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Protein-bound Male Urinary Pheromones: Differential Responses According to Age and Gender

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Abstract

The attractive properties of male urinary pheromones were tested on adult or prepubertal male and female mice. An androgen-dependent protein is present in adult male urine (major urinary protein, MUP) which has been suggested to be a pheromone-binding protein. We tested the pheromonal properties of the protein-bound volatiles in a test of attractiveness. These molecules, that co-purify with MUP, attract females and repel adult males. In prepubertal animals, females are repelled and males are attracted by the same stimuli. These results are similar to those obtained by others with adult male whole urine. Therefore MUP binds molecules with a pheromonal activity, and these molecules are sufficient to act as male signals.

Introduction

Pheromones are relevant signals in the ecology of nocturnal animals. In mice, the major source of pheromones is urine, which contains pheromones that indicate the reproductive and social status of the emitter (Keverne, 1983). Male mice usually mark their territory with urine, and urination is a social response to the presence of conspecifics (Reynolds, 1971). In males, a high level of circulating androgens results in the excretion of particular substances, e.g. major urinary protein (MUP) (Hastie *et al.*, 1979; Clissold *et al.*, 1984). MUP is a member of the lipocalin superfamily of proteins (Böcskei *et al.*, 1992) that are characterized by a double binding domain: one for a receptor and one for a ligand. In MUP there is a hydrophobic cavity that binds odorant molecules (Bacchini *et al.*, 1992). This binding is strong enough to resist several purification steps. Competitive displacement by an higher affinity ligand can remove the natural ligands from MUP (Cavaggioni *et al.*, 1990). The ligands in urine are continuously released from MUP as soon as the free ligand concentration decreases.

MUP accelerates the onset of puberty in female mice, while its ligands are not involved (Mucignat-Caretta *et al.*, 1995). On the other hand, male urinary odors induce strong behavioral reactions in conspecifics. Male urine elicits aggressive behaviors from other males (Mugford and Nowell, 1970), while adult females usually prefer male urine over a variety of stimuli (Coppola and O'Connell, 1988; Drickamer, 1989; Mossman and Drickamer, 1996). The most effective androgen-dependent stimuli are derived from highly aggressive, dominant males, since a submissive status is accompanied by a fall in gonadal functions, and therefore in a diminished excretion of male pheromones (Sandnabba, 1986).

The possible behavioral effects on conspecifics of the mixture of substances bound to MUP were never directly tested. It is possible that these odor molecules play a role in cueing the presence of a male mouse. A simple test of attractiveness involves the measure of the ratio of the time spent in the proximity of a stimulus over the time spent in the proximity of a blank (Mossman and Drickamer, 1996): the higher the ratio, the higher the preference for the stimulus. Should the presence of odorants bound by MUP modify this ratio, we could demonstrate their role as attractants or repellents, possibly involved in social communication. On the contrary, we could only regard these molecules as a random mixture of odors with no biological relevance. This work aims at investigating the role of MUP-bound substances as male chemosignals in modifying the behavior of male and female mice, at both adult and prepubertal ages.

Materials and methods

Animals

Swiss mice were reared under controlled conditions (light on from 6:00 to 18:00 h, temperature 24 ± 1°C) and fed *ad libitum* with mouse diet chows (Mucedola, Milan, Italy) and water. The litters were reared in plastic cages with both parents and weaned at 21 days of age. From this date onward, mice lived in groups of 4–5 animals, of the same

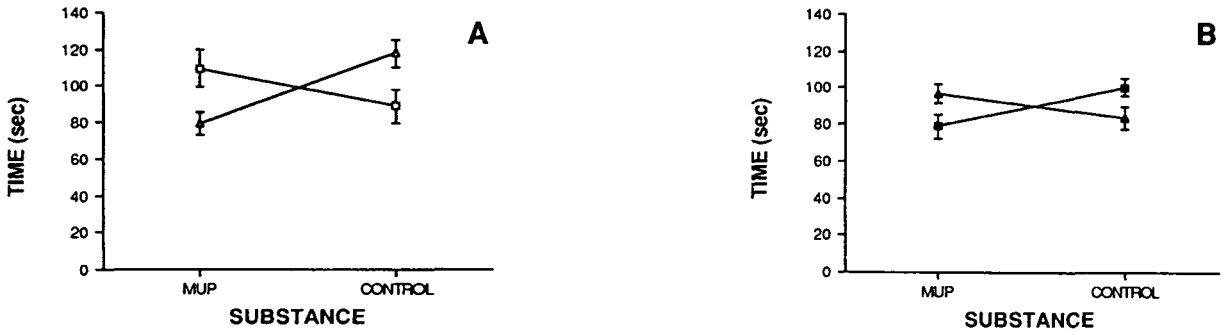

Figure 1 Time spent by each group of mice near or far the MUP area. **(A)** Means (± SE) of time spent by adult males (open triangles) and females (open squares) in the portion of the cage which contained the MUP-bound substances or in the control area. **(B)** Means (± SE) of time spent by prepubertal males (filled triangles) and females (filled squares) in the portion of the cage which contained the MUP-bound substances or in the control area.

sex and age. Prepubertal animals were tested at the age of 26–28 days and adult animals at the age of 2 months (55–65 days). All mice were naive with respect to sexual experience.

Protein purification

MUP was purified according to Cavaggioni *et al.* (1990), with some minor modifications. Briefly, groups of 20 adult male Swiss mice (3–5 months old) served as donors. Urine was collected overnight in metabolic cages, pooled, frozen and stored at –20°C. After thawing and centrifugation, urine was paper filtered to remove the largest aggregates and concentrated by pressure on semipermeable membranes (Amicon YM10, Milan, Italy; mol. wt cut-off 10 000 Da). The highest molecular weight fraction was chromatographed through a molecular sieve column (G50, Pharmacia, Milan, Italy). The fractions containing the protein were pooled, concentrated as above and re-chromatographed in the same way. The homogeneity of the protein was verified with polyacrylamide gel electrophoresis. A single band (apparent mol. wt 18 kDa) could be detected after Coomassie blue staining.

Behavioral assay

The experimental procedures conformed to the Italian law on animal experiments and were approved by the competent authority. Four groups of nine mice each were employed: prepubertal males, prepubertal females, adult males and adult females. Mice were tested basically according to Mossman and Drickamer (1996), with some adaptations. The test cage consisted of a plastic box (22 × 44 × 21 cm) with two small plastic vials (1.5 cm diameter, 0.5 cm height) sealed in the middle of the shorter side, at 3 cm from the edge. The vials contained MUP (10 mg) or the same volume (200 µl) of water. The side of presentation of the substances was randomized.

Each mouse was put in the center of the cage, and its behavior was recorded for five min with a videocamera (Panasonic, maximum sensitivity 0.5 lux). The light was similar to that normally turned on in the animal room, and therefore thought not to disturb the animals, presumed to be accustomed to it.

Data analysis

After the data were collected, an experimenter blind to the experimental conditions recorded the time spent by each animal in each side of the cage. The cage was divided in three zones: at the far ends, two rectangles (22 × 11 cm) defined the zones which contained the stimuli, while the central zone of the cage (22 × 22 cm) was the zone not scored. The ratio of the time spent by each animal in the MUP-containing area over the time spent in the control zone was calculated and transformed logarithmically (Mossman and Drickamer, 1996).

The transformed data were submitted to a between-subjects ANOVA for the factors Age (adult versus prepubertal) and gender (females versus males). Whenever appropriate, post-hoc analysis was performed (Duncan LSD or Newman–Keuls test).

Results

The mean times (± SE) spent in each area of the cage (in the MUP-containing area or in the control area) are shown in Figure 1 for the different groups (adult males and females, Figure 1A; prepubertal males and females, Figure 1B). ANOVA conducted on the logarithms of ratios (time near MUP/time near control) revealed no statistical difference for the factors Age and Gender. The interaction Age × Gender reached statistical significance: $F(1,12) = 12.729$, $P < 0.002$. Post-hoc analysis revealed that for both sexes, means from adult data are different from those of prepubertal animals ($P = 0.02$ for females and $P = 0.01$ for males), indicating that adult females spend more time in the MUP area than prepubertal females, and adult males spend less time in the MUP area than prepubertal males. In adults, males data are different from females ($P = 0.004$), with females spending more time than adult males near MUP. A strong tendence to

significance ($P = 0.055$) is observed in prepubertal animals, but in this group the males spend more time near MUP than the females.

Discussion

The main goal of the present experiments is to demonstrate that the volatile molecules bound by MUP act as male pheromones. The proteic fraction of adult male urine (Vandenbergh *et al.*, 1975) or MUP alone, with no ligands bound, induce an acceleration of puberty onset in female mice (Mucignat-Caretta *et al.*, 1995). Since the protein alone is sufficient to elicit this effect, a question arises on the possible role of the ligands bound by the protein: are they mere catabolites, collected by MUP in the bloodstream, with no significance for the recipient mouse, or do they possess a meaning for the conspecifics that smell urine? In this case, the structure of MUP, with an inner hydrophobic binding site, is functional to the transport of relevant molecules, and therefore MUP and its ligands are the major components responsible for the pheromonal effects of male urine. Although MUP has been suggested to be a pheromone-binding protein (Bacchini *et al.*, 1992; Böcskei *et al.*, 1992), the role of MUP natural ligands was never directly tested.

The present results, obtained with MUP-bound odorants, closely match those obtained using whole adult male urine samples as stimuli. Female mice spend more time investigating urine from adult intact males than urine from castrated males (Scott and Pfaff, 1970). Females are attracted by male urine (Jones and Nowell, 1974a), in particular if the donor male is aggressive ('dominant') and resident (Sandnabba, 1986; Hurst, 1990b). Adult females exhibit a preference for adult male odors when compared with juvenile odors, and this preference is present in both estrous and nonestrous females (Mossman and Drickamer, 1996). In prepubertal age, male signals are less attractive to females than females odors, while the opposite is found in adult animals (Coppola and O'Connell, 1988). Similar findings obtained in different tests are described by Drickamer (1989), who indicates that while prepubertal females avoided male olfactory cues, by about the time of puberty this preference is reversed, and females start to prefer the male cues. As adults, this preference is even stronger. The opposite behavior is shown towards female odors. All these data are consistent with the view of the male-associated olfactory cues as ecologically relevant, since they signal the presence of a possible mate.

The stimuli from male urine advertise the sex and social status of a male to other males (Hurst, 1990a). Male urine decreases investigatory behavior in males (Jones and Nowell, 1973). The urine of adult males elicits aggressive behaviors in other males, in particular when the donor mouse is dominant. Urine from submissive males is effective to a lesser extent and urine from castrated males has no effect (Mugford and Nowell, 1970). These results have been confirmed by tests in which urine from singly caged or dominant males is aversive, while the urine of cohabiting subordinate males is not (Rawleigh *et al.*, 1993; Jones and Nowell, 1989). In line with these findings are the results of androgen treatment on urine aversive potency: the higher the testosterone levels, the higher the aversive efficacy on males (Jones and Nowell, 1974b). In male urine there are several substances that are androgen-dependent. Some of them are hydrophobic volatile odorants, such as 2-sec-butylthiazoline and dehydro-exo-brevicomin (Schwende *et al.*, 1986), and under some conditions act together to elicit aggressive behaviors (Novotny *et al.*, 1985). Also the production and excretion of MUP is androgen-dependent (Clissold *et al.*, 1984). Noteworthy, the androgen-dependent volatiles are naturally bound by MUP (Bacchini *et al.*, 1992). Due to their relative hydrophobicity and binding constants, these substances in urine are almost all bound by MUP (Bacchini *et al.*, 1992). Since MUP is excreted in concentrations up to 10^{-3} M, it concentrates its hydrophobic ligands in urine, so that a larger amount of such substances can be excreted. This is a noticeable effect, due to the small size of mice and the comparably small amount of urine excreted per day: since more pheromones are concentrated in a small volume, a smaller amount of urine is needed to mark the territory. The binding of volatiles to MUP could also modify the release of these molecules to the air, a possibility currently under study.

The present data indicate that the subset of odorants bound by MUP is sufficient to induce behavioral modifications similar to those elicited by adult male whole urine. The changes consist mainly of the attraction of adult females and aversion for adult males, and the opposite pattern for prepubertal animals. The olfactory image represented by the MUP-bound odorants is therefore interpreted differently by the conspecifics, according to their age and gender. The present data support the idea that MUP also acts as a pheromone-binding (and releasing) protein.

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