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Review

Urinary proteins and the modulation of chemical scents in mice and rats

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Abstract

The urine of mice, rats and some other rodents contains substantial quantities of proteins that are members of the lipocalin family. The proteins are thought to be responsible for the binding and release of low molecular weight pheromones, and there is now good evidence that they discharge this role, providing a slow release mechanism for volatile components of scent marks. However, the proteins may function as chemosignalling molecules in their own right, contributing one or more roles in the communication of individual identity and scent mark ownership. In this review, we summarize current understanding of the structure and function of these urinary proteins, and speculate about their role as supporters or as key participants in the elaboration of the complex chemosensory properties of a rodent scent mark.
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1. Introduction

The presence of large amounts of protein in urine (proteinuria) is often considered to be a pathological condition, indicative of serious renal damage. While this is certainly the case in man, there are other species for which such a proteinuria is an obligatory condition. These urinary proteins were first observed in laboratory mice and rats, and it is the urinary proteins in these two species that have been most extensively understood. The proteins are known collectively as major urinary proteins (MUPs) or in the rat, as alpha 2u globulins (A2Us) (for recent reviews see [1,2]). There is good evidence for MUPs also being expressed in glandular tissues such as salivary and mammary glands, nasal tissue and in the respiratory epithelia [3–6] but we will not address the functions of these variants in this brief review.

Mice and rats deposit urine marks extensively around their territories (Fig. 1). Because scent marks are assessed in the absence of the depositing animal, the receiver must be able to glean a complex array of information from the scent signal – the sex, reproductive and social status of the donor, the time since the mark was deposited, and the identity and relatedness of the donor [7]. The receiver may also pick up information such as the health status of the donor [8], and the nature of the food they are consuming [9,10]. The complexity of information embedded in scent marks must be conveyed by a complex mixture of chemicals. Some responses can occur with an airborne signal, others require physical contact between the receiver animal and the scent mark. Additional dimensions to the reception of a chemical signal include the time since the mark was deposited, and the pattern of scent marks that are distributed through the territory. Understanding the interplay between behavioral and biochemical factors in deposition and reception of scents is challenging, and many aspects of this interaction remain elusive.

It is increasingly clear that the proteinaceous component in mouse urine is critical to some aspects of scent communication. Urinary MUPs contain bound molecules that are pheromonally active, establishing beyond all dispute their role in delivering chemical signals. Any endeavor to understand the function of MUPs in chemical communication

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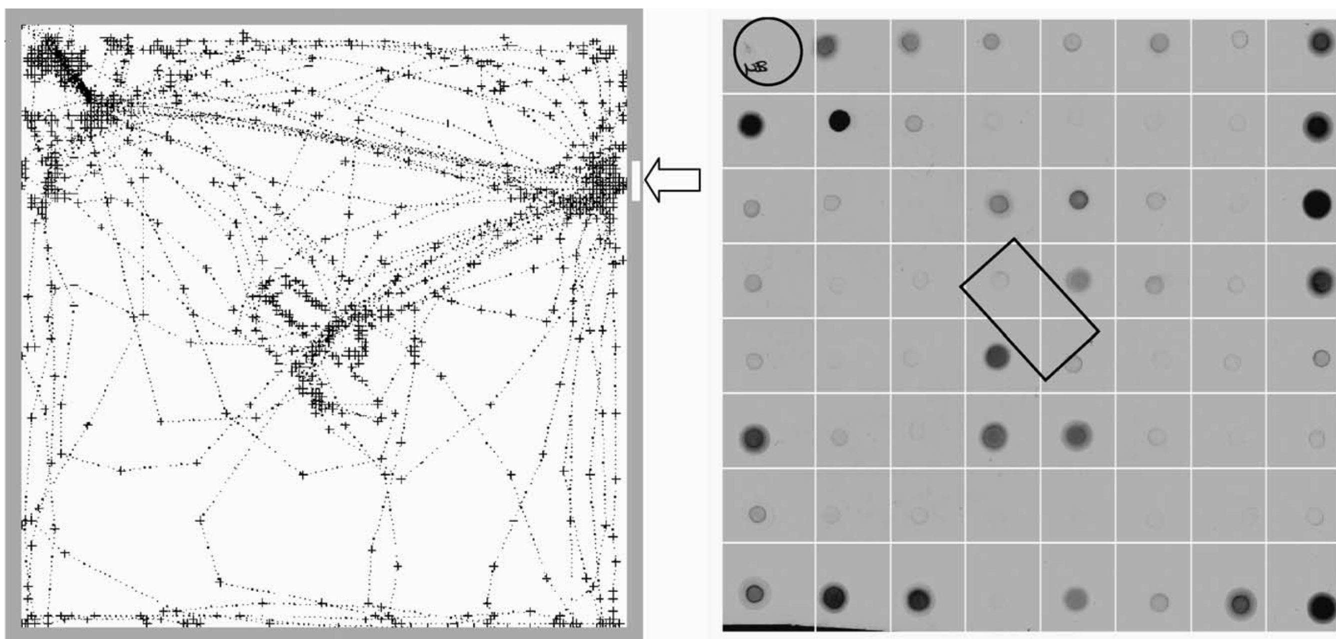

Fig. 1. Deposition or urinary protein by house mice. A male house mouse was allowed to enter a 1.2 m × 1.2 m clean territory at the position indicated by the arrow. For the next 15 min, the position of the mouse was recorded at 0.3 s intervals, and the ensuing path is displayed on the figure (left hand panel). At the end of this period, 15 cm × 15 cm areas were swabbed and subsequently tested for the presence of mouse major urinary protein using a polyclonal antibody specific for that protein. The 'dot blot' of antibody reactivity (not to scale) is shown in the right hand panel. Circle: nest box, rectangle: feed site.

must therefore include stringently designed behavioral experiments using appropriately manipulated biochemical samples.

Scent marks, notably those deposited by a dominant animal, are encountered by conspecifics of both sexes and of different status. The effects of male urine scents on female conspecifics can be dramatic, and include such phenomena as pregnancy block and puberty acceleration [11,12]. Moreover, there is good evidence that a female mouse uses the pattern and quality of male scent marks in selection of a mate [13–15]. Male conspecifics can be animals that are subordinate to the scent mark owner, in which case they will investigate and then tend to avoid the scent mark [16,17]. In particular, subordinates will avoid countermarking with their own scent, depositing their urine instead in larger pools away from sites marked by the dominant male, presumably to avoid arousing attack from the dominant territory owner [18,19]. Other males that are owners of neighbouring territories, or potential challengers to the current territory owner, will deposit their own urine countermarks (Fig. 2). This is the first skirmish in a 'scent war' [20] that is fought in part with chemical weapons – the scent marks themselves.

The protein concentration of wild-derived mouse urine is of the order of tens of milligrams per millilitre [21], which could be a substantial investment in protein synthesis that is lost irreversibly from the body. Additional costs associated with deposition of the marks include predation risk and the time and effort involved in continually refreshing scent marks throughout their territory. What is the evolutionary advantage to the mouse in incurring the cost of producing, disseminating and losing this protein? In this short review, we will summarize current knowledge of the biochemistry of MUPs, and show how the proteins can play multiple roles in the delivery of information in scent marks.

2. Structure of MUP and A2U

X-ray and NMR structures have been obtained for MUP, as have X-ray structures for A2U (Table 1). The structures are very similar, each having an eight-stranded beta-barrel fold that classifies them as lipocalins [27], and with a markedly similar structure (Fig. 3). A key feature of such lipocalins is a central cavity, lined with hydrophobic residues that constitute the site of binding of apolar ligands, especially pheromonal molecules (Fig. 4). In MUPs, this cavity has a volume of about 420–600 Å^3, depending on the MUP variant [21]. At the base of the cavity is the tryptophan residue (Trp 19), which is the only residue that is conserved in every lipocalin, and which may contribute to ligand binding but also to protein stability.

The most recent MUP structures have added considerably to our understanding of this protein. The original X-ray structure (1MUP) was of crystals produced from urine-derived MUPs, which is a heterogeneous mixture, and to which a range of pheromones might have been bound. Certainly, the electron density in the central calyx was weak. Further, there is no obvious opening to the cavity as a route for ligands to enter or leave the protein. More recent structures have been based on recombinant MUP produced in *Pichia pastoris* or *E. coli* (Table 1) and have supported the overall structure ob-

Table 1
MUP and A2U structures

PDB ID	Title	Reference, submission date, notes
1MUP	Pheromone binding to two rodent urinary proteins revealed by X-ray crystallography	[22], 21 September 1992, 2.40 Å crystal structure, also included structure of rat protein
2A2U	The crystal structures of A2U-globulin and its complex with a hyaline droplet inducer	[23], 19 November 1998, 2.50 Å crystal structure
2A2G	The crystal structures of A2U-globulin and its complex with a hyaline droplet inducer	[23], 19 November 1998, 2.90 Å crystal structure D-limonene 1,2-epoxide
1DF3	Solution structure of a recombinant mouse major urinary protein	[24], 17 November 1999, NMR: ensemble of 10 structures
1I04	Crystal structure of mouse major urinary protein-I from mouse liver	[25], 14 February 2001, 2.00 Å crystal structure
1I05	Crystal structure of mouse major urinary protein (Mup-I) complexed with hydroxy-methyl-heptanone	[25], 14 February 2001, 2.00 Å crystal structure
1I06	Crystal structure of mouse major urinary protein (Mup-I) complexed with *sec*-butyl-thiazoline	[25], 14 February 2001, 1.90 Å crystal structure
1JV4	Crystal structure of recombinant major mouse urinary protein (Rmup) at 1.75 Å resolution	[26], 28 August 2001, 1.75 Å crystal structure

tained in the original solution. The NMR structure [26] has provided some indication of regions of the protein chain that have a higher segmental mobility, which might indicate the degree of local unfolding to create the route of entry and exit of the ligand. Further information on ligand binding has come from X-ray structures where recombinant proteins were solved in the presence of bound synthetic pheromones [25] – in these structures the ligands are very well defined, and the position of the ligand is supported by additional NMR data [29].

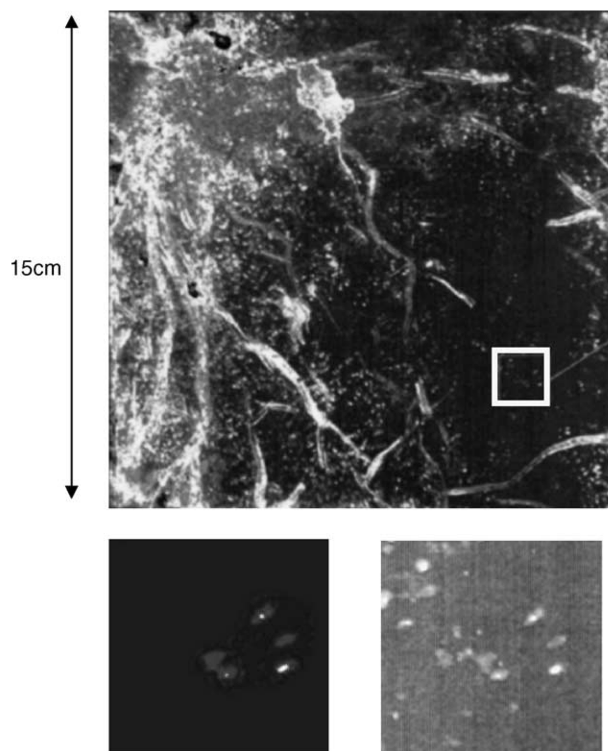

Fig. 2. Territory scent marking by house mice A 15 cm × 15 cm piece of clean Perspex tile was introduced into the territory of a male house mouse overnight. At the end of that time, the tile was removed and an imprint of the tile was made by blotting onto nitrocellulose sheet, which was subsequently probed with a polyclonal antibody specific for mouse major urinary protein. Close examination of the region outlined with the white box (bottom right hand area unprocessed, bottom left hand area, contrast enhanced) indicated that individual footprints could be detected by this method.

3. Ligand binding

There had been intermittent reports in the literature that the pheromonal qualities of mouse urine could be delivered in part by a high molecular weight fraction. In many of these early studies, the high and low mass fractions were separated from each other by simple methods such as dialysis or by ammonium sulfate fractionation to precipitate the proteinaceous material. The association of the biological effects with this high mass fraction was taken as evidence that the pheromones were associated with the protein. These early studies were followed by the observation that two male pheromones, 2-*sec* butyl 4–5 dihydrothiazole (‘thiazole’) and 3–4 dehydro-*exo*-brevicomin (‘brevicomin’), co-eluted with the urinary proteins on size exclusion and ion exchange chromatography [30,31]. The first three-dimensional structure for MUP, recovered from mouse urine (PBD:1MUP) confirmed that the proteins belonged to the lipocalin class, entirely in keeping with a putative role in pheromone binding. The electron density in the central cavity was sufficient to imply a bound ligand, although it was likely that cavity occupancy was low. The precise orientation of the ligand was updated by the subsequent publication of a structure of a recombinant protein with synthetic ligand [25]. Later studies also confirmed that the MUP cavity was flexible and was able to accommodate a variety of pheromonal [32,33] and reporter [34] molecules, as well as environmentally derived chemicals [35]. Binding studies on A2U have also confirmed the ability to accommodate a range of ligands [23,36].

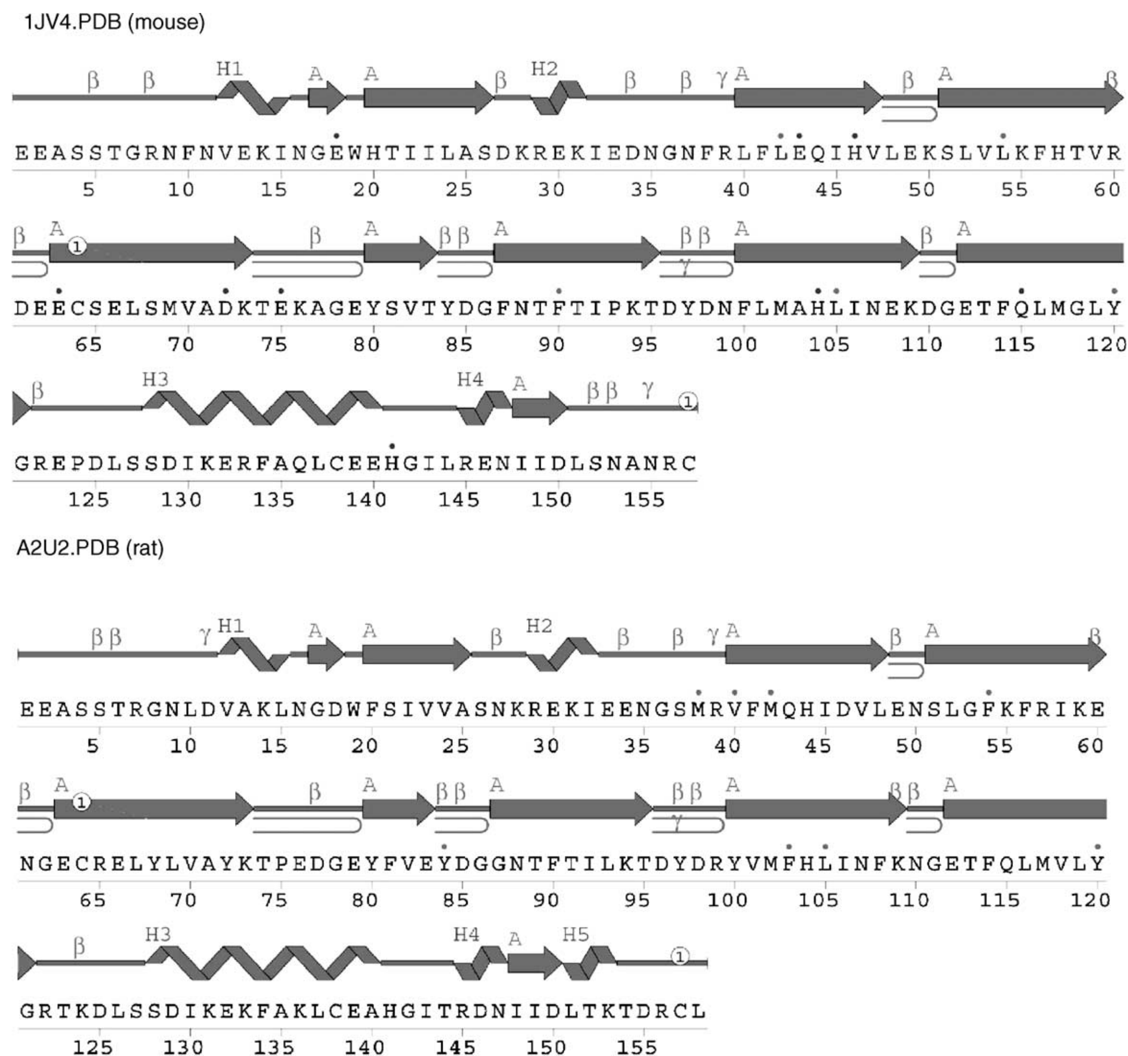

Fig. 3. Secondary structure maps of MUP and A2U. These diagrams were drawn with the aid of PDBSum [28] (http://www.biochem.ucl.ac.uk/bsm/pdbsum/) for structures 1JV4.PDB (mouse MUP) and A2U2.PDB (rat A2U). Helical, beta pleated sheet and turn regions are indicated diagrammatically, and emphasize the close similarity between the two structures.

Although it quickly became established that the MUPs bound pheromonal ligands, the reason for this association was less clear. In the X-ray or NMR structures of MUP, the cavity is enclosed by polypeptide chain, and unlike lipocalins such as retinal-binding protein, there is no obvious trajectory along which ligands could travel. The implication is that the structure must undergo substantive conformational changes, such that one segment of the protein chain acts as a flap, or the entire beta barrel flexes to permit access to the ligand. Thus the ligand is completely enclosed by the polypeptide chain in the MUP. Interestingly, ligand binding seems to be accompanied by an increase in backbone conformational entropy, an unusual binding mode [37].

4. Primary sequence polymorphisms in MUPs

MUPs recovered from the same inbred mouse strain consisted of several isoforms that could be resolved by electrophoresis or isoelectric focusing and indeed, co-inheritance of specific isoforms was used as a biochemical marker in genetics. Subsequently, the cloning of MUP cDNA sequences and the characterization of the gene structure confirmed that a region of mouse chromosome 4 encoded large numbers (over 30) of MUP genes and highly homologous but non-expressed pseudogenes in a complex arrangement [38–41]. The complexity of the gene structure and the recovery of multiple related cDNA sequences from mouse liver was consistent with the idea that the multiple urinary MUP isoforms were the products of discrete genes, rather than the outcome of complex post-translational modifications. There are now over 2000 MUP sequences in the GENBANK database, and it is a challenging task to ascertain how many of these reflect novel MUPs, how many are liver (and presumably urine) specific, and whether any of these are expressed at low levels that might be masked by the predominant species.

The original cDNA sequences were obtained from two inbred mouse strains: C57BL/6J and BALB/cJ. These well-

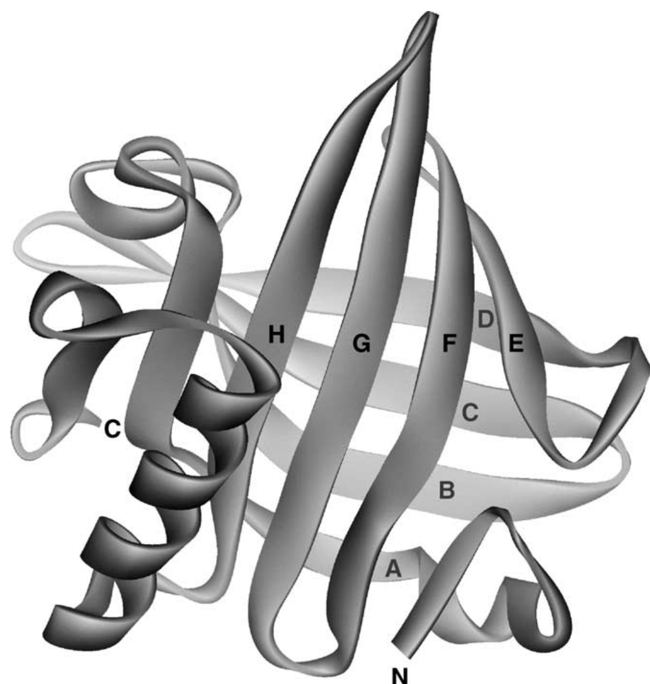

Fig. 4. Structure of MUP The main chain trajectory of structure 1I04.PDB is shown. The N- and C-terminal ends are marked, and each strand of the beta barrel (A–H) are labeled. The ligand-binding site is in a central calyx that is enclosed by all eight strands of the beta barrel.

established mouse strains are derived from different genetic lineages, and have been maintained as inbred populations for over 200 generations. In a detailed analysis of the MUPs from these and other inbred mouse strains, we separated the urinary proteins by high resolution ion exchange chromatography, analyzed the intact masses of the proteins by electrospray ionization mass spectrometry (ESI-MS), and located sites of polymorphism by matrix assisted laser desorption ionization time of flight (MALDI-ToF) mass spectrometry of MUP fragments generated by proteolysis with endopeptidase LysC [42]. From such analyses, we were able to confirm that several of the proteins predicted by the cDNA sequences were definitively expressed in urine, unmodified apart from the removal of the signal peptide and formation of a single disulfide bond. Other liver cDNA sequences predicted proteins that could not be located in urine, implying either sequencing errors leading to erroneous mass predictions or a level of expression of specific MUPs that was below detection thresholds.

Although inbred mouse strains are a valuable laboratory resource, presenting a constant genetic background to many biological experiments, this degree of inbreeding would never occur in wild house mice. Certainly, analysis of MUP cDNA and proteins from a number of *Mus* species implied a much greater degree of complexity than the inbred strains had revealed [39,43]. Accordingly, we also embarked on a study of MUP complexity in wild house mouse populations. Initial mass spectrometric surveys of a limited set of urine samples from wild mice revealed a complex pattern of MUPs in each animal [44]. A combination of high resolution ion exchange chromatography and electrospray ionization mass spectrometry provided further evidence for intact protein masses that had not previously been identified, implying the presence of many new MUPs in the wild population. Moreover, there was substantial variation between MUP patterns expressed by individual wild-caught animals. There was also some evidence for limited proteolysis, such that the AlaArgGlu C-terminal tripeptide was removed under some conditions. This degradation probably happens after urine has been released into the environment, although any function of this processing is unknown.

It seemed that the pattern of MUP expression in inbred mouse strains had only hinted at the true complexity of MUPs in wild populations. Further analysis of MUP expression, whether surveyed by isoelectric focusing or subjected to detailed ESI-MS and MALDI-ToF MS investigation, confirmed that the MUP complexity in wild mice was attributable to the presence of new genes or alleles [45]. Even mice from a closed island population in which there was substantial genetic homogeneity at other loci showed noticeable variation between individuals, raising the possibility of strong selection pressure maintaining MUP heterogeneity. Most of the variation between individual MUPs seems to be confined to a region in the sequence corresponding to the B, C and D strands of beta sheet, including the intervening turns [46].

5. Sexual dimorphism in MUP expression

It is a widely held notion that MUPs are produced exclusively by sexually mature male mice. This may have derived from studies of inbred laboratory strains where the difference between the sexes is most marked. Also, some analyses have concentrated on the level of MUP mRNA present in liver of the subjects, rather than evaluating urinary output. A second complicating factor is the concentration of urine. Dilute urine would give a MUP concentration that is apparently less than that from an individual where the volume of urine produced is lower. It is possible to correct for dilution by measuring creatinine in urine. Creatinine is created in muscle in a non-enzymic process and eliminated almost exclusively through the kidneys. The rate of production of creatinine is quite consistent for animals of similar body mass, and this parameter can be used as a reference value to assess urine dilution. A better parameter to assess MUP output is therefore protein:creatinine ratio [47]. Recent data from our laboratories gives the mean urinary MUP output (expressed as protein:creatinine ratios) of laboratory inbred strains as male: 10.7 (n = 140, range 1.6–26.5) and female: 3.01 (n = 140, range 0.7–7.6). On average, males produce between three and four times as MUP as females, but the ranges overlap substantially. For wild-caught adult mice, the equivalent figures are male: 37.3 (n = 24, range 3.2–83.7), female: 9.5 (n = 18, range 4.0–20.3), also consistent with this three to four-fold male:female ratio. The pattern of MUP isoforms expressed

in males and females differs ([47–49], unpublished observations), and in wild populations, patterns of MUP isoforms are shared more between females than males, consistent with a male-dispersal model [47]. Male and female wild mice have similar protein:creatinine ratios pre-puberty, but with the onset of puberty, male MUP output rises substantially.

6. The role of MUPs in mouse urine scent communication

Questions concerning the role that MUPs play in communication can only realistically be answered by combining biochemical studies with the assessment of behavioral and physiological responses to scent signals. Our experimental paradigm reflects a strong collaboration between biochemists and behaviorists, using natural responses to experimentally manipulated scent sources. Most of our research is targeted to wild-caught or wild-derived mice, as these exhibit a wider range of functionally relevant responses to scent marks. Inbred laboratory mice, by contrast, often exhibit impaired natural responses. In as much as these inbred animals have been derived by brother–sister mating through hundreds of generations, and are usually reared and housed in an unnatural social environment when the only scent profile they encounter is from genetically identical conspecifics, it is perhaps not surprising that their response can be limited. Moreover, subliminal selection of individuals that are tolerant to living in close confinement with conspecifics of their own sex and to mating with a closely related individual may have suppressed behavioral responses normally associated with competitive behavior and mate choice, for which olfactory cues provide strong drivers. A disadvantage specific to wild-caught wild mice is the lack of knowledge of their experiential history. However, this can be controlled in subsequent generations of mice bred from wild-caught parents. The degree of genetic heterogeneity of wild-derived mice is much greater than that for inbred strains, but the remarkable consistency of many responses to scent cues presumably reflects their importance in mouse social behavior.

6.1. Slow release of volatile signals

The binding of volatile pheromones to MUPs could function to protect the ligands from chemical degradation (for example, through oxidation) or MUPs might act as a vehicle to elicit the slow release of pheromones. The main ligands bound to MUPs in male mouse urine, thiazole and brevicomin, are known to have pheromonal priming effects on female reproductive physiology [32,50,51] as well as having a number of direct effects on both male [52–54] and female [50,55,56] behavior. Both thiazole and brevicomin are rather volatile, and might be expected to be lost rapidly once urine is deposited in the environment as a mouse scent mark. Could the protein therefore act as a slow release mechanism?

To assess whether MUPs are able to delay loss of pheromonal signals from scent marks over a timescale that would be functionally important to the animals, we made use of the observation that males are initially cautious in approaching the freshly deposited urine of another aggressive adult male [57]. This aversive effect is mediated, at least in part, by the male specific pheromones thiazole and brevicomin that had been recognized as protein bound. When the hesitancy to approach a scent mark was assessed over time, the decay in hesitancy matched the loss of thiazole and brevicomin from urine marks analyzed in parallel. To confirm that proteins mediated the longevity of the signal, we needed to compare responses when ligands were displaced from the protein. A suitable competitive displacer molecule was menadione (Vitamin K3) that we had shown to be capable of binding to the ligand cavity [35] and which had no effect itself on mouse behavior. When urine marks were treated with the displacer the hesitancy to approach the scent mark was virtually eliminated. Biochemical analysis of parallel samples confirmed that the ligands had been rapidly lost from the urine marks, consistent with their volatility once displaced from the protein [57]. A role for proteins in extending the time domain of male specific signals has therefore been demonstrated.

If the primary role of MUPs is to extend the time domain of pheromone signals, there could be significant advantages to the scent marking individual. Since scent mark signals need to be easily detected by conspecifics when the owner is elsewhere, volatile airborne components are essential to draw attention to the scent mark. However, volatile components will also be lost from the scent and require replenishment. Thiazole and brevicomin are rapidly lost from a surface, within minutes, if they are deposited in an organic solvent free of protein. The presence of proteins in a scent marks extends the release to several hours [57], and thus, the originating animal does not need to replenish the mark so often, and can use marks to delineate a wider territory. It has been suggested that the cost of production of between 10 and 20 mg of protein each day is substantial in evolutionary terms [58], but this may not be the case. In the first instance, an animal such as a mouse has a high basal rate of protein turnover, and there may be as much as 200 mg of protein synthesized and degraded every day in a mouse liver. The energetic cost of synthesis of protein as an irreversible loss may not be so great in that context. Further, a major ‘cost’ to an individual is the time and energy taken to traverse the territory to renew scent marks, together with the risk of exposure to dangers such as predators. Molecular mechanisms that reduce these costs while retaining a detectable chemical signal might have substantial advantages to the individual.

6.2. Competitive scent countermarking

The main purpose of scent marking by dominant male mice is to advertise territory ownership and their ability to defend the territory successfully to competitors and to fe-

males. By using scent signals deposited throughout the defended area, territory owners are able to provide physical proof of their ability to defend and scent mark the area. Territory owners exclude competitors and countermark any scents from competitors by depositing their own urine scent in the vicinity, returning repeatedly to deposit more scent marks by the competitor's scent over the next few hours [59]. Because scent marks remain in the environment, they therefore provide a record of competitive challenges between males and proof of which male won that is available to any other animals in the area [60]. Females appear to use these scent marks and countermarks when selecting a mate, preferring a territory owner whose scent is fresher than that of any intruders over an owner whose territory contains intruder scents that have not been countermarked [61,62]. However, to be used in this way, territory scent marks must contain information on the age of a scent mark and the owner's individual identity in addition to information indicating that scent marks come from a dominant male mouse.

To first establish whether MUPs and their ligands might provide some or all of this information, we fractionated male mouse urine into a high molecular weight fraction containing all of the urinary proteins and low molecular weight components that were not associated with proteins. When these were introduced into a resident male's territory, only the high molecular weight fraction stimulated countermarking [59]. Biochemical analysis confirmed that this high molecular fraction contained not only proteins but also the low molecular weight male signalling volatiles, thiazole and brevicomin, that would provide information that the scent came from an adult male mouse. Initially, this might have suggested that males detected signalling volatiles from another male and thus countermarked. However, we also found that the high molecular weight fraction remained just as potent in stimulating a competitive countermarking response even when aged by 7 days or when most of the signalling volatiles were displaced from the protein [59]. Further, when we prevented contact with urine scent marks, males still investigated volatiles emanating from the scent but failed to countermark [63]. The information that stimulates competitive countermarking thus not only involves volatile components but also non-volatile components. One possibility is that the involatile MUPs present ligands to the vomeronasal organ (VNO). Alternatively, the MUPs themselves might provide important information in their own right. Since the male signalling volatiles clearly have the capacity to indicate that scents come from another adult male, what additional information might be provided by the urinary proteins or protein–ligand complexes?

One possibility is that the protein–ligand complex allows animals to make an accurate assessment of scent mark age. Females discriminate between male scent marks and countermarks only when there is an age difference between them [62]. This may explain why males return repeatedly to an intruder's scent to keep depositing their own fresh scent – this gradually maximizes the difference in freshness between their own scent and that of the competitor [60]. Females must be able to assess the time that has elapsed since a scent mark was deposited. The age of a mark cannot be assessed simply by the concentration of a single molecule in the vicinity of the scent mark. A sensor animal would, when confronted with a strong signal, be unable to tell the difference between a recent mark and a mark deposited some time ago, but in a large quantity. The only feasible approach to assess scent age is by assessment of the ratio of two molecules that disappear from the mark at different rates. Because the MUPs are very refractory to proteolytic or other degradation, they might act as a stable and long lasting 'timebase' against which volatiles can be assessed. Of course, for this to be the case, the sensor animal must make contact with the scent, as proteins are far too involatile to be detected as an airborne signal.

6.3. Individual ownership signals

Whatever the role of MUPs in mediation or propagation of volatile scent cues, it is difficult to explain the extreme polymorphism observed in this class of molecules. However, for scent marks to provide information about an individual in the absence of the owner, the scent mark must provide reliable information about the identity of the depositor [7]. Could MUP polymorphism be involved in providing a stable individual ownership signal in scent marks? Examination of the variability in MUP patterns expressed by individuals even when captured from the same population [46,47] suggests that MUP polymorphism has the capacity to provide individual ownership signals. Further, MUP type is "hard-wired" in the genome such that individuals express the same pattern throughout life while the stability of MUPs once deposited would ensure that an individual's MUP pattern remained constant.

To investigate whether the urinary MUP pattern might contribute to an individual's scent mark ownership signal, we assessed whether wild mice could discriminate between their own scent marks and those of other males when urine marks were introduced into a male's territory [64]. Within a litter of wild house mice, some brothers inherit the same MUP pattern from their parents (same MUP) while others inherit a different pattern (different MUP). Unrelated males all inherit different patterns since their parents generally carry different patterns of MUP alleles. When a male's own urine was introduced, the territory owner paid little attention and scent marked no more than in response to a water control. If urine from an unrelated male (different MUP) was introduced on the other hand, territory owners countermarked and spent much more time in the vicinity of the intruder's scent. Adult male mice defending a territory are also highly aggressive towards their brothers – despite being kin, brothers are still competitors for territory ownership and mating opportunities. Urine from a brother with a different MUP pattern to the territory owner stimulated a similar competitive response to urine from an unrelated male, with owners spending much time near the urine and countermarking. However, urine from a brother of same

MUP pattern as the territory owner stimulated no such response. Although males briefly sniffed the urine when it was first introduced, indicating that they detected some difference compared to their own urine, they failed to spend more time near the urine or to countermark once they had investigated the scent mark closely. Since the only consistent difference between brothers was in the similarity of their MUP pattern to the territory owner, this suggests that MUP pattern was crucial in detecting that the urine was not own on investigation and stimulated a competitive response. However, in these experiments, males were F1 offspring derived from crosses between wild-caught animals. There would thus have been many genetic differences between brothers besides MUP type that might have contributed to their individual urine scent. The implication of the lack of response to a brother of same MUP type is that other genetic differences were not important in allowing males to recognize scent marks from another male in the absence of a difference in MUP type. We confirmed that MUP type was responsible for recognition of urine from another male by adding a recombinant MUP to a territory owner's own urine mark, while controlling the total protein concentration. The addition of a recombinant MUP altered the pattern of MUPs in a male's scent mark and stimulated a countermarking response [64].

How do animals detect differences in MUP type? One possibility is that the different MUP isoforms release ligands at different rates, and thus, can alter the profile of volatile release. Certainly, there is good evidence that the affinity of different MUPs for different natural or reporter ligands can vary, and this is particularly true of those isoforms where there are amino acid substitutions in the central hydrophobic calyx [33,34]. However, the profiles for loss of thiazole and brevicomin in drying urine samples were virtually identical when compared between two inbred strains of mice with different MUP isoforms [65], and a role for different MUPs in ligand release must remain somewhat conjectural at present.

7. MUPs and histocompatibility complex (MHC)

Another highly polymorphic system that influences scent signals is the major histocompatibility complex, which is involved in self–non-self recognition at the cellular level in the immune system. MHC haplotype affects the scents produced by individuals in a wide range of species including rodents [66,67]. Experiments utilizing MHC congenic strains of mice and rats have revealed that rodents can discriminate scents caused by single gene mutations in the MHC (e.g. [67–71]). Behavioral responses suggest that MHC-associated odors are used to assess the MHC similarity of conspecifics and elicit MHC disassortative mating preferences [72–74] or to associate preferentially with animals likely to be close kin [75].

Thus, both MUP and MHC are highly polymorphic multigene complexes that contribute to individual differences in rodent urinary scents, along with many other genetic differences that influence the urinary volatile profile [76–78]. Although non-volatile MUPs appear to be essential to the ownership signal in scent marks, low molecular weight components are important for individual recognition in other contexts. For example, pregnancy block occurs if a recently mated female mouse is exposed to male urinary chemosignals from a different male [79], even when the male differs genetically only at the MHC [80]. The components that induce pregnancy block (and thus presumably recognition of a strange male) are in the low molecular weight fraction of urine, although the strongest effects are induced by a combination of high and low molecular weight components. Thus, a generalized binding capability of MUPs may be vital for concentrating low molecular weight components and for their transport to receptors in the vomeronasal organ [81,82]. However, as yet the interaction between MUP and MHC-associated odors is unclear, particularly since the mechanism underlying the elaboration of MHC-associated odors is unknown.

MHC-type is detected through airborne odors in behavioral tests of discrimination in which an airstream is passed over rodent urine. However, fractionation of mouse urine revealed that MHC type could only be assessed from the high molecular weight fraction containing urinary protein [83]. Later eluting fractions that contained only low molecular weight components were not active. When the protein fraction was dialysed and ultrafiltered, MHC type was clearly discriminable in behavioral tests using the resultant low molecular weight material. MHC-associated odors appear to be a complex mixture of low molecular weight urine components that are bound and released by urinary proteins [83,84]. MHC-based developmental variations could give rise to distinct volatile profiles [76], which could then bind to urinary proteins. Differences in hormone levels, growth and behavior between MHC congenic laboratory strains could also influence metabolites in urine [85,86]. It has now been established that individual MHC odors are not associated with a unique population of bacterial flora. It is not known whether the urinary proteins involved are MUPs or fragments of MHC proteins in urine.

The "carrier hypothesis" [67], proposes that urinary volatiles bind to fragments of MHC proteins in urine. According to this hypothesis, large fragments of MHC class I proteins act as odorant carriers, binding a specific profile of volatiles from the complex mixture in urine or serum. Allelic differences that specify the MHC type are concentrated within the antigen-binding cleft formed between the $\alpha 1$ and $\alpha 2$ domain of class I MHC proteins. Soluble MHC class I molecules are filtered via the kidneys into urine where they undergo further proteolytic degradation. Enzymatic cleavage at the junction of the $\alpha 2$ and $\alpha 3$ domains allows relaxation of the binding platform, opening the cleft and resulting in the loss of the bound peptide. It is suggested that this vacant binding site then acquires small volatile molecules, the specificity of binding of which is dictated by the allelic variant. How low molecular weight volatiles could be specifically bound to class I protein fragments that normally bind peptides is unclear [84]. By contrast, the central calyx of MUPs

is designed to bind small odorant molecules. MUPs are also present in considerably greater concentration than MHC fragments in urine [21]. If the first hypothesis is correct and MHC-dependent volatiles are bound and released by MUPs, then two highly polymorphic multigene complexes inherited independently on separate chromosomes may act together to determine the main basis of an individual's volatile and involatile urinary profile. The capacity for unique individual combinations of these components is considerable.

8. MUP receptors?

There is an emerging body of evidence to support the idea that MUPs convey information in their own right, with or without the involvement of their bound ligands. However, the existence of specific receptors for MUPs remains somewhat controversial. The role of MUPs in mediation of pheromone signals strongly implicates the vomeronasal organ (for a recent comprehensive review of VNO see [87]). Certainly, there is now increasing evidence that a subclass of receptors (V1R) in the vomeronasal system (VNS) elicit the response to lipophilic, low molecular weight pheromones. These V1Rs are considered to recognize odorants by binding to the cavity created by the seven transmembrane spanning segments of the protein [88]. Lipophilic pheromones elicit a strong response in VNO slices in vitro [89]. However, in direct recordings made from VNS neurons in intact animals, low molecular weight pheromones were ineffective in stimulating a response [82]. Although not tested directly, it was suggested that MUPs might enhance detection either by direct recognition of the proteins themselves or by aiding delivery of lipophilic molecules to the VNO by enhancing solubility in the mucus flow that occurs as a consequence of the VNO pumping mechanism. A second subclass of VNO receptors, V2Rs (encoded by a multigene family of about 100 genes) differ from V1Rs in having an extended and highly variable N-terminal domain [90–92]. It is tempting to speculate that the N-terminal domain may provide an interaction domain that binds MUPs, although alternative mechanisms have been proposed [93].

Definitive proof of the existence or otherwise of putative MUP receptors is needed. Responses to single protein molecules, such as those used for low molecular weight pheromones, may not be the most appropriate test. It is possible that the protein component of a MUP-ligand complex provides a context, such as an 'identity tag', and that activation of an appropriate receptor requires a combination of MUP (mediated via the variable N-terminal extension of the V2Rs?) and a ligand. Physiological tests, such as puberty acceleration and pregnancy block should require that at least part of the stimulus advertises freshness of the scent mark – a role most readily discharged by volatile urine components. The protein fraction may accentuate that 'freshness' signal by increasing the contrast to olfactory images previously obtained, and the complexity of the MUP profile offers a straightforward, combinatorial approach to maximizing that contrast. It is not likely that the individual MUPs are responsible for delivery of specific lipophilic molecules, as the proteins do not show stringency in specificity of ligand binding. Further, the rarity of cavity-specific mutations and the imperfect occupancy of the calyx by ligands all suggest that differential ligand binding is not a primary function of the MUPs.

9. Conclusions

It is increasingly clear that the chemical signals in mouse urine elicit complex effects, and that multiple classes of molecules must be involved. Urine is such a critical source of complex information in mouse social behavior that it would be most unlikely that this role could be discharged by just a few compounds. It is now firmly established that proteins, specifically synthesized in the liver and passed through the kidney into urine, are critical to modulation of urinary signals. The stage is set for an integrated model of chemical communication that includes proteins in scent marks as critical components. Only then can we really claim to understand the interplay and dynamics of scent deposition, animal physiology and behavior.

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