Trial-by-Trial Discrimination of Three Enantiomer Pairs by Neural Ensembles in Mammalian Olfactory Bulb.

Ensemble Discrimination of Odorants

M.J. Lehmkuhle$^{3,2,1,*}$, R.A. Normann$^1$, and E.M. Maynard$^1$

$^1$Department of Bioengineering, University of Utah, Salt Lake City, UT 84112
$^2$Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI 48109
$^3$Kresge Hearing Research Institute, University of Michigan, Ann Arbor, MI 48109

*Correspondence should be addressed to Mark Lehmkuhle, Neural Engineering Lab, Biomedical Engineering Department, University of Michigan, 2200 Bonisteel Blvd., Ann Arbor, MI 48109-2099. E-mail: miehmkuh@umich.edu.
ABSTRACT

Populations of output neurons in the mammalian olfactory bulb (OB) exhibit distinct, widespread spatial and temporal activation patterns when stimulated with odorants. However, questions remain as to how ensembles of mitral/tufted (M/T) neurons in the mammalian OB represent odorant information. In this report, the single trial encoding limits of random ensembles of putative single- and multi-unit M/T cells in the anesthetized rat OB during presentations of enantiomers of limonene, carvone, and 2-butanol are investigated using simultaneous multielectrode recording techniques. The results of these experiments are: the individual constituents of our recorded ensembles broadly represent information about the presented odorants, the ensemble single-trial response of small spatially-distributed populations of M/T neurons can readily discriminate between six different odorants, and the most consistent odorant discrimination is attained when the ensemble consists of all available units and their responses are integrated over an entire breathing cycle. These results suggest that small differences in spike counts among the ensemble members become significant when taken within the context of the entire ensemble. This may explain how ensembles of broadly-tuned OB neurons contribute to olfactory perception and may explain how small numbers of individual units receiving input from distinct olfactory receptor neurons can be combined to form a robust representation of odorants.

Key words: multielectrode; electrophysiology; coding; olfaction; synchrony
It has been suggested that odorant stimulus identity is encoded in spatiotemporal neural activation patterns. This coding model has been examined in the invertebrate olfactory bulb (OB) and insect antennal lobe (OB equivalent) (Lei et al. 2004; Sachse and Galizia 2002; Stopfer et al. 2003). In the anesthetized mammalian OB, single mitral and tufted (M/T) neurons respond to multiple odorant stimuli (“broadly tuned”) and tend to have variable responses across individual stimulus trials (Chaput and Holley 1985; Lehmkuhle et al. 2003; Motokizawa 1996). The extent of this broad tuning to odorant stimuli and the formation of a uniform representation of odorants in real time from these neuronal responses are unclear. In this report we use a multielectrode array implanted in the OB of rat to investigate: 1) the discrimination of molecularly similar and dissimilar odorants by a random ensemble of M/T neurons, and 2) the effect of temporal integration on trial-by-trial, ensemble-level odorant discrimination.

Electrophysiological investigation of OB neuron encoding has been pursued with many technologies. Neural responses to simple odorant stimulation in the mammalian OB have been extensively studied with single microelectrodes (Chaput and Holley 1985; Leveteau and MacLeod 1966; Motokizawa 1996) as well as by arrays of microelectrodes (Bhalla and Bower 1997; Buonviso et al. 1992; Chalansonnet and Chaput 1998; Kashiwadani et al. 1999; Kay and Laurent 1999; Lehmkuhle et al. 2003). Additionally, local field potential recordings have been used to observe odorant-induced oscillations in the OB (Freeman 1978; Kay 2003; Martin et al. 2004; Ravel et al. 2003). Depending on the particular
methodology and technology used, these studies had practical limits on the number of elements in the studied ensemble, the spatial and temporal granularity of the ensemble members, and the duration over which the ensemble activity could be monitored. In every case, the capacity to investigate the contribution of individual neurons within the context of a simultaneously active ensemble to physiologically relevant stimuli is greatly diminished. Planar arrays of microelectrodes potentially overcome these limitations by directly recording single action potentials from many simultaneously active OB neurons (Anderson et al. 1989; Hoogerwerf and Wise 1994; Nicolelis et al. 2003; Nordhausen et al. 1996).

The propensity for multielectrode electrophysiological recordings to facilitate the investigation of neuronal populations is well established in both sensory and motor systems. Simultaneous and near-simultaneous electrophysiological recordings of multiple neurons in motor, sensory, and limbic systems have been shown to contain additional information about a stimulus that is only available at the ensemble level. As with these systems, the capacity of an ensemble of M/T units to reliably discriminate odorants based on spike count information likely benefits from both an averaging of signals from many broadly-tuned receptors and a reduction in the trial-by-trial response variability by concentrating on the differences present in the population response rather than any individuals’ response (Arabzadeh et al. 2004; Hampson et al. 2002; Katz et al. 2002a; Katz et al. 2002b; Maynard et al. 1999; Oram et al. 2001; Paninski et al. 2004). Likewise, small populations of M/T neurons receiving input from the
same glomerulus in OB have been shown to be temporally correlated which may be important in signal amplification or redundancy (i.e., robustness) in light of OB neuron plasticity and turnover (Buonviso and Chaput 1990; Luo and Katz 2001; Schoppa and Westbrook 2002). Temporally correlated activation of ensembles of spatially disparate M/T neurons could provide highly specific odorant feature information to higher olfactory centers mediated through coincident synaptic connections in areas such as anterior piriform cortex (Haberly 1985; Haberly and Price 1977; Mori et al. 1999; Wilson 2000). To date, little information is available about the extent of odorant information that can be conveyed by ensembles of M/T neurons in the mammalian OB.

In order to address questions of the representation of odorant information in ensembles of M/T neurons, we first establish the broadly-tuned nature of the unit responses to odorants. Parallel recordings from a number of random M/T neurons in both hemispheres of rat OB allow us to record the trial-by-trial ensemble spike counts of both intra- and inter-hemispheric M/T units evoked by a number of molecularly similar and dissimilar odorants. A Bayesian statistical analysis is used to assess the capacity of spike count responses from individual units to discriminate pairs of enantiomer odorants on a trial-by-trial basis. This analysis is then repeated on populations of M/T neurons to compare the discrimination capability of ensembles. Finally, the population of units is dissected to investigate the contribution of individual units to the ensemble odorant representations. It was found that: 1) odorant information is distributed across the entire population of units in an OB ensemble, 2) in all animals, the
recorded ensemble of OB neurons performed better than 90% at the trial-by-trial discrimination of pairs of enantiomers over individual neurons, 3) the ensemble responses to randomized trials of six different molecularly similar and dissimilar odorants could be reliably discriminated in over 82% of all trials, 4) the ensemble response of a small population of units could be used to predict the odorant trials as well as the ensemble responses of larger populations of units, and 5) the percentage of trials that were correctly classified increased from 50% when responses were integrated over a small time interval to >90% when responses were integrated over the entire breathing cycle. These results suggest that ensembles of broadly-tuned M/T neurons are robust encoders of odorant identity in the mammalian OB.

**MATERIALS AND METHODS**

*Array implantation and odorant delivery.* The electrode arrays used in this study are commercially available devices (Cyberkinetics, Inc., Salt Lake City, UT) constructed from a silicon wafer to form square matrices of electrodes 400 µm on center (Campbell et al. 1991; Jones et al. 1992). Each electrode was 500 µm in length and ~80 µm at the base tapering to a point. The tips of the electrodes were coated with platinum and the shanks insulated with either silicon nitride or Parylene-C. In a departure from earlier architectures with this particular structure, two arrays of sixteen electrodes each were implanted to a depth of ~300 µm in both hemispheres of the dorsal aspect of OB of seven urethane
anesthetized male Wistar rats. An additional PTFE-insulated platinum wire positioned adjacent to the OB was used as a recording reference electrode. Further details regarding surgical procedures and electrode array implantation have been described elsewhere (Lehmkuhle et al. 2003). All surgical procedures used in this study were conducted according to University of Utah Institutional Animal Care and Use Committee-approved protocols.

Three pairs of enantomers were used in these experiments based on their use in previous olfactory studies where they have been shown to elicit a response from both glomeruli and M/T cells within the dorsal aspect of OB. Specifically, Limonenes as +L (((R)-(+)−Limonene, > 97% pure, Sigma-Aldrich, Milwaukee, WI) and -L (((S)-(−)-Limonene, > 95% pure, Sigma), Carvones as +C (((S)-(+)−Carvone, 96% pure, Sigma) and -C (((R)-(−)-Carvone, 98% pure, Sigma), and 2-Butanols as +2B (((S)-(+)−2-Butanol, 99% pure, Acros Organics) and -2B (((R)-(−)-2-Butanol, 99% pure, Acros), were used in these experiments. Each odorant (or clean air control) was delivered to freely-breathing animals by one of two digitally controlled liquid-dilution olfactometers made in-house or available commercially (KNOSYS, Washington, DC). Only concentrations of 1% (vol/vol diluted in mineral oil) were used in this analysis. Odorant and clean air trials were randomly interleaved in 30 second intervals and delivered to the animal for 1 second through a rat-specific anesthesia nose cone at flow rates of 5 ml/min or 635 ml/min.

Electrophysiology. Signals from individual electrodes were amplified by 5000x gain, filtered from 0.250 kHz to 7.5 kHz, and digitized at 30 kHz.
Recordings from all simultaneously recorded channels were saved for offline analysis with a Pentium-based acquisition system (Neural Signal Acquisition System (NSAS), Cyberkinetics, Inc.). The animal’s phase of breathing and olfactometer solenoid position information were simultaneously recorded throughout the duration of the experiments (6.0 to 14.4 hours). The phase of the animal’s breathing cycle was monitored through a sensitive thermistor circuit situated near the animal’s mouth and sampled by the NSAS at 200 samples/sec. An automated routine was created to extract time stamps indicating the start of the inhalation and exhalation phases of the animals’ breathing; these were subsequently used for alignment purposes during analysis.

Spikes were clustered using a combination of an automated routine, which fits the observed waveforms using probabilistic models of spike waveform variability (Shoham et al. 2003), and manual principal component cluster analysis sorting methods (Offline Sorter, v. 2.3.1., Plexon, Dallas, TX). Mitral and tufted cells were identified by their distance from the surface of OB, the magnitude of their action potentials (typically > 50 µV peak to peak) and our expected inability to record extracellular action potentials from granule cells due to their small size, the low signal-to-noise ratio of the recorded units, and high impedance of the recording electrodes. Single- and multi-unit activity was analyzed with Neuroexplorer (Nex Technologies, v. 2.671, Littleton, Massachusetts) and Matlab (Mathworks, release 13, Natick, Massachusetts). Spiking events were reduced to an event time series. Each unit’s response to clean air or odorant presentation was aligned to the inhalation phase of the animal’s breathing in the presence of
odorant stimulus. Six stimuli were used in these experiments: 1) (+L), 2) (-L), 3) (+C), 4) (-C), 5) (+2B), and 6) (-2B), in which all responses to an odorant presentation were aligned to the inhalation phase of breathing.

Statistical analysis. Trial-by-trial rasters were created of all unit responses aligned to the inhalation phase of the animal’s breathing in the presence of each of the six odorants. For subsequent analysis these rasters of spike counts were integrated over five different time intervals within a single breathing cycle centered on the transition from exhalation to inspiration; 10, 50, 100, 250, and ~600 msec (consisting of the entire breathing cycle in the anesthetized animal).

To determine the statistical relationship between a units’ firing rate and the presented stimulus, Bayes’ rule was then applied to each unit on a trial-by-trial basis to obtain probability density estimates (PDE) for each of the odorants in each of the integrated time intervals for each animal (number of calculations = $n$ neurons * 6 odorants * 5 integrated time intervals). Probability density estimates were calculated using Bayes’ law for each individual unit where:

$$Pr(A | counts_i) = \frac{Pr(counts_i | A) \times Pr(A)}{Pr(counts_i)}$$

$Pr(counts_i | A)$ is the probability of a particular spike count when odorant “A” is present, $Pr(A)$ is the probability that odorant “A” was the stimulus (the number of odorant “A” trials out of the total number of trials, an independent variable), $Pr(counts_i)$ is the unconditional spike counts for all odorant stimuli, and $Pr(A | counts_i)$ is the probability that odorant “A” was present given a particular spike.
count. In each case, the current trial was not used when calculating \( \Pr(\text{counts}_i) \). At this step, all the PDEs were calculated for each combination of unit, odorant, and integrated time interval.

Once PDEs for each *individual* unit were obtained, they were combined into an *ensemble* PDE (Sanger 1996):

\[
\Pr(A \mid \text{population}) = \prod \Pr(A \mid \text{counts}_i)^n \times \text{“normalization constant”}
\]

where \( \Pr(A \mid \text{population}) \) is the probability that odorant “A” was present given a particular spike count based on the *ensemble* response from the entire population of neurons, \( n \). The normalization constant is a number that guarantees a “total probability” of 1.0 and is the probability that the ensemble has a particular set of firing rates.

Trials from one enantiomer pair or all six odorants were grouped together and the above analysis was performed to determine the ability of the individual units or ensemble of units to discriminate between trials of similar and dissimilar odorants. The PDEs of each individual unit and each ensemble of units were used to estimate from the single trial information which of the two or six odorants were present for any given trial. In a given trial, the maximum likelihood estimation (MLE) was used to collapse the probability function into a single likelihood. This MLE (the odorant prediction) was then compared to the actual odorant trial. From these data the percent of correct and incorrect responses were determined for *individual* units and the *ensemble* of units for each animal.
The extent to which olfactory information is distributed across units in an ensemble was determined by sequentially removing the “best” performing units from the ensemble and observing the ensemble performance at discriminating trials of all six odorants as described above. This analysis determines the amount of odorant information contributed by each unit and is described as the hallmark of distributed coding (Ghazanfar et al. 2000). Starting with the full ensemble, each unit is removed sequentially to find the unit that causes the greatest degradation in ensemble performance. This unit is considered the “best” unit and is removed from the ensemble and the entire process is repeated until only one unit is left. If the units are exhibiting distributed coding, the percent of trials correctly predicted should decay smoothly. Conjointly, starting with an ensemble consisting of only the “best” unit and subsequently adding the next “best” unit to the ensemble, will reveal two aspects of distributed coding; the number of units contributing useful information to the ensemble and how quickly the ensemble improves with each additional “best” unit. If units are independent encoders of odorant information, odorant classification should improve proportionally to the square root of the number of units.

RESULTS

In this report a multielectrode array was implanted in the OB of rat to investigate the discrimination of molecularly similar and dissimilar odorants by a random ensemble of M/T neurons. The results of this study address the representation
of odorant information in ensembles of M/T neurons in the mammalian OB. The analyses progresses from first establishing the broadly-tuned nature of the odorant responses for the individual units in the recorded ensembles to the role of broad tuning in individual units’ spike counts and their capacity to discriminate between presented odors in a single trial is examined by a Bayesian analysis. This analysis, with modification, is then repeated on ensembles of M/T neurons to compare the discrimination capability of ensembles. Finally, the ensemble responses are dissected to investigate the contribution of individual units to the ensemble response.

Unit Recordings
Ensemble recordings were made from up to 32 microelectrodes implanted in both hemispheres of the dorsal aspect of the olfactory bulb (OB) of seven urethane-anesthetized male Wistar rats for up to 14.4 hours. The average number of units recorded per animal was 32, including single- and multi-units recorded on the same electrode. The mean signal-to-noise ratio (SNR) of all recorded units was 3.5:1 (low 0.8:1, high 10.3:1, std dev 1.2) as calculated by dividing the peak-to-peak amplitude of the signal by the peak-to-peak amplitude of the noise. Single-units were classified based on waveform shape, interspike interval histogram, and signal-to-noise ratio (> 3.5:1) in order to isolate separate units recorded on a single electrode. Single- and multi-units (all recorded units) were used together in subsequent analyses and are hereafter termed “units”. Details of the electrophysiological characteristics of the recordings from these
data including recording stability, viability, and the dynamics of the spatio-temporal responses to odorants have been previously reported (Lehmkuhle et al. 2003).

**Recorded Parallel Responses of Olfactory Bulb Neurons**

Trial-by-trial rasters were compiled to compare the ensemble responses of units recorded simultaneously at different electrode positions; a process well suited to parallel multielectrode recordings. We recorded simultaneous spiking events from single- and multi-units from seven animals to random presentations of six enantiomer odorants (1% vol/vol) (table 1), or clean air. Time stamps from these spiking events were aligned to the inhalation phase of the animals’ breathing as derived from external instrumentation (see methods). The average breathing rate of all animals was 2.1 breaths/sec (std dev 0.3). Trial-by-trial raster plots were created for each of the odorants (figure 1). A clear modulation of the unit’s response to the phase of the animal’s breathing (~2.1 breaths per sec) is indicated by the consistent periodic increase in the density of the rasters. Baseline variability was observed as a global increase and decrease of the unit responses as a function of time and through a number of units ceasing and resuming firing action potentials over time (note rasters, figure 1). Additionally, temporal variability was observed in the trial-by-trial responses of each unit to a given odorant. This begs the question of how specific any one unit’s response can be to a particular odorant.
Units are Broadly-Tuned to Odorants

In many cases an individual unit responded to multiple odorants. The next step in this analysis was to quantify how multiple odorant responses were distributed throughout the population of units to identify domains where specific odorant information was represented. To investigate the degree to which individual units respond to odorants we first compared the distributions of unit spike counts aligned to the inhalation phase of the animals breathing in the presence of each of six odorant and clean air stimuli over one breath cycle using one-way ANOVA. If each unit response to each of the six odorants were indeed unique to that unit (i.e. “narrow” tuning), we would expect the majority of spike counts to be completely distinguishable across all odorants. Conversely, a completely redundant unit response to all six odorants would mean that the majority of spike counts were not significantly different across odorants. Each group of the ANOVA was each of six odorants (and clean air). Each treatment was trials of spike counts of each stimuli. Only animals A, B, D, and E were used in this analysis in which all six odorant stimuli were presented (table 1). Of those 117 units in this analysis 42 (35.9 %) had a significant difference (p ≤ 0.0001) in the means of spike count responses between groups. Stated another way, of the 117 random units recorded from these four animals, approximately 36 % had a response to at least one of the six odorants. Tukey’s HSD post-hoc analysis of those 42 units was used to determine how many unit responses to odorants were significantly different from air (p ≤ 0.05, figure 2a). The majority of units responded to more than one odorant, suggesting that these 42 units were not
narrowly tuned to the six available odorants. A similar post hoc analysis allowed us to determine the extent to which unit responses produced uniquely discernable responses to each odorant. These tests indicate that unit responses to odorants are not easily discernable \( p \leq 0.05 \), figure 2b; this is indicative of “broad odorant tuning”. If each unit’s response to each of the six odorants were unique to that unit, we would expect the majority of counts in figure 2b to be skewed towards the left. A completely redundant unit response to all six odorants would skew the counts in figure 2b to the right. In conclusion, these data indicate that units are broadly tuned to the odorant set, yet the question still remains as to how the olfactory system effectively discriminates between odorants.

**Trial-By-Trial Discrimination of Odorants by Individual Units**

The ANOVA in the previous analysis uses the units’ averaged responses to determine whether significant differences exist in their spiking activity to odors and clean air. A probabilistic approach was used to assess the 234 individual units’ tuning to enantiomers of limonene on a trial-by-trial basis. Probability density estimations were calculated for both \((R)-(+)\)-Limonene (+L) and \((S)-(+)\)-Limonene (-L) given each unit’s spike counts integrated over the entire breathing cycle and evoked by each of the respective odorant trials. For each trial in the data set Bayes’ Law returns a set of probabilities that the particular observed spike count results from a trial of a specific odorant; this probability space was collapsed into a single choice by means of a maximum likelihood estimator.
(MLE), taken as the maximum of the probabilities for (+L) and (-L). These results were then compared with the known odorant stimulus for each trial to determine if the “correct” choice was made. The percentages of correct and incorrect classifications were 51.2% and 44.4% for (+L) trials and 59.2% and 36.6% for (-L) trials, respectively (figure 3, circles). Additionally in 4.4% of (+L) trials and 4.2% of (-L) trials there was either no response (zero spikes) or there was an equal probability of the unit response representing (+L) or (-L). These trial predictions were categorized as not having enough information to correctly identify the odorant (shown as +L|? and –L|? in figure 3). In general, individual units performed slightly better than chance (50% correct) at discriminating between the enantiomers of limonene. In over half of all unit responses (trials) there was not enough information to discriminate between the odorants.

Trial-By-Trial Discrimination of Odorants by Ensembles of Units

It is an organizing principle in sensory and motor systems that broadly tuned individual elements combine in ensembles to form sharper neural representations. Ensembles of broadly-tuned units may contain additional information that could be used to discriminate between multiple odorant stimuli that is not represented by individual units. The hypothesis that an ensemble of OB neurons has a more sharply tuned response than those of its constituent individual units was tested by evaluating how each of the seven populations of units (one population for each of the seven animals) used in this analysis could predict the presented enantiomer of limonene on a trial-by-trial basis. For each
odorant presentation, we calculated the most probable odorant presented based on the ensemble of spike counts. This result was then compared with the known odorant stimulus for each trial. The percent of correct classifications and false classifications were 91.2% and 8.8% for (+L) trials and 95.9% and 4.1% for (-L) trials, respectively (figure 3, triangles). The average number of correctly and incorrectly classified trials for all 2,414 (+L) and (-L) odorant trials across seven animals was 2,264 (93.8%) and 162 (6.7%), respectively. In all animals, the ensemble of OB neurons performed significantly better than chance (50% correct) at the trial-by-trial discrimination of the enantiomers of limonene.

This analysis was then expanded to include other enantiomer pairs of carvone and 2-butanol using the same ensembles of units for each animal (figure 4). Similarly, the ensemble response was able to predict the odorant presentation well above chance (50% correct) at the trial-by-trial discrimination of enantiomers of the same odorant. Additionally it was found that in those animals in which a small number of units were recorded, the ensemble responses could be used to predict the odorant trials at least as well as those animals in which a larger number of units were recorded. Table 2 summarizes the average percentages of correct and incorrect predictions from these analyses.

**Odorant Information is Encoded Over the Entire Breathing Cycle**

The previous results were arrived at by integrating the response of the units over the entire breathing cycle. Since it is possible that the olfactory code may be featured only in particular epochs during the breathing cycle, we investigated the
role of the integration interval in the ensemble discrimination between the enantiomers of limonene. Specifically we questioned whether the integrated spike counts in a small time bin centered around the apex of the animal’s breathing cycle had more discriminating power on a trial-by-trial basis than a larger time bin encompassing the entire breathing cycle. The time of transition from exhalation to inspiration in of the animals’ breathing cycle was chosen as the alignment point based on earlier studies indicating that the peak of excitatory patterns of M/T responses are phase-locked on late inhalation and early exhalation (Chaput et al. 1992; Kashiwadani et al. 1999). The data were analyzed in five different time bins; the first four bins centered around the apex of the inhalation phase of the animals’ breathing and the last bin encompassing the entire breathing cycle (~600 msec) (figure 5a). The percentage of trials that were correctly classified increased from chance (50% correct) at the smallest interval to over 95% when responses were integrated over the entire breathing cycle. Further, the variability in the performance of the classification, defined as the separation in probability space of the choices in the set of probabilities, decreased with the additional integration time. We then realigned responses to the start of the breathing cycle for the same time epochs as before to compare these findings with data aligned to the apex of breathing (figure 5b). A decrease in the mean percentage of correct classifications for all time epochs was observed along with an increase in classification variability across animals for data aligned to the start of the breathing cycle. This suggests that integrating unit responses over an entire breathing cycle sets the “sampling rate” by which
animals explore their olfactory environment, a process that needs further investigation in awake animals where sniffing frequency is actively modulated.

**Ensemble Discrimination of Random Trials of Six Odorants**

All of the previous analyses were conducted on enantomer pairs. Given that we recorded from a random sample of units from which our previous results suggest are broad odorant encoders, we wanted to test the limits of ensemble coding with the entire stimulus set. The final analysis tested the ensembles’ discrimination among the 3 enantomer pairs (6 odors all together) on a trial-by-trial basis. For this analysis, trials from all odorant presentations were combined in each of the four animals in which all six odorant stimuli were presented; not all animals were presented the full range of stimuli. PDEs were calculated as described above on a trial-by-trial basis for each odorant. Subsequently, MLEs were determined and compared to the known odorant presentation. The MLE for each trial was determined as the maximum of the probability density for (+L), (-L), (+C), (-C), (+2B), and (-2B). The percent of trials correctly and incorrectly classified based on the ensemble response from each animal can be seen in figure 6. When given 4,275 trials of all six odorants across animals, the ensemble response predicted 3,539 (82.8%) trials of the odorant presentations, well above chance (16.7% correct) (table 2). These data indicate the ability of a randomly selected population of OB neurons to robustly discriminate odorants irrespective of their spatial position in the OB.
Odorant Information is Distributed Across a Population of Units.

The foundational assertion of this study is that populations of broadly-tuned units in OB form ensembles in order to effectively discriminate between low concentrations of molecularly similar, yet perceptually distinct odorants without the benefit of temporal averaging. Our approach is predicated on ensembles of broadly-tuned neurons, the presence of single units that “perfectly” coded for a single odorant would cast serious doubts on our hypothesis. Thus, our final set of analyses undertakes to assess the relative contribution of each unit in the ensemble to the overall performance of the ensemble at discriminating trials of six different odorants. Specifically, is there a single unit in the ensemble that is directly responsible for correct discrimination of each of the odorants? To test this hypothesis we first started with all the units in each ensemble and found the best predictor unit, the unit that contributed the most useful odorant information (see methods). This unit was removed from the ensemble and ensemble performance reevaluated. This process was repeated until only one unit remained and was repeated for each animal (figure 7a). Ghazanfar and colleagues used this analysis to quantify the performance of ensembles of somatosensory neurons. The smooth degradation that they observed in ensemble performance was described as a hallmark of distributed coding (Ghazanfar et al. 2000). Similarly, the results of figure 7a show a smooth decay in ensemble performance at predicting trials of the six odorants.

The second part of this analysis starts with an ensemble consisting of the best predictor unit and sequentially adds the next best predictor unit to observe
ensemble performance until all units have been added (figure 7b). This analysis will allow us to quantify the number of units contributing to ensemble performance and evaluate ensemble performance with each additional unit. If only a few units of the ensemble contribute the majority of useful information we would expect a quick rise in the performance metric to an asymptote. Likewise, if all units were contributing equally to the ensemble performance we would see a linear relationship between the number of units added to the ensemble and ensemble performance. The data in figure 7b suggest that ensemble performance is not being driven by only a few units. Taken together, figures 7a & 7b indicate that odorant information is nearly uniformly distributed across the population of units in the ensemble.

**DISCUSSION**

The ability to record simultaneously from multiple neurons is a requirement for investigating systems-level information processing in the mammalian olfactory system. Although studies have demonstrated that simple odorants produce concentration-specific spatio-temporal regions of activation of the dorsal OB (Johnson et al. 1998; Lehmkuhle et al. 2003; Rubin and Katz 2001; Spors and Grinvald 2002; Wachowiak and Cohen 2001; Xu et al. 2000) it is not clear whether or how these glomerular responses on the input side of OB interact to form a uniform perception of odor by the output neurons of OB. In the invertebrate olfactory system it has been suggested that spatial and temporal
correlations within oscillatory networks, perhaps modulated by local field potentials, are an important mechanism for such fine olfactory discriminations (Christensen et al. 2003; Laurent et al. 2001; Lei et al. 2004; Rabinovich et al. 2001; Stopfer et al. 1997). In this report we investigate the encoding of odorants through ensembles of random populations of mammalian M/T neurons; specifically, it was found: that individual units tend to represent multiple odorant stimuli, small ensembles of a random units are able to discriminate multiple odorant stimuli on a trial-by-trial basis, and no individual unit is able to reliably discriminate between multiple odorant stimuli.

We recorded simultaneously from up to 49 single- and multi-units of the major output neurons of OB. An ANOVA for response tuning classified units into two groups: those with significant odorant responses (ANOVA, \( p \leq 0.0001 \)) or those not responding to odorants (ANOVA, \( p > 0.05 \)). In those units with significant responses to odorants, paired post-hoc tests of individual unit responses were not significant across multiple odorants. This result supports the hypothesis that single unit encoders of olfactory information in a random selection of OB units are more likely the exception rather than the norm. Finally, to examine the assembly of ensemble responses from broadly-tuned individual contributors, a statistically-based method was employed to interpret the simultaneous multielectrode electrophysiological recordings (Sanger 1996). This analysis clearly demonstrates the ability of small, randomly selected populations of intra- and inter-hemispheric spiking neurons in OB to correctly classify
individual trials of molecularly similar odorants; something that cannot be inferred from spatial plots of OB activation.

The concentration of odorants (1 % vol/vol) used in these experiments is relatively high compared to behavioral threshold concentrations. It would be expected that the integrated spike counts of the recorded units to be similar for enantiomer odorants as glomerular responses saturate. Here, all recorded units are used in the ensemble regardless of the strength of any unit’s response to a particular odorant, including those units that may have saturated responses. However, slight differences in the spike counts on only a few units will amplify differences reflected in the ensemble of units in this analysis. This is a significant observation and may form the basis for how ensembles make decisions. Given that only a small population of M/T neurons are needed to make these odorant discriminations reflects recent studies indicating an animal’s ability to behaviorally discriminate enantiomer odorants after significant portions of OB have been ablated (Bisulco and Slotnick 2003; Slotnick and Bisulco 2003). These results support the conclusion that responses from a small number of M/T cells (representing functionally distinct olfactory receptor neuron populations as described below) integrated over a single breathing cycle produce distinct representations of odorants.

A significant assumption inherent in this analysis is that each unit in the population is an independent encoder of olfactory information. Within this context, our results maintain the likely organization of OB into smaller functional subunits. Each electrode in the recording array is separated from its neighboring
electrodes by at least 400 μm; the recorded units are likely projecting to non-neighboring glomeruli and thus represent functionally distinct olfactory receptor neuron populations. Similar to imaging techniques that have shown spatial and temporal selectivity to odorants in the glomerular layer of OB, the technique used in this report supports the existence of stimulus-induced differences in the ensemble M/T responses that can reliably discriminate between molecularly similar odorants. Further support for glomerular independence is born out by results comparing the response of simultaneously recorded units with the response of pseudo-serially recorded units. The process of shuffling the trials breaks any temporal structure to the ensemble responses that may be present (to simulate recording each unit serially) which did not degrade the ability of the ensemble responses to discriminate between trials of six different odorants (table 2, figure 6). Such a result supports an interpretation of each unit’s role in the ensemble as an independent encoder of information. The extent to which interactions within the ensemble were investigated was inherently limited by the analysis used. The absence of significant local temporal interactions in the ensemble in no way precludes other interactions within the population of recorded units. The strength of the independence assumption is an area for further investigation given that mitral cell lateral dendrites are synaptically connected with other mitral and granule cells (Rall et al. 1966; Schoppa et al. 1998).

Interactions within an ensemble of OB neurons can be on a global and local scale. The ensemble analysis presented in this report has investigated only
global scale temporal interactions (e.g., attentional modulation) while intentionally omitting fine scale temporal interactions (i.e., classical synchrony). Our use of urethane anesthesia could have profound effects on lateral inhibitory processing within OB, which may confound the interpretation of these data. Given these synaptic interactions begs the question of how temporally connected events (i.e., synchronous events) within the population response affect the information content of odorant responses on a trial-by-trial basis. It is possible that synchronous events driven by the serial two-stage inhibitory network of olfactory bulb activation fine-tune the ensemble response for a given concentration of an odorant (Friedman and Strowbridge 2003; Friedrich et al. 2004; Kashiwadani et al. 1999; Lei et al. 2002; Linster and Cleland 2001; Perez-Orive et al. 2004). Future research will allow us to extend this analysis to include synaptically connected events such as synchronous events in order to determine how dependent interactions between units affect systems-level odorant identification.

The analyses in this study use binned spike counts to predict odorants. Binned spike counts are affected by two factors: the length of the bin and the start and endpoints of the bin. Here we have demonstrated that a population of individual unit responses integrated over a large time bin encompassing an entire breathing cycle can be used to discriminate between enantiomer odorants with high fidelity on a trial-by-trial basis. This discrimination of single trial responses to odorants becomes more distinct when spike counts are integrated in increasingly larger time bins, the largest differences occurring over the entire breathing cycle. The subtle differences in the ensemble responses to the odors
that form the basis of the discrimination may be representative of the refinement process that renders the broadly tuned olfactory receptor neuron responses and noise introduced by the stochastic nature of M/T neurons into a coherent olfactory perception. Further, the process of aligning responses to the start of the inhalation phase of breathing provides less information about odorant identity than alignment to the apex of the breathing cycle (figure 5). This is to be expected given that information is present about an odor on both the inhalation and exhalation phases of breathing (Chaput 1986; Margrie and Schaefer 2003); it has also been shown that behaving animals tend to identify an odorant over a single fast-breathing cycle during odorant identification tasks and that subsequent breathing cycles do not contain additional odorant information (Uchida and Mainen 2003). Thus our finding of the significance of the information present in the spike counts of cells is congruous with the performance of the whole organism in a discrimination task. Yet, there is evidence in behaving animals of a trade-off between speed and accuracy (Abraham et al. 2004). There still remain the questions of whether these results hold true for faster breathing rates given that the average breathing rate of our anesthetized animals was 2.1 breaths per second, and the role of fast-timing events in odorant encoding.

In any discrimination process performed on the ensemble response of spiking neurons to presented stimuli, it is possible that the discrimination is driven by entities that are “tuned” to a single stimulus. In the case of the Sanger algorithm in which each member of the ensemble “votes” it is possible to
construct situations where all but one of the members of an ensemble are unaffected by the given stimuli and thus contribute nothing to the analysis outcome. The lone responder perfectly identifies a given stimulus situation and thus drives the “ensemble” outcome leading to a misinterpretation of the significance of the ensemble response. The smooth degradation of ensemble performance after dropping each successive best predictor unit as seen in figure 7a mirrors that of somatosensory neurons in somatosensory cortex and ventral posterior medial nucleus of the thalamus (Ghazanfar et al. 2000). This smooth degradation in performance indicates that there is not only a few units contributing useful information to the ensemble but that a population of units are needed to discriminate odorants based on spike counts. Likewise, sequentially adding the best predictor units to the ensemble produces a smooth increase in ensemble performance with additional units as can be seen in figure 7b. A caveat to this interpretation is that multi-units are predominately “driving” the performance of the ensemble, in the sense that multi-units could arguably contain more information than single units. However, an inspection of the first fourteen best predictor units in each of the four ensembles of figure 7b reveal ratios of single units to multi-units to be: 8:6, 7:7, 7:7, and 3:11, for animals A, B, D, and E, respectively. There was no visible pattern between the order of single and multi-unit best predictor units.

Additionally, figure 7b show that 14 – 16 units are contributing useful odorant information to the ensemble, whereas additional units provide seemingly redundant information. This is to be expected given that only 34 units of those
four animals had significantly different responses from clean air (ANOVA, $p \leq 0.0001$). Given the random nature of these recordings it is likely that we are recording from a number of units that are unresponsive to the odorant set. However, the data in figure 7b indicate that more than 34 units are contributing to the performance of all four ensembles, suggesting that units with insignificant differences in spike counts in the presence of an odorant at the individual unit level may be contributing useful information at the level of the ensemble. Together, these results suggest that despite the organization of the input side of OB into functional subunits (glomeruli), the output neurons may rely on ensemble encoding techniques that rely on the activity of a population of neurons to form unique representations of odorants.
ACKNOWLEDGEMENTS
This work was supported by a pre-doctoral National Research Service Award through the National Institute on Deafness and other Communication Disorders to (M.J.L.) entitled Spatio-temporal responses of olfactory bulb neurons. NIDCD NRSA 5 F31 DC5520. Additional funding provided through a subcontract from NIDCD SBIR Phase II 1R43DC04261-01 to Cyberkinetics, Inc., (formerly Bionic Technologies, LLC.) We thank two anonymous reviewers for their insight.

Drs. Richard Normann and Edwin Maynard have a financial conflict of interest with Cyberkinetics, Inc.
REFERENCES


Chaput MA. Respiratory-phase-related coding of olfactory information in the olfactory bulb of awake freely-breathing rabbits. Physiol Behav 36: 319-324, 1986.


### Table 1: Summary of statistics

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Electrode Array ID</th>
<th>No. of trials of each odorant</th>
<th>No. of multi-units</th>
<th>No. of single-units</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>a</td>
<td>(+L) 121 (-L) 99 (+C) 134 (-C) 113 (+2B) 144 (-2B) 109</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>B</td>
<td>a</td>
<td>(+L) 145 (-L) 177 (+C) 195 (-C) 163 (+2B) 184 (-2B) 152</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>C</td>
<td>b</td>
<td>(+L) 177 (-L) 193 (+C) 181 (-C) N/A (+2B) 202 (-2B) N/A</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>D</td>
<td>b</td>
<td>(+L) 195 (-L) 216 (+C) 236 (-C) 262 (+2B) 210 (-2B) 220</td>
<td>19</td>
<td>22</td>
</tr>
<tr>
<td>E</td>
<td>b</td>
<td>(+L) 200 (-L) 181 (+C) 201 (-C) 207 (+2B) 205 (-2B) 206</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>F</td>
<td>c</td>
<td>(+L) 155 (-L) N/A (+C) 157 (-C) N/A (+2B) N/A (-2B) N/A</td>
<td>19</td>
<td>29</td>
</tr>
<tr>
<td>G</td>
<td>c</td>
<td>(+L) 200 (-L) 198 (+C) N/A (-C) N/A (+2B) N/A (-2B) N/A</td>
<td>20</td>
<td>29</td>
</tr>
</tbody>
</table>

**Mean** 170.4 174.4 189.4 189.4 185.8 171.8 14.1 19.3

**Total** 1193 1221 947 947 743 687 99 135

**Grand Total** 5738 234

N/A, not available

Table 1 Summary of statistics. Animal ID, each letter corresponds to a single animal; Electrode array ID, each letter corresponds to two, 16-channel Utah Electrode Arrays implanted in each hemisphere of the dorsal aspect of olfactory bulb – each electrode was used in multiple animals; Limonenes as +L ((R)-(+)Limonene, > 97% pure, Sigma-Aldrich, Milwaukee, WI) and -L ((S)-(−)-Limonene, > 95% pure, Sigma), Carvones as +C ((S)-(−)+Carvone, 96% pure, Sigma) and -C ((R)-(−)-Carvone, 98% pure, Sigma), and 2-Butanols as +2B ((S)-(−)-2-Butanol, 99% pure, Acros Organics) and -2B ((R)-(−)-2-Butanol, 99% pure, Acros); No. of multi-units, the number of recorded unit waveshapes classified as originating from more than one unit (neuron); No. of single-units, the number of recorded unit waveshapes classified as originating from a single unit (neuron) with a signal-to-noise ratio > 3.5:1 and sharply defined interspike interval histogram. Each trial represents a one-second pulse of 1% (vol/vol) concentration of odorant saturated vapor (diluted in mineral oil) followed by thirty-seconds of clean air.
Figure 1  Rasters (top of each graph) and peri-stimulus time histograms (bottom of each graph) of one multi-unit aligned to the inhalation phase of the animal’s breathing at time zero seconds (vertical line) in the presence of (R)-(+) Limonene, (+L) odorant stimulation; (S)-(−)-Limonene, (−L); (S)-(+) 2-Butanol, (+2B); (R)-(−)-2-Butanol, (−2B); (S)-(+) Carvone, (+C); and (R)-(−)-Carvone, (−C), each 1% vol/vol. The unit response to a clean air stimulus is presented for reference. Each row of the raster represents a single trial of 195 (+L), 216 (−L), 210 (+2B), 220 (−2B), 236 (+C), and 262 (−C) odorant presentations. Each tick mark represents a spiking event at that time. The PSTH represents the mean frequency (spikes / sec) of all trials (rasters) combined in 5 msec bins. A clear modulation of the unit’s response to the phase of the animal’s breathing is indicated by the consistent periodic increase in the density of the rasters. Note the global increase and decrease of the unit responses as a function of time that may be an artifact of anesthesia. Data are representative of Animal D.
Figure 2a Units respond to more than one of the six odorants. These data were selected from Animals A, B, D, and E in which all odorant stimuli (+L), (-L), (+C), (-C), (+2B), (-2B), and clean air stimuli responses were obtained. One-way ANOVA were performed across all seven stimuli responses for each unit. Only those units that had ANOVA $p \leq 0.0001$ (hence removing units that did not respond significantly to the odorant set) were used to create this figure via Tukey's HSD post-hoc test ($p \leq 0.05$, see text). Data displayed are the number of odorant responses within each unit that had significant differences in mean spike counts from clean air stimulus. Those units described as “labeled-line” units are units that respond significantly to only one odorant. Those units described as “broadly-tuned” units are units that respond significantly to more than one odorant. This analysis indicates that the majority of units respond to multiple odorants, a premise that units are “broadly-tuned” to odorants. *Note, a case in which a unit's odorant response is different from air AND different from all other odorant responses could also be considered a "labelled-Line" Unit. However, no unit exhibited this type of response.
Figure 2b  Unit responses to odorants are not easily discernable. Data are the number of odorant responses within each unit that had significant differences in mean spike counts from the response to other odorants. There are 6|2 odorant response comparisons (15 combinations) within each unit. Data are derived from the same Tukey’s HSD post-hoc tests for each of the 42 units from Figure 2a (p ≤ 0.05). Counts in bin “15” indicates that unit spike counts from all 15 odorant response combinations had significant differences in their means and can be described as “narrowly-tuned” units. Count in bin “0” indicates that none of the unit spike counts had significant differences in their means for any odorant response and can be described as “broadly-tuned” units. Note that a count in bin “0” indicates that all six odorant responses had significant differences in their means from clean air stimulus response as derived from ANOVA.
Figure 3 Individual units versus ensembles of units. Data are the performance of individual units' spike counts on correctly or incorrectly predicting the odorant trial versus the population response. $+L|\text{true}$ and $-L|\text{true}$ are the number of trials correctly identified as $(+L)$ and $(-L)$ odorant presentations, respectively. $+L|\text{false}$ and $-L|\text{false}$ are the number of trials incorrectly classified as $(+L)$ and $(-L)$ odorant presentations, respectively. $+L|?$ and $-L|?$ represent the percentage of trials in which there was not enough information to correctly predict the odorant. These two categories include those trials in which there was an equal probability that the population density estimation represented $(+L)$ or $(-L)$ and those trials in which the unit was not active (zero spikes). Points represent mean and bars represent standard error of the mean, ($N_{\text{individual}} = 234$ units, $N_{\text{ensemble}} = 7$ animals).
Figure 4 Absolute percent of all trials correctly and incorrectly predicted based on the ensemble response from each animal. Odorant trials from each of the three enantiomer pairs were analyzed separately using population density estimations. Black shapes are representative of (+L) and (-L) odorant trials. Grey shapes are representative of (+C) and (-C) odorant trials. White shapes are representative of (+2B) and (-2B) odorant trials. Results from each odorant are presented slightly offset for clarity. Diamonds indicate the absolute percent of odorant trials that were correctly predicted. Squares are the percent of odorant trials incorrectly predicted as the opposite enantiomer (e.g. a (+L) trial incorrectly predicted to be a (-L) trial). Triangles are the percent of trials in which there was not enough information in the ensemble response to make an odorant prediction, including trials in which there was an equal probability that the population density estimation represented either of the enantiomers and trials in which there was no response (zero spikes). The number of units for each animal, A-G are: 14, 25, 30, 41, 37, 48, 49, respectively.
<table>
<thead>
<tr>
<th></th>
<th>Enantiomers by Pairs</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(+L) vs. (-L)</td>
<td>(+C) vs. (-C)</td>
<td>(+2B) vs. (-2B)</td>
</tr>
<tr>
<td>correct (std error)</td>
<td>93.8 (1.9)*</td>
<td>91.7 (2.4)**</td>
<td>91.7 (3.3)**</td>
</tr>
<tr>
<td>incorrect (std error)</td>
<td>6.7 (2.0)*</td>
<td>9.3 (2.8)**</td>
<td>10.1 (4.4)**</td>
</tr>
<tr>
<td>not enough information</td>
<td>0 (0)*</td>
<td>0 (0)**</td>
<td>0 (0)**</td>
</tr>
<tr>
<td></td>
<td>All Six Odorants Together</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Simultaneous Trials</td>
<td>Randomized Trials</td>
<td></td>
</tr>
<tr>
<td>correct (std error)</td>
<td>82.8 (4.5)**</td>
<td>84.9 (4.5)**</td>
<td></td>
</tr>
<tr>
<td>incorrect (std error)</td>
<td>17.2 (4.5)**</td>
<td>15.1 (4.5)**</td>
<td></td>
</tr>
<tr>
<td>not enough information</td>
<td>0 (0)**</td>
<td>0 (0)**</td>
<td></td>
</tr>
</tbody>
</table>

*N = 7 animals, **N = 5 (A,B,C,D,E), ***N = 4 (A,B,D,E)

Table 2  Summary of results. Data are in the format, mean (standard error). Enantiomers by Pairs, trials of odorants were tested on an enantiomer pair-by-pair basis. All Six Odorants Together, trials of all six odorants were analyzed together in two groups: Simultaneous Trials, all trials are made up of simultaneous recordings from all units in the population; Randomized Trials, trials of each odorant were randomized, unit by unit, and reconstructed into ensembles – the equivalent of serially recording many neurons.
Figure 5a Percent of trials correctly and incorrectly classified based on the population density estimation: the role of the integration period. Each time bin represents the integrated spike counts in a single epoch of time centered around the apex of the animals’ breathing. “Full breathing cycle” represents a single ~600 msec bin encompassing the entire breathing cycle. +L|true (black circles) and –L|true (light grey squares) are the number of trials correctly identified as (+L) and (-L) odorant presentations, respectively. +L|false (dark grey inverted triangle) and –L|false (white diamonds) are the number of trials incorrectly classified as (+L) and (-L) odorant presentations, respectively. Points represent mean and bars represent standard error of the mean (N = 7 animals).
Figure 5b Percent of trials correctly and incorrectly classified based on the population density estimation: the role of the integration period. Each time bin represents the integrated spike counts in a single epoch of time aligned to the inhalation phase of the animals’ breathing. “Full breathing cycle” represents a single ~600 msec bin encompassing the entire breathing cycle. +L|true (black circles) and –L|true (light grey squares) are the number of trials correctly identified as (+L) and (-L) odorant presentations, respectively. +L|false (dark grey inverted triangle) and –L|false (white diamonds) are the number of trials incorrectly classified as (+L) and (-L) odorant presentations, respectively. Points represent mean and bars represent standard error of the mean (N = 7 animals).
Figure 6  Trial-by-trial discrimination of six odorants. Each point represent the absolute percent of trials of all six odorants correctly and incorrectly predicted based on the ensemble response from each animal. Randomized trials of all six odorants were analyzed together using population density estimation. Filled circles are the percent of odorant trials correctly predicted. Filled squares are the percent of trials incorrectly predicted (e.g. a (+L) trial predicted as any of the remaining five odorant trials). Triangles are the percent of trials in which there was not enough information in the ensemble response to make an odorant prediction, including trials in which there was an equal probability that the population density estimation represented any two of the six odorants and trials in which there was no response (zero spikes). Open circles and squares are the absolute percent of trials of all six odorants correctly and incorrectly predicted based on the ensemble response of temporally shuffled units and trials from each animal. Shuffling the trials for each unit in the ensemble is representative of pseudo-serially recording each unit response separately. The distributions of correct and incorrect responses from simultaneously recorded units and pseudo-serially recorded units were not significantly different. *Animals C, F, and G do not have data because only a subset of the odorants were delivered to these animals (see table 1).
Figure 7a  Distributed coding: dropping best units from the ensemble.  Starting with the full ensemble, each unit was sequentially removed to determine the unit that had the most detrimental effect on ensemble performance.  This unit is termed the “best” predictor unit and is subsequently removed from the ensemble.  The analysis is then repeated until only one unit is left in the ensemble.  Each point on the graph represents the ensemble performance at discriminating six different odorants starting with the full number of units in the ensemble.  The smooth degradation in ensemble performance with each subsequent removal of the best predictor neuron to the six-choice task is indicative of distributed coding.
Figure 7b Distributed coding: adding best units to the ensemble. The performance of an ensemble consisting of the absolute best predictor unit is plotted for discriminating trials of six odorants. Subsequent best predictor units were added to the ensemble to determine how fast ensemble performance improves until all available units have been added. The smooth increase in performance to asymptote indicates distributed coding amongst the ensemble at predicting trials of the six odorants.