previously [25].

2.4. Immunohistochemistry

Brain sections were rinsed in 0.02 M potassium PBS (KPBS), pH 7.4, followed by a pretreatment with 0.3% hydrogen peroxide for 30 min. After rinses in KPBS, sections were blocked in 3% normal donkey serum for 1 h, followed by incubation in anti-Fos antibody (1:20.000, Millipore) or ERα-antibody (1:100.000, Millipore) for 24 h. Subsequently, sections were incubated with biotin-conjugated donkey anti-rabbit IgG (1:1000, Jackson Laboratories) for 1 h followed by an avidin–biotin complex (1:500, Vector Labs, Burlingame) for 1 h. The peroxidase reaction was performed using 0.05% 3,3′-diaminobenzidine (DAB), 0.25% nickel sulfate and 0.03% hydrogen peroxide. Sections were then incubated overnight with the primary antibody anti-hrGFP (1:2000, Agilent Technologies). Sections were then incubated with biotin-conjugated donkey anti-rabbit (1:1000, Jackson Laboratories), followed by an avidin-biotin complex and DAB as chromogen. Sections were finally mounted onto gelatin-coated slides and coverslipped using DPX mounting medium.

2.5. Image analysis

One representative rostrocaudal level of each subdivision of the MeA, anterior (MeAa) e posterior (MeAp), was used to perform the quantification. The approximate bregma coordinates of each rostrocaudal level (MeAa; bregma = −1.06 mm; MeAp; bregma = −1.58 mm) were obtained according to the mouse brain atlas [12]. Since MeA is composed by paired symmetrical nuclei only one side of each brain section was used for quantification. The percentage of double-labeled neurons (hrGFP/ERα or hrGFP/Fos) was determined by the average number of neurons in each subdivision of the MeA. Brain sections were analyzed using a Zeiss Axioimager A1 microscope (Zeiss, Germany) and photomicrographs were captured using a Zeiss Axiocam HRc camera (Zeiss). Image processing and lettering was carried out with the Photoshop software (Adobe Systems Inc., Mountain View, CA).

2.6. Statistical analysis

Differences between males and females in the average number of hrGFP neurons or average percentage of hrGFP neurons that co-express ERα were compared using the unpaired two-tailed Student's *t*-test. To analyze the effects of gender and/or pheromone, the two-way ANOVA was employed. GraphPad Prism software (La Jolla, CA, USA) was used for the statistical analyses and results were expressed as mean ± SEM. Only p values < 0.05 were considered to be statistically significant.

3. Results

3.1. high percentage of Kiss1-expressing neurons in the MeA co-express the ERα

Previous studies revealed that kisspeptin neurons in the MeA are sensitive to changes in estrogen levels [19,30]. However, it remains to be determined whether MeA kisspeptin neurons indeed express ERα. Using a reporter mouse that allows the visualization of *Kiss1*-expressing cells through the expression of hrGFP protein [8,9], we first determined the number of hrGFP cells in the current model. We observed that the average number of hrGFP cells was similar in the MeAa comparing data obtained between genders (Fig. 1A; $P = 0.5$). However, as previously reported [19], the average number of hrGFP cells in the MeAp were significantly higher in males than in female mice on diestrus (Fig. 1B; $P = 0.02$). Next, we analyzed the average percentage of hrGFP neurons in the MeA that co-express ERα (Figs. 1C, D and 2). We observed that approximately 49% of the hrGFP-positive neurons in males, and 40% of hrGFP cells in female mice co-expressed ERα in the MeAa (Fig. 1C; $P = 0.5$). In the MeAp, 41% of hrGFP neurons in males and 59% of neurons in females co-expressed ERα (Fig. 1D; $P = 0.5$). Thus, the average percentage of MeA *Kiss1*-expressing neurons co-expressing ERα is similar between gonadally-intact male mice and female mice in diestrus (Figs. 1C, D and 2).

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