Chem. Senses 31: 393–401, 2006

doi:10.1093/chemse/bjj043
Advance Access publication March 1, 2006

Mouse Major Urinary Proteins Trigger Ovulation via the Vomeronasal Organ

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Abstract

The major urinary proteins are a species-specific complex of proteins excreted by male mice that influence the reproductive behavior and the neuroendocrine condition of female mice through the olfactory system. The aim of this work is to determine their influence on ovulation. The major urinary proteins isolated from the urine of adult male mice were voided of bound odorants, dissolved at a physiological concentration in urine of prepubertal mice, and put on the nostril of reproductively cycling female mice housed in groups, the first day of estrus at 1100. The eggs shed in the oviducts were counted under dissection the morning of the second day of estrus. The results showed that 1) a single stimulus of the major urinary proteins increased ovulation nearly as much as the whole urine of male mice, 2) the effect was not elicited by male rat urine which contains different proteins, 3) a peptide with four residues of the amino-terminal sequence of the major urinary proteins stimulated ovulation, and 4) mice that had been isolated or had the vomeronasal organ (VNO) removed did not respond to the major urinary proteins and had a high spontaneous ovulation. The results suggest that the major urinary proteins activate the neuroendocrine system through the VNO and trigger ovulation.

Key words: eggs shed, estrus, isolation, oviduct, pheromones, urine

Introduction

Mice release olfactory cues with urine that are relevant to reproduction. The functional characterization and the chemical identification of these cues are still at an early stage (Novotny *et al.*, 1985, 1995, 1999a,b; Kimoto *et al.*, 2005; Stowers and Marton, 2005). The major urinary proteins, belonging to the lipocalin family (Sansom *et al.*, 1994; Flower *et al.*, 2000), are excreted in the urine of male and adult mice (Finlayson *et al.*, 1965; Marchlewska-Koj *et al.*, 2000). These proteins have two intriguing peculiarities. Firstly, they bind odorant molecules in the mouse and release them in the air conferring a characteristic odor to the spots of male urine (Hurst *et al.*, 1998, 2001; Cavaggioni *et al.*, 2003), and secondly, they are present in mouse urine as a polymorphic protein complex (Hurst *et al.*, 1998; Nevison *et al.*, 2003; Beynon and Hurst, 2004). While the odorants released in the air by a urinary spot can be perceived at a distance, thus, activating behavioral and perhaps also physiological responses in the female mice (Sam *et al.*, 2001; Baum and Keverne, 2002; Mucignat-Caretta, 2002), the olfactory function of the protein moiety would seem difficult to understand because there is no evidence that proteins stimulate the main olfactory epithelium. However, besides this epithelium, mice have a chemosensory organ also accessible to proteins. The vomeronasal organ (VNO) (Meredith, 1994; Cavaggioni *et al.*, 1999; Keverne, 1999) and vomeronasal receptor neurons can be stimulated by proteins (Wysocki *et al.*, 1980; Leinders-Zufall *et al.*, 2004; Kimoto *et al.*, 2005). It has been proposed that the polymorph complex of major urinary proteins may provide a female with an olfactory fingerprint of the releaser, carrying information about the species, gender, strain, and individuality of the releaser, all paramount features for the choice of a mating partner (Hurst *et al.*, 2001; Cavaggioni *et al.*, 2003). The major urinary proteins also release, through the VNO, neuroendocrine responses such as the anticipation of the first cycle at puberty (Vandenbergh, 1969; Mucignat-Caretta *et al.*, 1995) and the return of the cycle in noncycling mice (Whitten, 1956; Marchlewska-Koj *et al.*, 2000). However, the key event of the cycle is ovulation. For the first time, the effect of the urinary proteins on ovulation is here studied by counting the eggs shed in the oviducts. Under a well-defined set of laboratory conditions, it is shown that the major urinary proteins actively influence ovulation.

Materials and methods

General

Albino Swiss mice (*Mus musculus*) bred in the Institute of Physiology (University of Padova, Italy) were used. Mice were weaned 21 days after birth, and female mice from different litters were randomly housed six mice per cage ($42 \times 27 \times 15$ cm). When necessary, mice were isolated 8 days before the experiment. The animals were kept in a mixed sex room. The light–dark cycle duration was 12 h (lights on at 0500), and the temperature was 22 ± 1°C, with 10 air renewals per hour. The bed shaving of the cages was changed two times per week. Food and water were *ad libitum*.

The vaginal smears were taken daily at 0830–0930 and inspected under a microscope to characterize the cycle. The estrus phase was defined when more than 95% cornified cells were present.

Mice were killed with an overdose of Nembutal [500 μg/g body weight, intraperitoneally (i.p.)].

For the egg count, the uterus was dissected at the base and between the oviducts and ovaries (Hogan *et al.*, 1994) and cleaned of the fat tissue. The presence of liquid in the uterus and of swollen periovarial sacs (at least 2 mm in size) was annotated. The uterus was emptied of internal liquid, and the relative uterus weight (uterus weight/body weight) was determined.

The uterus was then placed in a solution of hyaluronidase (Sigma H4272) 30 μg/ml for 10 min to dissociate the egg cumulus (Mark and Long, 1912; Ozgunen *et al.*, 2001). The eggs were counted under a dissection microscope (32×) with light coming from below.

All the experiments were done according to the Italian law on animal experimentation (L. 116/92).

The characterization of the cycle

The cycle of 30 mice 90 days old housed six per cage was characterized by taking a vaginal smear twice a day at 0830–0930 and 1630–1730, for a period of 29.3 days. The smears were stained with hematoxylin and eosin and examined under a light microscope.

The percentage (h) of cornified cells, epithelial cells, and leukocytes was determined by counting at least 100 cells in a central microscope field of the smear. The profiles h (n) for each cell type were obtained, where n is the day number ($1 \le n < K = 30$). The estrus index has been calculated (Sanchez and Dominguez, 1995) as the percentage of the days in estrus. The autocorrelation function $R_h^2(m) = \sum_{n=1}^{K} h(n)h(n-m) / \sum_{n=1}^{K} h^2(n)$, where m is the delay, was determined for every cell type. R_h^2 (peak) was then calculated for m in correspondence with the first statistically significant peak ($\alpha < 0.05$ Box-Ljung test) of every cell type and $\sum R_h^2$ for the three cell types. The criterion for a regular estrus cycle was set empirically at $\sum R_h^2 \ge 0.75 (0 \le \sum R_h^2 \le 3)$. The cross-correlations $R_{hg}^2(m) = \sum_{n=1}^{K} h(n)g(n-m) / \sum_{n}^{K} h(n)g(n)$ were also determined, where m is the delay and g and h refer to two cell populations. The phase relation was obtained from the first cross-correlation peak.

Mice with a cycle period longer than 8 days, or an estrus lasting more than 3 days were not used in the following experiments.

Spontaneous ovulation

Both isolated than six per cage mice were used. The egg count was made in random order, the morning of the first day of estrus in half of the mice and the morning of the second day of estrus in the other half of the mice of either population.

Experiments with pheromones

The major urinary proteins were purified from the urine of male mice by differential precipitation (0°C) using a 50–70% saturated solution of ammonium sulfate, dialyzed extensively against 5.5 mM NaCl (molecular weight membrane cutoff 10^4), concentrated at 143 mg/ml by osmosis against polyethyleneglycol (molecular weight 20^3), and extracted three times with one volume of CH_2Cl_2 to remove odorants and the aqueous phase was stored in a plastic vial at −20°C. The organic phase was checked with gas chromatography–mass spectrometry (GC–MS) for the presence of major urinary proteins ligands. They were below the detection presence (see supplementary file). One hundred microliter of this solution was analyzed after the extraction of the volatiles by solid phase mass extraction (SPME, 30 min at 50°C and 30 min at 75°C, apolar polydimethylsiloxane fiber, film thickness 85 μm) by a gas chromatograph Varian GC 3800 coupled with a mass spectrometer Varian MS 2000 with ion trap (scansion ranged from 20 to 800 amu; software Varian MS Workstation for Windows 2000). A VA-5 capillary column (30 m long, 0.25-mm diameter, film thickness 0.25 μm) conditioned for 24 h by helium flow was used. Also a GC–MS in single ion mode, monitoring m/z 115, was performed in order to increase the detection level for 2-*sec*-butyl-4, 5-dihydrothiazole. It was below the detection presence (see supplementary file).

GC data were compared with fresh male mouse urine (containing the natural concentration of 12 mg/ml of major urinary proteins, obtained by gently squeezing the abdomen of one 6-month-old male housed in isolation). The injector temperature was 260°C, the temperature program was 15 min at 35°C, a ramp (3°C/min) to 60°C, then holding for 5 min, a ramp (10°C/min) to 150°C, holding for 1 min, and a ramp (25°C/min) to 290°C, holding this temperature for 18 min. The carrier gas was helium. The identification of substances in mass spectroscopy was via fragmentation (Bacchini *et al.*, 1992).

Major urinary proteins were dissolved at 10 mg/ml in urine of prepubertal mice. The stock used in these studies had been checked for pheromonal activity, by inducing estrus

in susceptible animals (the Whitten effect; Marchlewska-Koj *et al.*, 2000).

Synthetic peptides were synthesized using 9-fluorenylmethoxycarbonyl-amino acids and purified by high performance liquid chromatography. The peptide NH_2-Glu-Glu-Ala-Arg-Ser-Met (EEARSM, molecular weight 721.8) or its control MSRAEE was dissolved at 5 mg/ml in urine of prepubertal mice. Sodium dodecyl sulphate polyacrylamide gel electrophoresis in 15% acrylamide stained with Comassie brilliant blue did not show major urinary proteins in this urine. The solution (25 µl) was put with a syringe on the oro-nasal groove of mice in most of the experiments at 1100 (in experiment B at 1900 and in experiment C at 1100 and at 2300) of the first day of estrus (Figure 1).

Urine collection

Male mouse and male rat urines were collected overnight with a funnel placed under a cage with a grid on the floor. Female urine, either in estrus or diestrus, was collected by gently squeezing the abdomen once the estrus phase was determined. Prepubertal urine was collected by gently squeezing the abdomen from mice of 15–17 days old of either sex. Urines were rapidly centrifuged and stored at −20°C.

Ablation of the VNO

Forty-eight mice, 45–52 days old, underwent bilateral VNO ablation under anesthesia (chloral hydrate i.p. 600 µg/g body weight), according to the method of Wysocki *et al.* (2004). Mice were placed supine, and the palate was incised up to the end of the third palatal plica. Then the vomer was incised with a dental drill (cutter diameter 1 mm) in the junction point with the superior incisive teeth. Both the VNO and the vomer were rapidly removed using a couple of bone tweezers. The hall was filled up with surgical glue and the palate sutured. The ablation of the VNO was verified in all mice by histology of the nasal tissues after decalcification in 0.5 M ethylenediaminetetraacetic acid for 14 days and paraffin embedding. Serial sections, 7 µm thick, were stained with hematoxylin and eosin. Twelve sham operated mice housed six per cage, sisters of the mice with VNO ablation, served as a control. Sham control mice underwent the same procedure, but the vomer was not incised and the VNO was left in place and no surgical glue was used. Seventy-two hours after the ablation of the organ, the mice were tested for the absence of neurological signs to exclude surgical sequelae (Wolf *et al.*, 1996). In order to not modify the housing composition of the cage made at the weaning time, both vomeronasal ablated than sham operated mice, only cages where all the six animals went over the surgery without complications were used.

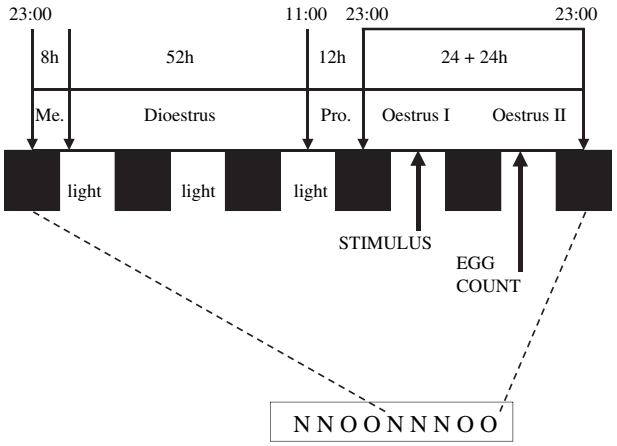

Figure 1 Timetable of the experimental procedure. The table shows the time of stimulation and of the dissection for the egg count in frame with the sexual cycle (metestrus, Me.; proestrus, Pro.; estrus, O; nonestrus: N) and the light–dark cycle.

Mating and pregnancy

Female mice were mated with fertile and experienced males taken from a pool of 12 mice, 100–120 days old, kept in isolation. A female was introduced in the cage of a male mouse from 0900 to 1100 on the second morning of estrus. Mating was checked by the presence of the vaginal plug. Each male was alternated with females of the experimental and control group every third day.

Nineteen days after mating, the female mice were killed with an overdose of Nembutal (500 µg/g body weight, i.p.), and the vital fetuses were counted. Fetuses were then killed with an overdose of Nembutal. The absence of ovarian cysts was accurately checked in all mice.

Statistics

The Mann–Whitney *U*-test with correction for equal scores for the number of eggs and the χ^2-test or Fisher exact probability test for the number of ovulating mice were used. The null hypothesis was rejected if $P < 0.05$.

Results

Preliminary experiments: assessment of the cycle in six per cage housed mice

Two mice did not show an estrus, eight mice had one to three estruses lasting more than 3 days, and 18 mice had four or five estruses lasting 3 days or less, over the entire period. The profile of the percentage of each cell type over the period made it possible to define an empirical criterion of cycle regularity based on autocorrelation.

The parameter $\sum R_h^2$ ranged between 0.18 and 1.69 with 0.81 ± 0.34 (mean ± SEM). Twenty out of 30 mice had cycles with $\sum R_h^2 \geq 0.75$ and were thus regular (66.7%). In order to select the experimental population and discard mice with irregular estrus cycles, only mice with a cycle ≤8 days were taken. Eighty-five percent of the mice selected in this way

were regular. Also mice with an estrus lasting more than 3 days were discarded (less than 5%). In the regular mice, cornified cells appeared in the smear nearly in phase opposition to leukocytes and epithelial cells, 156° and 153°, respectively (see an example in Figure 2), and the mean estrus index was 8.1%.

Eight out of nine cyclic mice housed six per cage had no eggs in the oviducts and one or two periovarial sacs swollen (≥5 mm), in the diestrus phase.

Four out of the 19 mice that were checked on the first morning had ovulated compared to 10 of the 21 that were checked on the second morning, when peak ovulation is expected. The difference between the number of eggs shed on the first and second morning was not significant ($2P$ = 0.094): 1.95 ± 0.92 versus 4.62 ± 1.42 (mean ± SEM), first and second morning of estrus, respectively. No mice had periovarial sacs swollen without eggs in the oviducts on the morning of the egg count.

In order to validate the number of eggs shed in the oviducts on the second morning of estrus, female mice were mated and the fetuses at term of pregnancy have been counted. The fetuses were 3.89 ± 1.20 (mean ± SEM, N = 19). When this number was compared to the number of eggs shed, 3.42 ± 1.35 (mean ± SEM, N = 18), the difference was not significant (P = 0.29).

It was concluded that anovulatory cycles are likely to occur, spontaneous ovulation is low and broadly tuned over estrus in mice housed six per cage.

The cycle and ovulation of isolated mice

It is known that the social factors in the laboratory modify the sexual cycle of female mice (van der Lee and Boot, 1955; Whitten, 1956); however, ovulation has never been studied before.

The cycle and the spontaneous ovulation of isolated mice were different from that of six per cage mice. Isolation increased ($2P$ = 0.002) the percentage of mice with a cycle equal or less than 8 days from 61.5 + 3.3% to 95.1 + 1.8%, mean + SEM, N = 587 versus N = 224. This observation is in agreement with earlier data showing that the cycle is longer in mice housed in a group in an environment free of male odor as compared to mice housed in isolation (van der Lee and Boot, 1956; Whitten, 1956; Bronson, 1979). More eggs were found on the second day of estrus versus the first, this difference was statistically significant ($2P$ = 0.004), 3.17 ± 1.31 versus 9.06 ± 1.23 (mean ± SEM), first and second morning of estrus, respectively, at variance of mice housed six per cage. Statistics over the entire population of 72 isolated versus 112 mice housed six per cage confirmed that more eggs were counted (7.63 ± 0.72 vs. 3.37 ± 0.51, mean ± SEM; $2P$ = 0.001) and more isolated mice ovulated on the second day of estrus (70.8% vs. 34.8%; χ^2 test: $2P$ = 0.001). Moreover, at least 80% of the cycles could be considered ovulatory since 15 out of 18 mice had shed eggs the second morning of estrus. Thus, in isolated mice, unlike mice housed six per cage, ovulation was finely tuned on the second morning of estrus.

It was also interesting to test whether the lower spontaneous ovulation observed in mice housed six per cage could be due to urinary chemosignals in urine released by other females that inhibit ovulation. This possibility was explored by stimulating isolated females. However, urine of neither estrus nor diestrus mice influenced the ovulation in these experimental conditions (Table 1I and 1L). It was concluded that a single stimulus with the urine of females was not sufficient to inhibit ovulation.

Isolated mice did not respond to the chemosignal present in the urine of male mice; the number of eggs found in the

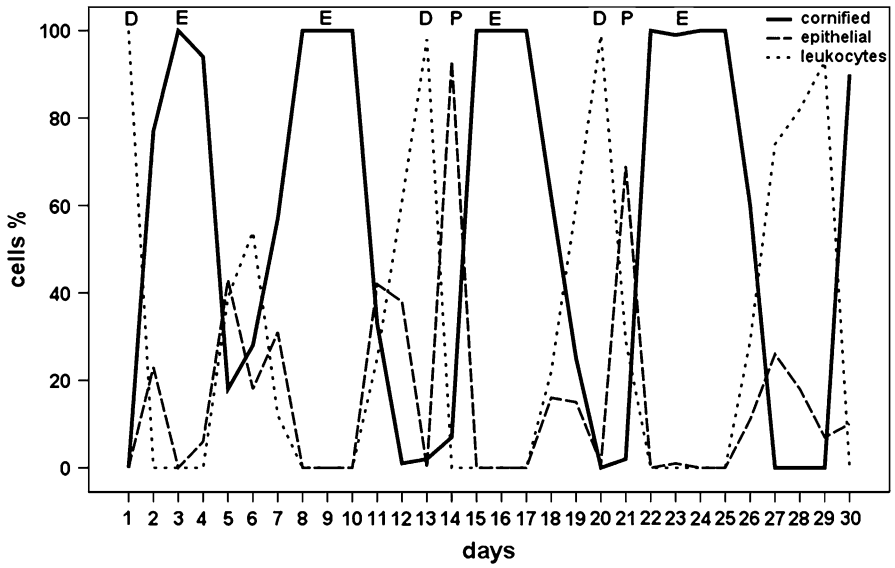

Figure 2 Cell percentage in the vaginal smear of a mouse. D, diestrus; P, proestrus; E, estrus. Cycle regularity $\sum R_n^2$ was 1.26 (see Materials and Methods).

Table 1 Experiments with pheromonal stimulation

Exp.	Mice/cage	Stimulus, type, and time	VNO	Egg in stimulated (mean ± SEM)	Egg in controls (mean ± SEM)	M–W, *P*	Mice with eggs in stimulated mice	Mice with eggs in control mice	Fisher or χ^2, *P*
A	6	MMU vs. water, E1, 1100 h	Yes	12.67 ± 2.22	3.72 ± 1.40	0.001	12/18	6/18	0.047
B	6	MMU vs. water, E1, 1900 h	Yes	5.33 ± 1.93	1.44 ± 0.83	0.07	6/18	3/18	0.22
C	6	MMU vs. water, E1, 1100 + 2300 h	Yes	8.89 ± 1.85	2.28 ± 0.95	0.003	12/18	6/18	0.047
D	6	RMU vs. water, E1, 1100 h	Yes	3.28 ± 1.13	4.11 ± 1.42	0.55	7/18	6/18	0.50
E[a]	6	MUPs + MPPU vs. MPPU, E1, 1100 h	Yes	10.56 ± 1.96	3.89 ± 1.20	0.005	12/18	8/19	0.12
F[a]	6	EEARSM + MPPU vs. MPPU, E1, 1100 h	Yes	7.94 ± 1.91	4.00 ± 1.36	0.045	11/18	7/18	0.16
G[a]	6	MSRAEE + MPPU vs. MPPU, E1, 1100 h	Yes	3.72 ± 1.29	4.56 ± 1.54	0.58	7/18	7/18	0.63
H	6	MMU vs. water, E1, 1100 h	No	5.00 ± 1.55	8.33 ± 1.70	0.93	8/19	12/18	0.12
I	1	MFOU vs. water, E1, 1100 h	Yes	7.17 ± 1.61	7.00 ± 1.68	0.50	12/18	11/18	0.47
L	1	MFDU vs. water, E1, 1100 h	Yes	7.11 ± 1.69	8.56 ± 1.62	0.68	10/18	13/18	0.24
M	1	MMU vs. water, E1, 1100 h	Yes	6.78 ± 1.90	5.89 ± 1.17	0.44	9/18	12/18	0.25

E1, first day of estrus; Exp., experiment; Fisher, Fisher exact probability test; MFDU, mouse female diestrus urine; MFOU, mouse female estrus urine; MMU, mouse male urine; MPPU, mouse prepubertal urine; MSRAEE, peptide NH_2-Met-Ser-Arg-Ala-Glu-Glu; MUPs, major urinary proteins without natural ligands; *P*, *P* value obtained from the comparison between stimulated and control group within the same experiment (distribution of eggs and number of mice with eggs); RMU, rat male urine; M–W, Mann–Whitney *U*-test with correction for equal scores; χ^2, Chi-square test.
[a]Blind experiment.

oviducts was not significantly different from the controls ($P = 0.54$; Table 1M).

Because of the result of these experiments, isolated mice have been considered a population not susceptible to this type of pheromonal stimulation; thus, only in the six per cage population has been tested whether the major urinary proteins increased ovulation.

Timing of ovulation by major urinary proteins

The effect of urine was tested first. An experiment was made to see whether the whole urine of adult male mice influenced ovulation in the experimental population of six per cage housed mice. A stimulus consisting of 25 µl of male mice urine was put on the oronasal groove of females on the first day of estrus at 1100, and the eggs in the oviducts were counted the next morning. The mice responded with an increase of eggs in the stimulated mice ($P = 0.001$; Figure 3; Table 1A). The time of stimulus presentation turned out to be important. When the stimulus presentation was delayed to 1900, the effect was not statistically different from the controls (Table 1B), and a double stimulus at 1100 and at 2300 gave a significant response ($P = 0.003$) which was similar to that of a single stimulus at 1100 (Table 1C). It was concluded that a single stimulus at 1100 of the first morning of estrus was sufficient to increase ovulation on the second day of estrus. The species specificity of the chemosignal present in the urine of males was tested by substituting

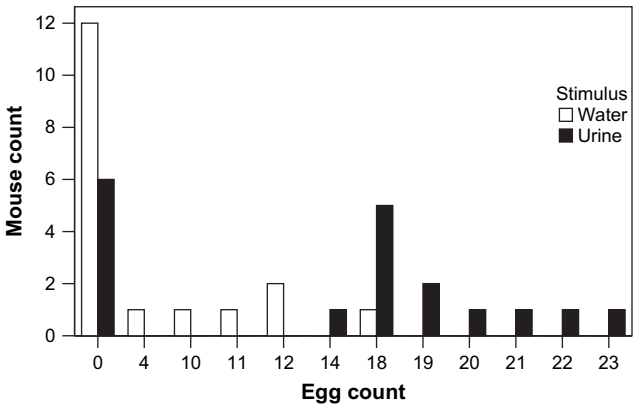

Figure 3 Distribution of the eggs recovered in experiment A. Egg count in the morning of the second day of estrus, stimulation on the first day of estrus at 1100 with mouse male urine versus water, mice housed six per cage.

male rat urine for mouse urine. Unlike mouse urine, the urine of male rats did not evoke an ovulatory effect (P = 0.45; Table 1D). Therefore, the active chemosignal of male mice was not present in the rat urine.

An experiment was then made trying to identify the chemosignal of male mice urine. The protein fraction of the male urine, the major urinary proteins, was isolated. The protein fraction was purified of the naturally bound urinary odorants, thus depriving the protein of the characteristic odor, and was given to test mice dissolved in a carrier of urine of prepubertal mice. It provided a urinary context lacking proteins. SPME–GC–MS showed that both the major urinary proteins preparation and the prepubertal urine did not contain a detectable amount of 2-*sec*-butyl-4,5-dihydrothiazole and 3,4-dihydro-*exo*-brevicomin that are the major ligands of major urinary proteins in adult male mouse urine. No peaks of volatiles were detected from 100 µl of major urinary proteins solution (143 mg/ml), whereas 100 µl of fresh male urine gave several peaks on the chromatogram (see supplementary file).

The mice responded (P = 0.005) to a single stimulus (Table 1E) nearly as well as to whole urine of male mice. Therefore, the major urinary proteins are a prominent stimulus for the effect on ovulation. To confirm this observation, experimental female mice were mated with fertile and experienced males on the second morning of estrus, and the fetuses were counted at term of pregnancy. The number of pregnant mice was twice when stimulated with major urinary proteins: 10 pregnant out of 19 experimental versus five out of 19 controls and the number of fetuses was also greater: 6.21 ± 1.48 versus 3.42 ± 1.35, respectively (mean ± SEM). These results are in agreement with the egg count. However, they were not statistically significant (P = 0.067), and the reason is not clear.

Since the volatiles bound to the protein had been removed below GC detection threshold, the determinants for sensory recognition reside probably in the protein moiety. An experiment was then made using the synthetic peptide NH_2-Glu-Glu-Ala-Arg-Ser-Met, EEARSM, as a stimulus that shares four residues (underlined) with the amino-terminal sequence of the major urinary proteins (Flower *et al.*, 2000). This peptide also increased ovulation (P = 0.045; Table 1F), whereas the peptide with the inverted sequence did not (Table 1G). This result shows that the amino-terminal sequence of the urinary proteins is needed for sensory recognition. Sensory recognition for proteins can take place in the VNO that is known to mediate pheromone signaling (Wysocki and Lepri, 1991; Wysocki *et al.*, 1991; Keverne, 1999; Stowers *et al.*, 2002; Pankevich *et al.*, 2004). To test this possibility, mice without the VNO were stimulated with male mouse urine. The urine failed to increase ovulation (P = 0.93; Table 1H), whereas it was active in sham operated mice (not shown).

On the whole, the results suggest that the urinary proteins evoke a neuroendocrine response (Johns *et al.*, 1978) aimed at timing ovulation via the VNO.

Mice with VNO ablation

In order to understand the role of the VNO for the reproductive physiology of mice, the cycle and spontaneous ovulation were further characterized in mice whose VNO had been removed (housed six per cage).

A posterior portion of the organ was left, less than 40%, in 23 out of 37 mice that underwent the operation. Statistics on the number of eggs and on the number of ovulating mice showed no significant differences between mice with total and partial vomeronasal ablation.

Thirty-seven out of 48 mice with VNO ablation showed a cycle with a period shorter than 8 days (77.1%), which is intermediate between 61.5 ± 3.3%, in mice housed six per cage (587 mice from nine experiments), and 95.1 ± 1.8%, in isolated mice (224 mice from five experiments), and was greater than 58.3% in 12 sham operated mice housed six per cage (from one experiment). The number of eggs spontaneously shed in mice with VNO ablation was greater (P = 0.011) than intact mice housed six per cage but similar to isolated mice (P = 0.24; Figure 4). This observation supports the hypothesis that isolated mice lack chemosignals relevant to ovulation mediated by the VNO.

Despite these differences, the cycle period was about the same in the three groups (5.17 ± 0.18 in six per cage, 5.13 ± 0.30 in mice with VNO ablation six per cage, and 4.96 ± 0.18 in isolated mice; mean ± SEM). The relative uterus weight (mg/g body weight) was greater in isolated mice versus mice housed six per cage (7.26 ± 0.37 vs. 5.93 ± 0.34; mean ± SEM; P = 0.005) and mice with VNO ablation (6.13 ± 0.35; mean ± SEM; P = 0.005), probably because of a greater stimulation of ovarian steroids.

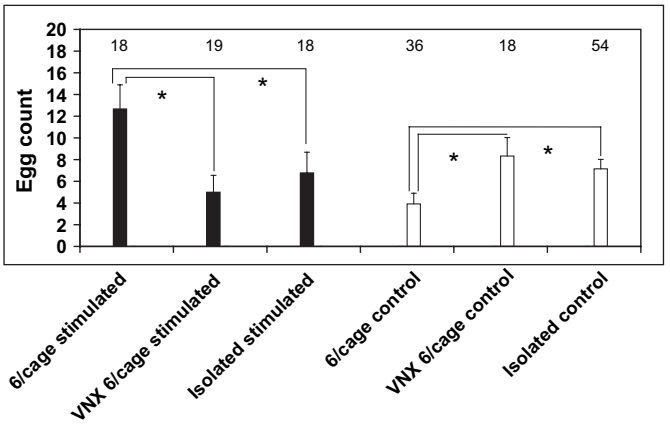

Figure 4 Comparison of ovulation (mean + SEM, *N*) between intact mice housed six per cage, mice without the vomeronasal organ (VNX) six per cage, and intact isolated mice. The stimulus was whole male mouse urine. Experimental data were from A, H, and M and controls were from A + D, H, and I + L + M of Table 1. Asterisks indicate statistically significant differences (P < 0.05). Among all the experiments, the greatest ovulation was observed after stimulation and the smallest in controls of mice housed six per cage.

Discussion

This study shows that a single stimulus of the urinary proteins of the male mouse is sufficient to actively influence ovulation on the second morning of estrus. In the laboratory, the effect requires a set of precise experimental conditions.

Firstly, the stimulus must be presented at 1100 of the first day of estrus, not later. An ordered chain of events determine ovulation timing in mammals. Luteinizing hormone plasma levels in mice are difficult to determine. However, in the rat they can be accurately determined. The luteinizing hormone surge that precedes ovulation takes place around 1500, and approximately 12 h later the rats shed eggs at about 0300 in the night between the estrus and the metestrus, when the rat also shows a behavioral activation (Fox *et al.*, 1984). Rats have only a single day of estrus (Everett, 1964, 1969). Luteinizing hormone must induce the expression of specific genes for the ovulation before those that reprogram granulose and theca cells for the luteinization in a precise time sequence. Any change in the programmed schedule may lead to either precocious or late ovulation. Moreover, if the events linked to the follicle rupture and luteinization are temporarily misaligned, the eggs may remain caught inside the corpus luteum (for a review see Robker *et al.*, 2000). Furthermore, exposing previously grouped ovariectomized females bearing estrogen implants to male mouse urine results in an immediate (1–3 h) elevation in luteinizing hormone (Bronson, 1976, 1979). It would thus appear that the olfactory signal in frame with a neural circadian signal provides the best chance for timing an ovulatory response in estrus mice.

Secondly, the ovulatory effect was seen in mice housed six per cage but not in isolated mice. The spontaneous ovulation also differed notably in the two populations. Spontaneous ovulation was inconsistent and, when present, fewer eggs were shed in the former compared to the eggs shed in the latter population. In mice housed six per cage, a number of eggs could be retained in the ovary at ovulation time (Long and McLean-Evans, 1922), and it is conceivable that the olfactory stimulus with major urinary proteins promote their shedding.

Thirdly, the urinary proteins without the natural ligands were given in a carrier of urine of prepubertal mice that, unlike water (Novotny *et al.*, 1999b), proved to be a suitable carrier without an appreciable amount of proteins (Mucignat-Caretta *et al.*, 1995; Marchlewska-Koj *et al.*, 2000). The reason why a urinary carrier is necessary is not known. Obviously, it is not possible to exclude the role of volatiles. It is also possible that the volatiles present in the prepubertal urine are different from previously identified pheromones, but this hypothesis seems to be unlikely. The volatile odorants of the carrier may stimulate the olfactory system and provide a urinary gestalt, or may combine chemically with the stimulus proteins, or finally may contribute to increase the pumping activity (Meredith and O'Connell, 1979) of the VNO carrying proteins in contact with the sensory epithelium.

Under this housing condition, that is, the animals were kept in a mixed sex room, it is not possible to exclude that mice could detect pheromonal volatiles from male mouse urine. An important role of male mouse urine volatiles could be to make a "male mousy" environment, and it is probable that this male mousy environment plays a fundamental role to load ovulation. Anyway, these experiments demonstrated that the trigger for egg shedding is the major urinary proteins or their peptide.

SPME–GC–MS excluded appreciable amounts of volatile pheromones in prepubertal mouse urine as well as in the major urinary proteins preparation after organic extraction. This observation makes a trigger effect of male mouse urine volatile pheromones unlikely. Thus, the pheromonal effect is conceivably due to the protein moiety of major urinary proteins.

Beside the urinary proteins, a synthetic peptide that shares four residues with the amino-terminal sequence of the urinary proteins of laboratory strains and wild-type mice (Finlayson *et al.*, 1974; Clark *et al.*, 1985; Mucignat-Caretta *et al.*, 1995) influenced ovulation at a molar concentration of about 12.5 times major urinary proteins concentration. This shows that the amino-terminal region contains a recognition determinant of the urinary proteins, namely, an epitope that is recognized by a receptor. Since a peptide with the inverted amino acid sequence was inactive, the sequence of the four residues is needed. However, the amino-terminal sequence of urinary proteins is common both to mice and rats and therefore is not species specific. The determinants of species specificity of the major urinary proteins should be found in the regions of the proteins not present in rat proteins, yet experimental evidence is lacking. It should be noted that a strong evolutionary pressure diversified mouse and rat urinary protein amino acid sequence along with species radiation, although they still share 65% sequence identity (Bishop *et al.*, 1982; Sansom *et al.*, 1994). Possibly, this signal was too weak in rat urine to elicit a response.

The presence of the VNO was necessary for the ovulatory effect of the major urinary proteins. The VNO is the receptor of the accessory olfactory system. It contains neurons that are stimulated by major histocompatibility complex class I peptides (Leinders-Zufall *et al.*, 2004; Stowers and Marton, 2005). An extensive search of the database (Swissprot) using the BLAST alignment tool showed no matches between major histocompatibility complex class I peptides of the mouse and the EEARSM peptide. It is unclear, however, whether major histocompatibility complex class I peptides are present in the prepubertal mouse urine that was used as a carrier in these experiments. Since both the major urinary proteins and major histocompatibility complex class I peptides are inactive as pheromones when presented to mice in water rather than in homologous urine (Marchlewska-Koj *et al.*, 2000; Leinders-Zufall *et al.*, 2004), it would be interesting to know whether they complement each other for the VNO of the mouse.

By virtue of a pumping system, substances dissolved in the mucus that fills the lumen of the VNO come into contact with the vomeronasal receptor neurons and can stimulate them (Wysocki *et al.*, 1980; Tirindelli *et al.*, 1998; Keverne, 1999). The vomeronasal neurons send their axons to the accessory olfactory bulb, and olfactory signals from here reach the vomeronasal amygdala, a relay station to the hypothalamus (Reynolds and Keverne, 1979; Brennan and Keverne, 1997). The hypothalamus can stimulate the release of gonadotropins from the pituitary and thus modulate ovulation (Schwartz, 1969; Zarrow *et al.*, 1973; Bakker and Baum, 2000). On the whole, the present experiments suggest that the urinary proteins influence ovulation through an integrative neuroendocrine mechanism linking the VNO to the ovary.

The integration of a permissive ovarian signal (estrogen) with a timed neural circadian signal and a neural signal in response to male-related stimulation solves three major problems in the control of ovulation. The permissive effect of estrogen ensures that ovulation takes place only after a sufficient maturation of a dominant follicle. The dependence of ovulation on a circadian neural signal ensures that ovulation occurs coincidentally with maximal behavior. The third adaptive advantage is derived from the neural signals specifying the timing of ovulation. The active influence of male-related sensory signals provides the greatest chance for conception and successful reproduction.

Olfactory signals may be particularly important in a species such as the mouse. These small mammals are natural prey, live a nocturnal, furtive life, and depend for survival on a high fertility rate. In this environment, efficient sexual communication through olfactory signals from the urine should improve reproductive capacity. Sexual communication among them takes place in the field especially through olfactory signals broadcasted via urine. The interplay of volatile and nonvolatile chemosignals present in male mouse urine is complex (Bigiani *et al.*, 2005), and the present experiments do not exclude the role of volatiles (Novotny *et al.*, 1999b). However, some of the signals are proteins (Leinders-Zufall *et al.*, 2004; Kimoto *et al.*, 2005). The major urinary proteins are considered a signal for individual recognition among mice that is essential for many aspects of the reproductive behavior such as inbreeding avoidance and the choice of the mating partner (Hurst *et al.*, 2001; Brennan, 2004). Besides behavior, the present results show that the mouse urinary proteins can actively influence ovulation.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org.

Acknowledgements

I thank Dr Fulvio Tognazzo for support with vomeronasal ablation; Marco Redaelli for support with GC; and Prof. Gianfranco Gabai, Prof. M. Midrio, and Dr Carla Mucignat-Caretta for helpful criticism. Greatest thanks to my Mentor, Prof. Andrea Cavaggioni.

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Accepted February 1, 2006