14. Y. Rong, S. R. Doctrow, G. Tocco, M. Baudry, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 9897 (1999).
15. S. Melov *et al.*, *Nature Genet.* **18**, 159 (1998).
16. S. Melov *et al.*, unpublished data.
17. J. Sulston and J. Hodgkin, in *The Nematode* Caenorhabditis elegans, W. B. Wood, Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988), pp. 587–606.
18. T. E. Johnson, *Science* **249**, 908 (1990).
19. N. Ishii *et al.*, *Mutat. Res.* **237**, 165 (1990).
20. N. Ishii *et al.*, *Nature* **394**, 694 (1998).
21. B. Lakowski and S. Hekimi, *Science* **272**, 1010 (1996).
22. Worms were transferred in 10 μl of medium onto a glass slide and then sealed under a glass coverslip. The area of image defined by the worm outline was measured from digitized images made with Scion Image (Scion, Frederick, MD). The grayscale image was transformed with a Hat 17×17 convolution, and automatic edge detection was used to define perimeter length and area after calibration with a stage micrometer. Measurement error was ±69.8 μm² (1 SD) for second-stage larvae (L2) and ±158.9 μm² for fourth-stage larvae (L4).
23. Worms were individually cultured for each treatment group. Worms were transferred to new culture wells daily during the fertile period. Eggs laid in each well were allowed to hatch and were cultured until the worms had reached adulthood.
24. We thank the Lithgow lab and A. Seyed Jazayeri-Dezfuly for critical and helpful discussions. M.S.G. is a Medical Research Council (MRC)–supported Training Fellow. D.W.W. is also supported by the MRC, and S. Malik was supported by Research into Aging. D.C.W. and S. Melov were supported in part by NIH grants AG-13154 and NS21328, awarded to D.C.W. All strains were obtained from the *Caenorhabditis* Genetics Center.

27 March 2000; accepted 11 July 2000

Responses of Vomeronasal Neurons to Natural Stimuli

Timothy E. Holy,[1]* Catherine Dulac,[1,2] Markus Meister[1]

The vomeronasal organ (VNO) of mammals plays an essential role in the detection of pheromones. We obtained simultaneous recordings of action potentials from large subsets of VNO neurons. These cells responded to components of urine by increasing their firing rate. This chemosensory activation required phospholipase C function. Unlike most other sensory neurons, VNO neurons did not adapt under prolonged stimulus exposure. The full time course of the VNO spiking response is captured by a simple quantitative model of ligand binding. Many individual VNO neurons were strongly selective for either male or female mouse urine, with the effective concentrations differing as much as a thousandfold. These results establish a framework for understanding sensory coding in the vomeronasal system.

Pheromones of mammals induce complex behaviors and neuroendocrine changes, such as the choice of a mate, territorial defense, the female estrous cycle, and onset of puberty (*1, 2*). It has been argued that pheromones are detected primarily by the VNO (*2, 3*). The identification of a large number of putative pheromone receptor genes, grouped into two divergent gene families, suggests that the population of sensory neurons is highly heterogeneous (*4–7*). Individual glomeruli of the accessory olfactory bulb collect projections from multiple types of VNO receptor neurons, and therefore the sensory code is likely to involve patterns of activity across the receptor population (*8, 9*). We reasoned that such a distributed population code should be observed by simultaneously recording the activity of a large number of VNO neurons in response to natural stimuli. This type of approach might reveal how sex, social dominance, or individual identity are represented by activity patterns in the VNO.

We recorded the action potentials of VNO neurons using a flat array of 61 extracellular electrodes (*10*). Even in the absence of stimulus, VNO neurons were spontaneously active (*11*), most of them firing intermittent bursts of spikes (Fig. 1A). When interpreting sensory responses, this pattern of maintained activity poses a hazard: A spontaneous burst may synchronize with the stimulus by chance and may be mistaken for a response. Such chance events may have confounded previous studies (*12–14*). We overcame this difficulty by delivering stimuli repeatedly, under precise temporal control (*15*). Of 221 neurons recorded in five preparations, 84 responded reproducibly to dilute urine by increasing their firing rate (see, for example, Fig. 1B). The sensitivity varied considerably across neurons, and the effective urine concentration (relative to undiluted urine) sufficient to elicit a response ranged from <0.0001 to 0.01. In no case did we observe a reproducible stimulus-induced inhibition (*13, 14*).

In addition to pheromones, mouse urine contains urea and potassium ions, which could potentially cause neurons to fire by direct membrane depolarization. Three lines of evidence establish that, instead, a specific chemosensory pathway underlies these responses. First, "artificial urine," containing the most abundant ionic and organic components of urine (*10*), did not affect firing, even at a relative concentration of 0.1 (*16*). Second, in any given VNO, some neurons were far more sensitive to female than to male mouse urine, whereas other neurons displayed an opposite selectivity (discussed further below). Third, responses to urine, but not to potassium ions, depend on a signal transduction cascade: 50 mM potassium excited the neurons, but the kinetics of the response differed sharply from that to urine (Fig. 2A). The onset of the urine response was delayed relative to the potassium response (by 0.33 ± 0.18 s, mean ± SD; $P < 10^{-7}$, if one assumes a gaussian distribution), and it also lasted considerably longer (by 3.1 ± 2.5 s, measuring the difference in exponential decay times; $P < 10^{-5}$). Presumably, potassium ions act directly to depolarize the membrane, whereas dilute urine achieves this only through a slower sensory transduction mechanism. A similar response delay occurs when odorants are presented to dissociated neurons of the main olfactory epithelium (*17*).

To obtain direct evidence for a signal transduction cascade, we applied pharmacological agents to the neuroepithelium. An inhibitor of phospholipase C, 10 μM U-73122 (*18, 19*), blocked spiking responses to urine but not to potassium (Fig. 2B). A nearly inactive structural analog, U-73343 (*18*), had no measurable effect on the response to urine. An inhibitor of phosphodiesterase, 500 μM isobutyl methylxanthine (IBMX), also had no effect on firing activity (44 cells). These results indicate that the response of VNO neurons to urine components involves the specific activation of an intracellular signal transduction pathway. Moreover, they identify phospholipase C–β (PLC-β) as a key element of the cascade and also confirm that cyclic nucleotides are not essential (*20, 21*). Molecular similarity has been found between the signaling pathways of mammalian VNO neurons and *Drosophila* photoreceptors, including specific expression of ion channels of the TRP family (*22*). Requirement for PLC-β function in the VNO parallels the involvement of the NorpA protein in the *Drosophila* eye (*23*) and provides additional support for similarity between the two pathways.

Having established the specificity of these VNO responses, we proceeded to a quantitative analysis of sensory coding. In most sensory systems (*24, 25*), a sustained stimulus causes the primary receptor cells to adapt by altering their sensitivity. For example, olfactory receptor cells change their dose-response relation within seconds (*26*). In contrast, we found little or no adaptation in

[1]Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA. [2]Howard Hughes Medical Institute, Cambridge, MA 02138, USA.

*To whom correspondence should be addressed. E-mail: timholy@mcb.harvard.edu

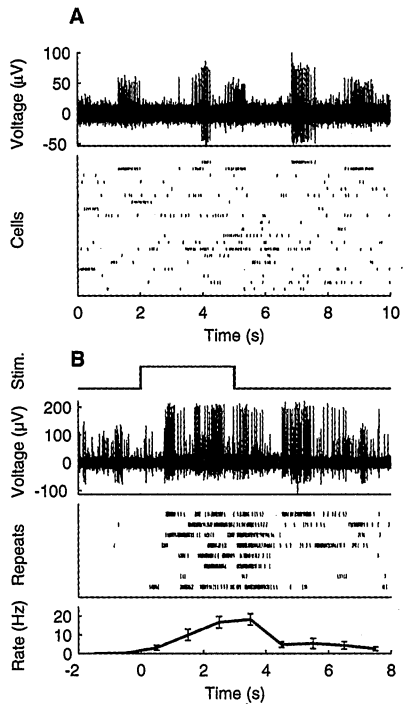
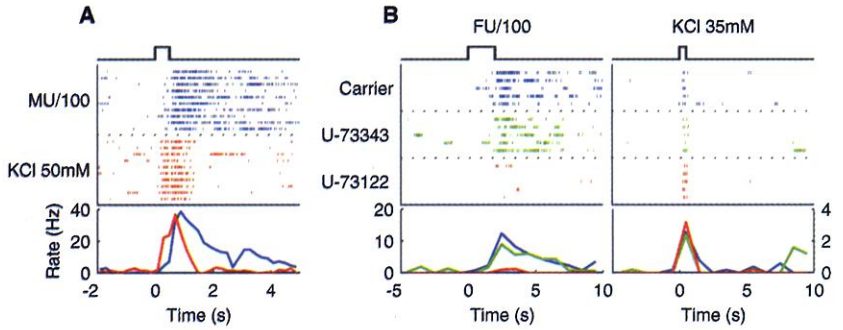


Fig. 1. Spontaneous and stimulated firing in a population of mouse VNO neurons. (**A**) Spontaneous activity. (Top) Recording at one site of the electrode array containing spikes from at least two cells, whose shapes are easily distinguished by the size of the undershoot. (Bottom) The spike times of 20 cells recorded simultaneously. Each row is a different cell, and every tick is an action potential; the first two cells were recorded in the trace shown on top. (**B**) Female mouse urine (diluted 100-fold) is presented for 3 s (stimulus trace, above). (Top) Recording at one site of the electrode array. Spikes from two neurons can be distinguished. (Middle) The large spikes were extracted from the raw trace above and displayed in the first row of this raster plot. Subsequent rows show the firing of this same cell in repeated trials of the stimulus. (Bottom) Average firing rate of the cell, in bins of 1-s width. Error bars represent the standard error in the mean.



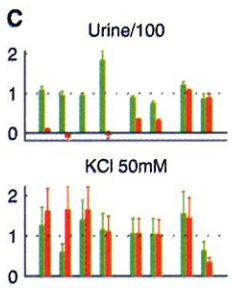


Fig. 2. Responses to urine are mediated through a signal transduction cascade, rather than by direct membrane depolarization. (**A**) Response of a neuron from male VNO to repeated trials of 0.5-s pulses (top trace) of male urine (diluted 100-fold, blue), or potassium (50 mM, red). Panels as in Fig. 1B. (**B**) Effects of U-73122, an inhibitor of phospholipase C, and its inactive structural analog, U-73343, on the spiking response to dilute female urine (left) and potassium (right). The carrier solution (0.67% dimethyl sulfoxide in Ringer's) served as a control (*15*). (**C**) Summary of experiments as in (B) for eight neurons in a female VNO. Bars represent the average increase in firing rate upon stimulation over eight trials, normalized to the response after control treatment. The inactive analog (green) was generally indistinguishable from the carrier control; the active inhibitor (red) reduced or eliminated the urine response for most neurons (top), although the KCl responses were unaffected (bottom).

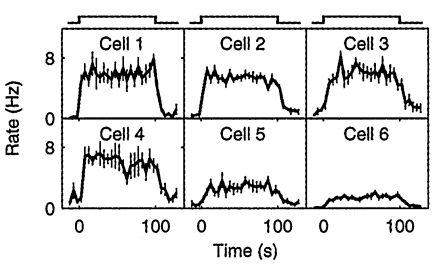


Fig. 3. Firing responses to urine showed little or no adaptation to prolonged stimuli. Firing rates of six neurons recorded from a female mouse VNO to a 100-s step of 300-fold diluted female urine (mean ± SEM). The firing rate of each neuron was calculated in 5-s bins, and averaged over seven trials.

VNO neurons during a 100-s presentation of 300-fold diluted urine (Fig. 3). Previous work showed that mouse VNO neurons fire at a steady rate when driven with intracellular current injection (*21*). Our results demonstrate that the entire chemo-transduction process fails to adapt. The primary purpose of adaptation in other sensory systems is to retain sensitivity to variations in stimulus intensity over a wide range of background intensities. Such a facility may not be biologically relevant for pheromone detection. Given the relatively slow access of stimuli to the VNO (*27*), the need to detect minute amounts of pheromones, and the long-lasting impact of pheromone detection on the organism, adaptation might indeed be undesirable.

These observations led us to formulate a model in which the firing rate directly represents the occupancy of a pheromone receptor, as determined by first-order binding kinetics. We suppose that each neuron has just one receptor type and that each receptor molecule exists in one of two states, either unoccupied or bound to its ligand. At ligand concentration C, transitions to the bound state occur at rate $k_+ = \kappa C$; reversion to the unbound state occurs at a rate k_- independent of ligand concentration. The firing rate r, averaged across trials, increases with the fraction p of occupied receptors as $r = r_0 + \alpha p$, where r_0 is the spontaneous firing rate, and the proportionality factor α is the firing rate increase at receptor saturation. From these kinetics, one readily derives the time dependence of the average firing rate following square pulses of ligand application [Eqs. 1 to 3 in (*28*)]. This model provides a pleasing fit to the kinetics of the response (Fig. 4, A and B). The steady-state firing rate during a prolonged step should follow the Michaelis-Menten law (Eq. 4), which is confirmed by the measurements (Fig. 4, C and D). Thus, one can characterize a neuron's sensitivity to a given stimulus with a single number: the Michaelis constant $K_m = k_-/\kappa$, which corresponds to the concentration that elicits a half-saturating response (*29*).

An individual neuron, faced with a urine sample of unknown concentration, cannot resolve the sex of the donor animal. On the other hand, a population of cells, with different chemical selectivities, can represent sex and concentration unambiguously. Thus, we measured the Michaelis constants of a population of neurons from a male mouse VNO (Fig. 4E). The sensitivities to both male and female mouse urine were distributed over 2 to 3 orders of magnitude. Nearly half of the neurons showed a clear preference for the sample from one or the other sex. This indicates that their specific ligands are present at different concentrations in male and female mouse urine, at ratios ranging up to 1000-fold. A similar result was obtained in a female mouse VNO (Fig. 4F).

To demonstrate that this observed selectivity is tied to the sex of the donor animal rather than to other characteristics, we tested the same VNO population with two urine samples from different animals of the same sex. The vast majority of neurons had similar Michaelis constants for the two samples: In Fig. 4, G and H, most points lie close to the diagonal (*30*), whereas they scatter far from

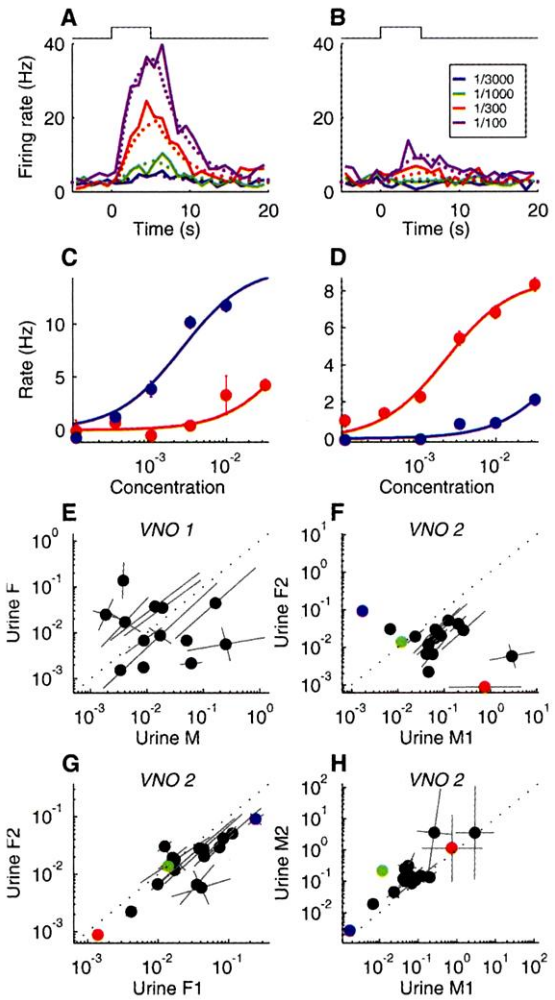


Fig. 4. (A to D) VNO responses can be selective for the urine of either sex. (**A** and **B**) Firing (solid curves) of a single neuron from a male mouse VNO to a 5-s pulse (top) of urine from female (A) and male (B) mice at a series of concentrations (averaged over 16 trials). Dotted curves: fit to the response using the kinetic binding model (Eq. 3). (**C** and **D**) Steady-state firing rates (averaged over eight trials) of two different neurons from the same female mouse VNO, plotted as a function of concentration of female (red) and male (blue) mouse urine (*28*). The solid curves correspond to a Michaelis-Menten relation (Eq. 4). (E to H) Distribution of Michaelis constants (K_m) for male and female mouse urine in a population of VNO neurons. Each point represents one neuron and plots its K_m for one stimulus against the K_m for another stimulus. Lower values of K_m indicate greater sensitivity. Neurons responding equally to the two samples would fall along the dotted line. Uncertainties in these measurements are represented by error bars along the principal axes of the error ellipse. (**E**) A population of neurons from a male mouse VNO was tested with samples of male and female mouse urine. (**F** to **H**) A population from a female mouse VNO was tested with two different samples of female mouse urine (F1 and F2) and two samples of male mouse urine (M1 and M2). The resulting Michaelis constants are plotted in a pairwise fashion: F2 versus M1 (F), F2 versus F1 (G), and M2 versus M1 (H). The colored symbols serve to track three cells across all three graphs. The red and blue points represent neurons highly selective for the female or male samples, respectively; the green point represents a neuron with clear preference for one of the two male mouse samples.

the diagonal in Fig. 4, E and F. We conclude that the population response of VNO neurons is very sensitive to the sex of the donor. In addition, though, some cells (Fig. 4H) show a clear preference for one of the two male mouse samples, suggesting that these neurons recognize pheromones that vary between individuals of the same sex. Such receptor neurons may contribute to the behavioral recognition of individual differences (*1, 2*).

The VNOs of both sexes were found to contain neurons specific for the pheromones of either sex. This result is consistent with the fact that all putative pheromone receptors examined so far are expressed in both males and females (*4, 5*). In addition, more than half of VNO neurons responding to urine stimuli detect cues that are independent of sex. The absence of any clustering of neuronal response types in Fig. 4, E to H, reinforces the notion of a large heterogeneity among VNO sensory neurons, consistent with the existence of over 100 different putative receptor genes (*4–7*). Pheromone-induced behaviors and endocrine changes clearly involve complex sensory recognition that goes beyond mere sex discrimination, requiring identification of the species, familial status, and even individual identity of animals. The population recording approach described here should help in unraveling the neural code for these variables.

References and Notes

1. M. Halpern, *Annu. Rev. Neurosci.* **10**, 325 (1987).
2. K. B. Doving and D. Trotier, *J. Exp. Biol.* **201**, 2913 (1998).
3. C. J. Wysocki and J. J. Lepri, *J. Steroid Biochem. Mol. Biol.* **39**, 661 (1991).
4. C. Dulac and R. Axel, *Cell* **83**, 195 (1995).
5. G. Herrada and C. Dulac, *Cell* **90**, 763 (1997).
6. H. Matsunami and L. B. Buck, *Cell* **90**, 775 (1997).
7. N. J. Ryba and R. Tirindelli, *Neuron* **19**, 371 (1997).
8. I. Rodriguez, P. Feinstein, P. Mombaerts, *Cell* **97**, 199 (1999).
9. L. Belluscio, G. Koentges, R. Axel, C. Dulac, *Cell* **97**, 209 (1999).
10. Adult DBA/2J mice between the ages of 6 weeks and 6 months were killed by CO_2 inhalation. The VNO was dissected out and immersed in ice-cold Ringer's solution. An intact sheet of neuroepithelium was mechanically detached from the blood vessel, connective tissue, and nerve layer. Neuroepithelium from the VNO was placed on a 61-electrode array (*31*) with the microvilli facing up, spread out, and held down with a 53-μm nylon mesh. The preparation was continuously superfused with fresh Ringer's solution and maintained at 34°C. Electrical signals were amplified and filtered (*31*) and then digitized at 10 kHz with a 12-bit 64-channel A/D card (National Instruments, Austin, TX) and written to disk. On each channel, spikes were sorted by their waveform, using custom software for cluster analysis. Spike trains that did not show a clear refractory period (typically 10 to 20 ms) were taken to be multiunit activity and omitted from further analysis. Ringer's (in millimolar concentration): NaCl, 115; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 2; $NaHCO_3$, 25; Hepes, 5; glucose, 10; equilibrated with 95% O_2/5% CO_2 to pH 7.4. All chemical stimuli were diluted with Ringer's. High-potassium solutions were made by substituting equimolar quantities of KCl for NaCl. Artificial urine (*32*) (in millimolar concentration): NaCl, 120; KCl, 40; NH_4OH, 20; $CaCl_2$, 4; $MgCl_2$, 2.5 NaH_2PO_4, 15; $NaHSO_4$, 20; urea, 333; with NaOH added to achieve pH 7.4.
11. Spontaneous firing was analyzed for 72 cells from two preparations in a 300-s stimulus-free period. Across cells, the mean firing rate had an exponential distribution, with an average value of ~1 Hz. Often the spikes occurred in bursts. For 49 cells, the cumulative distribution of interspike intervals showed a sharp "knee" at 150 ms; boundaries between bursts were therefore identified as gaps of at least 150 ms. The average spike burst across all cells contained about six spikes. This burst size was also distributed exponentially. The two preparations yielded nearly identical patterns of spontaneous firing.
12. K. Inamura, Y. Matsumoto, M. Kashiwayanagi, K. Kurihara, *J. Physiol. (London)* **517**, 731 (1999).
13. R. L. Moss *et al.*, *J. Neurophysiol.* **77**, 2856 (1997).
14. R. L. Moss *et al.*, *Chem. Senses* **23**, 483 (1998).
15. Chemical stimuli were delivered to the neuroepithelium through a 13-to-1 manifold pipette (DAD-12, ALA Scientific, Westbury, NY), and electrical recording was synchronized with the stimulus. The flow out of the pipette was switched between Ringer's and stimulus solutions, resulting in a constant flow across the epithelium and sharp concentration transients, undiluted by the bath Ringer's. Trials were spaced no less than 30 s apart. All stimuli were presented once in sequence, and the entire sequence repeated from 7 to 16 times. For urine collection, adult DBA/2J mice between the ages of 7 and 10 weeks were housed in same-age, same-sex metabolic cages, two per cage. Voided urine was immediately pumped to collection tubes and frozen in dry ice. At the end of the collection time (3 to 8 days), urine was thawed, passed through a 0.45-μm filter, frozen in aliquots, and stored at −80°C. Pharmacological agents were applied for 30 s, followed by five trials of stimulation, interleaving pulses of urine and potassium.
16. Artificial urine was repeatedly presented in 10-s pulses at concentrations ranging from 0.001 to 0.1. None of the 27 cells responded systematically to the stimulus; the change in the mean firing rate was statistically indistinguishable from 0.
17. S. Firestein and F. Werblin, *Science* **244**, 79 (1989).
18. J. E. Bleasdale *et al.*, *J. Pharmacol. Exp. Ther.* **255**, 756 (1990).
19. K. Inamura, M. Kashiwayanagi, K. Kurihara, *Neurosci. Lett.* **233**, 129 (1997).
20. A. Berghard and L. B. Buck, *J. Neurosci.* **16**, 909 (1996).
21. E. R. Liman and D. P. Corey, *J. Neurosci.* **16**, 4625 (1996).
22. E. R. Liman, D. P. Corey, C. Dulac, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 5791 (1999).
23. K. Scott and C. Zuker, *Curr. Opin. Neurobiol.* **8**, 383 (1998).
24. J. J. Falke, R. B. Bass, S. L. Butler, S. A. Chervitz, M. A. Danielson, *Annu. Rev. Cell Dev. Biol.* **13**, 457 (1997).
25. V. Torre, J. F. Ashmore, T. D. Lamb, A. Menini, *J. Neurosci.* **15**, 7757 (1995).
26. J. Reisert and H. R. Matthews, *J. Physiol. (London)* **519** (part 3), 801 (1999).
27. M. Meredith, *Physiol. Behav.* **56**, 345 (1994).
28. Under the assumptions stated in the text, a pulse of

concentration C, lasting from $t = 0$ to $t = T$ alters the receptor occupancy p with the following time course

$$p(t) = \begin{cases} 0 & \text{if } t < 0; \\ \frac{k_+}{k_+ + k_-}\left(1 - e^{-(k_+ + k_-)t}\right) & \text{if } 0 < t < T; \\ \frac{k_+}{k_+ + k_-}\left(1 - e^{-(k_+ + k_-)t}\right) e^{-k_-(t-T)} & \text{if } T < t \end{cases} \quad (1)$$

where the on-rate k_+ is given by

$$k_+ = \kappa C \quad (2)$$

We assume in addition that there is a small delay Δt between valve opening and onset of the response, such that the firing rate follows the form

$$r(t) = r_0 + \alpha p(t - \Delta t) \quad (3)$$

The steady-state rate during a long pulse is

$$r(\infty) = r_0 + \alpha k_+/(k_+ + k_-) \quad (4)$$

In fitting the model to the measurements, the neuron's firing rate was estimated by counting spikes in time bins and averaging across trials with the same stimulus. Standard errors were computed for each bin. Then Eq. 3 was fit to the data with the model parameters chosen to minimize chi-square. The values for r_0, α, k_-, and Δt, were common across all stimuli and concentrations. Different values for κ were used for chemically different stimuli. Uncertainties in the parameters were computed from the covariance matrix of chi-square at the minimum. In Fig. 4, C and D, the steady-state rate $r(\infty)$ was estimated from the data by extrapolating the response time course using Eqs. 1 and 3, but allowing an independent value of k_+ for each concentration. This is compared to the steady-state rate predicted from the assumption of first-order binding (Eq. 2). For Fig. 4, E to H, the above fits were performed for all recorded neurons. We excluded from the plots those cells for which the value of $K_m = k_-/\kappa$ could not be determined to within 2 log units for all stimuli. Invariably, these neurons lacked a recognizable response.

29. Mechanistically, this binding constant and the binding reactions (Eqs. 1 and 2) could reflect properties of the pheromone receptor or those of some subsequent limiting component of the signal transduction cascade. Note also that this model serves to predict only the average firing rate across trials, not the burst structure (Fig. 1) or any other statistic of the spike trains.
30. On closer inspection, the data points in Fig. 4G tend to fall on a line parallel to the diagonal. This could be explained if the two urine samples differed principally by an overall dilution of all the active components.
31. M. Meister, J. Pine, D. A. Baylor, *J. Neurosci. Methods* **51**, 95 (1994).
32. J. R. McClintic, *Physiology of the Human Body* (Wiley, New York, 1978).

33. We thank N. Holy, D. Hwang, P. Kharchenko, E. Soucy, T. Torello, and K. Wormstead for assistance and discussions. This work was supported by a Jane Coffin Childs Fellowship (T.E.H.), a grant from the Office of Naval Research to (M.M.), and the HHMI and NIDCD grant 3903-01 (C.D.).

20 March 2000; accepted 7 July 2000