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Physiology & Behavior 88 (2006) 160–166

PHYSIOLOGY & BEHAVIOR

Female odors stimulate CART neurons in the ventral premammillary nucleus of male rats

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Received 19 December 2005; received in revised form 23 March 2006; accepted 29 March 2006

Abstract

Olfactory information is known to influence both male and female sexual behavior. Chemosensory compounds known as pheromones activate distinct brain pathways, inducing innate and stereotyped behaviors, as well as hormonal changes. Studies have shown that female odors induce Fos expression in various brain nuclei of conspecific males, including the ventral premammillary nucleus (PMV). Although poorly investigated, previous studies have suggested that the PMV plays a role in aggressive and sexual behavior. In this study, we used Fos protein expression as a marker for neurons responsive to female odors in sexual inexperienced male rats exposed to soiled bedding. We observed that female odors induced intense Fos immunoreactivity throughout the PMV. Most of these neurons also express cocaine- and amphetamine-regulated transcript (CART) immunoreactivity. In addition, we used in situ hybridization and observed that, following exposure to female odors, CART mRNA increased only in the ventral PMV. Our results suggest that female odors stimulate CART production in the PMV of inexperienced males. Considering that the PMV CART neurons also express the leptin receptor, as well as the fact that they project to areas related to reproduction, we hypothesize that PMV CART neurons integrate nutritional and environmental (olfactory) information, being apt to modulate male reproductive behavior.

Keywords: Reproduction; Reproductive behavior; Pheromone; Vomeronasal system

1. Introduction

Olfactory information is known to influence male and female sexual behavior. Chemosensory compounds known as pheromones induce hormonal changes and behavioral responses through the activation of related neural pathways [1]. It is widely accepted that pheromones gain access to the brain via the vomeronasal organ, which sends input to the accessory olfactory bulb. From there, most olfactory information arrives at the medial nucleus of the amygdala (MeA), which in turn innervates hypothalamic nuclei such as the medial preoptic nucleus, the ventromedial nucleus and the ventral premammillary nucleus (PMV), all related to behavioral adjustments and hormonal secretion [1–3].

In response to female pheromones, males of different species present increased circulating levels of luteinizing hormone (LH) and testosterone [4–8]. These responses rely on the integrity of the circuitry. Therefore, sexually inexperienced male hamsters with vomeronasal organ lesion exposed to female pheromones exhibit reduced copulatory behavior and lower testosterone levels [9]. In addition, females with vomeronasal organ lesion show decreased receptivity and reduced LH secretion in response to male exposure, both of which are completely restored by administration of estrogen and GnRH [10]. These findings suggest that olfactory information and hormonal signaling are fundamental to normal sexual behavior [11].

In the pheromone pathway, the MeA is the first nucleus in which both pieces of information converge [12]. It contains a dense collection of gonadal steroid receptors and, in castrated males, steroid implantation in the MeA restores sexual behavior [13–15]. In females, MeA electrical stimulation precipitates LH

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doi:10.1016/j.physbeh.2006.03.032

secretion on the proestrous day [16], a response that is blocked by PMV lesion [17]. Both the MeA and the PMV express Fos protein in different paradigms of conspecific olfactory stimulation and are therefore engaged in pheromone-triggered arousal [18–21]. However, although the role played by the MeA in chemosensory and hormonal integration has been well described, little is known regarding that played by the PMV.

Neurons in the PMV express cocaine- and amphetamine-regulated transcript (CART) and are the main source of CART-immunoreactive fibers observed in areas related to reproductive control [22,23]. In addition, PMV CART neurons respond to circulating leptin, a hormone secreted by white adipose tissue that signals the brain the amount of energy stored [24–28]. Lack of leptin (*ob/ob* mice) causes morbid obesity, hypogonadism and infertility, but the neural pathways engaged by leptin involved in reproductive control are not well known [29,30]. Therefore, in the present study, we attempted to examine the participation of PMV CART neurons in female odor responses in order to determine whether such responses play a role in modulating male reproductive behavior.

2. Materials and methods

2.1. Animals

Normal adult Wistar rats, male and female, were housed one or two per cage in the animal care facility of our institution. Animals were maintained on a 12-h light/dark cycle (lights on at 7 a.m.) in a temperature-controlled environment (21±2 °C) and were given free access to food and water. All experiments were carried out in accordance with the guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996) and by the University of São Paulo Committee for Research and Animal Care. We attempted to minimize the number of rats used and every effort was made to ensure that no rat suffered unnecessarily.

2.2. Female odor test

In order to identify neurons responsive to female odors, we used twelve 75-day-old, sexually inexperienced adult male rats, separated into two groups: control (CT, $n=6$) and exposed to female odors (FO, $n=6$). All animals were housed individually in an isolated room. Between 4 p.m. and 5 p.m. on each day that preceded the day of the experiment (6 days total), animals were moved to clean cages with fresh bedding. This was done in order to reduce stress on the day of the experiment. On the seventh day, animals in the FO group were moved to cages with soiled bedding where normally cycling females had been housed (individually) for at least 5 days. Animals in the CT group were moved again to clean cages with fresh bedding. After 90 to 110 min, animals in both groups were perfused as described in the text that follows.

Animals were deeply anesthetized with intraperitoneal injection of 35% chloral hydrate (1 ml) and perfused transcardially with saline followed by 4% paraformaldehyde in borate-buffer (pH 9.5 at 4 °C, 900 ml over 25 min). Brains were removed, postfixed in the same fixative for 2 h and cryoprotected overnight at 4 °C in 0.1 M phosphate-buffered saline (PBS), pH 7.4, containing 30% sucrose prepared with diethyl pyrocarbonate (DEPC)-treated water. The brains were cut in the frontal plane into 30-μm sections on a freezing microtome. Five series were collected in antifreeze solution and stored at −20 °C.

One series from each animal was submitted to a standard immunoperoxidase reaction. Sections were pretreated with hydrogen peroxide and blocked in 2% normal donkey serum (Jackson Laboratories, West Grove, PA, USA) and 0.3% Triton X-100 (Sigma, St. Louis, MO, USA). The sections were then incubated in anti-Fos polyclonal primary antisera raised in rabbit (1:20,000; Ab5, Oncogene, Boston, MA, USA) overnight at room temperature. This was followed by incubation for 1 h in biotin-conjugated donkey anti-rabbit IgG (1:1000, Jackson Laboratories) and for 1 h in avidin–biotin complex (1:500, Vector Labs, Burlingame, CA, USA). The tissue was then submitted to immunoperoxidase reaction using 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) and nickel sulfate (Fisher Scientific, Pittsburgh, PA, USA) as chromogens and 0.03% hydrogen peroxide in 0.02 M potassium PBS (KPBS), pH 7.4, for 2–3 min. The reaction was terminated with rinses in KPBS. Sections were mounted onto gelatin-coated slides, dehydrated, delipidated and coverslipped with DPX mountant (BDH, Poole, England). The antisera specificity and adsorption control tests had been performed in advance [31]. One series from each animal was submitted to thionin staining (Nissl method) and used for cytoarchitectonic reference.

The forebrains of animals from both groups were analyzed and neurons expressing Fos immunoreactivity in the PMV were quantified. Cells were counted using a constant area and a scale grid positioned under a 10× or 20× objective. The position of the counting grid within each nucleus was defined through adjacent points of reference. We counted three PMV levels according to the distribution of responsive neurons and the extension of the nucleus, subdividing the PMV into dorsal, ventral and caudal components. Student's *t*-test was used to compare differences between groups (FO/CT, $p<0.05$).

2.3. Dual-label immunoperoxidase

In order to investigate whether CART neurons are involved in female odor responses, PMV sections from the FO and CT group animals were incubated in antisera against Fos (1:20,000) using DAB and nickel sulfate as chromogens. Sections were then submitted to an additional immunoperoxidase procedure with CART peptide antisera (1:10,000, rabbit anti-rat amino-acid sequence 55–102; Phoenix Pharmaceuticals, Belmont, CA, USA) as the second primary, using only DAB as chromogen, producing light brown cytoplasmic staining. Since both the primary antisera used were raised in rabbit, we then performed a control reaction that omitted the second primary antisera. The reaction was stopped with rinses in KPBS. Sections were mounted onto gelatin-coated slides, dried overnight, dehydrated in ethanol, cleared in xylene and coverslipped with DPX. Dual-labeled neurons were counted and percentages of the means (±S.E.M.) were estimated.