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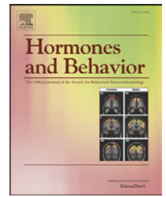

Effects of aromatase mutation (ArKO) on the sexual differentiation of kisspeptin neuronal numbers and their activation by same versus opposite sex urinary pheromones

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ABSTRACT

Pheromones have been shown to induce sexually dimorphic responses in LH secretion. Here we asked whether the sexually dimorphic population of kisspeptin neurons in the rostral periventricular area of the third ventricle (RP3V) could relay sexually dimorphic information from the olfactory systems to the GnRH system. Furthermore, we analyzed the effects of aromatase mutation (ArKO) and thus the role of estradiol on RP3V kisspeptin neuronal numbers and on the response of these kisspeptin neurons to same- versus opposite-sex urinary pheromones. Exposure to male but not female urinary odors induced Fos protein in kisspeptin neurons in the RP3V of female wildtype (WT) mice, suggesting that these kisspeptin neurons may be part of the neural circuitry that relays information from the olfactory brain to the GnRH system in a sexually dimorphic manner. Male pheromones induced Fos in kisspeptin neurons in ArKO females, albeit significantly less compared to WT females. The sexual differentiation of kisspeptin neuronal number was lost in ArKO mice, i.e. the number of kisspeptin-immunoreactive neurons in the RP3V of ArKO females was as low as in male mice, whereas male ArKO mice had somewhat increased numbers of kisspeptin neurons. These results suggest that the sex difference in kisspeptin neuronal number in WT mice reflects an organizational action of estradiol in females. By contrast, the ability of male urinary pheromones to activate kisspeptin neurons in WT females may not depend on the organizational action of estradiol since ArKO females still showed some Fos/kisspeptin co-activation.

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Introduction

Pheromones affect several aspects of reproduction in mice, including the induction of sexual maturation (Lombardi and Vandenbergh, 1977), estrus synchronization (Whitten, 1959), and the initiation of a pregnancy block (Bruce, 1959; Lloyd-Thomas and Keverne, 1982). Indeed, it has been shown that pheromones activate olfactory structures, i.e., induce the expression of Fos protein, such as the peripheral vomeronasal organ (VNO) or the accessory olfactory bulb (AOB), and downstream target-sites (Pankevich et al., 2006; Kang et al., 2006; Dudley et al., 2001). Recently, two research groups have used different tracing approaches to show that the main olfactory epithelium and main olfactory bulb (MOB) project to and activate GnRH neurons (Boehm et al., 2005; Yoon et al., 2005). Boehm et al (2005) also showed that some of these neural projections are sexually dimorphic, such as the ones including the VMHvl and the PMV (ventrolateral part of the ventromedial hypothalamic nucleus and ventral part of the premammillary nucleus, respectively), brain areas that are important for the expression of sexual behavior (Meisel and Sachs, 1994; Pfaff et al., 1994; Simerly, 2002). By contrast, no evidence of sexually dimorphic projections was found between the olfactory bulb and the GnRH population in the preoptic area. This is remarkable since only pheromones from opposite sex conspecifics induce sexually dimorphic responses in GnRH neurons and consequently LH secretion. For instance, pheromones derived from female mice significantly increased the percentage of activated GnRH neurons (Yoon et al., 2005) and induced a rapid and large increase in LH release in males (Coquelin and Bronson, 1979; Bronson and Desjardins, 1982) whereas pheromones derived from male mice induced LH release in female mice (Bronson and Maruniak, 1976). So at present it remains unclear how pheromones induce a sexually dimorphic activation of the GnRH system and consequently LH release.

In recent years, the *Kiss1* gene product, kisspeptin, has been proposed as an upstream regulator of the GnRH system as human patients did not enter into puberty due to a mutation in the *GPR54* gene (de Roux et al., 2003), which encodes the kisspeptin receptor, named GPR54 (now named Kiss1r). Similar results were obtained in mice with a targeted disruption of the *Kiss1* gene (d'Anglemont et al., 2007). Furthermore, kisspeptin is involved in integrating the positive feedback action of estrogens on the GnRH system in female mammals (Estrada et al., 2006; Adachi et al., 2007), whereas in male mammals,

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an injection of kisspeptin in discrete regions of the hypothalamus induced the release of LH (Patterson et al., 2006). Interestingly, kisspeptin expression is sexually dimorphic in rodent species, with females having a larger population of kisspeptin expressing neurons than males in the rostral periventricular area of the third ventricle (RP3V) (Clarkson and Herbison, 2006; Kauffman et al., 2007), suggesting that kisspeptin may play a sexually dimorphic role in controlling reproductive events. Due to this neural sexual differentiation and dimorphism, kisspeptin neurons in the RP3V could be part of the neural circuitry that relays sexually dimorphic information from the olfactory systems to the hypothalamic GnRH neurons. Therefore, in the present study, we determined whether pheromones are able to activate kisspeptin neurons in the RP3V of male and female mice in a sexually dimorphic manner. We used aromatase knockout (ArKO) mouse mice to determine whether these sexually dimorphic responses of kisspeptin neurons to pheromones reflect the developmental actions of estrogens in the central nervous system by comparing the number of kisspeptin neurons as well as its activation by pheromones in wild-type (WT) and ArKO mice of both sexes. ArKO mice have a targeted mutation in the *Cyp19* gene that encodes the aromatase enzyme which catalyzes the final step in the biosynthesis of C_{18} estrogens from C_{19} steroids, indicating thus a complete absence of estrogens during embryonic and postnatal development. We have observed several changes in olfactory functioning in ArKO mice. For instance, male ArKO mice resembled WT females in their ability to respond to lower concentrations of urinary odors than male WT mice, suggesting that the observed sex difference among WT mice in urine attraction thresholds (Baum and Keverne, 2002) results from the perinatal actions of estrogens in the male nervous system (Pierman et al., 2006). By contrast, the processing of sexually relevant odors by the olfactory bulbs was not affected in ArKO mice of both sexes (Pierman et al., 2008). These results indicate that estrogens may be important for the integration of odors into the reproductive system and thus may be involved in the organization and activation of sexually dimorphic responses of the hypothalamic GnRH system to pheromones. All subjects were treated with estradiol benzoate in adulthood so that any differences between ArKO and WT mice in kisspeptin neuronal number or activation reflect a difference in estradiol signaling earlier in life.

Materials and methods

Animals

In the present study we used aromatase knock-out mice with a targeted disruption of exons 1 and 2 of the *Cyp 19* gene (Honda et al., 1998). Heterozygous (HET) males and females of the *C57BL/6J* strain were bred to generate wild-type (WT) and knock-out (ArKO) offspring. Mice were genotyped by PCR analysis of tail DNA (Bakker et al., 2002a). All breeding and genotyping were performed at the GIGA Neurosciences (formerly known as Center for Cellular & Molecular Neurobiology), University of Liège, Belgium. Food and water were always available *ad libitum* and the temperature was maintained at 22 °C.

Adult WT and ArKO mice of both sexes were gonadectomized under general anesthesia through an intraperitoneal injection of a mixture of ketamine (80 mg/kg per mouse) and medetomidine (Domitor, Pfizer, 1 mg/kg per mouse). Mice received atipamezole (Antisedan, Pfizer, 4 mg/kg per mouse, subcutaneously) at the end of surgery in order to antagonize medetomidine-induced effects, thereby accelerating their recovery. Then mice were placed in individual cages and treated daily with estradiol benzoate (EB, 5 μg in 0.05 ml sesame oil/mouse) by subcutaneous injection in the neck at least 2 weeks before being exposed to odors. Males and females were housed in two separate rooms under a reversed light-dark cycle (12:12 light/dark). Subjects were gonadectomized and subsequently treated with estradiol in order to study the organizational and activational effects of estrogens on kisspeptin neurons. As previously shown by Fisher et al (1998), gonadally intact ArKO mice of both sexes lack detectable levels of estrogens as to be expected, but also show increased levels of testosterone, androstenedione, FSH and LH compared to WT animals. Therefore, to avoid confounding effects of these other hormones on kisspeptin expression in ArKO mice, all animals received the same estradiol treatment since we are primarily interested in the organizational role of this hormone. In that respect the ArKO mouse is unique in that they can be treated with estradiol at any time during their lifespan since they have functional estrogen receptors and thus this model can be used to distinguish between organizational and activational effects of estradiol on the brain. Finally, this estradiol treatment has shown to be effective to study the central processing of sexually relevant olfactory cues in mice (Pierman et al., 2008). All experiments were conducted in accordance with the guidelines set forth by the National Institutes of *Health Guiding Principles for the Care and Use of Research Animals* and were approved by the Ethical Committee for Animal Use of the University of Liège.

Urine collection

In the present study, we determined the ability of urinary odors that were applied directly onto their nose to activate kisspeptin neurons. We chose to apply the urine directly onto the nose instead of giving free access to the urine since we wanted to be sure that there would be no differences in activation due to differences in time spent investigating the urinary odors, since our previous studies (Bakker et al., 2002a,b; Pierman et al., 2006) showed that ArKO mice are less motivated to investigate sexually-relevant (opposite-sex) urinary odors. Furthermore, it has been shown that applying urine onto the nose directly activates both the main and the accessory olfactory system (Dudley et al., 2001; Kang et al., 2006; Pankevich et al., 2006) and by doing so we maximized the likelihood that any effect of the urinary pheromones on kisspeptin neurons would be seen.

Thus, urine was collected from 10 gonadally intact *C57BL/6J* males. Estrous female urine was collected from 10 ovariectomized *C57BL/6J* females which had an estradiol implant (diluted 1:1 with cholesterol; Bakker et al., 2002b) and which were injected with 500 μg progesterone (P) 2–4 h prior to urine sampling. Urine was collected by holding the mouse by the scruff of the neck over a funnel, taking care that no fecal contamination of the urine occurred. In order to collect the urine, animals were habituated to be handled and urine was collected at different days during several weeks, but always at the same time during the day (early afternoon). Same urine stimulus samples were pooled and subsequently aliquoted in 500-μL Eppendorf vials and stored at −80 °C until use.

Procedure for exposure to urine

After 2 weeks of EB treatment with a daily injection of 5 μg (dissolved in sesame oil), mice were trained daily for 1 week during the first hour of the dark phase of the light/dark cycle to the procedure used for urine exposure while continuing to receive EB treatment. Thus subjects were habituated to be taken out of their home cage and to receive 30 μl of deionized water onto their nose before being placed back in their cage.

On the day of testing, subjects were divided into three different groups depending on the urinary odor that they were going to be exposed to. Group 1 was exposed to intact male urine (WT: 6 males and 6 females; ArKO: 6 females and 9 males), group 2 to estrous female urine (WT: 8 males and 6 females; ArKO: 6 of each sex) and group 3 was exposed to deionized water to serve as control (WT: 6 males and 8 females; ArKO: 6 of each sex). All animals were exposed to the odor stimulus during the first 3 h of the dark phase of the light/dark cycle.

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