Toward an Estimate of the Number of Receptor Neuron Spikes Needed for Odor Identification

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ABSTRACT
We measured the concentration dependence of the ability of rats to identify odorants and compared these results with the calcium signals in the nerve terminals of the olfactory receptor neurons. Odorant identification remained far above random chance at all concentrations tested (between 0.0006% and 35% of saturated vapor). In contrast the calcium signals were much smaller than their maximum values at odorant concentrations less than 1% of saturated vapor. Extrapolation suggests that only a few spikes in olfactory sensory neurons may be sufficient for correct odorant identification.

INTRODUCTION
In the rodent olfactory system each glomerulus receives input from approximately 10,000 olfactory sensory neurons (1). All of the sensory neurons converging onto a glomerulus express the same receptor protein (2). The input from receptor neurons to glomeruli has been imaged using calcium indicator dyes located in the olfactory receptor neuron nerve terminals (3,4) or measuring the pH change monitored by synapto-pHluorin during synaptic vesicle fusion (5). Other imaging methods (intrinsic imaging, 2-deoxyglucose uptake, c-fos activation, and fMRI) report the combined pre- and post-synaptic activation of olfactory bulb glomeruli (e.g. 6-9).

While these imaging methods are able to monitor the activity of a large number of glomeruli, it is not known how well these signals correlate with the animal's ability to identify odorants over a wide range of odorant concentrations. We have compared the ability of rats to differentially identify five different odorants (10) and in these same animals we monitored glomerular calcium signals making both measurements as a function of odorant concentration. Previous studies in anesthetized rodents have shown that at higher odorant concentrations almost all monitored glomeruli are activated (3,4) raising the possibility that animals might make errors in identification at high concentrations. Conversely, at odorant concentrations less than 0.1% of saturated vapor the nerve terminal optical signals are small (3,4) suggesting that identification errors might occur at these concentrations as well.

Our behavioral measurements showed that the rats achieved greater than 80% correct odorant identification at all odorant concentrations we tested; from 0.0006% to 35% of saturated vapor. We then carried out measurements of the calcium signals in the same animals in an awake/restrained preparation. The rats were able to identify odors at concentrations where the calcium signals were too small to measure. And they made a high percentage of correct identifications at high odorant concentrations where a very large percentage of glomeruli are activated.
The concentration dependence of the calcium signals in the most sensitive dorsal glomeruli was fit by a Michaelis-Menten equation using a Hill coefficient of 1. When the fitted curve was extrapolated to an odorant concentration of 0.0006%, its amplitude was only 0.04% of the maximum signal. A calcium signal of 0.04% is equivalent to the high concentration odorant response of only four of the 10,000 sensory neurons innervating one glomerulus.

METHODS

Six adult male Long-Evans rats (labeled LC1 through LC6) were used in the behavior measurements. Following the completion of behavioral testing we attempted to measure calcium signals in these same six animals in an awake/restrained preparation. Successful measurements were carried out in five of the six. At the time of the optical measurements the rats were eight months old and had an average weight of 400 g. We also made similar calcium measurements in an additional group four naive Long-Evans rats. The experimental protocols were approved by the appropriate institution's Animal Care and Use Committee (SUNY Upstate Medical Center, Marine Biological Laboratory, and Yale University).

Choice of odorant set. As an initial screening we tested 17 odorants in rats. These odorants either had large dorsal signals in calcium measurements on mice (3,11) or had large dorsal signals in 2-deoxyglucose measurements (see Glomerular Activity Response Archive; leonserver.bio.uci.edu). From the nine odorants with the largest signal in rats we chose five for the present experiments; propanal, hexanal, octanal, propyl acetate and isoamyl acetate. Additional measurements of calcium signals were made with a sixth odorant, 2-hexanone.

Behavior

Using previously established operant training procedures (10,12), the six rats were trained on a five-odorant identification confusion matrix task that can directly extract both odorant identification performance and perceptual quality relationships across a set of odorants (10,12-14). Briefly, the rats were trained to break a photo-beam (trial initiating response) inside an odorant sampling port centrally located within a behavioral testing chamber (see Youngentob et al., 1990 for details) and sample the odorant presented. Following a minimum sampling period (300 msec), the rats were trained to register a response choice in one of five alternative response tunnels that, by training, the animal had learned to associate with reinforcement for the odorant presented. Each animal had a different set of odorant/response-tunnel associations. A response choice was made when the animal licked the reinforcement cup at the end of the response tunnel. At the completion of training, the animals were capable of differentially reporting the random presentation of five different odorants. Criterion training was set at an overall performance level of >90% correct
on 40 blocks of five randomized trials (one presentation of each odorant) and no less than 80% identifiability for each individual odorant. With this standardly applied criterion the trained rats are near maximal performance on the odorant identification task.

The five odorant stimuli were generated and delivered according to previously established methods using flow dilution olfactometry and computer-controlled electronic mass flow controllers (13). The training concentration for the odorants (expressed as percentage vapor saturation at 20°C) were 0.375%, 2.25%, 0.25%, 0.25%, and 0.375% for propyl acetate, octanal, amyl acetate, propanal, and hexanal, respectively.

Following criterion training, the animals were first tested stepwise on an ascending series of odorant concentration (maximum concentration of each odorant was 35.3 % of saturated vapor). At the completion of this testing, the animals were then returned to the odorant concentrations used for training. This, in turn, was followed by a decreasing stepwise series of odorant concentration (minimum concentration of each odorant 0.0006% (0.0012% for octanal)). In any one testing session only one odorant concentration of each odorant was used. Testing in any one session consisted of the randomized presentation of the odorants in blocks of five trials with each block consisting of the single presentation of each of the five different stimuli. Testing in any one session proceeded for 40 blocks of trials (200 trials total; 40 trials per odorant).

Imaging

Staining and surgery. Olfactory receptor neurons were loaded with Calcium Green-1 dextran (10 kD) using methods similar to those described previously (4,11). During dye perfusion into the nasal cavity the rats were anesthetized with Ketamine/Xylazine. The dye-Triton solution was injected as a mixture with final concentrations of 4% dye and 0.15% Triton-X100. We used volumes between 20 and 30 μl. Two days after the dye injection the rats were reanesthetized with Ketamine/Xylazine. After application of a local anaesthetic the skin covering the skull was retracted and the bone over the olfactory bulb was thinned and then covered with a thin layer of cyano-acrylate glue (Loctite) that prevented the bone from becoming opaque. A headpost was attached to the parietal and occipital bones using RelyX Luting cement (3M ESPE). The optical recordings were carried out between 4 and 8 days following dye injection.

Awake/restrained animals. The rats were restrained first by placing them in a cylindrical cloth sack with their head protruding from one end but with all four legs inside the sack. This sack was then taped to restrict the animal's movements and the headpost was fixed to a holder attached to the microscope stage. The rats seemed to accept the restraint without prior training to accommodate them to the experimental situation. These methods were demonstrated to us by Tomas Hromadka and Tony Zador (personal communication). During most of the recording
session the animals remained relaxed but even small movements would introduce obvious noise into the optical recordings. For higher odorant concentrations we recorded two or four trials, for lower concentrations we recorded eight trials. All of the individual trials were saved and each trial inspected for obvious noise. Noisy trials (12.5% to 50% of each group of 4 to 8 trials) were not included in the averaged response used for measuring signal amplitudes. For high odorant concentrations an interval between trials of 60 seconds or greater was used; for low concentrations the interval was 30 seconds or greater.

**Olfactometer.** To minimize the possibility of any mechanical response to the introduction of odorant into the airflow we used the olfactometer described earlier (15). This olfactometer was connected so that the odorant vapor, when introduced into the airflow, replaced the equivalent volume of diluent air so that the rate, humidity and direction of airflow over the nares was relatively constant throughout a measurement.

A two second odor pulse was delivered at a random time between 5 and 10 seconds prior to opening the shutter. If a fixed two second interval between shutter and odor was used, it seemed that the animal would often move in anticipation of the odorant.

**Optical recording.** The dorsal olfactory bulb was imaged through a Wild 10× 0.4 NA objective mounted onto a Leitz Ortholux II upright microscope using Ploem epi-illumination; the actual magnification of the lens was 15x. Excitation light from a 100 W tungsten filament lamp was passed through a 488/50 nm band-pass filter and reflected by a 515 nm dichroic mirror. The fluorescence above 530 nm was recorded with a NeuroCCD-SM256 camera using NeuroPlex software (RedShirtImaging, Decatur, GA) with a recorded frame rate of 31.25 frames per second and 128x128 pixel resolution. The apparatus was mounted on a Nano-K Biscuit vibration isolation table (Minus-K Technologies, Inc., Inglewood, CA).

**Data analysis.** Less obvious remaining noise resulting from movement of the animal in the X-Y plane was reduced using a MATLAB (The MathWorks, Inc.) program that maximized the correlation between frames by adjusting the X-Y position of one of the frames (Tay Netzog, Justus Verhagen, and Matt Wachowiak, personal communication). The results of analyses carried out before the movement correction were similar to those shown in Figures 2 and 3 and Table 1. The time course of the calcium signal was determined using NeuroPlex software (low pass Gaussian filter of 2 Hz) from traces like those illustrated in the right panel of Figure 2. We then determined the location of the signal by subtracting the average of 16 frames prior to the odor presentation from 16 frames measured at the peak of the response. The resulting frame subtraction images (illustrated in the left panel of Figure 2) were spatially smoothed by replacing each pixel value with the mean of 3x3 pixels centered on that pixel. These images were used to locate the most
sensitive glomeruli; those that were activated the most at low odorant concentrations. We measured the amplitudes of the six most sensitive glomeruli as the fractional fluorescence change, $\Delta F/F$, and plotted this value as a function of odorant concentration in Figure 2. No correction was made for the background fluorescence of an unstained bulb and thus the fractional calcium dye fluorescence changes will be larger than the fractional changes shown in Figure 2.

The measured amplitude vs. concentration data points for the most sensitive glomerulus for each odorant were fit with a Michaelis-Menten equation with the Hill coefficient fixed at 1.0 using the Solver function in Excel 2003 (Microsoft).

**RESULTS**

**Behavior**

Behavior measurements were carried out on 6 Long-Evans rats. Figure 1 is a plot of the mean % correct responses of the six animals for the five different odorants at concentrations between 0.0006% and 35% ($\log_{10}$ of -5.22 to -0.45) of saturated vapor. This range includes concentrations that are 50 times lower and 35 times higher than those used in previous odor identification measurements (10). The arrows indicates the odorant concentrations used for training (see figure legend). For all concentrations of all five odorants the percent correct is much higher than a random response (20%). For the 150 data points in Figure 1 the range of the Standard Error of the Means (SEM) was between 0.5% and 13%. For 60% of those data points the SEM error bar would be smaller than the symbol.

**Calcium signals.**

These same six animals were used for calcium measurements; results are given for only five animals because the staining procedure was unsuccessful for the sixth. Figure 2 illustrates the concentration dependence of the calcium signal for one of the five odorants (propanal) in one of the five rats (LC1). The two images on the left are frame subtractions of frames taken at the time of the signal peak minus frames prior to odorant presentation. The largest signal for the response to propanal at a concentration of 0.36% of saturation is localized near the center of the field of view. In contrast the signals in response to 11% propanal are very widespread. The amplitude and time course of the signals at four concentrations from the dark red active region near the center in the top image is illustrated on the right. There is little or no detectable signal in response to 0.12% propanal; the signal then progressively increases as the propanal concentration increases.
In this animal we measured the calcium signal amplitude as a function of odorant concentration for five odorants. In Figure 3 we have plotted (colored squares) the amplitudes as a function of the log$_{10}$ of the concentration. We show the result for the most sensitive glomerulus for each of the five odorants. Four of the odorants were those used in the behavior experiments; 2-hexanone is an additional odorant.

In addition we have plotted the mean of the behavioral results for the six animals and five odorants (black squares; for the 30 behavioral data points the range of the SEMs was between 0.4% and 3.9%). Clearly there is a very poor correlation between the ability of the rat to identify odorants and the calcium signal amplitude. The calcium signals decline markedly over the concentration range of 0.1% to 10% of saturated vapor while the ability of the rats to identify odors remains relatively constant and far above chance down to 0.001%.

In Figure 3 we also show fits to the data for the calcium responses using Michaelis-Menten kinetics (smooth curves). We used a Hill coefficient of 1.0; the k$_{1/2}$ was the fitted parameter. The data points are reasonably well fit with this Hill coefficient. The fits with a Hill coefficient of 0.67 and 2.0 were less good. This result is consistent with earlier fits where the Hill coefficient was a free parameter and its mean value was 1.2 (4).

The log$_{10}$ of the k$_{1/2}$ values from the fits in Figure 3 are given in the first line in Table 1. The values obtained using the other four animals where calcium signals were measured are also shown. The mean for each odorant was determined and the mean of these means was -1.82 (1.5% of saturated vapor). We made similar measurements on four untrained animals and obtained three measurements of k$_{1/2}$ for each of the five odorants. The means of these three measurements and the mean of these means, log$_{10}$ of -2.16, are also shown in Table 1. The means for trained and untrained animals are not significantly different; p>0.10. Results for a sixth odorant, 2-hexanone, are also shown. On average the most sensitive 2-hexanone glomerulus was slightly more sensitive than the most sensitive propanal glomerulus.

We also evaluated the difference in sensitivity between the most sensitive glomerulus in the response to an odorant and the 6th most sensitive glomerulus. For the trained animals the sixth
most sensitive glomerulus had a $k_{1/2}$ at a concentration that was 3.2 times higher than the most sensitive glomerulus; for untrained animals the factor was 2.8.

**DISCUSSION**

The results in Figure 3 and Table 1 show that as a function of odorant concentration there is a poor correlation between olfactory receptor neuron calcium signals and the ability of the rat to correctly identify odorants. Accurate identification occurs at odorant concentrations where the calcium signals are much smaller than the maximum signal. Similarly, several other signals (synapto-pHluorin, intrinsic imaging, 2-deoxyglucose, and fMRI) also become much smaller than the maximum signal when the odorant concentration is reduced to 0.1% of saturated vapor (5-7,9). Thus both of the imaging methods for monitoring the mean activity of the receptor neurons report very reduced activity at odorant concentrations where odorant identification remains highly accurate. And, the same holds true for the two methods which report generalized glomerular activity. The majority of olfactory sensory neurons have a response range that lies between 0.1% and 10% or saturated vapor. Relatively few neurons respond at concentrations below 0.1%.

One concern about the optical imaging measurements (calcium, synapto-pHluorin, and intrinsic imaging) is that they sampled only the dorsal glomeruli and thus could miss glomeruli that might be more sensitive but were located in other regions of the bulb. However, this concern does not apply to 2-deoxyglucose and fMRI techniques that measure activity everywhere in the bulb. Furthermore, 2-hexanone, which has large dorsal signals in 2-deoxyglucose measurements (7), has a $k_{1/2}$ only slightly lower than the other odorants (Table 1). Finally, many odorants elicit a widespread 2-doxyglucose uptake (16). Thus it is unlikely that our calcium measurements missed a hidden glomerulus with three orders of magnitude more sensitivity.

In contrast to the poor correlation (Figure 3) between identification performance and imaging signals as a function of odorant concentration, strong relationships between behavior and imaging signals have been seen in other settings at relatively high odorant concentrations (1% - 12% of saturated vapor). Using z-scored $[^{14}\text{C}]-2$-deoxyglucose (2-DG) glomerular uptake, Youngentob et al. (14) demonstrated that glomerular activity patterns predict perceptual quality relationships for odorants. Similarly, patterns of 2-DG glomerular uptake predict the differential ability of rats to perceive two chemically similar stimuli as different in an olfactory habituation/dishabituation task (17-19).

**Estimate of the number of olfactory receptor neuron spikes needed for correct odorant identification.**
Figure 4 illustrates a comparison of the mean behavior score and the Michaelis-Menten equation with a $k_{1/2}$ equal to the mean of means from Table 1 ($\log_{10} = -1.81$) of the most sensitive glomeruli. On the lower left this same fit is shown with the Y-axis expanded by a factor of 100 (dashed curve). At the lowest odorant concentration tested in the behavioral experiments the fit value is 0.04% of its maximum. With 10,000 axons innervating each glomerulus, a 0.04% of the maximum signal would correspond to four axons with the same spike activity as they had at high odorant concentrations. We don't know how much lower the odorant concentration has to be before the rat's ability to identify odorants approaches random chance (20%). If, for example, the ability to identify odorants remains high at $\log_{10} = -6$, the Michaelis-Menten curve predicts an activity of 0.005% of the maximum which corresponds to one axon with half of the spike activity that it had at high odorant concentrations. It seems reasonable to conclude that the rat can correctly identify odorants with relatively few action potentials in relatively few olfactory receptor neurons.

There are assumptions in this estimation. One is that the calcium signal is a linear reflection of the spike activity in the nerve terminals. Another is that the Michaelis-Menten equation will hold at concentrations that are several orders of magnitude lower than the concentrations where we can measure a signal. While these assumptions seem reasonable, their accuracy remains to be tested.

The above estimate leads to the prediction that monitoring many hundreds of olfactory sensory neurons would reveal a small number of neurons responding to odorant concentrations of $\leq 0.01\%$. The same might hold true for juxtaglomerular neurons. For these neurons the prediction could be tested by measuring the odorant responses of a large number of neurons using the bolus loading method and 2-photon microscopy (20).

We found that the $k_{1/2}$ for the sixth most sensitive glomerulus was 3 times more concentrated. These less sensitive glomeruli might also make a contribution at odorant concentrations of 0.0006% of saturated vapor. This contribution will not be easy to estimate because the Hill coefficient for less sensitive glomeruli may be higher then 1.0. In rodents the number of activated glomeruli increases with increasing odorant concentration, an effect that has been uniformly reported for calcium, synapto-pHluorin, intrinsic imaging, and 2-deoxyglucose signals (4-7, 11). For those glomeruli that are activated only at higher concentrations the Hill coefficient may be greater than 1.0 (e.g. Figure 7B in (5)). Hill coefficients larger than 1.0 result in smaller estimated responses at low odorant concentrations.
Our finding that rats successfully identify odorants even at concentrations where the number of activated receptor neurons is small is especially striking considering that in awake rodents the spontaneous activity of mitral cells is increased (21-23) and odorant responses can be difficult to detect.

REFERENCES


ACKNOWLEDGEMENTS
We are grateful to Thomas Hromadka and Tony Zador for demonstrating the method for recording from awake rats, to Robert Sachdev for helpful suggestions about the surgical and restraint procedures and to Matt Wachowiak for advice about staining. We thank Tay Netzog, Justus Verhagen, and Matt Wachowiak for the MATLAB program for correcting for movements in the X-Y plane. Supported by NIH grants DC05259 and AA014871.

AUTHOR CONTRIBUTIONS
The behavioral experiments were carried out by S.L.Y. The optical measurements were carried out by R.H., L.B.C., and E.K. All of the authors contributed to the preparation of the manuscript.
**Table 1: Sensitivity (log\(_{10}\) of \(k_{1/2}\)) of the most sensitive glomerulus.**

**Trained Animals**

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<th></th>
<th>propanal</th>
<th>hexanal</th>
<th>octanal</th>
<th>propyl acetate</th>
<th>isoamyl acetate</th>
<th>2-hexanaone</th>
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<tr>
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<tr>
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<td></td>
<td>-2.22</td>
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</table>

Mean  | -2.27    | -1.76   | -1.66   | -1.96          | -1.45           | -2.35       |

Mean of means = -1.82 ± 0.14 (SEM; N= 5)

**Untrained Animals (N=3)**

Mean  | -2.56    | -2.43   | -1.76   | -1.99          | -2.07           |

Mean of means = -2.16 ± 0.15 (SEM; N= 5)
FIGURE LEGENDS

Figure 1

Percent correct odor identification as a function of odorant concentration. At all tested concentrations the percent correct was far above random chance (20%). Five odorants were tested. Each data point is the average result from six animals. The left arrow indicates the training concentration for isoamyl acetate and propanal; the middle arrow indicates the training concentration for propyl acetate and hexanal; the right arrow indicates the training concentration for octanal.

Figure 2

Calcium signals in the most sensitive dorsal glomerulus in response to four concentrations of propanal. Little or no signal could be detected at an odorant concentration of 0.12% of saturated vapor. At low odorant concentrations (0.36% of saturation) large signals were restricted to a localized region of the olfactory bulb (top left). At high odorant concentrations (11%) the signals were widespread (bottom left). The traces are the average of 2 to 8 trials. They were temporally low-pass filtered with a 2 Hz Gaussian filter to reduce noise. The traces were also corrected for a sloping baseline by fitting the pre-stimulus period with an exponential and then subtracting that exponential curve from the entire time course. The images on the right were spatially smoothed by replacing each pixel value with the mean of 3x3 pixels centered on that pixel. Rat LC1.

Figure 3

Normalized amplitude of the calcium responses (colored squares) for the most sensitive glomeruli and the mean percent correct behavior response (black squares) as a function of the log_{10} odorant concentration. The calcium signals are very small at the odorant concentration of 0.12% of saturated vapor. The data represent the results from one animal (LC1) whose response to five different odorants was tested. The smooth curves represent fits of the Michaelis-Menten equation using a Hill coefficient fixed at 1.0. The maximum value of the fitted curves were normalized to 1.0. The calculated curves fit the experimental points reasonably well.

Figure 4

The mean percent correct behavior response (black squares) and the Michaelis-Menten equation (red curves) as a function of odorant concentration. The k_{1/2} of for the Michaelis-Menten equation was the mean of the most sensitive glomeruli (Table 1). On the left the Michaelis-Menten
equation is shown in the dashed curve with a Y-axis expansion of a factor of 100. At the lowest odorant concentration tested in the behavior experiment the calcium signal is 0.04% of the maximum.
Figure 1

ODOR IDENTIFICATION BEHAVIOR

Behavior (% correct)

log Concentration (% of saturated vapor)

hexanal
propanal
iaa
cotanl
propyl acetate

LCH-LGG-ID296-301-3: behavior FI-rat
Figure 2

CONCENTRATION DEPENDENCE OF THE CALCIUM SIGNAL

0.12%

0.36%

1.8%

11%

ΔF/F
1%

propanal

1 sec

0.5 mm
Figure 3

Behavior and Response of Highest Sensitivity Glomerulus

Behavior Normalized Calcium Signal

log Concentration (% of saturated vapor)

-6 -5 -4 -3 -2 -1 0

Species:
- hexanal
- fit to hexanal
- behavior
- propanal
- fit to propanal
- 2-hexanone
- fit to 2 hexanone
- propyl acetate
- fit to propyl acetate
- isoamyl acetate
- fit to isoamyl acetate

17
Figure 4

Behavior and Mean Highest Sensitivity Glomerulus Response

- Behavior and Calcium Signal
- Log Concentration (% of saturated vapor)

- Fit to mean
- Behavior
- Fit to mean X 100

0.005% 0.04%