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Major Urinary Proteins: Functional characterization of a novel pheromone family that
promotes intermale aggression

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Neurosciences

by

Tobias Francis Marton

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it is acceptable in quality and form for publication on
microfilm:

Co-Chair

Chair

University of California, San Diego

2008

DEDICATION

For Mo and Gidget.

EPIGRAPH

“A rabbit has been calculated to possess one-hundred-million olfactory receptors – small wonder its little schnozz is always twitching, it is trapped in an undulating blizzard of aromatic stimuli...”

Tom Robbins

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LIST OF ABBREVIATIONS

1. MUPs: Major Urinary Proteins
2. uMUP: Urinary MUP
3. VNO: Vomeronasal Organ
4. MOE: Main Olfactory Epithelium
5. OB: Olfactory bulb
6. VN: Vomeronasal sensory neuron
7. ESI-MS: Electrospray ionization mass spectrometry
8. GPCR: G-protein coupled receptor
9. OSN: olfactory sensory neuron
10. OBP: Olfactory Binding Protein
11. PLC: Phospholipase C
12. SBT: 2-secbutyl-4,5dihydrothiazole
13. DHB: 2,3-dehydro-exo-brevicomin
14. IEF: Iso-electric focusing
15. PAGE: Polyacrylamide gel electrophoresis
16. HMW: High Molecular Weight
17. LMW: Low Molecular Weight
18. ESP: Exocrine secreted peptide
19. MHC: Major Histocompatibility Complex
20. MTMT: (methylthio)methanethiol
21. ORF: Open reading frame
22. cAMP: Cyclic adenosine mono-phosphate

- 23. DNA: Deoxyribonucleic acid
- 24. IP₃: Inosine triphosphate
- 25. VA: 11-*cis* vaccenyl acetate
- 26. GH: Growth hormone
- 27. MS: Mass Spectrometry
- 28. FPLC: Fast Pressure Liquid Chromatography
- 27. T₄: Thyroxine
- 28. OMP: Olfactory marker protein
- 29: GC/MS: Gas chromatography/Mass Spectrometry

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Chapter 4, in full, is reproduced from: Stowers L, Marton TF. What is a pheromone? Mammalian pheromones reconsidered. *Neuron*. 2005 Jun 2;46(5):699-702. The dissertation author was the primary investigator and author of this paper.

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Stowers L, **Marton TF**. What is a pheromone? Mammalian pheromones reconsidered. *Neuron*. 2005 Jun 2;46(**5**):699-702.

Hu H, **Marton TF**, Goodman CS. Plexin B mediates axon guidance in *Drosophila* by simultaneously inhibiting active Rac and enhancing RhoA signaling. *Neuron*. 2001 Oct 11;32(**1**):39-51.

ABSTRACT OF THE DISSERTATION

Major Urinary Proteins: Functional characterization of a novel pheromone family that
promotes intermale aggression

by

Tobias Francis Marton

Doctor of Philosophy in Neurosciences

University of California San Diego, 2008

Professor Lisa Stowers, Chair
Professor Jing Wang, Co-Chair

Mice use pheromones, chemical cues emitted in bodily secretions and detected by the olfactory system of conspecifics, to regulate important innate social behaviors, such as aggression and mating. Neurons that detect pheromones are thought to reside in at least two separate organs within the nasal cavity: the vomeronasal organ (VNO) and the main olfactory epithelium (MOE), with each pheromone ligand activating a dedicated subset of these sensory neurons. Despite the molecular and anatomical characterization of these two olfactory pathways, little is understood about the identity of the pheromones that regulate critical behaviors, nor the identity of the sensory neurons that are activated to initiate a behavioral response. Urine is a rich source of pheromones, and male urine has been shown to be sufficient to generate intermale aggression in a VNO dependent manner (Stowers et al., 2002). Using direct activation of VNO sensory neurons (VNs)

with behavioral analysis, this study identifies a novel family of genetically encoded pheromones, the Major Urinary Proteins (MUPs), which are sufficient to generate intermale aggression and activate VNs. MUPs are male-specific lipocalin proteins expressed in large quantities in urine that bind organic small molecules thought to be pheromones. This study shows that MUP activation of VNs is entirely protein dependent, in contrast to prior hypotheses that MUPs are merely passive carriers for their bioactive pheromone ligands. In addition, the VNs activated by MUPs are identified as the Gao, V2R pheromone receptor expressing class of neurons. Genomic analysis indicates species-specific co-expansions of MUPs and V2Rs, as would be expected among pheromone-signaling components.

MUPs are expressed in unique strain and individual specific combinations of four to six of the 18 highly homologous MUP open reading frames arrayed on chromosome four. Here, we report that the VNO is tuned to discriminate between MUPs, despite the high homology in this gene family, indicating that the MUPs may activate a wide range of VNO sensory circuits, providing a rich source of information coding in the pheromone system. Finally, we have identified a discrete VNO sensory circuit activated by a single MUP that generates aggressive behavior. These findings are a fundamental step forward in elucidating the pheromone ligands that initiate important behaviors in mice, as well as understanding the neural circuitry that drives these critical social interactions.

Chapter 1: Introduction

Introduction

Most terrestrial vertebrates such as rodents are critically dependent on the emission and detection of compounds known as pheromones to drive appropriate behavioral interactions, such as mating and aggression. The term pheromone was first coined by Karlsson & Luscher in 1959: “Substances secreted to the outside of an individual and received by a second individual of the same species in which they release a specific reaction, for example, a definite behavior or developmental process” (Karlsson and Luscher, 1959). In the mouse, putative pheromones have been identified in an array of bodily secretions including urine, saliva and tears and can take the form of either small molecule metabolites or proteinacious compounds such as peptides (reviewed in Stowers and Marton, 2005, see chapter 4).

There are two sensory systems in the mouse dedicated to the detection of chemical stimuli in the environment, including pheromones. The main olfactory epithelium (MOE) contains olfactory sensory neurons (OSNs), each one tuned to respond to specific volatile odorants through the expression of distinct G-protein coupled receptors (GPCRs). Mice and other lower mammals contain an additional chemosensory organ known as the vomeronasal organ (VNO), which resides in the floor of the nose and has been historically thought to be specialized for the detection of non-volatile pheromone compounds. Molecular characterization has revealed that the primary signal transduction machinery of MOE neurons is distinct from that of VNO neurons (reviewed in Dulac and Torello, 2003). Although ligands for both structures activate specific GPCRs, the MOE receptors are evolutionarily distinct from all identified VNO receptors.

Furthermore, GPCR activation in the MOE leads to the production of cAMP to gate CNGA2 channels. These signaling components are not expressed in VNO neurons that instead utilize a phospholipase C (PLC) pathway to activate TrpC2 channels. Most interesting, however, is the apparent segregation of the neuronal circuitry. MOE neurons project axons to the olfactory bulb and synapse on mitral cells that in turn signal to the cortex and the olfactory amygdala. In contrast, VNO neurons project to the accessory olfactory bulb (AOB) and relay their signal to the anatomically distinct medial amygdala. While it was originally thought that these segregated pathways reflected a functional specialization for the two sensory systems (MOE for volatile odor detection, VNO for non-volatile pheromone detection), recent evidence suggests that there is considerable functional cross-over between these two systems (Reviewed in Stowers and Marton, 2005 chapter 4).

Despite the molecular characterization of these two sensory systems, few pheromone-mediated behaviors have been associated with their requisite chemosensory pathway. This lack of clarity arises largely from the fact that the majority of VNO receptors remain orphaned, and the functional anatomy of the VNO circuitry is still not understood. Stowers et al., 2001 shed some light on the question of VNO function when they reported genetically ablating the TRPC2 channel, required for vomeronasal sensory neuron (VN) evoked activity, finding that male TRPC2 $-/-$ mice were deficient in displaying aggression towards other males. Others have reported that the aggression pheromone is a constituent of wild-type male urine, although the pheromone itself has not been further purified or identified. Hence, intermale aggression provides a convenient platform for beginning to understand the molecular and functional anatomy of the

vomer nasal sensory system: it is a VNO dependent behavior with a defined and easily obtainable pheromone source (male urine). How could the aggression pheromone be detected by the VNO? All VNs express a discrete pheromone receptor of either the V1R (~100 receptors) or V2R (~221 receptors) family. V1R expression is anatomically restricted to the apical half of the VNO and uses *G α i* to signal via PLC and activate TRPC2 channels. In contrast, V2Rs are expressed more basally and utilize *G α o* to drive activity through TRPC2. The anatomical segregation of these two classes of VNs is maintained through their projections to the AOB, with the V1R neurons synapsing with more anterior glomeruli, and V2R neurons synapsing in the posterior half (reviewed in Dulac and Torello, 2003). To date, the functional logic of this anatomic organization remains a mystery; in fact, only one pheromone receptor (V1Rb2) has been paired with its ligand, and no pheromone-mediated behaviors have been described at the level of ligand-receptor interactions (Boschat et al., 2002).

In this study, we coupled behavioral assays with calcium imaging of dissociated VNs to determine the specific constituents of urine that drive intermale aggression through the VNO, as well as molecularly characterize the responding VNs. In doing so, we determined that the high molecular weight (HMW) fraction of urine contains aggression-promoting activity, and that this HMW fraction is composed exclusively of a previously orphaned class of proteins: the Major Urinary Proteins (MUPs).

Major Urinary Proteins: a novel protein pheromone family?

MUPs belong to a large superfamily of small (~20kD) ligand-binding proteins known as lipocalins. The members of the lipocalin family subserve many diverse

functions, including roles in co-factor transport in the blood, immune modulation, invertebrate coloration, and odorant binding (reviewed in Flower, 1996). However, despite their varied roles across and within organisms, as well as their rather low primary sequence homology (~20%), the lipocalins all share the fundamental tertiary structure of an eight-stranded anti-parallel β -barrel open at one end with α -helices at both the N and C termini (figure 1) (Ganfornina et al., 2000). The result of this “glove” shape is that all lipocalins bind specific small molecules that have high affinity for the hydrophobic binding pocket. As a group, lipocalins may act solely as carriers for these small-molecule ligands, or may themselves bind receptors or oligomerize to act in concert or completely independently of their cargo (figure 1). Elucidating the relationship between MUP and its ligand(s) and how this interaction modulates MUP function is a fundamental question we shall address in this study.

MUPs have been classically described as male specific proteins synthesized in the liver and secreted in milligram per milliliter quantities in the urine of male mice (Timm et al., 2001). Structural studies reveal that MUPs possess the essential structural properties of the lipocalin family: a β -barrel open at one end flanked by N and C terminal α -helices (figure 2). The urinary small molecule ligands carried in the hydrophobic MUP core have been identified and include combinations of all six of the previously identified male specific pheromones known as the “Novotny Compounds” (Chapter 4, table 1) depending on the particular mouse strain examined (reviewed in Stowers and Marton, 2005).

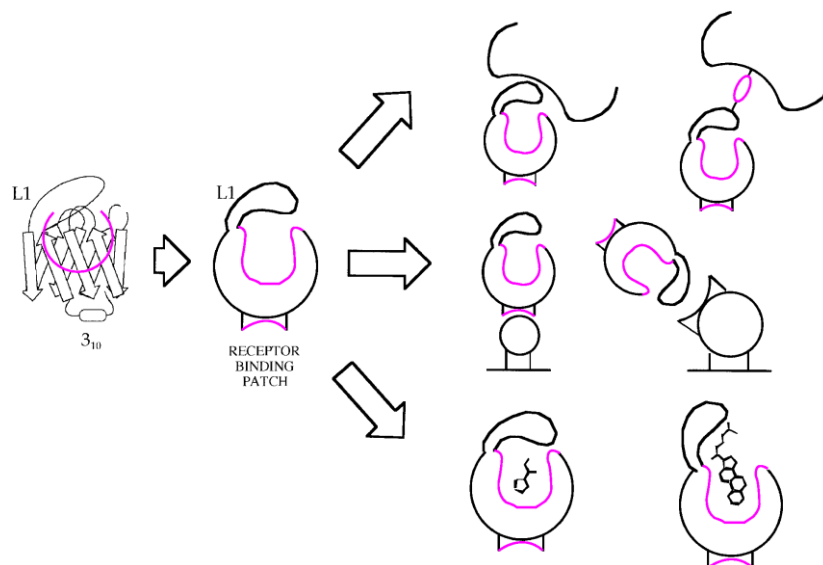


Figure 1.1 Schematic diagram showing the structure of lipocalin proteins with surface binding residues, ligand binding pocket and flexible gate (c/d loop) to binding pocket (left of arrows). Lipocalins can associate with macromolecules (top), and bind cell surface receptors (middle). Lipocalins associate with small molecules which may alter the conformation of the protein (bottom). Adapted from: Flower DR. The lipocalin protein family: structure and function. *Biochem J.* 1996 Aug 15;318 (Pt 1):1-14. Review.



Figure 1.2 Ribbon diagram showing the structure of MUP protein showing a β-barrel open at one end flanked by N and C terminal α-helices. Permission obtained. Lücke C, Franzoni L, Abbate F, Löhr F, Ferrari E, Sorbi RT, Rüterjans H, Spisni A. Solution structure of a recombinant mouse major urinary protein. *Eur J Biochem.* 1999 Dec;266(3):1210-8.

Hence, different MUP proteins in male urine can be complexed with a number of possible ligands which are thought to be pheromones, or may carry no ligand at all (Bacchini et al., 1992).

In Chapter 4, Stowers and Marton, 2005 review findings suggesting that the classical definition of pheromones as small molecules detected through the VNO effecting conspecific behavior may comprise an overly simplistic model. Indeed, Lin et al., 2005 had shown that the MOE may have a role in driving innate behavior through the detection of the semiochemical MTMT, and Leinders-Zufall et al., 2004 had extended the definition of the non-volatile VNO activating pheromones to include the proteinacious MHC class I peptides present in urine. Recently, Kimoto et al., 2005 demonstrated that the exocrine gland-secreting peptide (ESP) family secreted in tears from the extra-orbital lacrimal glands also have the ability to activate the VNO, identifying yet a second group of proteinacious VNO ligands. With these findings in mind suggesting a novel role for peptide pheromones driving murine behavior through the VNO, and given the ability of MUPs to complex with putative urinary pheromones, it is intriguing to ask what possible role MUPs may play in pheromone signaling between mice. To this end, the first goal of this study is to elucidate the specific mechanism MUPs may use to interact with the pheromone sensory system: Are MUPs merely passive carriers for their bioactive small molecules, or does the protein itself have intrinsic signaling capability?

However, the full complexity of MUPs as potential pheromones cannot be appreciated only by understanding their potential mechanism of action. Analysis of MUP genomic structure reveals a gene cluster of at least 18 putative open reading frames arrayed on chromosome 4 with a high degree of homology and gene duplication (figure

3) (Clark et al., 1985). In addition, analysis of urine from wild mice and inbred strains by iso-electric focusing gel (IEF) reveals that MUPs are expressed in different individual and strain-specific combinations of four to seven distinct MUPs per mouse (figure 4) (Robertson et al., 1996; Hurst et al., 2001). The result of this differential combinatorial expression of MUPs, as well as their ligands, is the potential that MUPs could encode discrete behavioral information either on their own or in combination (figure 6). Therefore, the second goal of this study is to determine the extent to which different MUPs, MUP complexes or MUP ligands may encode distinct, behaviorally relevant information between mice.

MUPs: protein pheromones or passive carriers?

The question as to what, if any, role MUPs play in pheromonal communication between mice is hardly new to the field of chemo-behavioral biology. The combinatorial expression of MUP proteins in urine, as well as the diversity of urinary small molecule MUP ligands expressed between mouse strains suggests abundant coding potential for MUPs in the pheromone system. Indeed, MUPs have been implicated in a large number of classical pheromone-mediated behaviors including puberty and estrus acceleration in females, the Bruce effect, and individual recognition (due to their unique expression profiles between individuals). While these studies lacked strong statistical significance as well as suffering from other methodological issues in their behavioral assays, the literature is further complicated by conflicting data regarding the exact role MUPs play in mediating these behaviors. Are MUP proteins merely passive carriers for their bio-active small-molecule cargo, which in turn interact with either MOE or VNO receptors to

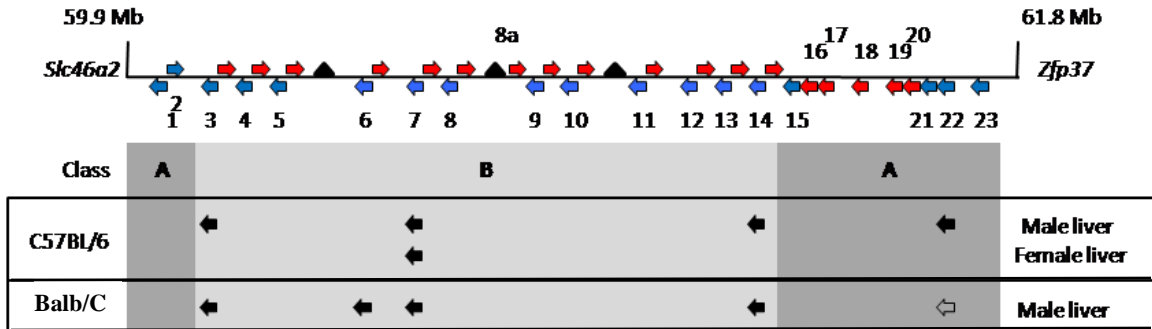


Figure 1.3 Genomic structure of MUP gene cluster on chromosome 4 showing known loci expressed in C57Bl/6 versus Balb/C. Blue arrows mark ORFs, while red arrows mark pseudogenes. Class A and class B MUP loci are marked underneath. The MUPs expressed in male and female C57Bl/6J as well as Balb/C are marked below.

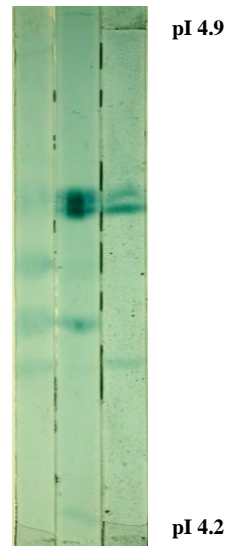


Figure 1.4 Iso-electric focusing gel of urine showing the differential MUP expression patterns of the C57Bl/6J, Balb/C and CD-1 strains (left to right).

convey behavioral information? Alternatively, are MUP proteins themselves pheromones engaged in protein-receptor interactions in the VNO to drive behaviors and endocrine changes? Finally, is there information encoded by the MUP-small molecule ligand complex itself, or are these autonomous components, each conveying specific information independently of the other? Determining which of these three models (passive pheromone carriers, autonomous protein pheromones, or protein-ligand complexes) describes the mechanism by which MUPs interact with the pheromone system to drive behavior is a central question we shall address in this study (figure 5).

The small molecule MUP ligands, known as the Novotny compounds, have been shown to be involved in regulating a number of pheromone mediated behaviors, such as intermale aggression and estrus acceleration (Novotny et al., 1999). In particular, it was shown that the two Novotny compounds established to be the MUP ligands in C57Bl/6J mice (2-secbutyl-4,5dihydrothiazole (SBT) and 2,3-dehydro-exo-brevicommin (DHB)) are sufficient in combination to drive intermale aggression in the resident-intruder assay (Bachinni et al., 1992, Novotny et al., 1985). These findings, which suggested that bioactivity resides in the MUP ligands, was supported by subsequent reports that these compounds derived synthetically were sufficient to activate discrete populations of VNO sensory neurons (VNs) by calcium imaging (Leinders-Zufall et al., 2000). Given this apparent small molecule activity in the fluid filled VNO, it was postulated that the MUPs functioned to serve as carrier molecules for these hydrophobic ligands, protecting them from the aqueous environment of the VNO and delivering them to their receptors. This hypothesis was consistent with previous findings which first demonstrated that the high molecular weight non-volatile constituents of urine (e.g. the proteinacious component)

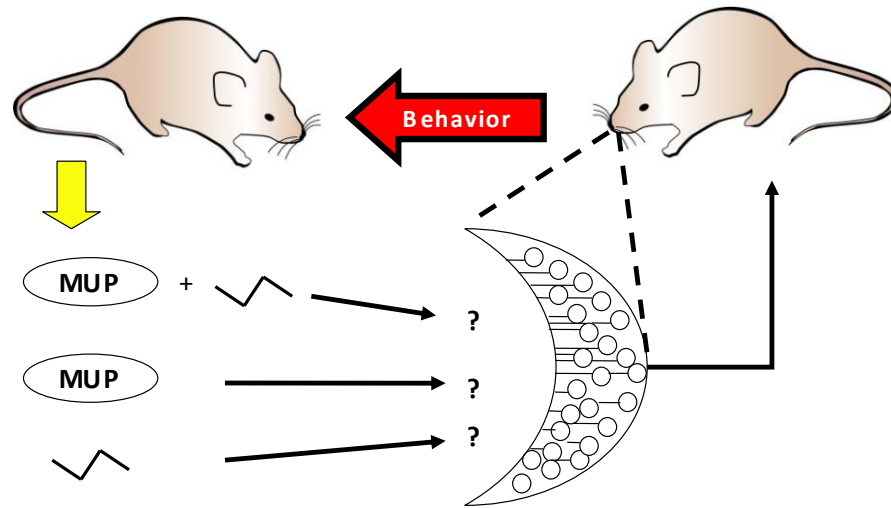


Figure 1.5 Three models for MUP activity at the VNO. MUP-ligand complexes, MUP protein alone, or MUP ligands alone secreted in the urine of male mice may interact with VNO sensory neurons in receiving animals to drive pheromone mediated behaviors.

were capable of accessing the mucus filled sensory duct of the VNO (Wysocki et al., 1980). In addition to functioning as passive pheromone carriers, it has also been proposed that MUPs serve to stabilize volatile territorial marking signals deposited in the environment. Hurst et al., 1998 showed that mice are less likely to avoid these deposited signals over time when the volatile cues are competitively displaced from their associated MUP, suggesting that the MUP helps to stabilize these volatile compounds in the environment so as to increase the longevity of a territorial chemosignal. It should be noted, however, that while these studies propose a carrier/stabilization role for the MUP protein, they do not exclude the possibility that the MUP or MUP-ligand complex conveys additional behaviorally relevant information.

Indeed, numerous conflicting studies have attempted to address the extent to which MUPs may possess activity independent of their carrier function. Brennan et al., 1999 examined the pattern of immediate early gene *egr-1* expression in the accessory olfactory bulb (AOB), the site of VN projection from the VNO, in female mice exposed to male urinary constituents. They found that while MUP ligands SBT and DHB activated mitral cell nuclei in the posterior-lateral and medial divisions of the AOB, MUPs proteins stripped of their ligands activated more anterior mitral nuclei. These findings indicated that that the MUP can initiate specific activity distinct from its small-molecule cargo. However, subsequent studies have been unable to reproduce these findings, reporting that MUP activation of AOB mitral cells is small-molecule dependent (Peele et al., 2003). A number of studies have addressed the question of MUP protein sufficiency from the standpoint of male pheromone induced changes in female physiology. Both Mucignat-Caretta et al., 1995 and Marchlewska-koj et al., 2000

reported that ligandless MUP protein conveys sufficient information to induce puberty and accelerate the estrus cycle in exposed female mice. However, in both cases the MUP protein had to be presented to the female dissolved in normally inactive pre-pubertal male urine in order to have an effect on reproductive physiology, indicating that the MUP requires some urinary context and is not completely sufficient to drive bioactivity in these assays. This lack of sufficiency has also been bolstered by reports that MUPs produced recombinantly, and so devoid of pheromone ligands, do not mediate the pregnancy-block effect (chapter 4, figure 2) when dissolved in water (Peele et al., 2003). Indeed, the literature provides a conflicting picture as to the extent to which MUP proteins convey specific information in a manner autonomous from their ligands or other low molecular weight constituents present in urine.

Can the pheromone sensory system of other species provide any clues as to the potential mechanism of action of MUPs in the mouse? Genes orthologous to the MUP genes are expressed in several other species including rat, cat, horse, cow, and boar. However, with the exception of rat, these MUP orthologs are restricted in their expression to the epidermis and saliva, and are largely notable for being the primary human allergen in these species (Gregoire et al., 1996; Loebel et al., 2000; Smith et al., 2004). The rat MUPs, known as the α_{2u} -globulins, share many of the same expression characteristics of the mouse MUPs: they are a gene cluster (~9 ORFs) expressed dimorphically and combinatorially in urine, lachrymal and submaxillary gland (MacInnes et al., 1986). The solution structure of a α_{2u} -globulin has also been resolved and shows striking homology to mouse MUP, including the ability to bind small hydrophobic molecules thought to be pheromones (Bocskei et al., 1992). This genetic, expression and

structural homology with mouse MUPs is unique to rat α_{2u} -globulins, raising the possibility that these homologous gene families may have similar mechanisms of action in effecting pheromone signaling. Indeed, supporting the hypothesis that MUPs may carry activity independent of their ligand, Krieger et al., 1999 used a biochemical assay to show that recombinant α_{2u} -globulin was sufficient to drive increases in G-protein second messenger IP₃ in preparations of rat VNO membranes, and that this activity was restricted to the V2R, G α_o expressing sensory neurons.

Several other species utilize lipocalin small molecule binding proteins (non-MUP orthologs) in their pheromone sensory systems, which may provide some insight into MUP activity. Female golden hamsters express a lipocalin known as aphrodisin in their vaginal secretions, which has been shown to be sufficient to drive male copulatory behavior in a VNO dependent and protease sensitive manner (Singer et al., 1986; 1987). Notably, no small molecule ligand has shown to be associated with the protein, and recombinant aphrodisin has been shown to cause increases in VNO IP₃ signaling as well as induce c-fos in AOB mitral cells, but appears insufficient on its own to drive copulatory behavior, although no confirmation of proper folding of the recombinant protein has been presented (Kroner et al., 1996; Jang et al., 2001). The olfactory binding proteins (OBP) of *Drosophila melanogaster* may also provide some insight into MUP function in mice. These lipocalin, small-molecule binding proteins are secreted into the fluid surrounding olfactory neuron dendrites in the trichoid sensilla of the fly. A particular OBP, LUSH (OBP 76a), was shown to be necessary for evoked activity of pheromone sensitive olfactory neurons by male pheromone 11-*cis* vaccenyl acetate (VA), and LUSH mutants demonstrate defects in VA induced behavior (Xu et al., 2005). It

was postulated that LUSH binds VA and acts as protein-ligand complex to evoke olfactory neuron activity and drive behavior. This model supports the hypothesis that in the mouse, MUPs may act in a complex with their ligand to bring about activity in the VNO. In summary, analysis of MUP ortholog function in other species reveals evidence that is consistent with all three of the possible mechanisms of MUP function under examination in this study.

MUP function: Clues from structure?

Structural studies of the MUP protein and MUP protein-ligand complex provide some interesting insights as to the potential mechanism of action of MUPs in the VNO. What is known about the structure of MUPs and the nature of their small molecule ligands? Structures for both wild type MUPs purified from mouse urine and a recombinant MUP have been resolved by X-ray crystallography (Bocskei et al., 1991) and in-solution NMR (Lucke et al., 1999). As discussed previously, both 2-secbutyl-4,5-dihydrothiazole (SBT) and 2,3-dehydro-exo-brevicommin (DHB) have been identified as the small molecule ligands carried by MUPs in C57Bl/6J urine (Bachinni et al., 1992). Additional Novotny compounds, including farnesene and 2-heptanone, have been shown to associate with the MUPs expressed in the CD-1 strain (Marchlewska-koj et al., 2000). These compounds have also been assigned a number of potential pheromonal functions that have been discussed, and have been shown to activate VNO sensory neurons independent of their MUP carriers (Leinders-Zufall et al., 2000).

Structural studies have further sought to elucidate the specific interaction between the MUP protein and its small molecule ligand. Timm et al., 2001 crystallized a common

MUP made recombinantly and loaded with different synthetic Novotony compounds, including SBT, in order to study how these small molecules associate with the hydrophobic MUP binding pocket. In addition to identifying key binding residues, it was observed that binding small molecule ligands resulted in a conformational change in the MUP protein largely effecting the Bc, Bd and intervening c/d loop (figure 2). It has been suggested that mobility in this loop may serve as a gate to the entrance of the B-barrel's hydrophobic interior (Timm et al., 2001). These observations raise the question as to how changes in MUP conformation due to ligand binding may affect the global tertiary and quaternary structure of the MUP protein and, subsequently, the extent to which the MUP-ligand complex may have the ability to differentially effect VNO signaling as opposed to the MUP or ligand alone.

Polymorphisms between MUP genes have also been shown to result in differential ligand affinities between individual MUP species expressed in mouse urine. Using an optical probe (n-phenyl-naphthylamine) that fluoresces strongly when bound to the MUP binding pocket as a reporter for ligand binding affinity, it was shown that all class B MUP variants except one (MUP 3) expressed in C57 Bl/6J show similar binding characteristics to the probe (Chapter 3, figure 1). X-ray crystallography followed by molecular modeling studies revealed that MUP 3's differential binding was due to a single polymorphism (val to phe) at position 56 that resides within the binding cavity, resulting in altered ligand affinity (Darwish-Marie et al., 2001). Sharrow et al., 2002 extended these findings to show that MUP 3 shows differential affinities for natural ligands SBT (~2X higher) and DHB (~2X lower) as compared to the other C57Bl/6J and Balb/C class B variants (MUPs 6, 7, 14). While the functional significance of MUP 3's

differential ligand affinity is unclear, the ability of certain MUPs to bind the small molecule repertoire differentially supports a carrier role for these proteins. However, it is of interest to note that the other three polymorphic sites (N/K 50, Q/K 136, E/K140) present in C57Bl/6J and Balb/C class B MUPs all reside on surface residues not effecting the small molecule binding pocket, raising the question as to whether the functional pressure driving MUP variation is more related to direct MUP-receptor interactions as opposed to MUP-small molecule ligand binding. As with the earlier evidence examined, these structural studies can be interpreted as supportive of all three of the hypotheses regarding MUP function: Differential ligand binding by different MUPs could support a carrier role, ligand dependent conformational changes are consistent with a MUP-ligand complex specificity, and the comparatively large number of surface polymorphisms between individual MUPs could support a role for MUP protein activity independent of its carrier function. A thorough study combining physiological and behavioral techniques will be necessary to fully resolve the question of how MUP proteins effect pheromone signaling between mice.

MUP Expression: Combinatorial coding in a protein pheromone family?

The MUP gene family consists of at least 18 unique ORFs under a complex regulation leading to differential gender, tissue and strain-specific combinatorial expression (figure 3). Given the potential for MUPs to be an active component of pheromone signaling as ligand carriers and/or protein ligands in their own right, it is logical to ask next what possible role the expression of different MUPs, both dimorphically and combinatorially, may play in pheromone signaling between animals.

As has been discussed, distinct MUPs display differential affinities for MUP ligands, which may explain the need for diversity in this gene family. However, it has also been noted that most of the polymorphisms between MUPs fall on surface residues that have little or no effect on ligand binding. This variability on the outer surface of MUPs raises the possibility that single MUP proteins may have the ability to interact with discrete populations of sensory neurons through specific pheromone receptors, and thereby encode unique behavioral information either on their own or in combination (figure 6). Indeed, determining the extent to which this is the case will be a primary goal of this study.

What regulatory mechanisms give rise to the differential and combinatorial expression patterns of MUPs which may effect pheromone signaling? MUPs were initially shown to be synthesized in the liver and excreted in urine in large quantities (10-30 mg/ml) (Rumke and Thung, 1964; Finlayson et al., 1965). In the male liver, MUP mRNA is the most abundant transcript present constituting 4% of the total liver mRNA (30,000 copies per cell) (Hastie and Held, 1978; Hastie et al., 1979). It was further observed that urinary MUP expression was much higher (~30X) in male than female mice, and that androgenization of females with testosterone resulted in male levels of MUP protein. Additionally, castrated male mice showed MUP expression levels in urine similar to female mice, which could be rescued to reflect that of intact males by administration of testosterone, indicating a significant contribution of sex hormone axis in regulating MUP expression (Finlayson et al., 1965; Szoka and Paigen, 1978).

However, testosterone is not the only hormone shown to exert endocrine control over MUP expression; both growth hormone and thyroxine axes are also necessary for

proper regulation of MUP expression. Knopf et al., 1983 showed that mice genetically deficient in the peptide growth hormone (GH) (*little* mice) express MUPs at levels 150-fold lower than wild-type males by Northern blot analysis. This phenotype was completely reversible with exogenous GH administration. Ectopic administration of testosterone to *little* mice was further shown to be insufficient to restore wild-type expression levels, indicating that GH and testosterone regulation of MUP expression are truly complementary (Al-shawi et al., 1992). Further extending our understanding of this multi-hormonal regulation, Knopf et al. 1983 examined the induction of MUP expression by administration of various combinations of hormones in hypophysectomized females, which lack all pituitary hormones and exhibit no liver MUP expression. They found that neither GH alone nor GH + testosterone were sufficient to induce wild-type male expression levels. Surprisingly, testosterone alone had no effect on MUP mRNA levels in these hypophysectomized females. Only exogenous administration of GH and thyroxine (T4) together resulted in wild-type male levels of MUP expression, and additional administration of testosterone to this GH/T4 cocktail had no further measurable effect on expression.

These data, which suggest an additional testosterone-independent mechanism for MUP expression raise a troubling question: How to explain the complete transformation from female to male MUP expression in wild-type females (which have GH and T4 serum levels similar to males) simply by the administration of testosterone? The answer likely lies in the dimorphic secretory rhythm of GH between male and females, as well as complex epigenetic interactions between these three hormonal axes which are poorly understood. Indeed, it has been shown that the pulsatile GH release present in males (and

used in the complementation experiments described above) results in male-levels of MUP expression, whereas the more continuous release of GH in females appears to actually repress MUP expression (Norstedt et al., 1984; Johnson et al., 1995). Hence, there are at least two hormone axes controlling the dimorphic regulation of MUP expression in a non-redundant, but apparently complementary manner. The exact way in which these axes interact to achieve proper MUP regulation, as well as the apparent necessity of T4 to the system, is unknown.

The complexity of this multi-hormonal regulation is further compounded by the fact that different MUP genes appear to be differentially regulated by testosterone, GH and T4. This phenomenon was first observed by Szoka and Paigen, 1978 who reported that just as male mice of different strains demonstrated unique urinary MUP expression patterns, females expressed unique subsets of the MUPs expressed by males of the same strain. In the context of the multi-hormonal control described by Knopf et al., 1983, this finding suggested strongly that those MUPs whose expression persisted (although at lower levels) in females were likely less sensitive to the dimorphic hormonal environment in females (i.e. less testosterone and sustained GH pulses) and were therefore under differential endocrine regulation as compared to male-specific MUP species (Johnson et al., 1995). Indeed, the fact that MUPs can be organized into two groups based on dimorphic expression raises the question as to the extent to which the non-dimorphic MUPs may encode behavioral information distinct from their dimorphically expressed counterparts, and more generally the extent to which differential regulation of MUP species may predict differential activity in the VNO.

Subsequent studies have attempted to elucidate the extent to which specific MUPs are differentially regulated by the three hormone axes, and thus may provide another means of expression and functional grouping. While these studies did demonstrate differential endocrine regulation of specific MUPs in the C57Bl/6J and Balb/C strains, their methodologies assayed MUP expression indirectly through *in vitro* translation of liver cDNA (as opposed to direct analysis of urinary MUPs *in vivo*) making it difficult to formulate solid conclusions (Knopf et al., 1983; Clissold et al., 1984; McIntosh et al., 1989; Johnson et al., 1995).

While this study is primarily concerned with the function of MUPs expressed in the liver and excreted in the urine (e.g. urinary MUPs (uMUPs)), it should be noted that MUP expression has been observed in multiple other secretory tissues, including the parotid, sublingual, submaxillary glands (salivary MUPs) as well as the lachrymal gland (tear MUPs) (Shaw et al., 1983). Like the liver, these tissues show combinatorial MUP expression, however it is interesting to note that MUPs are expressed in partially overlapping but differential tissue specific combinations within a strain (Shahan et al., 1987). The extent to which a particular MUP is truly tissue specific (e.g. submaxillary only) across all strains is unknown, but does raise intriguing functional questions. In addition, it should be noted that unlike the liver, the submaxillary gland has been shown to express MUPs in a non-dimorphic manner, and is in fact devoid of any hormonal regulation whatsoever (Shaw et al., 1983). Again, the functional significance of this endocrine-independent regulation of salivary MUPs is unknown, but just as with the dimorphic versus non-dimorphic MUPs, does suggest a potentially differing role for these

MUPs as compared to urinary MUPs in regulating intra-specific communication and physiology.

MUP expression between strains and individual: Common themes?

While the preceding studies examined the complex regulation of MUP expression in general terms, it is important to understand the specific MUP loci expressed in common laboratory strains used for physiological and behavioral studies, and how this relates to expression patterns in wild mice, before one can begin to address the potential function of this protein family. Additionally, it would be of great value in elucidating MUP function to understand if there is any obvious logic or stereotypy in the specific MUPs expressed between individuals and laboratory strains. Until recently, understanding MUP expression at this level of resolution proved to be quite difficult. The large number of pseudogenes and high sequence homology between MUP loci arrayed in the genome made identification of the precise genomic structure of this gene family particularly challenging. Early studies aiming to identify MUP transcripts expressed in C57Bl/6J and Balb/C liver relied on Northern Blot analysis using full-length cDNA probes. However, due to the high sequence homology in the MUP family, this method was only able to parse transcripts crudely into the two groups (“group 1” and “group 2”) based on cDNA probe hybridization (Kuhn et al., 1984, Knopf et al., 1983).

The exact expression profile of urinary MUPs in various strains and wild mice was only elucidated when the Hurst and Beynon groups collaborated using biochemical techniques including FPLC purification, mass spectrometry (MS), and de novo peptide sequencing to purify and precisely identify individual MUP proteins directly from urine.

Using this biochemical approach allowed them to directly visualize by anion exchange chromatography and identify by MS the MUP protein species actually present in the urine. This technique is preferable to inferring MUP expression in urine from the presence of MUP mRNA in the liver, which has been shown to be unreliable due to apparent post-transcriptional regulation. When Hurst and Beynon purified and identified MUPs from two inbred strains (C57Bl/6J and Balb/C) they confirmed the earlier conclusions derived from Northern Blot analysis: Most expressed uMUPs are highly homologous and differ at only one or two residues (class B MUPs) and there is also occasionally present divergent MUP proteins (class A MUPs) with only about ~80% homology with the class B MUPs (Robertson et al., 1996; Armstrong et al., 2005). It is of interest to note that the class B MUPs expressed in C57Bl/6 and Balb/C differ at only four residues: N/K50, F/V56, Q/K136, E/K140 and share two MUPs in common, including MUP 3, which has the differential ligand affinity discussed earlier (figure 3) (Robertson et al., 1996).

What about the class A uMUPs? This MUP “sub-family” demonstrated several unexpected expression attributes in laboratory strains and wild mice. One class A MUP was found to be expressed in C57Bl/6 urine in a male-specific manner. Surprisingly, a survey of wild field-caught mice using ESI-MS (which determines the masses of all expressed MUPs) revealed that class A uMUP expression is a male-specific phenomenon and is never observed in female urine (Armstrong et al., 2005). The sequence divergence from class B in these MUPs, coupled with their gender dimorphic expression, raises the question as to their potential function in driving gender dimorphic behaviors. However, it should be noted that class A MUP expression in urine is not universal in male mice. A

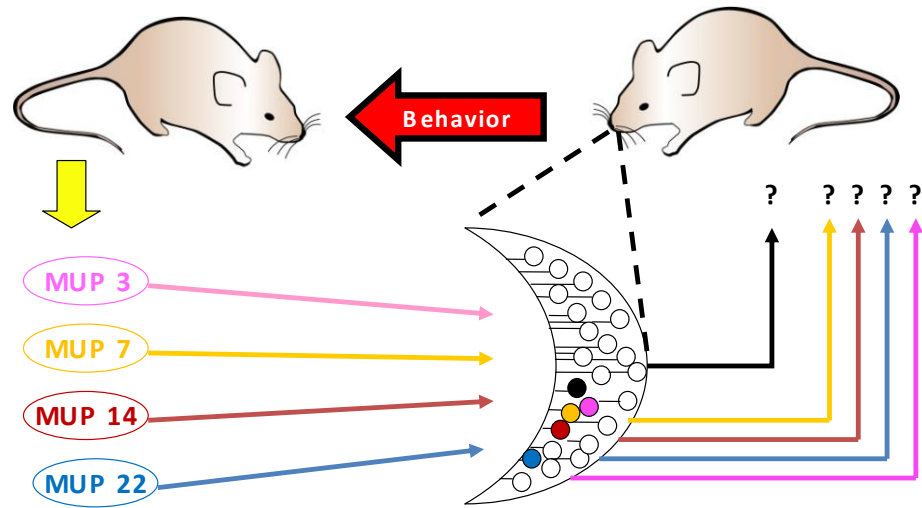


Figure 1.6 The individual MUPs expressed in strain-specific combinations in male urine may encode discrete or redundant behavioral information. The four MUPs expressed in male C57Bl/6J urine may each activate discrete sensory circuits in the VNO of receiving animals to drive specific behaviors (colored pathways), or may be detected non-discriminately (black pathway).

survey of 20 wild-caught male mice demonstrated that only a minority expressed a class A MUP, whereas all expressed different combinations of class B MUPs (Robertson et al., 1997). In short, class A MUP expression, while male-specific, is not ubiquitous unlike the combinatorial expression of class B MUPs which is observed in all mice of both genders (although the combinations are less complex in females and inbred strains). Indeed, a careful analysis by ESI-MS of both class B and class A MUP expression in wild male mice revealed that there is no single MUP or obvious pattern of MUPs that is expressed in all wild mice or inbred strains (Robertson et al., 1996; Beynon et al., 2002). The apparent complexity and lack of obvious stereotypy in the expression pattern of uMUPs between individuals and genders raises intriguing questions about how this protein family may be involved in driving stereotyped behaviors in mice.

Strain and individual specific MUP expression: To what end?

Clearly, a complex regulatory scheme gives rise to the expression of unique combinations of uMUPs in laboratory strains and individual mice in the wild. What is the purpose behind this combinatorial expression? Indeed, if as postulated, single MUPs can convey unique information about the excreting animal, is it possible that the expression of MUPs in unique strain or individual specific combinations could encode the specific identity of an individual? A number of studies have addressed precisely this question using a variety of behavioral paradigms that assess individual recognition.

One such paradigm exploits the fact that males will deposit more counter-marks in the environment if they encounter a strange male's territorial markings as opposed to their own, presumably to cover up a competitor's chemo-signal. Nevison et al., 2003

showed that this ownership signal is involatile; mice will only show increased counter-marking if they are allowed direct contact with their competitor's scent mark, but not if direct contact is blocked by nitrocellulose, which still allows volatile cues to be detected. Hurst et al., 2001 implicated MUPs directly in this process by showing that males demonstrate increased counter-marking to their own scent mark if the composition of the mark is altered by the addition of a single non-self recombinant MUP. These studies suggest a role for MUPs encoding intermale individual recognition, and provide further evidence supporting the hypothesis that MUP proteins convey information independent of their small-molecule ligands.

MUPs have also been implicated in female to male individual recognition. The ability of females to distinguish between males has been shown to be critical in mediating the Bruce effect, in which a female will abort her pregnancy if she encounters a male non-congenic with the original stud male within a critical post-conception time window (reviewed in Stowers and Marton, 2005, see Chapter 4 figure 2). Exposure of the newly pregnant female to non-stud urine has been shown to be sufficient to terminate the pregnancy (exposure to the original stud urine has no effect), indicating that urine-derived cues provide the individuality substrate which the female uses to drive memory formation of the stud. Do MUPs provide the individuality cue in this case? There is some evidence that spiking non-familiar MUPs into normally inactive stud male urine will transform this urine such that it can cause the female to block her pregnancy (Brennan et al., 1999). However, the MHC class I peptides secreted in the urine in individual-specific combinations (discussed in chapter 4) have been shown to have the same effect when non-familiar MHC are spiked into stud urine (Leinders-Zufall et al.,

2004). The presence of both MUPs and MHC class I peptides as potential individuality signals raises the question as to whether these combinatorially expressed molecules subserve redundant or specific functions in the mouse. Two studies that have attempted to distinguish between MUP and MHC activity in mediating individual recognition indicate that MUPs are solely responsible for inbreeding avoidance and female-male recognition in out-bred mice living in a semi-wild environment (Sherborne et al., 2007; Cheetham et al., 2007). However, to date, only MHC class I peptides have been shown to directly activate VNO sensory neurons (Leinders-Zufall et al., 2004).

MUPs and aggressive behavior: Significance of this graduate thesis.

The complex regulation and expression of the MUP gene family pose intriguing questions as to their potential function in mediating intra-specific communication. The excretion of such large quantities of MUP protein in urine coupled with the combinatorial, gender dimorphic and additional exocrine tissue expression of these proteins are all supportive of a role for MUPs in driving pheromone-mediated behaviors either directly as protein pheromones or indirectly as pheromone carriers. While a multitude of studies have implicated MUPs in mediating behaviors from estrus induction to individual recognition, to date there has not been strong evidence to make any solid conclusions regarding their behavioral function. Importantly, it has yet to be shown the degree to which and by what mechanism MUPs activate VNO sensory neurons (VNs) to drive behavior. Therefore, it is the primary goal of this graduate thesis to determine the extent to which MUPs activate VNO sensory neurons, the coding potential of this gene family, and the behaviors which are mediated by this MUP-dependent activity.

The biochemical nature of the pheromone code that induces innate behavior in mammals is not known, and the sensory system responsible for detection is a matter of controversy. This study identifies MUPs as novel mouse pheromones that mediate aggressive behavior and further, determines the identity of the aggression-promoting chemosensory neurons. We have taken a unique approach to decode olfaction by working forward from the behavioral activity using calcium imaging on dissociated VNs to identify MUPs as genetically encoded pheromones. These pheromone proteins reproducibly activate a subset of VNs enabling the molecular identification of those neurons that mediate the aggression-promoting behavior.

In addition, we have shown that, despite the high sequence homology in the MUP gene family, certain VNs are tuned to respond to specific MUPs or specific combinations of MUPs. We further show that individual MUP species encode specific behavioral information through the activation of discrete VNO sensory circuits, which suggests that the individual and strain specific expression of MUPs in unique combinations could constitute a pheromone code that drives behavior. This finding has allowed us to understand how aggressive behavior is encoded by the MUP gene family, as well as identify the MUPs as a novel pheromone gene family with a rich coding potential to drive additional behaviors. These findings represent a fundamental step towards understanding intra-specific communication in mammals and characterizing the neuronal circuits involved in behavior.

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Chapter 2: Identification of protein pheromones that promote aggressive behavior

Identification of protein pheromones that promote aggressive behavior

Mice use pheromones, compounds emitted and detected by members of the same species, as cues to regulate social behaviours such as pup suckling, aggression and mating (Stowers and Marton, 2005). Neurons that detect pheromones are thought to reside in at least two separate organs within the nasal cavity: the vomeronasal organ (VNO) and the main olfactory epithelium (MOE) (Dulac and Torello, 2003). Each pheromone ligand is thought to activate a dedicated subset of these sensory neurons. However, the nature of the pheromone cues and the identity of the responding neurons that regulate specific social behaviours are largely unknown. Here we show, by direct activation of sensory neurons and analysis of behaviour, that at least two chemically distinct ligands are sufficient to promote male–male aggression and stimulate VNO neurons. We have purified and analysed one of these classes of ligand and found its specific aggression-promoting activity to be dependent on the presence of the protein component of the major urinary protein (MUP) complex, which is known to comprise specialized lipocalin proteins bound to small organic molecules (Stowers and Marton, 2005; Cavaggioni et al., 2000; Flower, 1996). Using calcium imaging of dissociated vomeronasal neurons (VNs), we have determined that the MUP protein activates a sensory neuron subfamily characterized by the expression of the G-protein $G\alpha_o$ subunit and V2R putative pheromone receptors. Genomic analysis indicates species-specific co-expansions of MUPs and V2Rs, as would be expected among pheromone-signalling components. Finally, we show that the aggressive behaviour induced by the MUPs occurs

exclusively through VNO neuronal circuits. Our results substantiate the idea of MUP proteins as novel pheromone ligands that mediate male–male aggression through the accessory olfactory neural pathway.

Male–male territorial aggression in mice is a robust, innate, social behaviour. However, the aggression-promoting pheromone(s) and the responding neural circuits that mediate aggression are unknown. Castrated males no longer produce the aggression pheromone and fail to stimulate aggressive behaviour from recipient males. However, whole urine from intact males is sufficient to promote aggression when swabbed on the backs of castrated animals, providing a bioassay for the identification of urinary pheromones (Fig. 1a) (Mugford and Nowell, 1970). We used this behavioural assay to determine which components of urine act as pheromones that cause aggression. We first fractionated male urine over size-separation columns and tested these fractions in the castrated-male bioassay. We found that fractions comprising molecules of low (LMW; less than 3 kilodaltons (kDa)) and high molecular mass (HMW; greater than 10 kDa) both contained aggression-promoting activity (Fig. 1b). The behavioural characteristics of the observed aggression promoted by LMW and HMW fractions were indistinguishable from each other and from the behaviour promoted by whole urine (data not shown). These findings suggest that at least two distinct molecules promote aggression.

To further assay pheromone activity, we established an *ex vivo* system using primary sensory neurons suitable for screening many heterogeneous cells for biologically active ligands. Our previous studies revealed that VNs are required for the aggression response, as mice lacking the primary sensory transduction channel, TrpC2, are unable to

detect and respond to the aggression-promoting pheromone (Fig. 1a) (Stowers et al., 2002; Bean, 1982; Leybold et al., 2002). We found that dissociated primary VNs loaded with fura-2 responded to male whole urine with robust and reproducible intracellular Ca^{2+} transients (Fig. 1c). A battery of controls and a dose–response curve (Figs 1f and 2e, and Supplementary Fig. 1), based on the molecular characteristics and physiology of VNs, show the response specificity of urine and, importantly, establish that dissociated VNs provide a biological platform to analyse the activity of potential pheromone ligands.

To further investigate the LMW and HMW fractions, we analysed the activation of dissociated VNs by each size fraction. VNs are a highly heterogeneous population, with each neuron expressing one of approximately 250 different G-protein coupled receptors (GPCRs), providing a mechanism for individual neurons to respond to different compounds (Shi and Zhang, 2007). We next determined whether the LMW and HMW fractions activated distinct or overlapping populations of dissociated VNs. Our calcium imaging method allows us to record calcium transients to repetitive exposure of multiple substances at a resolution of the single cell. This enables us to precisely determine which ligands are biologically active as well as the response profile of individual neurons. When assayed, one population of the responding cells was activated by the HMW stimulus whereas a second distinct population showed calcium transients in response to the LMW ligands (Fig. 1d–f). This indicates that two chemically distinct ligands activate separate subsets of VNs. When considered with the bioassay, it suggests that at least two populations of neurons are capable of detecting urinary aggression pheromones and that each are sufficient to promote male–male aggression.

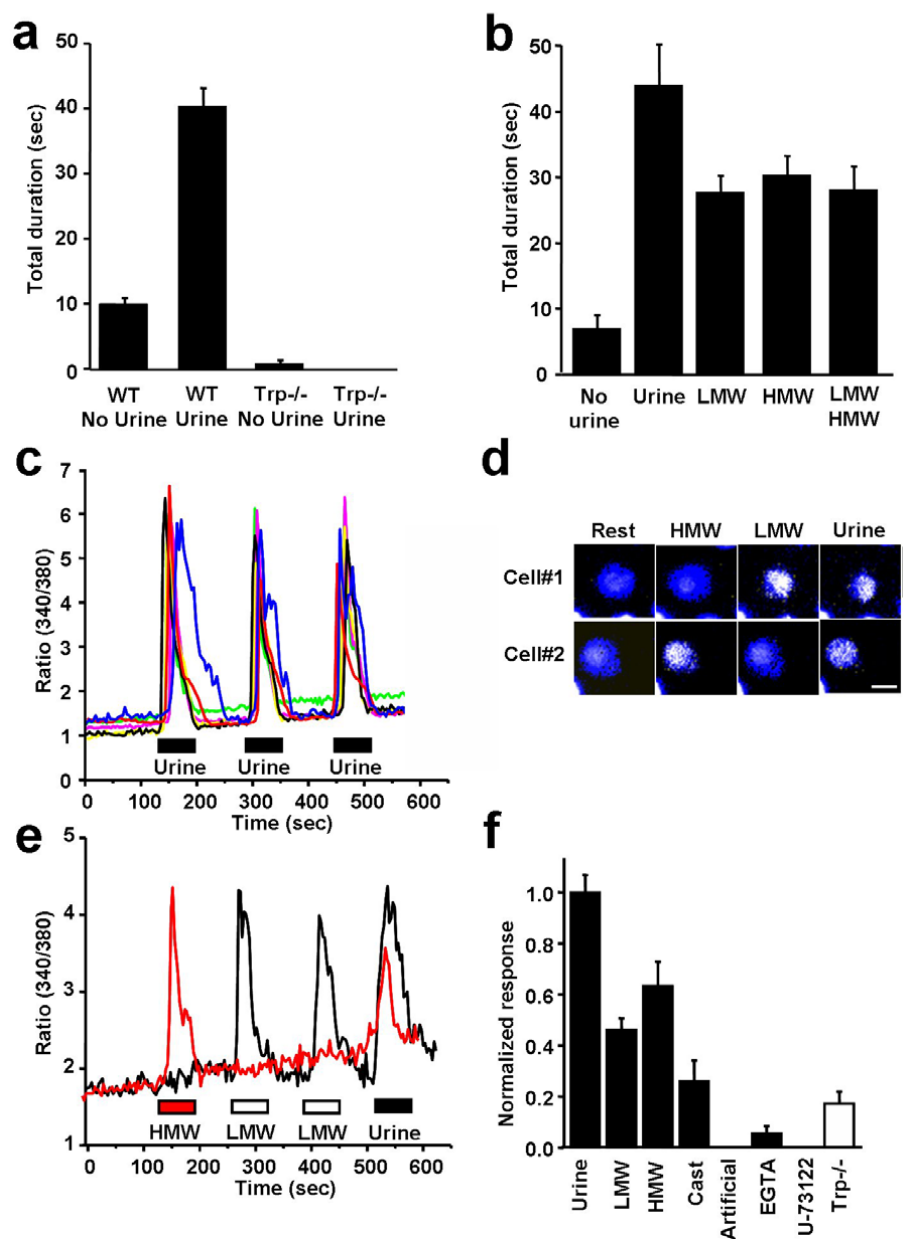


Figure 2.1 Male urine contains two aggression pheromones. **a**, Male urine swabbed on castrated mice stimulates aggression ($P < 0.0001$; Student's *t*-test) in WT (73 trials/36 animals) but not *TrpC2*^{-/-} mutants (36 trials/6 animals, mean \pm SEM). **b**, Aggression with urine, LMW, HMW or both ($N = 16$ trials/6 animals each; urine/HMW $P = 0.1$, urine or HMW/no urine $P < 0.0001$). **c**, Repetitive application of male urine (1:300) induced Ca²⁺ transients in dissociated VNs. Six representative traces. **d**, Fura-2 ratio images of two VNs of the same experiment. Cell #1 responds to LMW, cell #2 to HMW. Scale bar, 10 μ m. Pseudocolour: low (blue) to high (white) [Ca²⁺]. **e**, Separate populations of VNs are activated by LMW (black) and HMW (red) fractions. **f**, Summary of VN activation (mean \pm SEM normalized to the urine response): WT (black bars) stimulated with urine, 1,951 of 28,289 cells; LMW, 548 of 17,260 cells; HMW, 885 of 21,096 cells; 80 of 12,679 to both. Castrated urine, 44 of 2,153. Artificial Urine, 0 of 1,224. EGTA, calcium-free media, 9 of 2,426. PLC inhibitor; U-73122 (50 μ M), 0 of 2,205. *Trp*^{-/-} (white bar), *TRPC2*^{-/-} VNs in response to urine, 38 of 3,312.

There are very few HMW components in mouse urine; none have been identified as pheromones (Schwende et al., 1986; Hastie et al., 1979). Therefore we chose to focus our subsequent purification and characterization only on the robust HMW bioactivity. We used anion-exchange fast protein liquid chromatography (FPLC) to separate the HMW components into 40 fractions over a 0–1 M NaCl gradient (Fig. S2). Only five fractions (fractions 15–19) induced calcium transients in VNs. This activity overlapped with and accounted for all the HMW activity. Polyacrylamide gel electrophoresis (PAGE) revealed that the five active fractions contained proteins of 19–24 kDa, which can be further resolved into four major bands by isoelectric focusing (Fig. 2a); these features closely mirror the size and isoelectric point (pI) of MUPs (Flower, 1996). Indeed, western blot (Fig. 2a) and electrospray ionization mass spectrometry (ESI-MS) of these five fractions confirmed their identities as MUPs. Of the 20 identified MUP-encoding genes arrayed in the genome, males are known to express unique combinations of four to six MUPs in a strain-dependent pattern (Robertson et al., 1997). Figure 2a identifies the four MUPs excreted by C57BL/6J males. Importantly, we did not detect any other proteins in these purified fractions. When used in the behavioural assay, we observed that the MUPs purified by FPLC are sufficient to mediate robust male–male territorial aggression (Fig. 2f).

MUPs are β -barrel in structure, bearing a central hydrophobic binding pocket that has been shown to carry small organic ligands (Timm et al., 2001). Gas chromatography followed by (mass spectrometry (MS) revealed that our isolated MUPs primarily bind 2-s-butyl-4,5-dihydrothiazole (SBT) (Supplementary Fig. 3). Previous studies have implicated SBT as a pheromone capable of activating a subset of VNs (Novotny et al.,

1985; Leinders-Zufall et al., 2000); however, the role of the MUP itself without ligands, MUP protein, remains elusive (Flower, 1996; Hurst et al., 1998; Hurst et al., 2001).

To further investigate the function of the MUP protein, we first eliminated the protein component of the purified MUP complex by protease digestion. This treatment abolished the ability of the purified complex both to activate VNs and to promote aggressive behaviour (Fig. 2b, f). Next, we investigated the specificity of the small molecule ligand in promoting aggression. We tested synthetic SBT in our bioassay and found no aggression-promoting activity (Fig. 2f). The binding affinities and infinite characteristics of potential small-molecule ligands preclude the definitive dissociation of all ligands from MUPs. Therefore, we incubated fractions 15–19 with menadione, to competitively displace MUP small-molecule ligands, as analysed by gas chromatography – mass spectrometry (GC–MS) (Supplementary Fig. 3) (Xia et al., 2006). This displaced fraction retains 40% of its original activity, as determined by calcium imaging (Fig. 2d); however, importantly, it retains all of the behavioural aggression-promoting activity (Fig. 2f). This indicates that the MUP protein determines neuronal activation that encodes male–male aggressive behaviour, irrespective of the specificity of its small molecule. Lastly, we prepared the four MUPs excreted in urine from C57BL/6J mice as recombinant maltose–MUP fusion proteins in *Escherichia coli* (rMUPs), and determined by GC–MS that they are not bound with mouse urinary small molecules (data not shown). These pooled rMUPs both induce intracellular calcium transients in VNs and promote aggressive behaviour, demonstrating the functional necessity and sufficiency of the MUP protein as the HMW activity (Figs 2c, d, f). Finally HMW, rMUPs and urine all show similar dose–response activation profiles as analysed by the number of responding

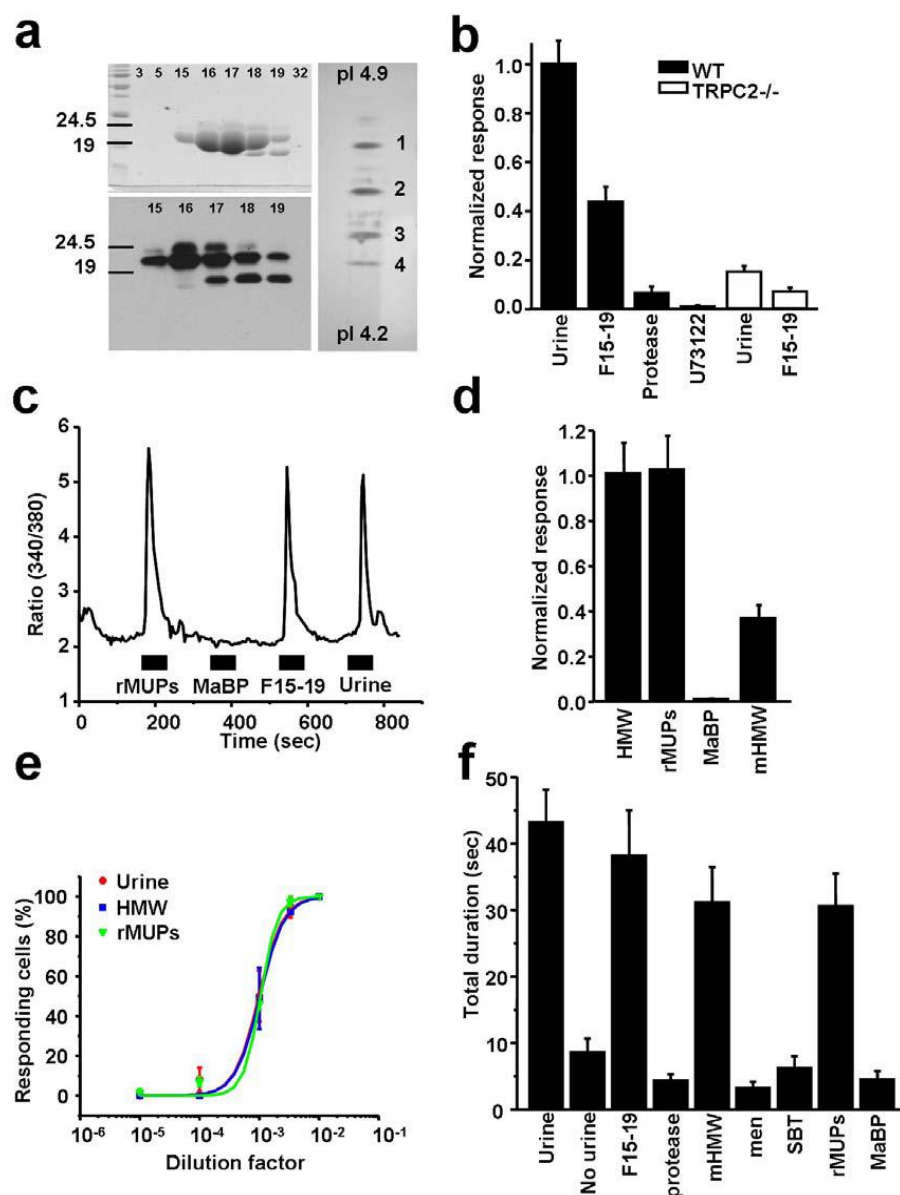


Figure 2.2 HMW aggression activity is dependent on MUPs. **a**, Purification of bioactivity. Top, PAGE of FPLC fractions (Figure S2). Bottom, anti-MUP western blot. Right, IEF gel (pI range 4.1–4.9) of the fractions (F15–19); accession numbers: 1, AAI00587; 2, CAM19799; 3, AAH13649; 4, AAH19965. **b**, Calcium imaging of VNs. F15–19 activated one half (72 of 1,220 cells) of the same VNs activated by whole urine (133 of 1,220 cells). Proteinase treatment of F15–19 (8 of 1,220 cells) and U-73122 (50 μ M; 2 of 1,563 cells) ablated activity. TRPC2^{-/-} VNs show decreased activity; whole urine (28 of 1,737 cells), F15–19 (13 of 1,737 cells) (Lucas et al., 2003). **c**, Calcium transients in a single VN induced by rMUPs, F15–19, and whole urine but not maltose-binding protein (MaBP) alone. **d**, VN activation normalized to the HMW response. rMUPs (573 of 4,613 cells); MaBP (1 of 4,613 cells); menadione-displaced HMW (mHMW) (190 of 3,997 cells) and HMW (808 of 6,573 cells). **e**, Dose–response fitted to a sigmoid curve using the Hill equation of VN activation by urine. **f**, Aggressive behaviour measured as total attack duration in resident-intruder assay ($n = 21–50$). rMUPs/no urine $P = 0.00002$; F15–19/rMUPs $P = 0.3599$.

cells (Fig. 2e). Together, these data reveal a role for the MUPs without ligands as independent pheromones.

The mouse VNO is composed of two molecularly distinct populations of sensory neurons, defined by the expression of $G\alpha_{i2}$ and $G\alpha_o$, that project to two physically separate domains of the accessory olfactory bulb (Dulac and Torello, 2003). The functional significance of these two neuronal classes has yet to be determined. However, the small-molecule ligands alone, such as SBT, are thought to activate the $G\alpha_{i2}$ -expressing neurons (Leinders-Zufall et al., 2000). Therefore, we aimed to establish the extent to which the MUPs initiate aggression through the activation of one or both classes of VNs. We used calcium imaging followed by immunostaining for $G\alpha_{i2}$ and $G\alpha_o$ to identify the molecular characteristics of those cells activated by MUPs. Figure 3a, b reveals that MUPs specifically activate the $G\alpha_o$ -positive VNs that also express V2R receptors. None of the MUP-activated cells were immunoreactive for $G\alpha_{i2}$. Previous studies have shown that $\beta 2$ -microglobulin^{-/-} mice do not properly traffic putative pheromone V2R receptors in the $G\alpha_o$ -expressing neurons and fail to display male–male aggression (Loconto et al., 2003). To functionally assay if the MUPs are indeed signalling through $G\alpha_o$ -expressing neurons, we examined the ability of the purified MUPs to evoke activity in VNs dissociated from the $\beta 2$ -microglobulin^{-/-} animals. Unlike wild-type (WT) neurons, we found MUP-mediated activity to be abolished in the mutant neurons (Fig. 3c). Together, these results demonstrate that the VNs activated by the MUPs belong to the $G\alpha_o$ subset of VNs. Moreover, the neurons activated by the MUP protein are different to the $G\alpha_{i2}$ expressing neurons shown to be activated by the MUP-

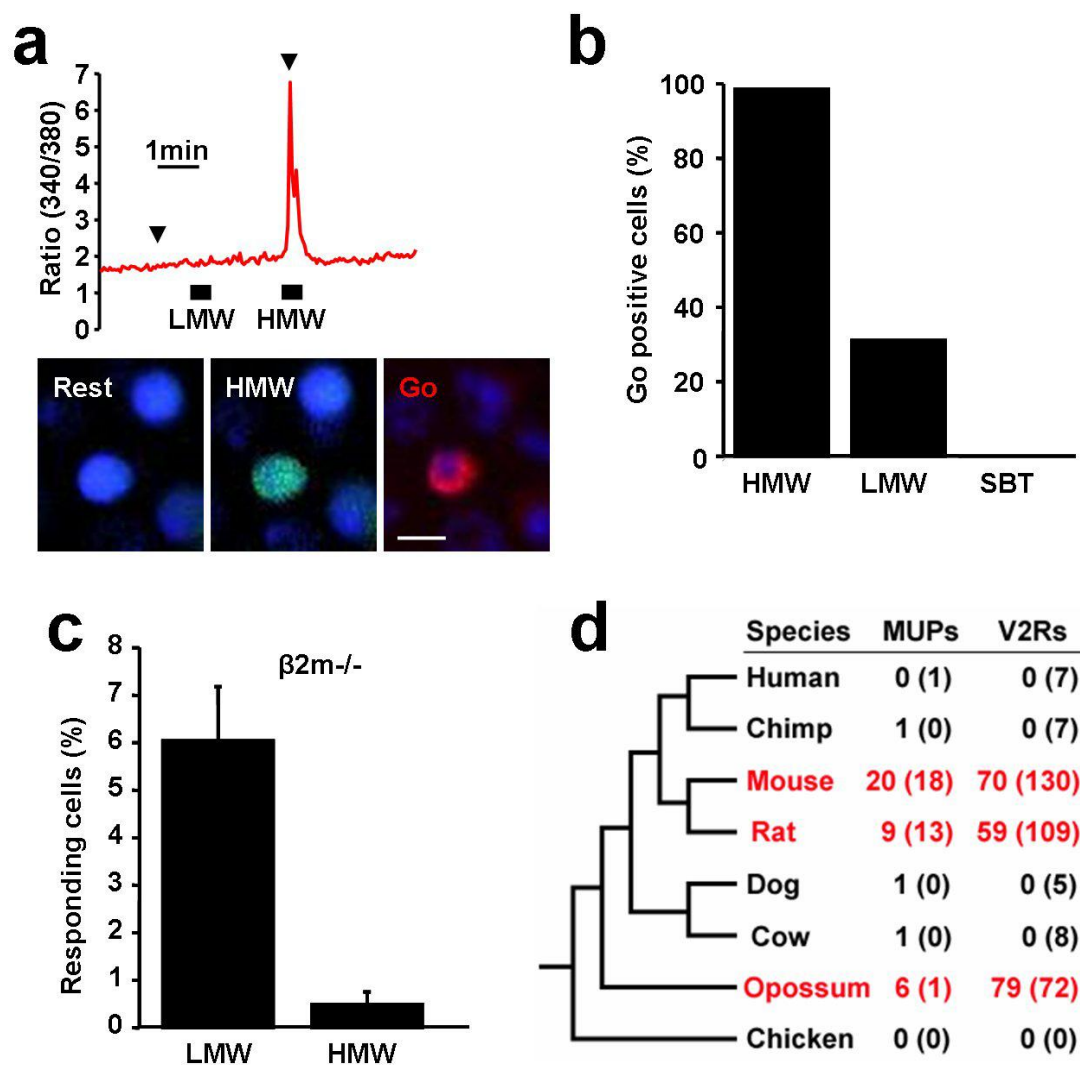


Figure 2.3 MUPs activate a subset of VNs that express $G\alpha_o$. **a**, HMW responsive cell (red trace) is labelled by anti- $G\alpha_o$ immunostaining (red) and DAPI (blue) immediately after Ca^{2+} imaging (right). Fura-2 ratio images (pseudocolour) during rest (left) or HMW activation (middle). Arrows show exact image time. **b**, Percentage of activated cells positive for $G\alpha_o$: HMW 98.6% (75 of 76 cells), LMW 29% (27 of 92), SBT (0 of 4). **c**, β -2m $^{-/-}$ VNs activated by LMW (99 of 1,673) and HMW (8 cells to both HMW and LMW). No cells responded only to HMW (mean \pm SEM of six experiments). **d**, Co-expansion of MUP and V2R gene families. The numbers of genes and pseudogenes (brackets) are indicated. V2R data are as reported, with the addition of chimpanzee (Shi and Zhang, 2007).

associated small-molecule ligands alone (Fig. 3b) (Leinders-Zufall et al., 2000).

Together, our results indicate that MUPs act as male–male aggression pheromones that specifically stimulate the $G\alpha_o$ -expressing subpopulation of VNs.

Recent comparative genomic and morphological analyses have shown that not all terrestrial vertebrates express markers and functional receptors of the $G\alpha_o$ neurons, including the family of V2R putative pheromone receptors (Shi and Zhang, 2007; Takigama et al., 2004). We analysed sequenced genomes and identified the presence of V2R and MUP gene expansion only in the genomes of rat and mouse, and a parallel expansion of V2R and MUP-like genes in the evolutionary divergent opossum lineage (Fig. 3d and Supplementary Fig. 5). All other mammals studied contain a single, intact MUP gene within the syntenic region, except humans, which have a single pseudogene. The species-specific co-expansion of MUPs and V2Rs further underscores the likelihood that they are both components functioning in species-specific processes, as would be predicted in pheromone communication.

Previous behavioural experiments have found that, like $TrpC2^{-/-}$ animals, male mice defective in MOE signalling do not initiate male–male aggression (Mandiyan et al., 2005; Wang et al., 2006). This prompted us to ask whether the MUP complex was additionally activating MOE neurons. Calcium induced by whole urine and the LMW fraction increases in dissociated MOE neurons; however, we were unable to detect any activation by the purified MUPs (Fig. 4a, b). Our results suggest that the MUP protein mediates male–male aggression exclusively through VNO circuitry. The previously identified necessity of MOE signalling may compose a second, independent pheromone-

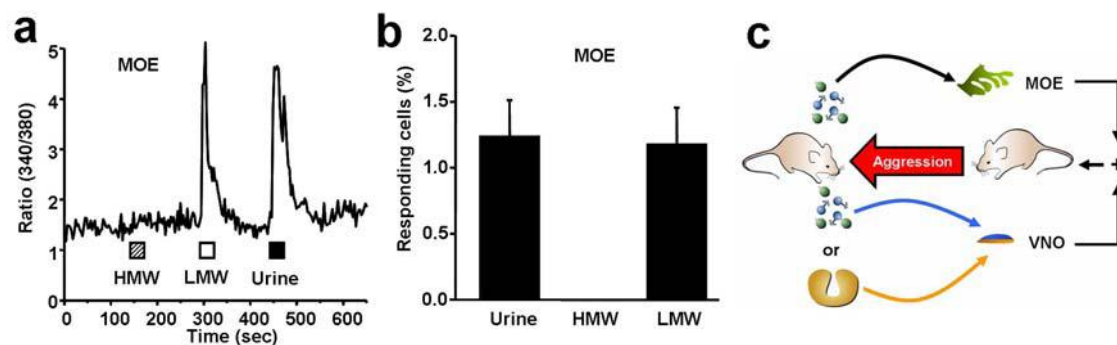


Figure 2.4 MUP activation is specific to VNs. a, MOE-dissociated cells are not activated by HMW. **b**, Summary of MOE cell activation by urine 1.23% (40 cells), HMW (0 cells), LMW 1.17% (38 cells) of 3,250 total cells sampled (mean \pm SEM of six experiments). **c**, Male–male aggression is mediated by at least two sufficient pheromones: MUPs through $G\alpha_o$ /V2R VNs (orange arrow) and unidentified LMW pheromones that stimulate either the VNO or both the VNO and the MOE (blue and black arrows). Previous genetic experiments indicate that both a functional VNO and MOE are necessary for aggressive behaviour (Stowers et al., 2002; Leypold et al., 2002; Mandiyan et al., 2005; Wang et al., 2006).

responsive circuit. Purification and analysis of the LMW aggression-promoting pheromone will enable us to further address the nature of this dual processing.

Behavioural analysis followed by direct VN activation has allowed us to begin to unravel the nature of the aggression-promoting pheromone code. We found that at least two pheromone cues independently promote aggressive behaviour (Fig. 4c). The underlying neuronal logic that promotes an animal's behaviour is not well understood. Several characteristics such as gender, age, status or individuality may be transmitted in the pheromone profile, each acting as equal cues triggering male–male aggression. The MUPs and the unidentified LMW ligands may encode any of these characteristics, independently promoting aggression when encountered by another adult male. Identification of the entire repertoire of aggression-promoting neurons will allow investigation to determine the logic and integration of multiple aggression-promoting circuits that underlie the regulation of behaviour.

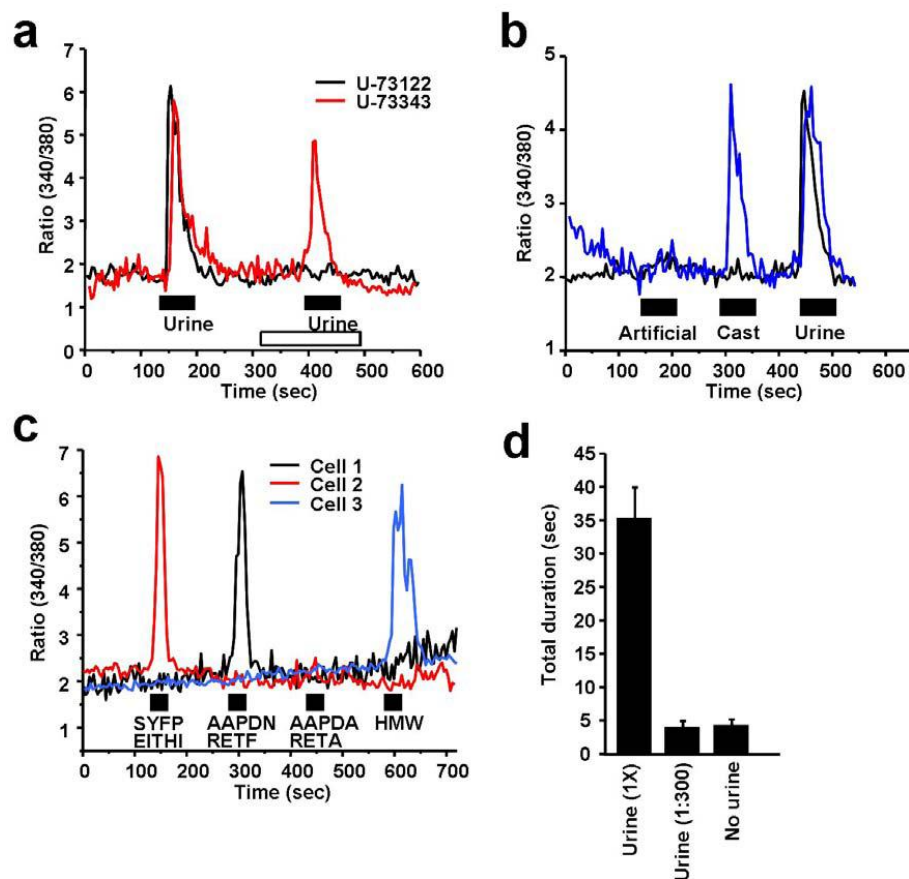


Figure 2.S1 On average, 7% of all cells were activated by urine (figure 1f). To determine whether the responding cells were neurons, we analyzed calcium transients in cells from a transgenic mouse line in which the olfactory marker protein (OMP) drives GFP expression exclusively in VNs. OMP-GFP was expressed in 70% of the dissociated cells and 100% of responding cells (data not shown). Therefore, we estimate approximately 10% of all VNs can be activated by male mouse urine in our system. **a**, Two sample traces of urine-induced Ca transients, black trace was inhibited by 10 μ M PLC blocker U-73122, 100% of all responding cells screened (0 out of 40). Red trace was a parallel experiment in the presence of 10 μ M of the inactive analog U-73343, none of the cells activated by urine (45 out of 45) were blocked by U-73343. White box indicates duration of compound application. **b**, Only a subset of VNs are activated by castrated mouse urine which does not contain aggression promoting activity. A urine responsive cell (black line) and a cell that responds to both urine and castrated urine (blue line) are shown. **c**, MHC peptide-induced activation of VNs does not overlap with MUPs. Three different cell traces from the same experiment are shown: single cells responsive to the peptides SYFPEITHI (red trace), AAPDNRETf (black), or HMW (blue). Of 3874 total VNO cells screened, 6 cells responded to SYFPEITHI (10^{-11} M), 10 cells to AAPDNRETf (10^{-11} M), 1 cell to both, and 112 to HMW (1:300 dilution). No cells were activated by the inactive analog peptide AAPDARETA (10^{-11} M). **d**, Aggressive response to urine is concentration dependent. As there are no precise means of determining the effective concentration in vivo of the natural stimuli that reaches the VNO epithelium, behavior experiments are performed with 1X urine (purified and recombinant samples adjusted to 1X urine concentration). Using the calcium imaging concentration curve (figure 2e) as a guide we evaluated a 300-fold dilution in the behavioral assay. Total aggressive behavior measured as attack duration time in resident-intruder assay after swabbing castrated mice's backs with 40 μ l of normal male urine, urine diluted at 1:300 (vol/vol), and unswabbed intruders. N=36, 12 individuals. Urine 1X/urine1:300 p<0.00001; urine 1:300/ no urine p=0.831 (student's T-test).

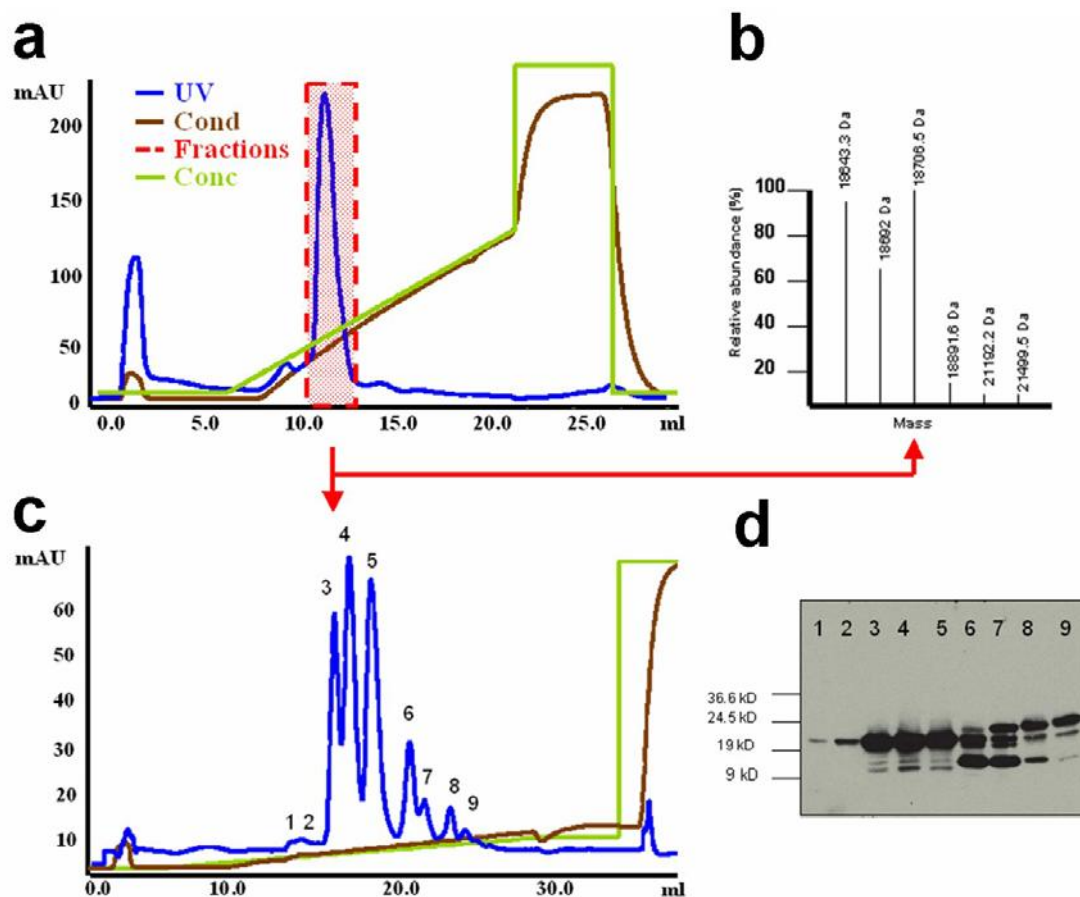


Figure 2.S2 FPLC anion exchange chromatography purification of MUP complexes from C57BL/6J urine. **a**, HMW fraction (>10kD) of urine was injected onto a HiTrap anion exchange column and 40 fractions were collected over a salt elution gradient (green line) of 0-1M NaCl. A single large peak as monitored by UV 280nm absorbance corresponded to five eluted fractions (shaded box, fractions 15-19) that were subsequently pooled and used for calcium imaging and behavioral assays. PAGE analysis of the five eluted fractions (fractions 15-19) revealed proteins with the mass of MUPs (19kD) and western blot of the five eluted fractions using polyclonal anti-MUP antibody confirmed the identity of the eluted proteins as MUPs (see figure 2a). The five MUP containing fractions eluted from the HiTrap column were further analyzed for purity. **b**, The five fractions were pooled and analyzed by LC/MS followed by ESI-MS to determine the protein composition of the fractions. Six peaks were identified by this method, and their masses matched those of C57BL/6J MUPs reported previously by this method (Cavaggioni et al., 2000; Flower, 1996). **c**, To further determine purity, the five fractions were desalted, pooled and injected onto a high resolution UnoQ strong anion exchange column and 40 fractions were collected over a gradient of 0-200mM NaCl. Monitoring of eluted fractions off this column by UV 280nm absorbance revealed nine peaks containing protein. **d**, Western blot analysis of the protein containing fractions corresponding to these nine UV peaks revealed that all nine fractions contained proteins immunoreactive to the anti-MUP antibody.

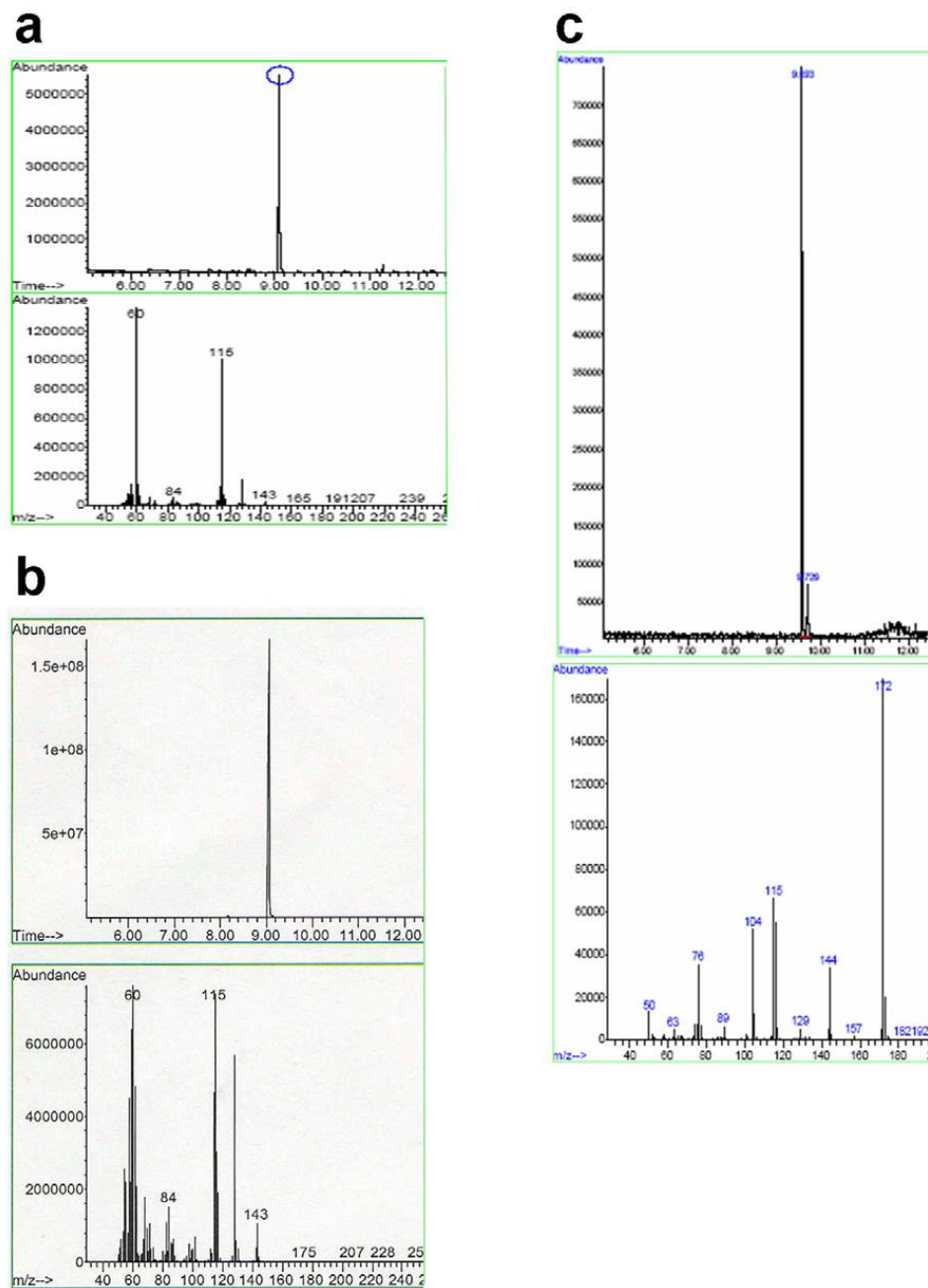


Figure 2.S3 MUP ligand SBT is fully displaced from MUP protein by treatment with competitive displacer menadione. **a**, GC/MS of an organic extraction with chloroform of the HMW fraction of urine shows a compound at retention time 9 minutes with relative abundance of 5×10^6 (top) and corresponding mass spectra of this compound (bottom). **b**, Validation using GC/MS of pure synthetic SBT which shows identical retention time (top) and mass spectra (bottom) mirroring the isolated compound. **c**, SBT is completely displaced from MUP protein when HMW fraction is treated with competitive displacer menadione. Note: loss of peak at retention time 9 minutes associated with SBT and change in Y-axis scale indicating complete displacement of this compound by menadione. Peak at retention time 9.5 minutes (top) is identified as menadione by mass spectra (bottom) as compared to NIST mass spectra databank.

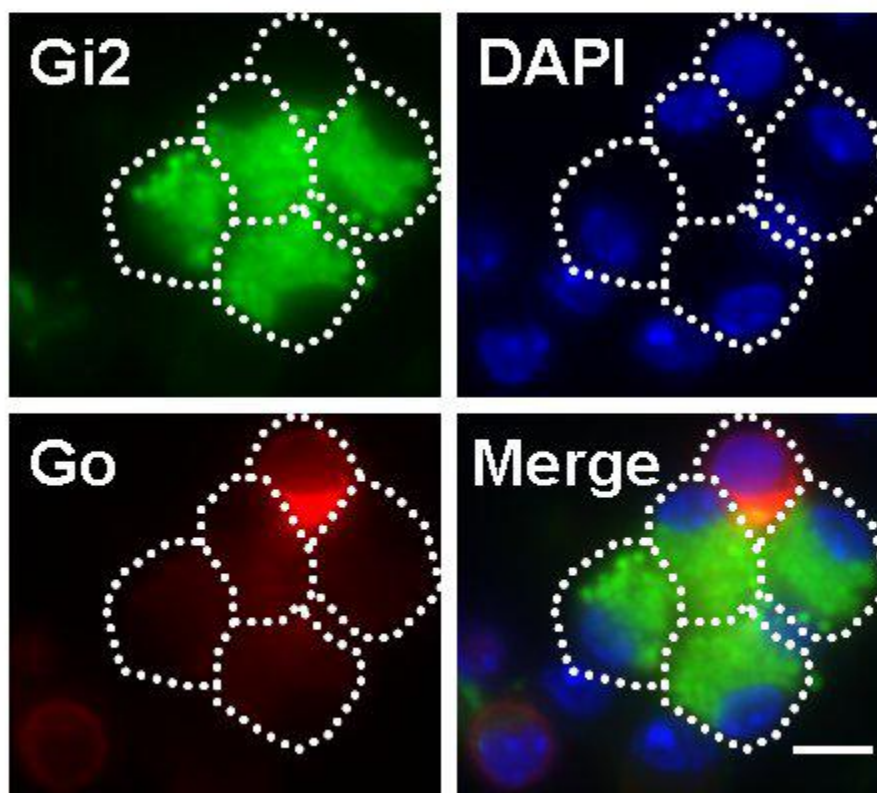


Figure 2.S4 Fluorescence images of the staining with anti-G α o (red), anti-G α i2 (green) antibodies and DAPI (blue) and merging of both, in VNO dissociated cells. A white dotted line has been included to differentiate individual cells. The scale bar equals 10 μ m.

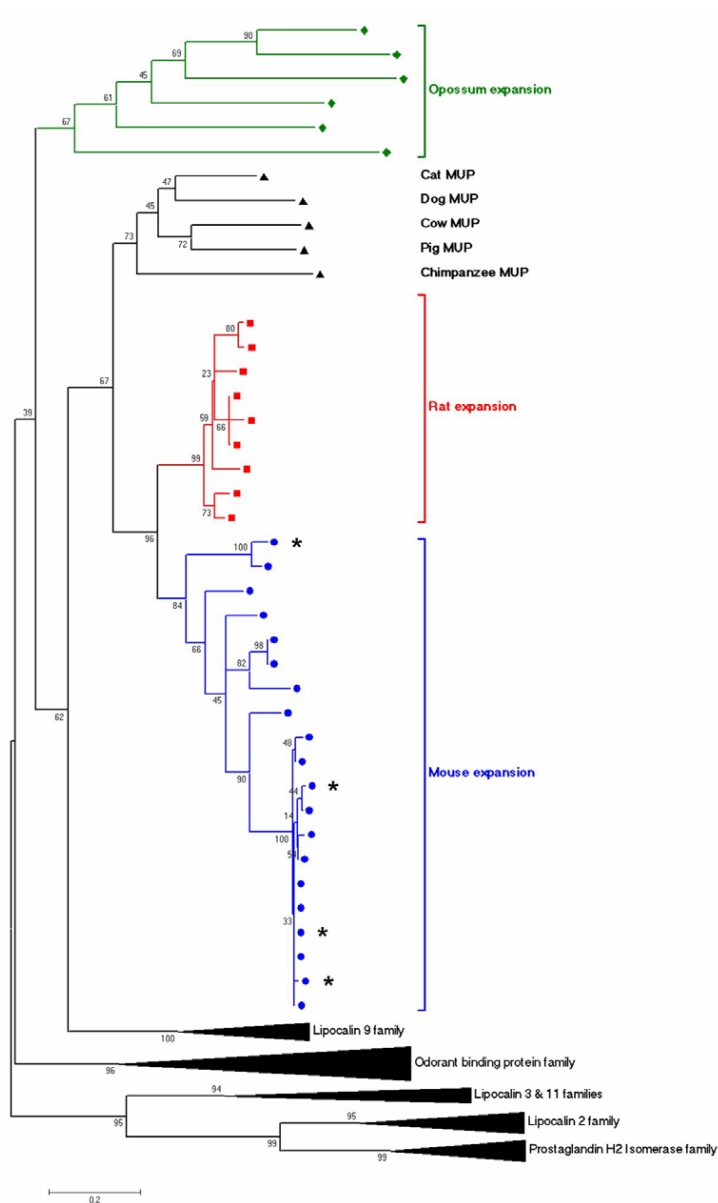


Figure 2.S5 Neighbor-joining tree of MUP-like proteins from terrestrial vertebrates. The tree was reconstructed using Poisson correction to estimate evolutionary distance; the scale bar indicates 0.2 amino acid substitutions per site. Stability of the phylogeny was evaluated by the bootstrap method with 1000 pseudo-replications. The 79 sequences included are: translations of all 38 intact MUP-like genes described in Figure 3d, 2 single intact MUP-like genes from the low coverage cat and pig genome sequences and 38 related intact genes identified from human, mouse, rat, dog, cow and opossum genomes. Clades in black are polyphyletic, and coloured clades are monophyletic. Condensed clades contain related lipocalin families. Blue circles indicate the expanded mouse MUP repertoire, red squares the expanded rat MUP (also known as α -2 μ -globulin) repertoire, black triangles the single MUP orthologues from other placental mammals and green diamonds indicate an expansion of MUP-like proteins the evolutionary divergent opossum lineage. Asterisks (*) indicate relationships of MUPs expressed in C57BL/6J adult males. Genbank accession numbers are indicated in figure 2a.

Methods.

Calcium imaging. VNs were prepared from male C57Bl/6J mice by dissection followed by dissociation with papain and plating on coverslips coated with concanavalin A. Dissociated VNs were perfused with stimuli, and intracellular calcium was monitored using fura-2/AM (Molecular Probes) in a Zeiss Axiovert 200M inverted microscope with a 20× fluar 0.75 objective lens. Urine was collected from 8- to 12-week-old C57Bl/6J males and used or further fractionated for behavioural and physiological experiments. Cells were loaded with HBSS supplemented with 10 mM HEPES, and incubated for 30–60 min at room temperature. Coverslips were placed in a temperature-controlled (37 °C) laminar-flow perfusion chamber (Warner Instrument Corp.) and constantly perfused with HEPES-buffered HBSS. Fura-2-loaded cells were excited alternating between 340 and 380 nm, and light of wavelength greater than 540 nm was captured with an Orca-ER camera (Hamamatsu). After subtraction of background fluorescence, the ratio of fluorescence intensity at the two wavelengths was calculated and analysed using MetaFluor (Universal Imaging Corporation) and NIH Image J. Urine was diluted 1:300 in HBSS; test fractions and purified MUPs were normalized to 1× urine and then diluted 1:300 before experimentation.

MUP Purification. Size fractionation of urine was performed using Centricon filtrating columns (3 kDa and 10 kDa, Millipore). Purification of MUPs from the HMW fraction was completed by using a HiTrap Q HP anion exchange column (GE) fixed to an AKTA FPLC apparatus (Amersham Pharmacia). Isoelectric focusing of MUPs was performed on a LKB 2117 Multiphor II Flatbed Electrophoresis Unit using Immobiline

dry-plate gel, pH range 4.2–4.9, and cooled to 10 °C. Protease treatment of FPLC purified MUPs was performed by overnight incubation at 37 °C with proteinase K and papain. Recombinant MUP proteins were generated using the pMAL Protein Fusion and Purification system (New England Biolabs), and normalized to 1× urine by molarity for all calcium imaging and behavioural experiments.

Cell preparation. Male 8- to 12-week-old C57BL/6J mice were used for all the experiments. The VNO was removed to dissect the epithelium. The tissue was incubated for 20 min at 37 °C in cation-free 0.22 units ml⁻¹ papain, 5.5 mM cysteine-HCl and 10 u ml⁻¹ DNase I in PBS. The papain was inactivated with 10% FBS containing D-MEM and the dissociated cells were plated on 12 mm round coverslips coated with concanavalin-A. For dissociated MOE cells, the whole MOE was first dissected and dissociated in 1 ml PBS containing 40 mM urea, 0.22 u l⁻¹ papain and 10 u ml⁻¹ DNase I for 20 min at 37 °C. β 2-microglobulin^{-/-} mice were purchased from Taconic.

Urine fractionation. C57BL/6J male mice of 8–12 weeks age were used as the source of urine. Between 0.5 and 1 ml of urine was size fractionated by centrifugation (5,000g, 30 min), using Centricon molecular weight cut-off filtrating columns (3 kDa and 10 kDa, Millipore). The first centrifugation flow-through was collected as the LMW fraction. The HMW retentate was washed with one volume of PBS three times and re-concentrated to reach the same initial concentration of urine. The composition of artificial urine was (in mM): 120 NaCl, 40 KCl, 20 NaH₄OH, 4 CaCl₂, 2.5 MgCl₂, 15 NaH₂PO₄, 20 NaHSO₄, 333 Urea, pH 7.4 (Holy et al., 2000).

Protease treatment of MUPs. The four pooled FPLC-purified MUPs were incubated overnight at 37 °C with a protease cocktail of 0.22 u ml⁻¹ proteinase K and 0.22 u ml⁻¹ papain. PAGE showed the digestion to be 95% complete. The digested proteins were spun in a Centricon 3 kDa molecular weight cut-off filtration column to remove undigested and partly digested MUPs.

IEF. Isoelectric focusing of C57BL/6J MUPs was performed on a LKB 2117 Multiphor II Flatbed Electrophoresis Unit using an Immobiline dry-plate gel, pH range 4.2–4.9, and cooled to 10 °C. Male C57BL/6J urine was de-salted over a G-50 Microspin Column (GE) and 5 ul of sample was applied directly to the gel. Samples were loaded into the gel at 200 V, 5 mA and 15 W for 200 V h. The gel was electrophoresed at 3500 V, 5 mA and 15 W for 14.8 kV h and then fixed and stained with Coomassie brilliant blue.

Behaviour. C57Bl/6J male mice (8–12 weeks old) were isolated for one week. The mice were exposed to castrated adult mice swabbed with 40 µl of test solution (1× male urine; fractions and FPLC-purified MUPs were normalized to 1× urine) and assayed for 10 min. Tests took place in the home cages of isolated mice, and at least 48 h was allowed before a new test was conducted. Tests were videotaped and analysed at quarter speed using Observer software (Noldus Technology) to measure aggression parameters including tail rattling, biting, chasing, cornering, tumbling and kicking. Total duration was defined as the total duration of aggressive contact behaviour consisting of kicking, biting, wrestling or tumbling. One round of urine and no-urine controls was performed with each resident mouse before and after sample testing.

Production of recombinant MUP. Recombinant MUP protein was produced using the pMAL Protein Fusion and Purification System (New England Biolabs). Full-length MUP complementary DNAs (cDNAs) corresponding to the four C57BL/6J MUPs expressed in urine were cloned from a male C57BL/6J liver cDNA library and subcloned into pMAL bacterial expression vector pMAL-c2X. The starter culture was diluted into 1 litre LB/AMP/2% glucose, grown for 1 h at 37 °C followed by 2 h of induction with 0.3 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). Cells were centrifuged at 4,000g, 20 min and resuspended in 25 ml Column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA) plus protease inhibitors (Roche) and incubated for 30 min on ice with 1 mg/ml lysozyme. The sample was sonicated and then centrifuged (9,000g for 30 min). The supernatant was incubated overnight at 4 °C with 2 ml bed volume amylose resin and subsequently washed three times with 50 ml cold column buffer. rMUPs were eluted with 2 ml column buffer plus 25 mM maltose for 2 h at room temperature. rMUPs were assayed by SDS-PAGE. All rMUPs were pooled and normalized to 1 \times urine for behavioural analysis and further diluted 1:300 for calcium imaging.

Dose-response curve. For all calcium imaging experiments, stimuli were normalized to the concentration of MUPs in 1 \times urine (20 mg/ml as determined by Bradford assay) and then diluted 1:300 in Hanks/HEPES buffer. The four rMUP fusion proteins were pooled together using the estimation that each MUP is present in urine at one quarter of the concentration (5 mg/ml) of all MUPs. The pooled rMUPs were normalized to 1 \times urine by molarity. The dose-response curve was generated by presenting the stimuli (urine, HMW or pooled rMUPs) to VNs serially in the following

dilutions: 1:100,000, 1:10,000, 1:1,000, 1:300, 1:100. The number of responding cells was counted for each dilution and normalized to the maximum number of responding cells observed. The dose–response was fitted to a sigmoid curve by using the Hill equation. Urine $EC_{50} = 0.00099$, slope = 2.15, $n = 135$ cells in four experiments; HMW $EC_{50} = 0.001$, slope = 2.14, $n = 52$ cells in two experiments; rMUPs $EC_{50} = 0.0011$, slope.

Genomics. MUP genes were searched in the genome assemblies of the mouse (*Mus musculus*, NCBI m36), rat (*Rattus norvegicus*, RGSC 3.4), human (*Homo sapiens*, NCBI 36), chimpanzee (*Pan troglodytes*, PanTro 2.1), dog (*Canis familiaris*, Canfam 2.0), cow (*Bos taurus*, Btau 3.1), opossum (*Monodelphis domestica*, Mondom 4.0) and chicken (*Gallus gallus*, WASHUC2) using a modification of the methods used by Shi and Zhang (2007) (Shi and Zhang, 2007).

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The dissertation author was the primary investigator and author of this paper.

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Chapter 3: Single MUPs encode discrete information through the VNO

Single MUPs encode discrete information through the VNO

The biochemical nature of the pheromone code that induces innate behaviors such as aggression and mating in mice is not well understood, and the chemosensory systems responsible for pheromone detection are largely uncharacterized. The Major Urinary Proteins (MUPs), a class of small-molecule binding proteins expressed in large quantities in male mouse urine, were recently identified as a novel family of genetically encoded protein pheromones that promote intermale aggression through activation of VNO sensory neurons (VNs) (Chamero et al., 2007). However, the MUPs constitute a highly homologous gene family consisting of 18 open reading frames, and unique strain specific combinations of four to six MUPs are expressed in the urine of male mice. While it has been proposed that these unique expression patterns may constitute an individuality code detected by the VNO, the extent to which individual MUP isoforms can encode discrete information remains a matter of controversy (Hurst et al., 2001; Cheetham et al., 2007; Sherborne et al., 2007). We report here that, despite the high sequence homology in the MUP gene family, VNO sensory circuits can discriminate between individual MUPs that carry discrete behavioral information. By calcium imaging dissociated VNs exposed serially to the four MUPs expressed in C57Bl/6J urine, we show activation of at least six distinct populations of VNs, comprising both single MUP specific and multiple MUP responding neurons. Further, we show that single MUPs can carry unique behavioral information, as aggressive behavior in C57Bl/6J is coded for by a single, male-specific MUP (MUP 22), which shares relatively low sequence homology with the other three

non-aggression promoting MUPs (MUPs 3,7,14) expressed in C57Bl/6J urine. Our results demonstrate that single MUP isoforms can encode discrete information through the activation of specific VNO sensory circuits, suggesting that the MUP gene family comprises a rich source of VNO dependent information coding with the potential to regulate an array of pheromone mediated behaviors in addition to aggression. Elucidating the coding logic behind MUP expression will provide greater insight into both the nature of the secreted pheromone chemosignal, as well as the identity of the numerous sensory circuits that are tuned to respond to MUPs and drive critical behaviors.

Introduction.

Mice are critically dependent on pheromones, chemical cues secreted by one individual and detected by olfactory system of another, to properly regulate social behaviors such as aggression and mating (Stowers et al., 2002). While the importance of these pheromone cues in generating murine social behaviors is well established, only a handful of pheromone ligands that drive behavior have been identified and shown to activate sensory neurons in either the main or accessory olfactory system (Leinders-Zufall et al., 2004; Lin et al., 2005; Kimoto et al., 2005). Recently, we identified the Major Urinary Proteins, a previously orphaned class of lipocalin small-molecule binding proteins present in urine, as protein pheromones that activate VNO sensory neurons (VNs) and promote intermale aggression (Chamero et al., 2007). We further characterized the responding VNs, and showed that they belong to the V2R/G α o expressing class, each expressing at least one of 221 unique V2R G-protein coupled receptors. While these findings were significant in identifying a novel class of

genetically encoded pheromones that drive intermale aggression, they raised intriguing questions as to coding potential of individual MUP isoforms.

The MUP gene family consists of 18 putative open reading frames, and previous studies have shown that unique strain-specific combinations of four to six MUPs are expressed in the urine of male mice (Robertson et al., 1996; Armstrong et al., 2005). Some evidence suggests that this individual and strain-specific combinatorial expression plays a role in regulating individual recognition between conspecifics, however no one has assessed the extent to which individual MUP species carry the differential activation specificity required to encode specific information to the VNO (Hurst et al., 2001; Cheetham et al., 2007; Sherborne et al., 2007). Previously, we generated the four urinary MUPs (uMUPs) expressed in C57Bl/6J recombinantly, and showed that they activate VNs and promote aggression when pooled as a single stimulus (Chamero et al., 2007). To determine the individual coding potential of single MUPs, we have assayed the activation specificity of each of the four C57Bl/6J MUPs, as well as elucidated how aggressive behavior is encoded by these four MUPs.

Several lines of evidence that have been discussed in previous chapters are suggestive of VNs being tuned to respond to specific MUPs species, as opposed to a more promiscuous mechanism by which all MUPs carry the same activation specificity. Chamero et al., 2007 reported that the activity of the four uMUPs expressed in the C57Bl/6J strain accounted for close to 50% of the total urine activity on dissociated VNs by calcium imaging (figure 2). Based on VN counts in which a specific V1R was genetically labeled with GFP, the relatively large number of pooled MUP responding VNs seems inconsistent with activation of a single receptor (Boschat et al., 2002).

Further, the co-expansion of V2Rs in species that have polygenic MUP families (Chapter 2, figure 4) provides additional evidence suggesting that MUP activity may be generated through a number of V2R expressing neurons (Chamero et al, 2007). Finally, the majority of polymorphic sites differentiating three of the highly homologous MUPs expressed in C57Bl/6J reside on surface, as opposed to cavity binding residues, raising the possibility that single MUPs may have differential receptor binding affinities (Darwish-Marie et al., 2001).

Here, we report that the MUPs demonstrate a range of activation specificities and stimulate numerous MUP-specific, as well as non-selective, populations of VNs which greatly enhances the coding potential of the MUP gene family. Finally, we demonstrate that single MUPs can encode discrete behavioral information, suggesting that this gene family could encode behavioral information in addition to aggression.

Results.

Previously, we identified the four C57Bl/6J MUPs present in urine and expressed them recombinantly in e.coli in order to assess the protein versus small-molecule ligand dependence of VN activation by the MUP complex (Chamero et al., 2007). We further demonstrated that MUPs activate the Gao, V2R expressing class of VNs. Here, to determine the activation specificity of single MUPs, we first performed a genomic characterization of the MUP gene cluster, as well as a proteomic alignment of the MUPs expressed in C57Bl/6J in order to better predict the coding potential of this gene family. Using a Hidden Markov Model of expressed rodent MUPs, we analyzed a 1.9 Mb region of Chromosome 4 between *Slc46a2* and *Zfp37* in the NCBI m37 C57BL/6J mouse

genome assembly. We identified 18 open reading frames (ORF) encoding putative MUPs, and a further 18 presumptive pseudogenes (figure 1a). Analysis of the ORFs demonstrated that the genes can be placed in two groups based on sequence similarity and structural arrangement: Class A and Class B. Class A (marked in green, figure 1a,c) consists of 6 similar genes that are 82-94% identical at the cDNA level. In Class B (marked in red, figure 1a,c) we identified 12 highly similar genes, all greater than 97% identical at the cDNA level, some differing at only a single amino acid residue. The four C57Bl/6J uMUPs consist of three highly homologous class B MUPs (MUPs 3, 7 and 14), as well as one more divergent class A MUP (MUP 22). Alignment of the predicted translation products reveals that the three expressed class B MUPs differ at only three amino acid residues: F/V 56, N/K 50, Q/K 136 (figure 1b). Previous structural analysis indicates that two of these polymorphisms reside on surface residues (N/K 50, Q/K 136) while the third is located in the small molecule binding pocket, where it has been shown to alter MUP 3's ligand binding affinities (Darwish-Marie et al., 2001; Sharrow et al., 2002). Importantly, MUP 3 carries an additional surface polymorphism (Q/K 136), the result of which is that all three class B C57Bl/6J uMUPs differ at a minimum of one surface residue. MUP 22, the class A MUP expressed in C57Bl/6J urine, shares only ~80% homology with the three expressed class B MUPs and has been previously described as male-specific, based on the fact that no class A MUPs have been observed in a survey of wild-caught female mouse urine by ESI-MS (Armstrong et al., 2005). The expression of male-specific class A MUPs, while not ubiquitous across all strains and individuals, could be suggestive of a gender dimorphic function for these MUPs.

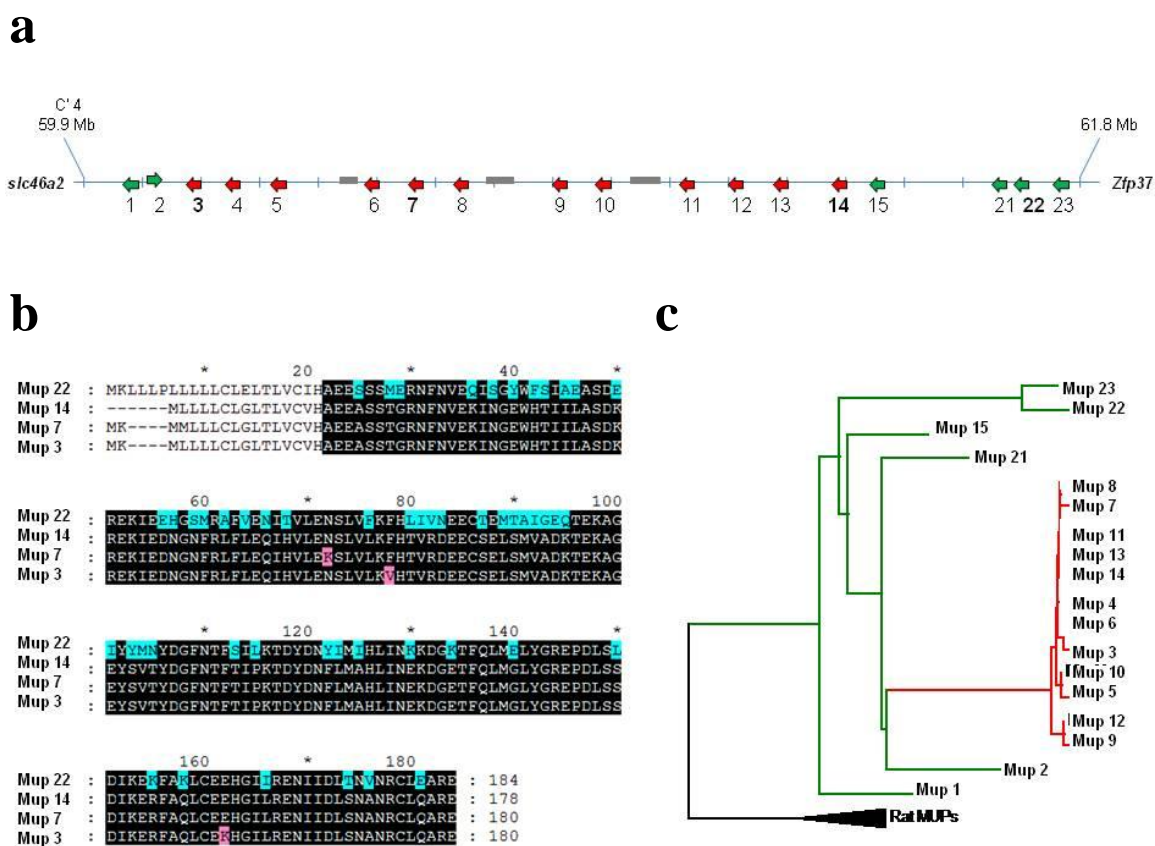


Figure 3.1 The MUP gene family has 18 highly homologous putative genes. **a**, MUP gene loci are located in a 1.9 Mb cluster on chromosome four between *slc46a2* and *Zfp37*. The eight class A MUPs (green arrows) are situated on the periphery of the cluster and share 80-90% homology, while the class B MUPs (red arrows) share close to 97% homology, in some cases differing at only a single amino acid. **b**, alignment of the protein sequence of the four MUPs expressed in male C57Bl/6J urine. The three expressed class A MUPs (MUP 3, MUP 7, MUP 14), differ at only three amino acid residues, while the male-specific class B MUP (MUP 22) is more polymorphic. All three class A MUPs carry at least one polymorphism on a surface (N/K 50, Q/K 136), as opposed to cavity binding (F/V 56), residue. **c**, Phylogenetic tree anchored by rat MUPs (α -2u globulin) showing the grouping of the 18 MUP genes into two classes based on homology: Class A (green) and class B (red).

Having characterized the MUP gene family and uMUP expression in C57Bl/6J more fully, we next set out to determine the activation specificity of the four C57Bl/6J uMUPs on dissociated VNs by calcium imaging. In order to assess activation specificity, we used a calcium imaging approach on dissociated VNs loaded with calcium indicator Fura-2 as performed in chapter 2 (Chamero et al., 2007). VNs were exposed to each of the four single recombinant MUPs (rMUPs) in series followed by the four rMUPs pooled as a single stimulus (figure 2a). Surprisingly, despite varying in some cases by only a single amino acid, we identified four populations of VNs based on activity each responding specifically to one of the four single MUPs, as well as three additional, less specific populations (figure 2a-c).

The proportion of single MUP responding VNs was roughly consistent across the four MUPs tested (each comprising about 10% of the total MUP activity), suggesting that a similar number of molecularly discrete VNs are dedicated to recognition of single MUPs (figure 2d). This finding is particularly unexpected given the near identity in protein sequence between MUP 3, MUP 7 and MUP 14, and indicates that the receptors dedicated to MUP detection are capable of discriminating their MUP ligands based on only one or two amino acid polymorphisms, which establishes a level of discrimination in pheromone detection not previously described in VNO sensory neurons. Further, when this remarkably specific activity is extrapolated across all 18 putative MUP genes, it is possible that a significant proportion of the basal/Gao expressing VNO is dedicated to specific MUP discrimination, which would greatly expand the coding potential of this gene family. To date, only a small number of specific pheromone compounds have been shown to activate a subset of the at least 250 molecularly distinct VNs, including the six

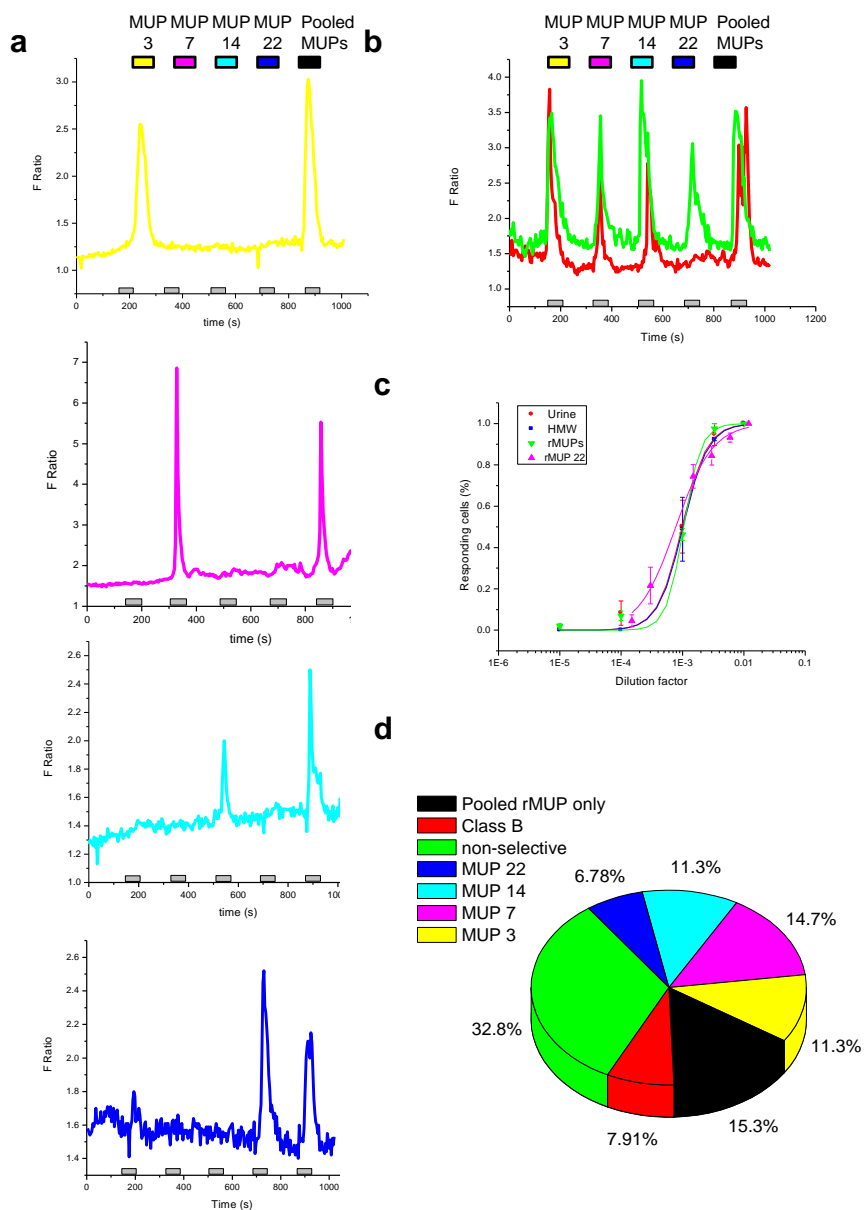


Figure 3.2 Single MUPs activate discrete populations of MUP-specific and non-selective VNs. **a**, Sample calcium transients of VNs serially exposed to each C57Bl/6J rMUP followed by the four rMUPs pooled as a single stimulus. Four populations of VNs were identified based on activity, each responding specifically to a single rMUP (MUP 3 yellow, MUP 7 magenta, MUP 14 cyan, MUP 22 blue). **b**, Two additional populations of non-selective VNs were also identified. A large population of non-selective VNs (green) and a class B MUP specific population (red) that responded to MUPs 3, 7, and 14, but not MUP 22. **c**, Dose-response curve fitted to a sigmoid curve using the Hill equation generated with rMUP 22, pooled rMUPs, HMW and urine showing proportion of VNs activated at several dilutions. **d**, Quantification of the VN populations, including an additional population of VNs that responded only to the pooled rMUP stimulus (black). 6766 total cells imaged, 176 responded to rMUPs; MUP 3, 20/176; MUP 7, 26/176; MUP 14, 20/176; MUP 22 12/176; Non-selective, 58/176; class B specific, 14/176; pooled rMUP only, 27/176.

identified “Novotny compounds,” several class I MHC peptides and the ESP peptides (table 1, chapter 4). Our results suggest that MUP specific activity may define an additional, and potentially abundant, set of dedicated VNO sensory circuits.

However, the MUP specific VN populations only accounted for about half of the total observed MUP activity; the remaining 50% of MUP activated VNs were less discriminant. Indeed, the largest population of activated VNs observed (about 33% of total MUP activity) comprised non-selective VNs that showed specificity to all four expressed MUPs (figure 2b,d). These non-selective VNs were two to four times more common than the MUP specific VNs, suggesting that either a single non-selective MUP receptor is expressed at a higher rate than MUP-specific receptors, or an additional number of MUP receptors are expressed which do not discriminate between specific MUPs.

Our analysis revealed additional intriguing structure-function relationships. In compliment to the VNs capable of discriminating between the highly homologous class B MUPs, we identified an additional population of VNs that appear to discriminate between class A and class B MUPs (figure 2b,d). These VNs responded non-selectively to the three expressed class B MUPs, but did not respond to MUP 22, the single expressed class A MUP. This class B specific population was represented at a rate roughly equal to that of the MUP specific VNs, suggesting that similar numbers of molecularly discrete VNs are involved in recognition of both groups. The identification of VNs that discriminate between class A and class B MUPs, when coupled with the relative sequence divergence between these two groups and the male-specificity of class A MUP expression, raises the

possibility that these two MUP classes may be functionally specialized to encode different qualities of behavioral information.

Finally, we observed a population of VNs that responded only to the pooled rMUP stimulus, but not to any of the single rMUPs. While it is tempting to postulate about additional, molecularly distinct VNs that respond only to MUPs when present in combination, it is possible that this population represents a concentration artifact due to the 4x total protein concentration of the pooled rMUP stimulus as compared to the single rMUP stimuli. To address this issue, we produced a concentration curve (figure 2c) with a single rMUP (MUP 22), which shows that the single rMUP concentrations used in these experiments activate 90% of the VNs activated if the same single MUP is presented at 4x concentration. This result gives us confidence that our dissociated VNs were presented with single rMUPs at concentrations that generate transients close to the population saturation for activity. Further, the single rMUP 22 dose-response curve overlays almost exactly with the dose response curves for urine, HMW and pooled rMUP stimuli (figure 2c). These stimuli show the same proportion of total VN activity as rMUP 22 at each point, despite having 4x the total protein concentration at each dilution used. This strongly supports our findings that the single MUPs carry discrete activation specificities, as well as suggesting that the pooled MUP-specific activity is not sufficiently explained by concentration artifacts. In total, our results show that the VNO contains circuits tuned to discriminate between specific MUPs, as well as circuits tuned to respond non-selectively to all MUPs or classes of MUPs. These findings both greatly expand the coding potential of the MUP gene family as protein pheromones acting through the VNO,

as well as highlight the numerous *Gao/V2R* expressing VNO sensory circuits dedicated to MUP detection and discrimination.

Having identified both MUP specific, and multiple MUP responding sensory circuits in the VNO, we next set out to identify the specific MUP responding VN population responsible for the generating the MUP dependent aggressive behavior reported previously (Chamero et al., 2007). Using our prior strategy of swabbing normally non-aggression promoting castrated intruder males with pheromone stimuli to induce an aggressive response in intact male residents, we assayed the ability of each of the four uMUPs expressed in C57Bl/6J to generate aggressive behavior. We found that only MUP 22, the male-specific class A MUP expressed in C57Bl/6J urine, can generate aggression equivalent to that of the four rMUPs pooled into a single stimulus (figure 3). None of the three expressed class B MUPs were sufficient on their own to generate significant levels of aggression, nor were they sufficient when combined as a single stimulus (figure 3). Based on these results, we conclude that the identity of the VNs responsible for driving aggression in response to C57Bl/6J MUPs, is the population tuned specifically to respond to MUP 22 (figure 2a). To our knowledge, no VNO mediated behaviors have been described at the level of VN activation by a single pheromone ligand. By using behavior coupled with calcium imaging of dissociated VNs, we have identified a discrete VNO sensory circuit responsible for generating intermale aggression. Further, our data suggest a potential functional specialization for the male-specific class A MUPs. It will be of interest to determine how this aggression code is stereotyped across individuals with differential MUP expression patterns.

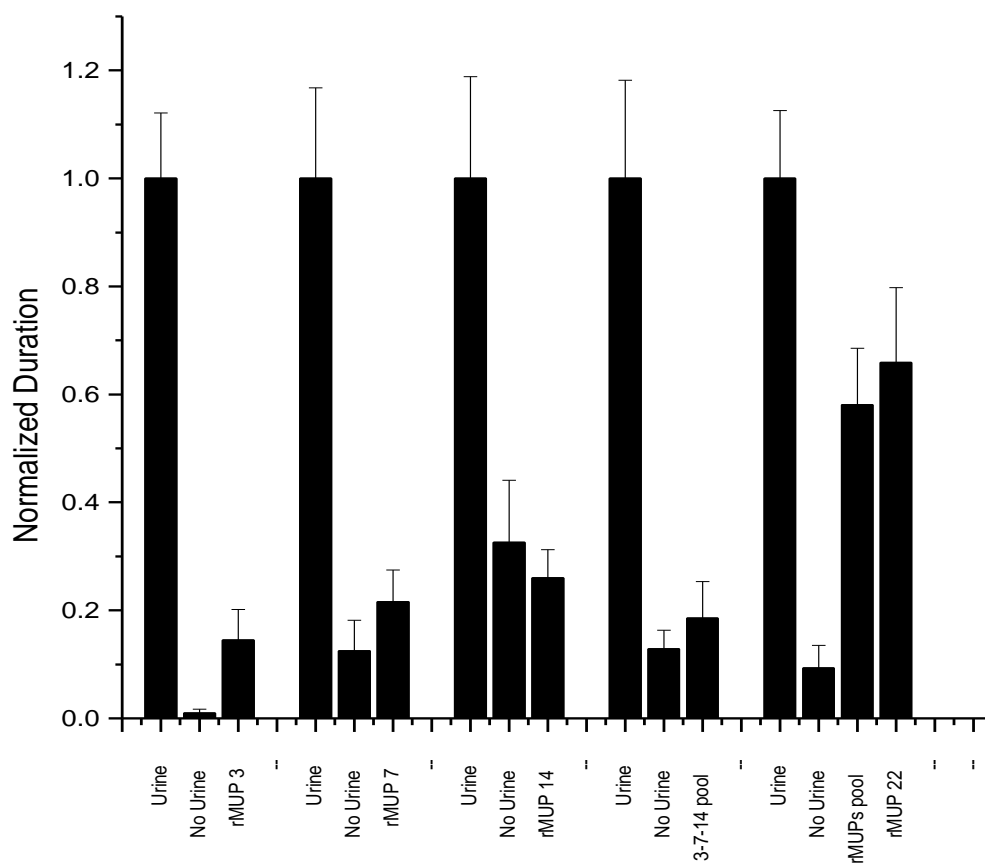


Figure 3.3 A single C57Bl/6J MUP specifically encodes for aggressive behavior. Each MUP was tested separately for aggression promoting activity in the resident-intruder assay. Duration of aggressive behavior is normalized to the duration of aggression generated by whole urine in each group. MUP 3 (n=33 trials), MUP 7 (n=18 trials), and MUP 14 (n=21 trials) did not generate significant levels of aggression when presented separately or when pooled as a single stimulus (3-7-14 pool) (n=18 trials). Only MUP 22 (n=44 trials) was sufficient to generate aggression equivalent to the pooled rMUP stimulus (n = 33 trials). One-tailed student's T-Tests: rMUP 22/rMUPs pooled $P = 0.327$; rMUP 22/no urine $P = 0.00013849$; rMUP 22/urine $P = 0.03633$; rMUP3/no urine $P = .01245$; rMUP 7/no urine $P = 0.1416$; rMUP 14/no urine $P = 0.3378$; rMUPs 3-7-14 pooled/no urine $P = 0.2422$. rMUP 3, while statistically significant compared to no urine, only generated four seconds of total aggression.

Discussion.

We have identified the MUP gene family as a source of diverse pheromone activity; It appears likely based on our data, and the number of additional MUP genes that may carry specific activity, that up to 20 distinct VNO sensory circuits are dedicated to sensing and discriminating between MUPs based on both highly specific as well as more general structural criteria. Given our earlier findings that MUP activity is restricted to the basal V2R/*Gαo* expressing VNs, it is worth considering what molecular mechanisms may underlie the observed populations of activity in these VNs. Several different classes of molecules have been described that are expressed in varying proportions in the sensory dendrites of *Gαo* expressing VNs, including non-classical MHC, and V2Rs. Nine non-classical MHC genes (*H2-Mv*) have been shown to be expressed in VNs, and were originally thought to be required for stabilization of V2R receptors at the plasma membrane, where it was postulated they could play a role in ligand recognition (Loconto et al., 2003). At first glance, these molecules seem attractive candidates as MUP protein receptors, as they are structurally capable of associating with peptide molecules (Olson et al., 2006). However, recent evidence suggests that the surface MHC may not be expressed in the VNO as widely as originally believed, and may not be as critical for pheromone recognition as previously assumed (Ishii et al., 2008). In addition, the nine *H2-Mv* genes are not expressed discretely enough across the VNO to account for the full range of MUP specific activity observed, nor the relatively small numbers of VNs in each population of activity.

How, then, to explain the recognition of single MUPs? We observed activation of about 0.5% of VNs by each single MUP, which is consistent with prior reports of single

pheromone compound activity in the VNO (Boschat et al., 2002; Leinders-Zufall et al., 2000). We find it most probable, based on the co-expansion of V2Rs and MUP genes, that single MUPs are discriminated by a sub-group of dedicated V2R receptors. The V2R gene family consists of 221 receptors divided across four families and 13 distinct phylogenetic clades. Assuming each of the 18 putative MUP genes activates a discrete V2R, several clades have numbers of receptors consistent with being dedicated to single MUP discrimination, including family A clade I (18 receptors), clade IV (25 receptors) and clade V (15 receptors) (Silvotti et al., 2007). The more numerous VNs that recognize MUPs non-selectively could express a V2R from a clade with only a single, over-expressed receptor (family A clades II, VI and X), or a clade with only a few receptors that all recognize MUPs indiscriminately (family D, family A clade IX). Alternatively, MUP signaling could be generated through an as yet unidentified class of receptors that co-express with V2Rs. Further study will be required to fully elucidate the molecular mechanism underlying MUP activity in the VNO.

Our finding that aggressive behavior is encoded by a single, phylogenetically distinct MUP (MUP 22) raises questions as to how behaviors are encoded by the MUP gene family across different strains and individuals with their own unique MUP expression patterns. It is possible that the male-specific class A MUPs are specialized to encode aggression, however as we stated, previous biochemical analyses of urine collected from wild caught mice and laboratory strains reveals that class A MUP expression is not ubiquitous in male mice (Robertson et al., 1996; Robertson et al., 1997). Given our previous findings that aggression is encoded redundantly by the low molecular weight (LMW) constituents of urine independently of MUPs, it is presumably not

necessary for every male to express an aggression promoting MUP (Chamero et al., 2007). Under this scenario, aggression is encoded redundantly by the small molecules in urine, and the MUPs specialized for aggression are expressed in some individuals, but not under strong enough selection to be expressed in all males. Of course, it is equally likely that our delineation of MUPs into class A and class B does not reflect any functional specialization as relates to aggression, and that a number of different MUPs across both classes encode aggression through the same or independent VNO circuitry. Finally, it should be noted that we have identified three MUPs activating four discrete populations of VNs that apparently do not encode aggression, suggesting strongly that the MUP gene family can encode for additional pheromone mediated behaviors. Certainly, the ability of single MUPs to activate discrete VNO sensory circuits lends weight to prior reports that individual and strain-specific MUP expression patterns function in behaviors related to conspecific recognition, such as the Bruce effect, counter-marking, and in-breeding avoidance (Hurst et al., 2001; Cheetham et al., 2007 Sherborne et al., 2007). Further studies will elucidate the full range of MUP bioactivity generated through the VNO.

Our characterization of MUP function reveals a protein pheromone family with diverse signaling capabilities through the VNO. Further, we have identified a VNO sensory circuit specifically activated by a single MUP that generates aggressive behavior. These findings mark significant progress in understanding the array of pheromone signaling generated through the VNO, and provide a springboard for further molecular characterization of the VNO circuitry that drives behavior.

Methods.

Behavior. Behavioral assays and analyses were conducted as described in Chamero et al., 2007 (see chapter 2 methods). Single rMUPs were presented as a 40ul stimulus on the backs of castrated males at a molar concentration equivalent to the relative concentration of that single MUP in urine assuming a roughly equal concentration of each MUP in urine (1/4X the concentration of the pooled rMUP stimulus for each single rMUP. See IEF gel, figure 2a, chapter 2). Behavioral data was normalized to the duration of aggression induced by whole urine for each group.

Calcium imaging. Calcium imaging was conducted as described in Chamero et al., 2007 (see chapter 2 methods). Single rMUPs were prepared at a molar concentration equivalent to the relative concentration of that single MUP in urine assuming a roughly equal concentration of each MUP in urine and then diluted 1:300 so as to remain consistent with our prior study. The pooled rMUP stimulus was prepared by pooling the single rMUP stimuli in an equimolar ratio and then diluting 1:300, so as to reconstitute the total MUP concentration in urine. Therefore, the pooled rMUP stimulus was 4x the total protein concentration of the single rMUP stimuli.

Dose-response Curve. The dose–response curve was generated by presenting stimuli urine, HMW or pooled rMUPs to VNs serially in the following dilutions: 1:100,000, 1:10,000, 1:1,000, 1:300, 1:100. The single rMUP 22 was presented at the following dilutions: 1:1,200, 1:600, 1:300, 1:100, 1:75. 1:300 represents the dilution used for all stimuli in calcium imaging experiments. The number of responding cells was

counted for each dilution and normalized to the maximum number of responding cells observed. The dose–response was fitted to a sigmoid curve by using the Hill equation.

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Chapter 4: What is a Pheromone? Mammalian Pheromones Reconsidered

What is a pheromone? Mammalian pheromones reconsidered

Pheromone communication is a two component system; signaling pheromones and receiving sensory neurons. Based on the number of pheromone receptors, hundreds of pheromones themselves are expected to be emitted by mice. Currently, pheromones remain enigmatic bioactive compounds as only a few have been identified, but classical bioassays have suggested that they are non-volatile, activate vomeronasal sensory neurons, and regulate innate social behaviors and neuroendocrine release. Recent discoveries of potential pheromones reveal that they may be more structurally and functionally diverse than previously defined. These new developments blur rather than refine the working definition of pheromones while providing hints to the complexity of the pheromonal regulation of behavior.

Pheromones are a mystery.

Pheromones are unlike the familiar chemical odorants that generate our perception of smell and subtly guide our behavior. With experience, we learn to be drawn to the aroma of finely prepared food and repelled when it has spoiled. But, in addition to the seemingly limitless odorant combinations that we associate with certain behavioral outcomes, most terrestrial vertebrates also respond to pheromones. These semiochemicals are classically defined as chemical cues emitted and detected by individuals of the same species that influence social and reproductive behavior. A naive animal responds behaviorally to the presence of pheromones without any prior experience or exposure: pups suckle, males fight, and estrus cycles are altered. And yet, despite the importance of

these chemical cues in regulating essential animal behaviors, the nature of these elusive ligands remains largely unknown. A growing body of evidence indicates that the structural and functional characteristics of pheromones may be far more diverse than revealed by classical experiments. Recent studies (Leinders-Zufall et al., 2000; Lin et al., 2005) in conjunction with prior evidence, suggests that the working definition of pheromones as non-volatile molecules that regulate innate social behavior by activating vomeronasal organ (VNO) sensory neurons may be too restrictive. Indeed, it appears that pheromones may be volatile or ephemeral, activate VNO or main olfactory epithelium (MOE) neurons, and may have their effects altered by context as opposed to being strictly innate.

Are pheromones simply ligands that activate the vomeronasal organ?

Two-hundred years ago Jacobson described an anatomically distinct organ within the nasal cavity filled with chemoreceptive cells and, without supporting evidence, dubbed it the 'sexual nose' as a potential mediator of the pheromone response (Cuvier, 1811). Subsequent experiments have suggested that the 'sexual nose', now referred to as the vomeronasal organ (VNO), responds to pheromones while chemoreceptive neurons that reside in the main olfactory epithelium (MOE) initiate the perception of odorants (figure 1).

More recently, molecular characterization has revealed that the primary signal transduction machinery of MOE neurons is distinct from that of VNO neurons (reviewed in Dulac and Torello, 2003). Although ligands for both structures activate specific G-protein coupled receptors (GPCRs), the MOE receptors are evolutionarily distinct from

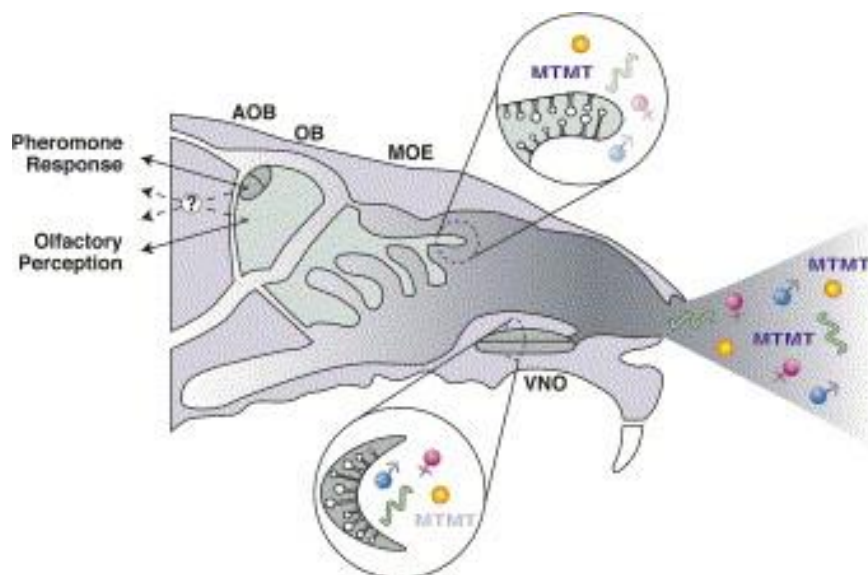


Figure 4.1 The Functional Organization of the Pheromone-Sensing System. Chemical cues in the environment are detected by two anatomically distinct chemosensory organs in the mouse nasal cavity. The location of the main olfactory epithelium (MOE) at the far end of the nasal cavity makes it well suited to detect volatile odorant ligands (yellow icons, MTMT), while the location of the fluid-filled vomeronasal organ (VNO) was thought to be better suited to detect nonvolatile pheromones (blue and red icons) as well as peptides (green icons). However, traditional odorants have also been shown to activate the VNO, and MTMT (shaded to denote uncertainty) may activate as well. Likewise, traditional pheromones, including those shown to be volatile, as well as peptides may act through the MOE (icons shaded to denote uncertainty). The mitral cell second-order neurons that are located in the olfactory bulb (OB) receive input from the MOE and are involved in the perception of odorants but may also be involved in the pheromone response. Second-order neurons in the accessory olfactory bulb (AOB) receive inputs from the VNO and transduce the classical pheromone response but may also be involved in odorant perception.

all identified VNO receptors. Furthermore, GPCR activation in the MOE leads to the production of cAMP to gate CNGA2 channels. These signaling components are not expressed in VNO neurons that instead utilize a phospholipase C pathway to activate TrpC2 channels. Most interesting, however, is the apparent segregation of the neuronal circuitry. MOE neurons project axons to the olfactory bulb and synapse on mitral cells that in turn signal to the cortex and the olfactory amygdala. In contrast, VNO neurons project to the accessory olfactory bulb and relay their signal to the anatomically distinct medial amygdala. Together, this molecular and anatomical evidence supports Jacobson's theory that the MOE and the VNO are designed for different functions.

However, it is becoming clear that the biological role of these two different chemoreceptive populations may not be as simple as Jacobson originally proposed. While it is true that the MOE responds to odorants and VNO neurons respond to pheromones (reviewed in Dulac and Torello, 2003 and Brennan and Keverne, 2004), it appears that the converse may also occur. Experiments in swine, which display a robust pheromone response, indicate that some pheromone-mediated behaviors are generated by MOE neurons (Dorries et al., 1997). Additionally, there are reports that humans respond to pheromones, yet we do not possess a functional VNO (Liman and Innan, 2003; Savic et al., 2001; Stern and McClintock, 1998). Recent experiments utilizing molecular genetics and behavioral analysis in mice clearly indicate that not all pheromone behaviors are initiated through the VNO. In particular, mice defective for VNO activity (*TrpC2*^{-/-}) continue to display some pheromone-mediated behaviors such as pup suckling (Leypold et al., 2002; Stowers et al., 2002), a behavior which is defective in mutant mice lacking

classical MOE activity (CNGA2^{-/-} formerly known as OCNC1 Brunet et al., 1996). Together, these findings indicate that pheromones can be detected by populations of neurons outside of the VNO that are molecularly similar to those MOE neurons generally thought to mediate odorant perception. Additional complementary experiments reveal that mouse VNO neurons can be stimulated by odorants not emitted from other animals such as floral and woody smelling compounds (Sam et al., 2001), and that mice defective in MOE signal transduction (Adenylate Cyclase III^{-/-}) are capable of behavioral responses to certain odorants (Trinh and Storm, 2003). These findings, which stand in contrast to the original hypothesis of Jacobson, inspire one to reconsider the function of these two different ‘noses’. To date, the biological relevance of the evolution of two separate chemosensory organs remains unknown. However, it is clear that a pheromone is not simply a ligand that activates VNO sensory neurons.

Recently, Katz and colleagues reported a novel semiochemical isolated from mouse urine that activates MOE neurons (Lin et al., 2005). To determine the precise volatiles in urine that activate the main olfactory system, Katz’s group accomplished a technical tour de force by combining single-unit electrophysiological recordings from MOE mitral cells with solid-phase microextraction and gas chromatography of urine. This ambitious experimental paradigm allowed for the characterization of mitral cells (located in the olfactory bulb in figure 1) that were specifically activated by individual compounds within urine. A novel compound was identified from male urine that is absent in female urine and excites neurons of the MOE. This compound, (methylthio)methanethiol (MTMT), elicits an attractive behavioral response from females. Is MTMT the first identified mouse pheromone acting through MOE neurons? It

does transmit behavioral information between species members and at first glance could be considered as a potential pheromone. Though the electrophysiology reveals that this socially relevant compound activates MOE neurons, it is not clear that these are the same neurons that mediate the behavior. The behavioral effect may be generated through additional MTMT responsive neurons in the VNO or elsewhere. Assays with *TrpC2*^{-/-} and *CNGA2*^{-/-} mice can confirm if MOE-type neurons are indeed the ones mediating the MTMT-induced behavioral response.

Are pheromones non-volatile chemical cues?

Everyday experience confirms that odorants are volatile, and that non-volatiles do not convey a sense of smell. Initially, it was presumed that most pheromones were non-volatile, since direct physical contact with the stimulus (by licking or inhalation of droplets) was observed to be necessary for activation of certain pheromone-mediated behaviors (O'Connell and Meredith, 1984). Although it is clear that mouse urine contains pheromone activity (for example, male urine elicits aggressive behavior from other males), the molecular identities of urine pheromones have not been defined. A small number of interesting volatile compounds have been purified based on their dimorphic presence in male and absence in female mouse urine (Table 1, and reviewed in Dulac and Torello, 2003). These compounds have been shown to directly activate VNO neurons *in vitro* (Leinders-Zufall et al., 2000). Currently, the biological function of these purified compounds is subtle, yet, based on their presence in bioactive fluids and their ability to activate VNO sensory neurons, they should be considered candidate pheromones. As the field begins to unravel the logic of chemosensation it will be interesting to address the

extent to which one molecule can activate both the VNO and the MOE. These isolated compounds along with MTMT, which is also volatile, suggest that there is no inherent biophysical difference between the molecular features of an odorant and a pheromone.

However, previous behavioral experiments clearly identified non-volatile pheromone activity and recently, a great step has been made towards identifying these cues. A second class of molecules was found to be present in mouse urine, activate VNO neurons, and alter reproduction: MHC class I peptides (Leinders-Zufall et al., 2004). These non-volatile ligands, which represent the “self-peptides” expressed on MHC class I molecules during thymic selection of T-cells, are implicated in turning off the normal pheromone response between males and females to allow for a pregnancy to proceed. Are these peptides pheromones? They are emitted and detected within a species, they activate chemosensory neurons, and serve to block a neuroendocrine response ensuring pregnancy. Thus, they possess many of the accepted functions of pheromones. In total, it appears that the structural nature of pheromones is heterogeneous from volatile small molecules to non-volatile peptides.

Do pheromones initiate innate responses?

Olfactory perception is associative; we learn to correlate odors with specific objects or situations based on experience. Moreover, our output behavior in response to odorants can be altered. A smell that was once unpleasant may become attractive when associated with a rewarding experience. In contrast, the response to pheromones is thought to be hardwired; the cues convey an intrinsic meaning. When a naïve male that is isolated after weaning is placed in the presence of another male’s pheromones, he

instinctively displays the predicted behavior of aggression that is thought to be unaffected by experience, learning, or memory (Connor, 1972). Based on these functional observations, it is not clear whether MTMT, the substance in male urine that activates MOE neurons and is attractive to females, can be classified as a pheromone. Since the females used in the behavioral analysis of MTMT were sexually experienced (Lin et al., 2005), their prior exposure to males provided ample opportunity for associative learning to male-specific cues that may not otherwise convey behavioral information when presented to naive females alone. It will be of interest to determine whether MTMT initiates attraction in females without sexual experience or rather functions as a learned cue that females associate with males after exposure.

Do MHC I peptides convey intrinsic information? It is first necessary to understand their biological function. Specifically, unknown pheromones in male urine initiate a female's estrus cycle (Figure 2a, Marsden and Bronson, 1964). This becomes an obvious problem for reproduction, since the presence of a male's pheromones after mating would trigger estrus and subsequent loss of the uterine lining rather than allowing for hormonal profiles conducive to embryo implantation and pregnancy. Female mice circumvent this problem by forming an 'olfactory memory' (Bruce effect, Bruce, 1959) specific to the MHC class I peptide profile of the mating partner which subsequently prevents entry into estrus normally evoked by his pheromones (figure 2b). However, this mechanism is specific to the mating partner as the pheromone profiles (small molecules and MHC peptides) of other males retain the ability to induce estrus (figure 2bi, Leinders-Zufall et al., 2004).

Table 4.1 Characteristics of candidate mouse pheromones.

Potential Mouse Pheromones ^a	Sensory Neuron Activation		Molecule Volatile	Behavior	Behavior Innate
	VNO	MOE			
2,5-dimethylpyrazine	yes	?	yes	puberty delay	yes
2-sec-butyl-4,5-dihydrothiazole	yes	?	yes	estrus induction, intermale aggression, and female attraction	yes
2,3-dehydro-exo-brevicomin	yes	?	yes	estrus induction, intermale aggression, and female attraction	yes
E,E- α -farnesene, E, β -farnesene	yes	?	yes	estrus induction, intermale aggression	yes
2-heptanone	yes	?	yes	puberty delay	yes
6-hydroxy-6-methyl-3-heptanone	yes	?	yes	puberty acceleration	yes
MHC class I peptides	yes	?	no	olfactory memory	yes/associative
(methylthio) methanethiol (MTMT)	?	yes	yes	female attraction	?

^aReviewed in Dulac and Torello, 2003.

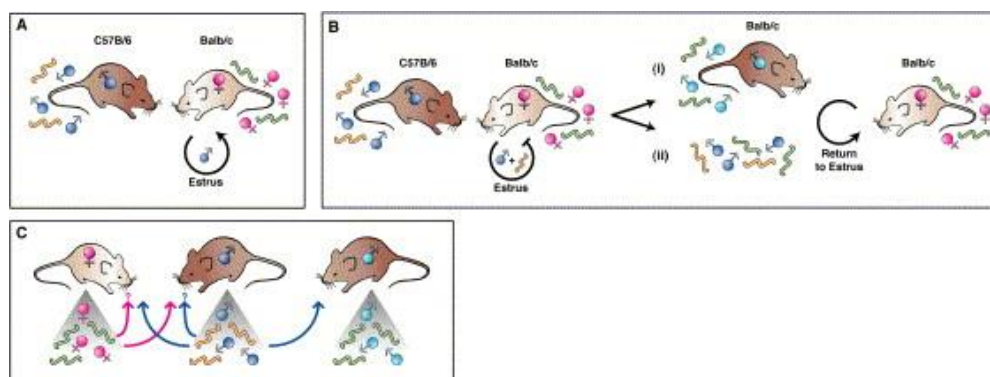


Figure 4.2 Role for Small Peptide and Chemical Pheromones in Mediating Reproduction. a, Unidentified chemical cues (blue icons) in the C57B/6 male urine induce estrus in the Balb/C female. **b,** After mating of the C57B/6 stud male to the Balb/C female, she forms a memory to the stud male's urinary peptides (yellow icons), inhibiting the estrus-inducing effect of his own chemical pheromones and ensuring successful pregnancy (left). If the pregnant Balb/C female is subsequently exposed to a male of a different strain as the mating male (Balb/C), his urinary peptide profile (green icons) is not recognized by the female, and his chemical cues induce estrus resulting in termination of the original pregnancy (i). MHC peptides are sufficient for this effect since, after mating to C57B/6 male, the female can be induced to return to estrus simply by exposure to C57B/6 urine spiked with BALB/c peptides (ii). **c,** Mice do not demonstrate behavioral responses to their own pheromones in the absence of contextual cues.

This demonstrates that the MHC peptides intrinsically alter behavior, but only after a form of learning. The mating male's pheromones induce estrus prior to mating, yet do not initiate a behavioral response after mating. Since all MHC peptides activate VNO sensory neurons *in vitro* (Leinders-Zufall et al., 2004), it is interesting to contemplate the possible mechanism of this memory dependent inhibition of normal male pheromone action. Memory formation has been shown to require the activity of inhibitory interneurons in the accessory olfactory bulb (Kaba et al., 1994) providing a general method to modify pheromone circuitry. Alternatively, mate specific peptides may be prevented from activating neurons *in vivo* after the memory is established. This exciting discovery of MHC peptide ligands as a direct mediator of this process provides the tools to elucidate the underlying molecular mechanisms that generate specific memory formation to the appropriate male. One wonders if MHC peptides are indeed pheromones in their own right, capable of inducing a behavioral response in the absence of other compounds or are instead accessory molecules that modify, in this example block, the response of other pheromones. In total, these studies reveal that unlike odorants, murine pheromones are intrinsically instructive. However, the exception of the mating dependant response to MHC peptides indicates that this definition is not absolute. In fact, the behavioral response to pheromones may indeed be altered by some limited forms of learning and memory.

Does the presence of pheromones always generate behavior?

The response to pheromones is thought to be unalterable. One imagines an animal's actions to be robotically dictated by pheromones. In reality, the response to

pheromones may be context dependant. Our current understanding of MTMT does not inform this aspect of pheromones, but MHC peptides reveal contradictions about the effects of ligands on behavior. MHC class I peptides are not gender specific therefore the female is continuously exposed to her own peptide ligands. However, this presents a potential problem; why does she not form a memory to her own peptides? Indeed figure 2B illustrates (Leinders-Zufall et al., 2004) that a female's BALB/c peptides are present during the critical period when the memory is being formed to the mate's C57B/6 peptides creating a molecular situation that is theoretically similar to the subsequent presentation of BALB/c peptide spiked C57B/6 urine that is sufficient to initiate estrus (figure 2Bii). This phenomenon suggests that there is a molecular mechanism that differentiates between the female's and the male's MHC peptides or alters access of the female's peptides to the sensory neurons.

The contradiction of the action of pheromone cues selective to appropriate context can be extended when one considers the refractive nature of an individual to their own pheromones. For example, a male excretes pheromones sufficient to induce aggression from other males, yet he does not continuously display aggressive behaviors in response to his own pheromones (figure 2C). In unpublished experiments, we have observed that a male can exhibit aggression in response to his own urine when it is presented on the body of another mouse (that has been castrated to prevent the release of pheromones). In this example, the presence of pheromone ligands in the cage environment without the proper context of another male is not sufficient to induce aggressive behavior. As with the MHC peptides, this suggests that additional environmental stimuli can regulate the behavioral response generated by pheromones. In rodents, learned odorant cues detected by MOE

neurons may be providing the contextual information. Both MOE and VNO circuitry converge in the amygdala (reviewed in Meredith, 1998) providing an opportunity for the integration of pheromone and non-pheromone cues.

Much progress has been made towards identifying the mechanisms underlying the mammalian pheromone response. Molecular genetics have revealed the immense potential for information coding by murine chemosensory neurons and identification of the pheromones themselves is the next important step necessary to elucidate the mechanisms underlying behavior. Indeed, recent findings of candidate murine pheromones have broadened our understanding of their role in mediating intra-species behavior. With the addition of small volatiles acting through the MOE and the VNO, and urinary MHC peptides joining the list of potential pheromones, it is clear that the family of pheromone molecules and their mechanism of action is far more diverse than previously thought. Consequently, the working definition of pheromones is now in flux. Continuing elucidation of the pheromone ligands promises more surprises and exciting advances.

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