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The advertisement role of major urinary proteins in mice

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Abstract

The variation of expression of major urinary proteins was studied in laboratory mice to further the understanding of the role of these proteins in social and reproductive contexts. Mouse major urinary proteins (MUPs) are known to carry volatile substances and protect them during their passage from the liver, through the kidneys into the urine. However, most studies on the role of MUPs were carried out on males. Using densitometry analysis of total MUP concentration in the urine, our present study clearly demonstrates that (i) individuals of both sex up-regulate MUPs during social contact, and that (ii) females use these proteins to advertise their reproductive state by varying the concentration of MUPs during the oestrous cycle. As the concentration of MUPs was normalized by the concentration of creatinine – a marker of glomerular filtration – the corrected concentration of MUPs represents instantaneous expression on the level of proteins. Cross-correlation analysis between oestrus quantification and MUP expression revealed that the oestrous curve is delayed by 1 day behind the MUP curve so that the expression of MUPs is up-regulated immediately at the beginning of oestrus. To conclude, the regulation of pheromone-carrying MUPs is directly linked to reproduction and, thus, enables female honest signalling.

Keywords: Oestrus; Oestrous cycle; Pheromone-carrying proteins; MUP; Major urinary protein; Mouse; Mice

1. Introduction

Pheromones are substances that trigger various physiological responses and are specific for each (signalling and/or receiving) sex. For example, the Whitten effect is a temporal change of female reproductive physiology that is switched on and off in response to the presence or absence of individual males or just their bedding material. This socially dependent mechanism has largely been demonstrated on the level of oestrus induction. Since the original discovery of this phenomenon [1] various authors have provided evidence that there exist specific oestrus-inducing pheromones and that these pheromones may have a different effect upon individuals of the same or the opposite sex. Female pheromones are involved in the suppression of oestrus and prolongation of the oestrous cycle is a typical response of females that are housed together [2,3]. This Lee-Boot effect has been described in various species of mice of the genera *Mus* and *Apodemus* [4–6]. However, there is scarce scientific evidence on what female pheromones trigger in males and how.

The Whitten and Lee-Boot effects may also be viewed as two aspects of one phenomenon. Stopka and Macdonald [5] presented evidence that both effects can be demonstrated in one species and one experiment with different social setups or in varying social contexts. For example, female wood mice (*Apodemus sylvaticus*) shorten their oestrous cycle and prolong oestrus when housed with males (separated by a metal grid), but prolong the cycle and shorten the oestrus when caged alone and even more so when caged with other females. According to their study social modulation of the oestrous cycle has further implications regarding mating systems. In this promiscuous species [7] daily alternations of unfamiliar males behind the grid stimulated an accelerated onset of oestrus that was even more prolonged while the oestrous cycle was significantly shortened, so much that oestrus seemed almost continuous. Therefore, these physiological changes are dynamic responses to specific social environments, which vary across species with different

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mating systems and, thus, are clearly tuned to optimise individual fitness benefits across varying social environments.

Potential benefits of being informed and informing are classically viewed in terms of reproductive benefits, where transmitted information on sex, relatedness, reproductive and social status is an important prerequisite for mate choice. However, many studies on social modulation show that the same information that is used in the process of mate choice stimulates responses with extensive physiological and reproductive consequences. For example, female puberty may be accelerated in sub-adult mice simply in response to the presence of adult males [8] whilst the presence of unfamiliar males may cause spontaneous abortion of early embryos [9] in female mice. All the above described cases of social modulation, including the Whitten, Lee-Boot, Bruce, and Vandenbergh effects, are important physiological phenomena where social organization, mate competition and mate choice have presumably acted as important selective pressures that shaped the evolution of specific signalling and physiological responses.

Original research on socially modulating and modulated effects has been guided by a new paradigm following the discovery of the function of major urinary proteins and their role in transporting pheromones from the liver through the kidney into the urine [10,11]. After being excreted, MUPs release pheromones while drying up and, thus, individual odours are manifested even when the individual is temporarily absent from the territory [12]. Milestones in the understanding of MUPs in the social context include: the discovery of binding sites for pheromones that have specific effects upon targeted individuals [13,14], individually specific and behaviourally relevant expression patterns [15], tissue-specific expression and, therefore, their different function [16,17]. Furthermore, MUPs are an essential part of the odour delivery system that enhances the onset of ovulation in female mice [18]. To complement Morè's [18] finding that male MUPs are important in ovulation induction, this paper highlights the role of female MUPs in oestrus advertisement. We present evidence that during the oestrous cycle female MUPs undergo up-regulation of MUP expression which is tightly bound to the beginning of the oestrous cycle and, therefore, plays an important role in honest reproductive signalling.

2. Methods

2.1. Animals

We used strain C57BL/6 mice as subjects for this experiment. Twenty females and twenty males aged 7 weeks were kept on a 12:12-h light cycle with lights off at 1900 h. Water and food was provided ad libitum. All mice were caged individually in compartmented cages during individual treatment; in social treatment two individuals were separated by a metal grid to allow communication but suppress any behavioural interactions. Urine samples and vaginal smears were collected every day in the first half of the light period. Urine was obtained by gentle abdominal massage and frozen immediately at −20 °C. The experiment lasted 17 days. Samples for cross-correlation analysis were collected from all females throughout the duration of individual treatment. Of these 17 days day 10 was taken as a reference point for individual treatment. The first day of the 4-day social treatment was taken as a reference for the analysis of expression variation due to social treatment.

2.2. Oestrus identification

Vaginal smears were taken by vaginal lavage with 10 μl of Dulbecco's phosphate-buffered saline (Sigma-Aldrich, St. Louis, USA) and put into an Eppendorf tube containing 90 μl of PBS. The whole volume was pipetted into a plastic chamber, placed in a Cytospin 2 slide centrifuge (Shandon Southern Instruments, Sewickley, USA) and spun down (1000 rpm, 10 min) onto a microscope slide. The microscope preparations were stained by the Pappenheim method (May-Grünwald, Giemsa) and analysed under a light microscope.

Different oestrous stages were determined using a modified method of Natynczuk [19]. Randomly selected fields of fixed cells were counted to a total number of 200 cells per slide. The cell types counted were leukocytes (L), cornified non-nucleated epithelial cells (C) and nucleated epithelial cells (N). The index $E=C/(C+N+L)$ was calculated to obtain a continuous and smooth oestrus curve. Females were categorized as: oestrous if $E>0.7$ or dioestrous if $E<0.3$.

2.3. Protein analysis

Major urinary proteins from urine samples were separated on SDS-PAGE according to a modified Laemmli [20] protocol. Samples were thawed, stirred and centrifuged for 5 min at 16,000 g. The volume of 0.4 μl of urine was loaded onto a 15% Tris–Gly gel. To allow quantification of MUPs, four different concentrations of standard (Carbonic Anhydrase, 29 kDa, marker for SDS-PAGE, Sigma-Aldrich, St. Louis, USA) were run together with samples on each gel and used for the calculation of the regression curve. The gels were stained with Coomassie® G-250 (SimplyBlueTM Safe Stain, Invitrogen life technologies, Paisley, UK). Gel images were acquired using GS-800 Calibrated Densitometer and analysed with Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Hercules, USA). Bands containing major urinary proteins were detected by western blotting with commercially available antibody YNGMMUP (Accurate chemical and Scientific Corporation, New York, USA). The antibody was tested against pooled normal mouse urine and purified mouse MUP and gives a reaction of full identity.

As the dilution of the urine can vary across different individuals or between days, we employed the creatinine assay to normalize all concentrations in the samples by using a LKreatinin kit (BioVendor, Brno, CZ). Creatinine is a reliable indicator of renal activity showing the volume of liquid filtered through the kidney. Based on the creatinine concentration, the dilution coefficient (kdil) was calculated using the formula kdil=Ccreat/Cref, where Ccreat is the creatinine concentration of each sample and Cref is the reference creatinine concentration. The reference concentration was defined as the highest concentration of all measured creatinine concentrations. Corrected volumes were obtained by division of