

Identification of protein pheromones that promote aggressive behaviour

Pablo Chamero^{1*}, Tobias F. Marton^{1*}, Darren W. Logan¹, Kelly Flanagan¹, Jason R. Cruz¹, Alan Saghatelian³, Benjamin F. Cravatt² & Lisa Stowers¹

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 mating¹. 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)². 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^{1,3,4}. 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_{α_o} subunit (also known as G_{nao}) and $Vmn2r$ putative pheromone receptors (V2Rs). 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 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 animals⁵, providing a bioassay for the identification of urinary pheromones (Fig. 1a). 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 (HMW; greater than 10 kDa) molecular mass 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 assay pheromone activity further, 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, because mice lacking the primary sensory transduction channel, TRPC2, are unable to detect and respond to the aggression-promoting pheromone (Fig. 1a)^{6–8}. 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 investigate the LMW and HMW fractions further, 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)⁹, providing a mechanism for individual neurons to respond to different compounds. 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 in response to repetitive exposure of multiple substances at a resolution of the single cell; this enables us to determine precisely 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.

There are very few HMW components in mouse urine^{10,11}; none has been identified as a pheromone. Therefore we chose to focus our subsequent purification and characterization on only 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 (Supplementary Fig. 2). 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);

¹Department of Cell Biology, ²Department of Chemistry, The Scripps Research Institute, La Jolla, California 92030, USA. ³Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138, USA.

*These authors contributed equally to this work.

these features closely mirror the size and isoelectric point (pI) of MUPs⁴. Indeed, western blot (Fig. 2a) and electrospray ionization mass spectrometry (ESIMS) 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 pattern¹². 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 ligands¹³. Gas chromatography followed by mass spectrometry (GC-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 pheromone¹⁴ capable of activating a subset of VNs¹⁵; however, the role of MUP itself without ligands, MUP protein, remains elusive^{4,16,17}.

To investigate the function of the MUP protein further, 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

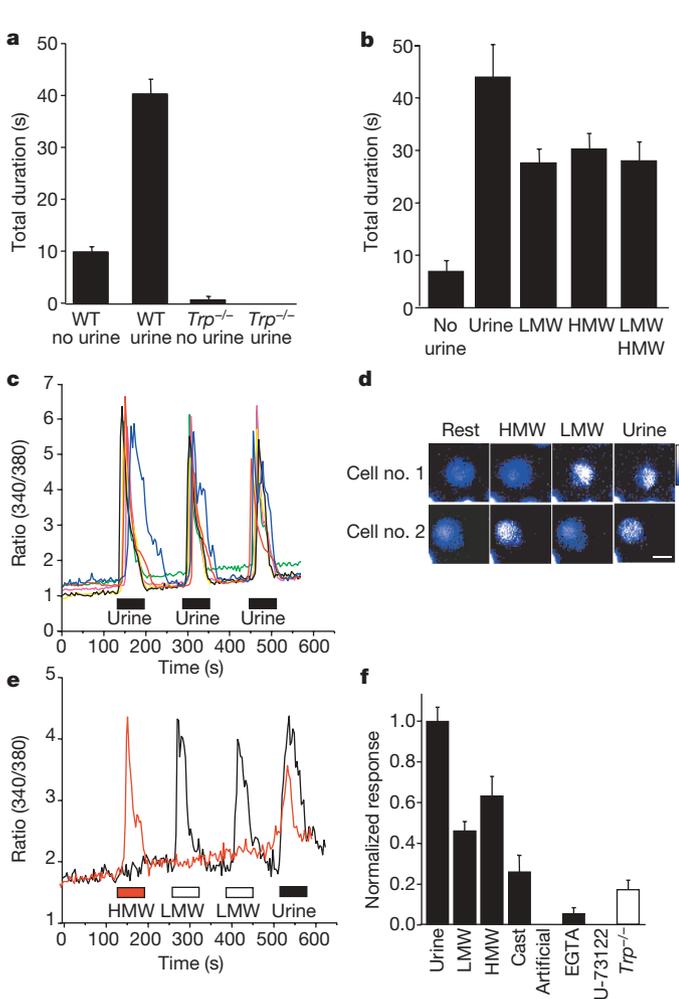


Figure 1 | Male urine contains two aggression pheromones. **a**, Male urine swabbed on castrated mice stimulates aggression ($P < 0.0001$; Student's *t*-test) in WT (73 trials/36 animals) but not *Trpc2*^{-/-} mutants (36 trials/6 animals, mean \pm s.e.m.). Aggression, total attack duration. **b**, Aggression with urine, LMW, HMW or both ($N = 16$ trials/6 animals each; urine/HMW $P = 0.1$, urine or HMW/no urine $P < 0.0001$). Error bars, s.e.m. **c**, Repetitive application of male urine (1:300) induced Ca^{2+} transients in dissociated VNs. Six representative traces. **d**, Fura-2 ratio images of two VNs of the same experiment. Cell no. 1 responds to LMW, cell no. 2 to HMW. Scale bar, 10 μm . Pseudocolour: low (blue) to high (white) $[\text{Ca}^{2+}]$. **e**, Separate populations of VNs are activated by LMW (black) and HMW (red) fractions. **f**, Summary of VN activation (mean \pm s.e.m. 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; common to both, 80 of 12,679; 'castrated' urine, 44 of 2,153; artificial urine²³, 0 of 1,224; EGTA, calcium-free media, 9 of 2,426; PLC inhibitor U-73122 (50 μM), 0 of 2,205. *Trp*^{-/-} (white bar), *Trpc2*^{-/-} VNs in response to urine, 38 of 3,312 (ref. 24).

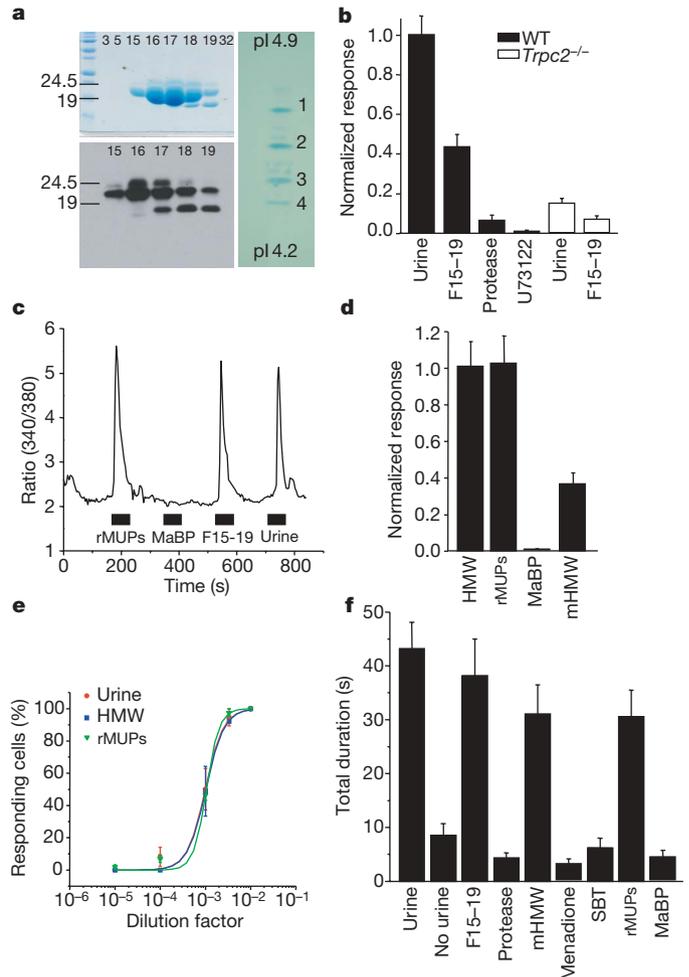


Figure 2 | HMW aggression activity is dependent on MUPs. **a**, Purification of bioactivity. Top, PAGE of FPLC fractions (Supplementary Fig. 2). Bottom, anti-MUP western blot. Right, isoelectric focusing gel (pI range 4.1–4.9) of the fractions (F15–19); accession numbers: 1, AAI00587; 2, CAM19799; 3, 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 μM ; 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)²⁴. **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 $P = 0.3599$. Error bars in **b**, **d** and **f**, s.e.m.

menadione, to competitively displace MUP small-molecule ligands¹⁸, as analysed by GC-MS (Supplementary Fig. 3). 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 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 bulb². 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 neurons¹⁵. 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.

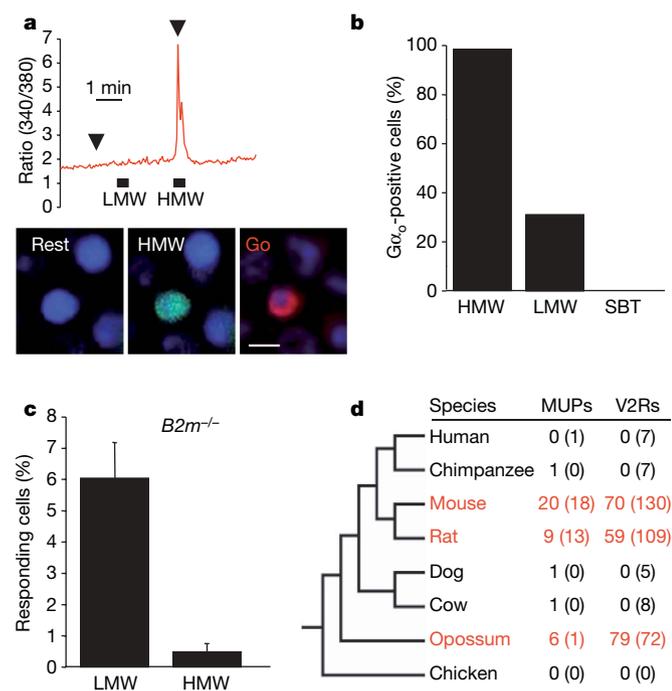


Figure 3 | MUPs activate a subset of VNs that express $G\alpha_o$. **a**, HMW-responsive cell (red trace) is labelled by anti- $G\alpha_o$ immunostaining (red) and DAPI (blue) immediately after Ca^{2+} imaging (right). Fura-2 ratio images (pseudocolour) during rest (left) or HMW activation (middle). 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**, $B2m^{-/-}$ 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 s.e.m. 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 reported⁹, with the addition of chimpanzee.

None of the MUP-activated cells was 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 aggression¹⁹. To assay functionally 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-negative ($B2m^{-/-}$) 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-associated small-molecule ligands alone (Fig. 3b)¹⁵. 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^{9,20}. 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^{21,22}. 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-responsive circuit. Purification and analysis of the LMW aggression-promoting pheromone will enable us to address the nature of this dual processing further.

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

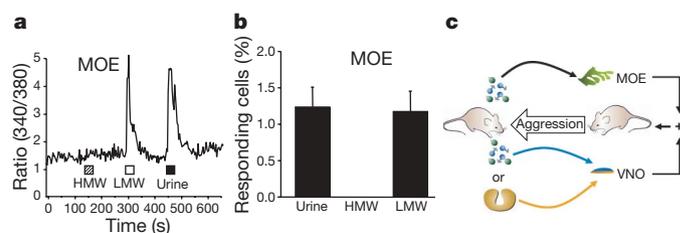


Figure 4 | MUP activation is specific to VNs. **a**, MOE-dissociated cells are not activated by HMW. **b**, Summary of MOE cell activation by urine 1.23% (40 cells), HMW (0 cells), LMW 1.17% (38 cells) of 3,250 total cells sampled (mean \pm s.e.m. of six experiments). **c**, Male-male aggression is mediated 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^{6,8,21,22}.

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.

METHODS SUMMARY

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. Urine was collected from 8- to 12-week-old C57Bl/6J males and used or further fractionated for behavioural and physiological experiments.

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 an 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.

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)⁹.

Behaviour. The resident–intruder assay was performed as previously described using 40 µl of stimulus normalized to 1× urine with protocols approved by the Institutional Animal Care and Use Committee⁶.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 26 July; accepted 12 October 2007.

1. Stowers, L. & Marton, T. What is a pheromone? Mammalian pheromones reconsidered. *Neuron* **46**, 699–702 (2005).
2. Dulac, C. & Torello, A. T. Molecular detection of pheromone signals in mammals: from genes to behaviour. *Nature Rev. Neurosci.* **4**, 551–562 (2003).
3. Cavaggioni, A. & Mucignat-Caretta, C. Major urinary proteins, α_{2U} -globulins and aphrodisin. *Biochim. Biophys. Acta* **1482**, 218–228 (2000).
4. Flower, D. R. The lipocalin protein family: structure and function. *Biochem. J.* **318**, 1–14 (1996).
5. Mugford, R. A. & Nowell, N. W. Pheromones and their effect on aggression in mice. *Nature* **226**, 967–968 (1970).
6. Stowers, L. *et al.* Loss of sex discrimination and male–male aggression in mice deficient for TRP2. *Science* **295**, 1493–1500 (2002).
7. Bean, N. J. Modulation of agonistic behavior by the dual olfactory system in male mice. *Physiol. Behav.* **29**, 433–437 (1982).

8. Leypold, B. G. *et al.* Altered sexual and social behaviors in trp2 mutant mice. *Proc. Natl Acad. Sci. USA* **99**, 6376–6381 (2002).
9. Shi, P. & Zhang, J. Comparative genomic analysis identifies an evolutionary shift of vomeronasal receptor gene repertoires in the vertebrate transition from water to land. *Genome Res.* **17**, 166–174 (2007).
10. Schwende, F. J. *et al.* Urinary volatile constituents of the house mouse, *Mus musculus*, and their endocrine dependency. *J. Chem. Ecol.* **12**, 277–296 (1986).
11. Hastie, N. D., Held, W. A. & Toole, J. J. Multiple genes coding for the androgen-regulated major urinary proteins of the mouse. *Cell* **17**, 449–457 (1979).
12. Robertson, D. H. *et al.* Molecular heterogeneity of urinary proteins in wild house mouse populations. *Rapid Commun. Mass Spectrom.* **11**, 786–790 (1997).
13. Timm, D. E. *et al.* Structural basis of pheromone binding to mouse major urinary protein (MUP-I). *Protein Sci.* **10**, 997–1004 (2001).
14. Novotny, M., Harvey, S., Jemiolo, B. & Alberts, J. Synthetic pheromones that promote inter-male aggression in mice. *Proc. Natl Acad. Sci. USA* **82**, 2059–2061 (1985).
15. Leinders-Zufall, T. *et al.* Ultrasensitive pheromone detection by mammalian vomeronasal neurons. *Nature* **405**, 792–796 (2000).
16. Hurst, J. L., Robertson, D. H. L., Tolladay, U. & Beynon, R. J. Proteins in urine scent marks of male house mice extend the longevity of olfactory signals. *Anim. Behav.* **55**, 1289–1297 (1998).
17. Hurst, J. L. *et al.* Individual recognition in mice mediated by major urinary proteins. *Nature* **414**, 631–634 (2001).
18. Xia, J. *et al.* Urinary pheromones promote ERK/Akt phosphorylation, regeneration and survival of vomeronasal (V2R) neurons. *Eur. J. Neurosci.* **24**, 3333–3342 (2006).
19. Loconto, J. *et al.* Functional expression of murine V2R pheromone receptors involves selective association with the M10 and M1 families of MHC class Ib molecules. *Cell* **112**, 607–618 (2003).
20. Takigami, S., Mori, Y., Tanioka, Y. & Ichikawa, M. Morphological evidence for two types of mammalian vomeronasal system. *Chem. Senses* **29**, 301–310 (2004).
21. Mandiyan, V. S., Coats, J. K. & Shah, N. M. Deficits in sexual and aggressive behaviors in *Cnga2* mutant mice. *Nature Neurosci.* **8**, 1660–1662 (2005).
22. Wang, Z. *et al.* Pheromone detection in male mice depends on signaling through the type 3 adenylyl cyclase in the main olfactory epithelium. *J. Neurosci.* **26**, 7375–7379 (2006).
23. Holy, T. E., Dulac, C. & Meister, M. Responses of vomeronasal neurons to natural stimuli. *Science* **289**, 1569–1572 (2000).
24. Lucas, P., Ukhanov, K., Leinders-Zufall, T. & Zufall, F. A diacylglycerol-gated cation channel in vomeronasal neuron dendrites is impaired in TRPC2 mutant mice: mechanism of pheromone transduction. *Neuron* **40**, 551–561 (2003).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank F. Papes, U. Mueller, A. Patapoutian, K. Baldwin and C. Zucker for discussions and critical reading of the manuscript. This work was supported by funding from the NIDCD, Pew Charitable Trust, Skaggs Institute, and Helen Dorris Foundation (to L.S.), a National Institute on Deafness and Other Communication Disorders (NIDCD) pre-doctoral fellowship (T.F.M.) and The Basque Government Post-Doctoral Research Fellowship (P.C.).

Author Contributions The behavioural analysis was performed by P.C., J.R.C. and L.S.; calcium imaging was done by P.C. and T.F.M.; and biochemical purification of MUPs by T.F.M. and advised by B.F.C. SBT synthesis was performed by A.S. and B.F.C.; construct preparation and immunostaining by P.C., T.F.M. and K.F.; and genomic analysis by D.W.L. All authors participated in data analysis and writing of the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to L.S. (stowers@scripps.edu).

METHODS

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 ml⁻¹ papain and 10 U ml⁻¹ DNase I for 20 min at 37 °C. β 2-microglobulin^{-/-} mice were purchased from Taconic.

Calcium imaging. Intracellular Ca²⁺ was monitored using fura-2/AM (5 μ M, Molecular Probes) in a Zeiss Axiovert 200M inverted microscope with a 20 \times fluar 0.75 objective lens. 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 with wavelengths 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 \times urine and then diluted 1:300 before experimentation.

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 flowthrough 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 (ref. 23).

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 μ l 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 3,500 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 μ l of test solution (1 \times male urine; fractions and FPLC-purified MUPs were normalized to 1 \times 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⁻¹ 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⁻¹ 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⁻¹) 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 effector concentration for half-maximum response (EC₅₀) = 0.00099, slope = 2.15, n = 135 cells in four experiments; HMW EC₅₀ = 0.001, slope = 2.14, n = 52 cells in two experiments; rMUPs EC₅₀ = 0.0011, slope = 3.0, n = 209 cells in four experiments.