Temporal structure of receptor neuron input to the olfactory bulb imaged in behaving rats

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Acknowledgments: We thank Alfredo Fontanini, Don Katz, and Howard Eichenbaum for advice with the behavioral paradigm, Rainer Friedrich for providing code for the deconvolution analysis, Kamal K. Sen for advice on coherence analysis, and members of the Wachowiak lab for comments on the manuscript. This work was supported by grants from the National Institute of Deafness and Communication Disorders and Boston University.

3Present address: The John B. Pierce Foundation, New Haven, CT, USA.

Disclosures: The authors state no conflicts of interest.
Abstract

The dynamics of sensory input to the nervous system plays a critical role in shaping higher-level processing. In the olfactory system, the dynamics of input from olfactory receptor neurons (ORNs) is poorly-characterized and depends on multiple factors, including respiration-driven airflow through the nasal cavity, odorant sorption kinetics, receptor-ligand interactions between odorant and receptor, and the electrophysiological properties of ORNs. Here, we provide a detailed characterization of the temporal organization of ORN input to the mammalian olfactory bulb (OB) during natural respiration, using calcium imaging to monitor ORN input to the OB in awake, head-fixed rats expressing odor-guided behaviors. We report several key findings. First, across a population of homotypic ORNs, each inhalation of odorant evokes a burst of action potentials having a rise-time of approximately 80 ms and a duration of approximately 100 ms. This rise-time indicates a relatively slow, progressive increase in ORN activation as odorant flows through the nasal cavity. Second, the dynamics of ORN input differs among glomeruli and for different odorants and concentrations, but remains reliable across successive inhalations. Third, inhalation alone (in the absence of odorant) evokes ORN input to a significant fraction of OB glomeruli. Finally, high-frequency sniffing of odorant strongly reduces the temporal coupling between ORN inputs and the respiratory cycle. These results suggest that the dynamics of sensory input to the olfactory system may play a role in coding odor information and that, in the awake animal, strategies for processing odor information may change as a function of sampling behavior.

Keywords: imaging, sniffing, coding, behavior, glomerulus, olfaction
Introduction

Olfactory information is initially represented by patterns of neural activity across a large and functionally diverse population of receptor neurons (ORNs). In mammals, the ORN population includes nearly 1000 functionally distinct cell classes defined by the particular odorant receptor protein they express, and ORNs – located deep inside the nasal cavity – are distributed across a physically complex space through which odorant molecules must flow in order to reach the receptor neuron. The axons of ORNs expressing the same odorant receptor converge onto one of two target glomeruli in the olfactory bulb (OB). Sensory neurons therefore present olfactory information to the brain in the form of stimulus-specific combinations of input to OB glomeruli. These patterns of glomerular input serve as the basis for all subsequent processing of olfactory information.

Odorant-evoked patterns of sensory input to OB glomeruli are temporally dynamic. First, the dynamics of ORN activation are strongly shaped by the inhalation of odorant into the nasal cavity and through the complex folds of tissue (turbinates) on which the olfactory epithelium lies (Schoenfeld and Cleland 2005). These dynamics are further shaped by the kinetics of odorant sorption into the mucus layer of the epithelium, a process which is dependent on intranasal flow rate and direction, odorant concentration, and odorant chemistry (Mozell and Jagodowicz 1973; Mozell et al. 1987; Scott-Johnson et al. 2000; Scott et al. 2000). Finally, the dynamics of ORN activation may be further shaped by receptor-ligand interactions, the transduction process, or intrinsic physiological properties of ORNs (Bobkov and Ache 2007; Hallem et al. 2004; Reisert and Matthews 2001). In anesthetized mice, ORN input to different glomeruli shows distinct temporal dynamics, with the same odorant eliciting input to glomeruli with different onset latencies, rise-times and decay kinetics (Spors et al. 2006). Thus, the fine-scale temporal pattern of sensory input to the OB may contribute to coding odor information.
In addition, the dynamics of sensory input to the OB likely plays a major role in shaping postsynaptic processing in the OB and beyond. Temporally-structured inputs linked to respiration are a key feature of most models of OB network function (Buonviso et al. 2006), and many experimental and computational studies have suggested that olfactory information is encoded in the precise timing of mitral cell spiking relative to the respiratory cycle (Chaput 1986; Hopfield 1995; Macrides and Chorover 1972; Margrie and Schaefer 2003; Schaefer and Margrie 2007). Finer-scale temporal dynamics are also likely important in OB processing: even within a single respiratory cycle, odorant-evoked postsynaptic activity shows latency and rise-time differences in different glomeruli (Spors and Grinvald 2002), and mitral/tufted cells (M/T cells; the principal OB projection neurons) show distinct temporal sequences of excitation and inhibition for different odorants and odorant intensities (Bathellier et al. 2008; Buonviso et al. 1992; Chaput et al. 1992; Meredith 1986; Wellis et al. 1989). In addition, synaptic interactions thought to be important in shaping odor representations – for example, dendrodendritic inhibition between mitral and granule cells – depend on the kinetics of sensory input to the OB (Arevian et al. 2008; Balu et al. 2004; Schoppa 2006a; Young and Wilson 1999). Finally, neurons in piriform cortex – a major target of M/T cells – show fine-scale temporal patterning that is likely shaped by the dynamics of ORN input (Rennaker et al. 2007).

Despite the importance of sensory input dynamics in odor coding and postsynaptic processing, little is known about the temporal structure of ORN input to the OB during natural sampling of odorants in the behaving animal. To date, the only recordings of odorant-evoked action potential firing in mammalian ORNs in vivo have used step pulses of odorant applied directly to the olfactory epithelium (Duchamp-Viret et al. 1999; Duchamp-Viret et al. 2000), revealing little about their natural response dynamics. In a series of recent studies we have used calcium imaging from the presynaptic terminals of ORNs to characterize sensory input to the OB during natural as well as artificial odorant inhalation (Pírez
and Wachowiak 2008; Spors et al. 2006; Verhagen et al. 2007; Wachowiak and Cohen 2001; Wesson et al. 2008a). The presynaptic calcium signals reflect action potential firing across the population of ORNs converging onto a glomerulus, as well as modulation of calcium influx into the axon terminal by presynaptic inhibition (Wachowiak and Cohen 1999; Wachowiak et al. 2005). These studies have revealed that ORN inputs are strongly shaped by the respiratory cycle and, even within a single inhalation-evoked response, show diverse temporal dynamics. These dynamics are not shaped by feedback presynaptic inhibition onto the ORN terminal (Pírez and Wachowiak 2008), and so primarily reflect complexity in the temporal patterns of odorant-evoked activation of ORNs. We also recently demonstrated that, in the awake rat, the strength and temporal structure of ORN inputs to the OB is dramatically altered during high-frequency sniffing of odorant (Verhagen et al. 2007). Here, we extend that study to provide a detailed characterization of the temporal structure of ORN inputs to the OB in the awake rat as it breathes naturally and expresses odor-guided behavior. We show that, during natural odor sampling, each inhalation evokes a volley of action potentials in ORNs whose activity envelope roughly matches that of odorant-evoked EPSPs recorded from mitral/tufted cells in vivo (Cang and Isaacson 2003; Margrie and Schaefer 2003); a significant fraction of glomeruli receive inhalation-evoked ORN inputs even in the absence of odorant. We also find that ORN inputs to individual glomeruli can be activated with distinct dynamics in an odorant-specific manner. Finally, we report that the coherence between the respiratory cycle and the dynamics of ORN input is greatly diminished during high-frequency sniffing, suggesting that the nature of postsynaptic processing in the OB may change qualitatively as a function of odorant sampling strategy.

**Materials and Methods**
Data were acquired from 7 adult female Long-Evans rats. Different analyses of data from a subset of these animals have been published in two earlier studies (Verhagen et al. 2007; Wesson et al. 2008a). Detailed descriptions of the surgical, behavioral, and recording procedures can be found in those studies.

**Head-fixed behavioral paradigm**

Naïve animals were outfitted with a head bolt for restraint (Katz et al. 2001) and an intranasal cannula for chronic measurement of respiration in a single surgical procedure. Behavioral training began 1 – 2 weeks after surgery, during which rats were shaped to perform a lick/no-lick odor discrimination task (Verhagen et al. 2007). The purpose of the discrimination paradigm was not to test perceptual ability, but mainly to ensure that the rat was attending to the stimulus and to minimize changes in behavioral state and stimulus context during data collection. In the final paradigm, odorants were presented for 4 – 5.5 s with an ITI varying randomly from 15 – 24 s. In 3 of 7 rats, a 1-s tone preceded odorant presentation by 2 sec. During the imaging experiments reported here, rats were used in a single daily session consisting of 50 – 140 trials (30 – 60 minutes). Rats were well-trained in discriminating most of the odorants presented. However, novel odorants or different concentrations of the same odorant were occasionally presented during an imaging session; in all cases the novel stimulus was presented as a CS−. Licking in response to a novel odorant occurred in approximately 20% of trials; rats were not rewarded for licking to the novel odorant.

Odorants were monomolecular hydrocarbon compounds known to reliably evoke ORN input to dorsal OB glomeruli (Bozza et al. 2004; Wachowiak and Cohen 2001). For some experiments, a four-component odorant mixture was used which contained benzaldehyde, eugenol, butyraldehyde, and ethyl butyrate (at a ratio of 2:10:1:1 to account for vapor pressure differences). The mixture was used to evoke input to a larger number of dorsal OB glomeruli; prior work has found little significant mixture
interactions at the level of ORN input to glomeruli, although mixture suppression may occur at the level of ORN spike generation (McGann et al. 2005; Pirez and Wachowiak 2008; Tabor et al. 2004; Rospars et al. 2008). Odorants were presented using a computer-controlled flow-dilution olfactometer that allowed precise control of odorant concentration, identity, and onset timing (Verhagen et al. 2007). Concentrations are reported as percent dilution of saturated vapor (s.v.), and ranged from 0.5 – 5% (1% s.v. was typical). Total flow rate of air presented to the animal was 0.5 – 2 L/min.

**Optical recordings**

ORNs were loaded with calcium-sensitive dye and optical signals imaged from the dorsal OB of awake, head-fixed rats as described previously (Verhagen et al. 2007; Wachowiak and Cohen 2001). Briefly, for ORN loading, rats were anesthetized with ketamine/xylazine (56mg/kg, resp. 6 mg/kg, i.p.) and placed on their back with the dorsum of the nose angled at 60º from horizontal, and an infusion canula was inserted 17 mm into one naris. 25 – 40 μl of a solution containing the dye (3% w/v) and 0.2% Triton X-100 (ICN Biomedicals Inc., Aurora, OH) was then infused into the naris and left for 10 min. The animal was then placed on its front for five minutes before repeating the procedure for the other naris. Imaging was performed through an optical window consisting of thinned bone sealed with ethyl-2-cyanoacrylate glue. Optical signals were recorded in select trials over 1 – 5 behavioral sessions. Signals were collected using an Olympus epifluorescence illumination turret (BX51) and full light from a 150W Xenon arc lamp (Opti-Quip) and appropriate filter sets (Verhagen et al. 2007). The imaged area covered a region of ~3 mm (ant-post) x 1.5 mm (med-lat) over one OB. Images were acquired using a 256 x 256 pixel CCD and digitized at 25 Hz along with respiration and behavioral (licking) signals using an integrated hardware/software package (NeuroCCD SM-256 and NeuroPlex, RedShirtImaging LLC).
Optical signals evoked by electrical stimulation of the olfactory nerve were recorded in 3 urethane-anesthetized rats, delivered as described previously (Pirez and Wachowiak 2008; Verhagen et al. 2007). The bone and dura overlying the dorsal OB were removed and a concentric bipolar electrode (OP: 125 μm SS, IP: 25 μm Pt/Ir; Frederick Haer &Co., Bowdoinham, ME) was placed in the anterior-medial region of the OB. Brief (100 μsec) constant current stimuli were delivered through a linear constant current stimulus isolator unit (Neurolog System, NL800A, Digitimer Ltd). Stimulus trains consisting of pulses of varying amplitude were generated using custom software written in LabView and sent to the stimulus isolator via an analog voltage output device (PCI-6722; National Instruments, Austin, TX). At least 4 trials were averaged for each stimulus protocol.

Data analysis

Respiration was measured using a chronic intranasal cannula attached to a pressure sensor (Honeywell, model# 24PCAFA6G). The opening of the cannula was fixed in the dorsal recess of the naris on one side of the animal. Intranasal pressure signals were high-pass filtered at 1 kHz and digitized and saved to disk at 100 – 500 Hz. Pressure decreases are displayed as positive voltages. The beginning of each inhalation was identified using a custom algorithm that involved filtering (2nd order Butterworth, 1 – 25 Hz) and integrating the raw pressure signal, then identifying inflections in the integrated signal that had a slope above an empirically-derived threshold (Wesson et al. 2008a). The algorithm was validated using simultaneous recordings of both intranasal pressure and airflow measured with a thermocouple (Verhagen et al. 2007).

Optical signals were processed to remove widespread intrinsic signals and movement artifacts, then regions of interest (ROIs) representing one or a few glomeruli were chosen and signals from each ROI were spatially averaged, as described previously (Verhagen et al. 2007). For most optical data,
signals were digitized at 25 Hz and upsampled to 100 Hz for analysis using the 'resample' function in Matlab (Mathworks, Natick, MA). This function effectively interpolates between sampled points using a polynomial waveform fit to the 10 samples around each time-point, and generates an interpolation that is close to the Nyquist-limited ideal. Thus, the temporal precision of these measurements was 10 ms but only included temporal frequencies below 12.5 Hz. For some data, optical signals were recorded at a frame rate of 50 Hz or 100 Hz; these data yielded quantitatively similar results to the data sampled at lower frequency (Verhagen et al. 2007). Optical data acquired during electrical stimulation experiments were sampled at 100 Hz. Temporal deconvolution of optical signals was performed using the algorithm of Yaksi and Friedrich (2006), as we have described previously (Verhagen et al. 2007). A decay constant of 260 ms (the mean decay time-constant of optical signals evoked by a single ON shock) was used and signals were low-pass filtered (4th-order Butterworth) at 15 Hz prior to deconvolution.

Response latency, rise-time and decay measurements were made using a custom algorithm that fit the optical signals to a double-sigmoid function (Wesson et al. 2008a). The goal of this algorithm was to allow objective and robust measurements of response timing and amplitudes. The algorithm is described in detail in Wesson et al. (2008). The general process was to first denoise the optical signal with a combination of band-pass filtering (2nd order Butterworth, 0.4 to 8 Hz) and wavelet decomposition-based denoising (4th order Daubechies wavelet decomposition), then to define the onset time (latency) of a response based on the time of the peak in the product of the first and second derivatives of the optical signal, which corresponds to a strong upward inflection in the signal waveform. Starting at this time, the denoised optical signal was fit, first to a sigmoid rise, and (when possible) a sigmoid fall; rise-times and response amplitudes were defined by the kinetics and peak of this fitted function. Some responses failed to yield full fits, generally because the response was “interrupted” by the next response in that glomerulus. When this occurred, the responses were not included in the dataset; thus, the number of measurements for
onset times, rise-times and durations could differ. A signal-to-noise ratio measure, defined as the fitted peak amplitude divided by the ‘noise’, was used to identify significantly-responsive ROIs (Wesson et al. 2008a). Here, noise was defined as the standard deviation of the peak response amplitude evoked by all inhalations made during periods preceding odorant presentations.

To compare optical signals elicited by patterns of electrical stimulation with those elicited by odorant inhalation in awake animals, an average odorant-evoked response was calculated. This analysis excluded glomeruli that showed large responses to inhalation alone and excluded all responses to inhalations with intersniff intervals < 400 ms. Individual traces were normalized to their maximum values and temporally aligned to their response onset times before averaging. For each electrical stimulation protocol, the average response of each glomerulus was compared to the odorant-evoked average by calculating the mean squared error (m.s.e.) between the two traces over the first 200 ms after response onset. Each stimulation-evoked response was scaled so as to minimize the difference between the waveforms; thus, m.s.e. values reported are comparable and independent of variation in response amplitude. To measure the variance in responses across different glomeruli and odorants, the average inhalation response for each glomerulus/odorant pair was calculated, and each of these traces was compared to the overall average using the m.s.e. technique.

Coherence between respiration and the presynaptic calcium signal was measured by first replacing each respiratory cycle in the presence of odorant with an identical, simplified waveform representing an average 'sniff'. The simplified waveform was made by averaging the first inhalation of each trial (n = 837; same as Fig 1C) over a time-window 250 ms from the start of each inhalation (defined as above) and then rectifying this average waveform. A simplified 'sniff trace' was then constructed by convolving the waveform with an event raster marking the time of each inhalation. Thus, the coherence analysis
effectively measured the coupling between inhalation timing and presynaptic calcium signals rather than respiration waveform.

All analyses were performed using custom software written in Matlab or LabView. Statistical tests were performed with Matlab.

Results

Odorant-evoked receptor inputs to the olfactory bulb of awake rats

We used calcium-sensitive dyes to image ORN input to glomeruli of the dorsal OB in awake, head-fixed rats while they performed a simple odor discrimination task. This preparation has been described previously (Verhagen et al. 2007). As noted previously, respiratory behavior was typical of unrestrained, quiescent rats in a familiar environment (Walker et al. 1997), occurring mostly within 1.5 – 2 Hz with occasional pauses and bouts of high-frequency sniffing. As we previously reported, rats typically (i.e., in most trials) showed little or no change in respiration frequency at the time of odorant onset, such that the mean frequency increase across all trials was less than 1 Hz (Verhagen et al. 2007). For the 3 rats in which a tone preceded odorant onset, the tone evoked similar slight (< 1 Hz) increases in respiration frequency; we did not observe any difference in performance accuracy or inhalation-evoked optical signals when the tone preceded odorant presentation (not shown, but see supplementary Figure 3, Verhagen et al. 2007).

Odorant-evoked presynaptic calcium signals in awake rats had peak signal amplitudes of 2 – 10% \( \Delta F/F \) and appeared as odorant-specific patterns of discrete foci corresponding to single glomeruli and, possibly in some cases, groups of several adjacent glomeruli that were not easily resolved (Figure 1A). While the basic characteristics of these signals have already been described (Verhagen et al. 2007), here
we focus on the detailed temporal structure of ORN inputs to the OB, with a particular emphasis on the potential for these dynamics to shape odor information processing in the awake rat.

Our initial analysis focused on the temporal structure of ORN inputs during low-frequency (1 - 3 Hz) respiration; in our head-fixed paradigm such respiration patterns are expressed during discrimination of familiar odorants. The example traces in Figure 1 illustrate the most prominent features of ORN inputs driven by resting-level respiration in the awake animal. First, ORN input to most (but not all) glomeruli is tightly coupled to respiration. Temporal deconvolution of these calcium signals (see Methods) to estimate patterns of action potential firing (Verhagen et al. 2007; Yaksi and Friedrich 2006) suggests that, during 1 – 3 Hz respiration, each inhalation-driven calcium transient in the presence of odorant reflects a burst of firing across the population of ORNs that lasts 100 – 200 ms. Second, there is variability in temporal response patterns across glomeruli, with some glomeruli (e.g., glomeruli 9 and 10, Figure 1B) showing more tonic responses during odorant presentation, with little or no modulation by individual inhalations. Close inspection reveals that the onset latency, rise-time, and time-to-peak of the inhalation-evoked input varies somewhat across glomeruli. Third, as we have previously reported (Verhagen et al. 2007), most glomeruli show little response adaptation for subsequent odorant inhalations – at least at respiration frequencies below 2.5 Hz. Fourth, input to some glomeruli occurs after each inhalation even in the absence of odorant (i.e., prior to odorant presentation). Finally, inhalation-evoked responses disappear or return to pre-odorant levels even in the first inhalation after odorant presentation ends (e.g., glomeruli 4 – 8, Figure 1), suggesting that odorant is cleared from the nasal cavity within one breathing cycle.

Figure 1C shows the mean response evoked by the first inhalation of odorant, averaged across all trials, glomeruli and odorants (n = 5776 responses, 837 trials, 7 animals). We used only the first inhalation after odorant presentation for this calculation to eliminate confounds from decaying responses evoked by preceding inhalations of odorant. To avoid confounds from delays in odorant onset kinetics and from
responses driven by inhalations beginning just prior to odorant onset, we eliminated all trials in which the first inhalation occurred within 30 ms of odorant onset (Wesson et al. 2008a). The consistency of this basic response waveform across successive inhalations of odorant is addressed in later analyses. The variance (standard deviation) in this initial response waveform is relatively small and does not increase in size relative to the baseline variance, indicating that the response to the first inhalation of an odorant is relatively stereotyped. Estimates of ORN firing by deconvolution were similarly stereotyped (Figure 1C, right) and had a half-width of 110 ± 29 (mean ± s.d.) ms. Thus, each initial inhalation of odorant evokes a burst of ORN input to a glomerulus lasting 100 – 200 ms (Figure 1C, E).

Odorant- versus inhalation-driven ORN inputs to glomeruli.

Respiratory patterning of postsynaptic neurons in the olfactory bulb (for example, mitral/tufted cells) can occur even in the absence of odorant stimulation (Adrian 1942; Chaput et al. 1992; Macrides and Chorover 1972; Rinberg et al. 2006a); whether this patterning is driven by input from ORNs remains an open question (Grosmaitre et al. 2007; Khan et al. 2008; Sobel and Tank 1993). We addressed this question by analyzing respiration-linked presynaptic calcium signals occurring prior to odorant presentation. As shown in the example in Figure 1, 'odorant-free' inhalations (i.e., before odorant presentation) drive ORN inputs to at least some dorsal OB glomeruli (e.g., glomeruli 1, 2, 3 and 7). Inhalation-driven responses were observed even when charcoal-filtered air was blown over the nose during this time-period. We evaluated the strength and extent of such responses in all dorsal glomeruli by generating ‘sniff-triggered average’ waveforms using the pre-odor time period for all trials in a session (one session in each of 7 animals) and for each glomerulus identified as responding to any tested odorant (see Methods). This method allowed for averaging of optical signals across many 'odorant-free' inhalations per glomerulus (range, 100 – 231 inhalations), maximizing the ability to detect even small
responses. As a control, we also generated average waveforms using an equal number of samples taken at random times (i.e., not inhalation-triggered) during the pre-odor period. A glomerulus was counted as showing a response to odorant-free inhalation if its sniff-triggered average waveform had a peak value that was 4 times greater than the standard deviation of the control trace (Figure 2A). Using this significance cutoff, we found that 50% of all glomeruli showed significant odorant-free responses; a greater fraction of glomeruli appear responsive to inhalation using a less conservative cutoff (Figure 2B).

The amplitude of odorant-free responses varied strongly across glomeruli, with responses in most glomeruli being much smaller than the amplitude of odorant-evoked inputs to the same glomerulus (Figures 2A, B). However, in each preparation, a few glomeruli showed strong odorant-free responses that were similar in amplitude to odorant-evoked responses (odorant-evoked response amplitudes were measured from all inhalations in the presence of odorant). A comparison of odorant-free and odorant-evoked response amplitude distributions is shown in the receiver-operating characteristic plot in Figure 2C. This plot shows, for example, that approximately 10% of glomeruli showed odorant-free responses that were above the 20th percentile of odorant-evoked response amplitudes. Thus, certain glomeruli appear to be much more strongly driven by odorant-free inhalation than others. We asked where on the dorsal OB the most strongly inhalation-driven glomeruli were activated by making maps of the pre-odor sniff-triggered average waveform. Examples from three preparations are shown in Figure 2D. Odorant-free inhalation-driven glomeruli were distributed across the dorsal OB, although in every preparation (n = 7), the most strongly-activated included several glomeruli in the same region of the anterior-medial dorsal OB (Figure 2D). Thus, while weak inhalation-driven ORN input appears to occur for many – and perhaps most – glomeruli in the absence of odorant stimulation, strong inhalation-driven input occurs for a few specific glomeruli.
Odorant- and glomerulus-specific dynamics of ORN input to glomeruli

Earlier work in anesthetized rodents has shown that the temporal dynamics of ORN inputs to glomeruli vary in a glomerulus- and odorant-specific manner (Spors et al. 2006). We observed similar variation in the dynamics of ORN inputs in awake rats, with inhalation-evoked calcium signals varying in latency, rise-time and duration, both for different glomeruli and for input evoked to the same glomerulus by different odorants. An example of this diversity is shown in Figure 3, in which responses for six glomeruli to three different odorants are shown. Four of these glomeruli are activated by all three odorants, while the remaining two glomeruli are activated by two of the odorants. Qualitative differences in the waveform evoked by each inhalation are clear (compare, for example, glomerulus 2 and 5 responses to benzaldehyde, and glomerulus 1 responses to methyl valerate and ethyl butyrate in Figure 3B). These differences appeared consistently across successive inhalations, as revealed by sniff-triggered averaging of each response in the presence of odorant (Figure 3C). The relative latency and fine-scale temporal structure does not appear to be determined by location on the dorsal OB surface; nearby glomeruli could show distinct response latencies and rise-times (compare glomeruli 3 and 4, response to ethyl butyrate, Figure 3), while distant glomeruli could show similar temporal response patterns.

To quantify the variance in the temporal dynamics of ORN inputs, we measured several parameters of the odorant-evoked calcium signal: response latency, rise-time and duration. Response latency (Figure 4A) was measured from the time of onset of the first inhalation in the presence of odorant to the time of onset of the calcium signal (defined using a custom algorithm, see Methods and (Wesson et al. 2008a)). Rise-time was defined as the time from 10 – 90% of the peak response amplitude (Figure 4B), and duration was defined as the time over which the response was greater than 50% of peak amplitude (Figure 4C). Peak amplitudes, rise-times and durations were determined by fitting responses to a double-sigmoid function (see Methods), as described previously (Wesson et al. 2008a). Measurements were made
for all responsive glomeruli and for all inhalations occurring at an intersniff interval of 400 ms (< 2.5 Hz). We also excluded all glomeruli showing large responses to inhalation alone (defined as > 10 times the s.d. of the baseline signal). For this dataset, mean response latency was 154 ± 59 (s.d.) ms (n = 18865 responses, 7 rats), rise-time was 91 ± 40 ms (n = 18865 responses), and duration was 392 ± 149 ms (n = 15172 responses). We also compiled these measurements as a function of inhalation number within each trial. All three parameters showed roughly similar mean values across successive inhalations, though response duration was slightly longer and response latency slightly shorter for the first inhalation (Table 1). Finally, we compared inhalation-evoked response dynamics across animals, using the 5 animals with the largest number of trials and responsive glomeruli (Table 2); again, response latency, rise-time and duration all showed similar values in different animals.

We next evaluated the diversity of these parameters first among individual glomeruli, then for individual glomeruli in the presence of different odorants. For each odorant in each imaging session, we selected the five glomeruli that responded most strongly (averaged across multiple inhalations), measured the latency and rise-time of all inhalation-evoked responses (there were approximately 4 – 8 inhalations per trial), and performed a two-way ANOVA for each measured parameter to test for a significant effect of glomerulus identity on both latency and rise-time, using a significance cutoff of p < 0.01. For all three odorants shown in Figure 3, glomerulus identity had a highly significant effect (p < 10^-4) on both latency and rise-time. Overall, 29/35 odorant groups (82.9%) showed a significant effect of glomerulus identity on latency, and 27/35 (77.1%) showed a significant effect of glomerulus identity on rise-time (grouped by odorant and imaging session; n = 35 groups from 5 animals, 10-69 inhalations/group). Thus, the dynamics of ORN input to the OB differs across glomeruli but remains reliable across successive inhalations. Similarly, we evaluated the effect of odorant on response onset and rise-times for each glomerulus. For this analysis, we selected 60 glomeruli (from 10 imaging sessions in 4 animals) that responded to at least
three odorants (at least 10 inhalations/odorant, > 400 ms ISI), and performed a separate two-way ANOVA for each of the latency and rise time parameters. Overall, odorant had significant (p < 0.01) effect on onset latency in 36% of glomeruli and on rise-time in 33%.

We also measured the temporal spread over which ORN inputs occur following an inhalation, defined as the time from the onset of the earliest-responding glomerulus to the time to 90% of peak for the latest-responding glomerulus in the same trial (Figure 4D). This value was calculated for each trial, and only included trials in which at least 5 glomeruli were activated by an odorant. The temporal spread of ORN inputs had a mean of 242 ± 72 ms (n = 1382 inhalations, 194 trials). Thus, glomerular response maps evolve for approximately 200 ms after each inhalation; this time may be important for defining temporal windows for postsynaptic integration during natural odorant sampling.

Finally, we compared response dynamics with those from urethane-anesthetized, freely-breathing rats. We measured rise-time, duration, and temporal spread in two rats (26 glomeruli; we did not compare onset latencies due to differences in respiration waveform in anesthetized and awake animals). Inhalation-evoked response waveforms were qualitatively similar (not shown), although rise-time, duration and temporal spread of responses were all slightly but significantly longer in anesthetized rats (p < 0.05, unpaired t-tests; see Table 2).

Temporal structure of ORN inputs evoked by inhalation

Understanding the temporal structure of action potentials arriving at the ORN axon terminals in a glomerulus is important for understanding postsynaptic information processing in the olfactory bulb. For example, a number of recent studies in OB slice preparations have revealed novel network phenomena that emerge during patterned electrical stimulation of ORN axons designed to mimic natural respiration (Balu et al. 2004; Hayar et al. 2004; Schoppa 2006b; Schoppa and Westbrook 2001). These studies have
used either single ON shocks or brief, 30 msec-long trains of isointensity ON shocks to mimic input evoked by a single ‘sniff’. However, our deconvolution estimates of the envelope of action potential firing (Figure 1C) suggested that an inhalation evokes a volley of ORN input lasting significantly longer (100 – 200 msec). We thus performed a series of experiments in which we imaged presynaptic calcium signals evoked by patterned electrical stimulation of ORN axons in the anesthetized rat and compared these responses to those evoked by odorant inhalation. The purpose of these experiments was twofold: First, to test the accuracy with which commonly-used electrical stimulus paradigms mimic inhalation-driven ORN input dynamics; and second, to verify the prediction from the deconvolution estimates that inhalation-driven inputs are spread over a relatively long (100 – 200 msec) time-window.

We tested several different ON shock patterns (Figure 5A) and compared response waveforms of the shock-evoked signal with those of the mean inhalation-evoked signal shown in Figure 1C. We focused in particular on the first 200 ms of each response, because this time-window appears to be critical for odor identification (Abraham et al. 2004; Rinberg et al. 2006b; Uchida and Mainen 2003; Wesson et al. 2008a) and because initial input dynamics are likely the most important determinants of postsynaptic activity patterns. Single (0.1 ms) ON shocks elicited calcium signals with an initial rise that was extremely rapid, reaching peak amplitude in 1 – 2 frames (i.e., less than 20 ms) after ON shock (n = 22 glomeruli, 2 animals). We also used a train of three pulses delivered at 100 Hz, as this protocol has been used to mimic naturalistic ORN inputs in several recent studies (Gire and Schoppa 2008; Schoppa 2006b; Schoppa and Westbrook 2001). This protocol elicited responses only slightly slower than that of the single-shock protocol (Figure 5A, pattern 2), with a time-to-peak of 20 - 30 ms (n = 13 glomeruli, 2 animals). We quantitatively compared initial response waveforms by calculating the mean squared error (m.s.e.) between individual response traces and the mean odorant-evoked response (shown in Figure 5B) over the first 200 ms after response onset (see Methods for details). As a measure of variance in response
dynamics across different glomeruli, the m.s.e. between the mean odorant-evoked response and that from individual glomerulus-odorant pairs was $0.0061 \pm 0.015$ (n = 374, 7 animals). Both the single-shock and three-shock response waveforms varied by several orders of magnitude from the mean inhalation-evoked waveform (m.s.e. = $0.157 \pm 0.037$ for pattern 1; m.s.e. = $0.109 \pm 0.021$ for pattern 2). Increasing the length of the stimulus train to 28 pulses at 100 Hz (Figure 5A, pattern 3) evoked presynaptic calcium signals that more closely approximated the inhalation-evoked response but still showed a significantly different initial response waveform (m.s.e. = $0.014 \pm 0.004$; n = 7 glomeruli, 2 animals; p = 0.0011, unpaired t-test).

The deconvolution estimates of inhalation-evoked responses suggest a more gradual activation of ORNs over the course of approximately 100 msec (see Figure 1C). We attempted to mimic this response by using a train of pulses delivered at different current intensities, with the goal of recruiting new subsets of ORNs over the course of the train, mimicking the presumed progressive activation of ORNs by odorant flux across the nasal epithelium. The train consisted of three stages: a low- but constant-intensity phase (3 pulses set to near- or just sub-threshold for evoking detectable responses with single shocks), a ramp phase (19 pulses) in which stimulus intensity increased with each pulse, and a final phase of 6 decreasing intensity pulses (Figure 5A, pattern 4). The most important feature of this protocol is that the ramp phase spans the range of recruitment of different ORNs innervating a glomerulus. Because we expected that the initial, low-intensity portion of the protocol would likely activate only a few ORNs, we used a protocol that was longer in total duration (280 msec) than our estimates of inhalation-evoked action potential bursts. This protocol was more effective than the simple train of iso-intensity shocks at eliciting an initial response waveform similar to that evoked by odorant inhalation (Figure 5B). The m.s.e. between pattern 4-evoked responses and the sniff-evoked response (m.s.e. = $0.0044 \pm 0.0019$; n = 9 glomeruli, 2 preparations) was an order of magnitude lower than that of the iso-intensity train and not significantly
different from the m.s.e. representing variance in odorant-evoked response profiles (p > 0.05, unpaired t-test). Overall these results agree with the temporal deconvolution estimates in suggesting that odorant inhalation evokes a “slow burst” of ORN firing whose envelope rises and falls over a time-window of at least 100 ms. In addition, while other stimulus patterns might be even more effective at mimicking inhalation-evoked calcium signals, these results suggest that the dynamics of ORN input are qualitatively different from that evoked by repeated, synchronous activation of ORNs using trains of isointensity nerve shocks.

Effect of odorant intensity on response maps and dynamics

In anesthetized mice, spatial patterns of glomerular input change dramatically as odorant concentration increases, with increasing numbers of glomeruli being activated and large (approximately five-fold) increases in the amplitude of the input signal (Bozza et al. 2004; McGann et al. 2005; Wachowiak and Cohen 2001). Studies visualizing patterns of glomerular activation using 2-deoxyglucose and fMRI imaging have reported less dramatic changes (Johnson and Leon 2000; Xu et al. 2000). Thus, we asked how odorant concentration shapes the magnitude and spatial pattern of ORN input in awake rats. Figures 6A and 6B show 'sniff-triggered average' maps and example traces of responses to increasing concentrations of the odorant benzaldehyde. In total we tested four odorants (two different animals) at a minimum of three concentrations spanning a range of at least one log unit. All measurements were made from the first inhalation of odorant and taken from trials in which the odorant was not novel and during low-frequency (1 -3 Hz) respiration. In all, increasing concentration increased the number and spatial distribution of glomeruli receiving odorant-evoked ORN input – a result similar to that seen in anesthetized mice (Bozza et al. 2004; McGann et al. 2005; Wachowiak and Cohen 2001). Increasing concentration also, on average, increased the magnitude of the inhalation-evoked response (Figure 6C),
although the relationship between concentration and magnitude often appeared non-monotonic and differed for different glomeruli. Interestingly, moderate changes in concentration of a familiar odorant typically did not evoke the same high-frequency exploratory sniffing as did novel odorants (not shown).

We next asked how odorant intensity affected response dynamics, focusing on the latency and rise-time of odorant-evoked responses. Again, we restricted the main analysis to the first inhalation after odorant onset in each trial and to trials in which animals were breathing at frequencies below 3 Hz. We included only those glomeruli that responded to all tested concentrations. A particular odorant concentration was presented in multiple trials and the parameter was averaged across trials (2 – 6 trials per measurement). The most consistent effect of increasing odorant concentration was a slight decrease in response latency and rise-time (on average, a decrease of ~20 – 30 ms in both parameters over a ten-fold increase in concentration; Figures 6D, E). Thus, compared to the relative increase in response amplitude, the effect of increasing odorant concentration on the initial dynamics of the odorant-evoked response was modest.

**Effect of frequency on the temporal structure of ORN inputs**

In active rodents, respiration rarely occurs at regular low rates but instead varies across a frequency range of 2 to 10 Hz (Kepecs et al. 2007; Macrides 1975; Verhagen et al. 2007; Welker 1964; Wesson et al. 2008b; Youngentob et al. 1987). We recently reported a strong effect of sniff frequency on the nature of ORN inputs to the OB, but focused in that study on magnitude effects (Verhagen et al. 2007). Here, we focus on the effect of sniff frequency on the temporal structure of ORN inputs – in particular the coupling of inputs to the respiratory cycle. As in our previous study (Verhagen et al. 2007), we used presentations of novel odorants to evoke sustained high-frequency (typically, 4 – 7 Hz) investigative sniffing of an odorant, and compared responses to those evoked during low-frequency
sampling of the same odorants in subsequent trials. We limited this analysis to data from 4 animals showing responses in the largest number of glomerulus/odorant pairs.

Figure 7A shows an example of optical signals measured from one glomerulus during low- and high-frequency sampling of the same odorant (ethyl butyrate). While low-frequency sampling evokes inhalation-coupled responses that return rapidly to baseline, high-frequency sniffing (elicited by the first presentation of this odorant earlier in the session; (Verhagen et al. 2007)) evokes sustained responses that show no clear coupling to respiration. Importantly, the degree to which respiration-locked responses were lost during high-frequency sniffing was odorant-specific and was independent of the magnitude of the initial response. Figure 7B shows an example of optical signals imaged from the same glomerulus as in Figure 7A, but evoked during low- and high-frequency sampling of a different odorant (valeric acid). Despite the fact that the initial response to this odorant is as large as that evoked by ethyl butyrate, individual inhalations during the high-frequency bout still elicit clear responses. Thus, the loss of respiration-linked responses during high-frequency sniffing is not due to response saturation or an inability of the calcium-sensitive dye to follow high-frequency inputs; instead this effect appears to reflect odorant-specific differences in the dynamics of ORN activation at different sampling frequencies.

To quantify the coupling between presynaptic calcium signals and respiratory cycle, we measured the coherence between the optical and respiratory signals. The coherence function is a continuous function indicating the correlation between these two signals as a function of frequency. In this analysis we were concerned with the relationship between ORN responses and the occurrence of an inhalation rather than its waveform. To avoid confounds in the coherence measurement caused by variation in sniff waveform, we constructed simplified ‘sniff traces' that consisted of the same waveform representing an average 'sniff’ repeated at the time of each inhalation in the raw intranasal pressure signal (see Methods for details). The relationship between the measured pressure signal and the simplified sniff trace is shown
graphically in Figure 7A, B. For trials in which rats showed only low-frequency respiration (defined as all inhalations occurring at a minimum interval of 400 ms, or less than 2.5 Hz), a coherence function was calculated using the time period from the second inhalation after odorant onset to the last one before the end of odorant presentation (using the second inhalation avoided confounds caused by the initial response to odorant, which is independent of frequency). For trials showing high-frequency sniffing (defined as 9 s per 2 sec time-window, or 4 Hz average frequency), coherence was calculated from the second sniff after odorant onset to either the end of the high-frequency sniff bout or the end of odorant presentation, whichever came first. We defined the degree of coupling between the respiratory cycle and optical signals as the value of the coherence function at the mean respiration frequency in a given trial. As expected, mean respiration frequency had a corresponding peak in the power spectrum of the simplified trace and, for low-frequency trials, the optical signal trace (Figure 7C) (other peaks in the power spectra reflect high-frequency components of the sniff and optical signal waveforms). The coherence function also showed a peak at or near the mean respiration frequency, both for low- and high-frequency trials (Figures 7C, D).

We measured coherence with respiration in this way using trials from 4 sessions (24 high-frequency sniffing trials, 380 low-frequency trials, 4 animals) and including all glomeruli showing an initial odorant-evoked response having a signal-to-noise ratio greater than 4 (measured from the first odorant-evoked response during low-frequency respiration and calculated as described in the Methods). On average, the coherence between respiration and the presynaptic calcium signal was higher for low-frequency trials (0.53 ± 0.23, n = 2755) than for high-frequency trials (0.31 ± 0.22, n = 140; p < 1×10^{-27}, unpaired t-test). Comparing mean coherence for low- versus high-frequency sampling of the same odorant in the same glomerulus (Figure 7E) revealed that nearly all glomeruli showed frequency-dependent decreases in coherence (p < 2×10^{-19}, paired t-test, n = 109). The magnitude of this effect varied, however
across glomeruli and odorants, as already described for the glomerulus shown in Figure 7A. Overall, there was no significant relationship between the decrement in coherence with respiration and the magnitude of the response evoked by low-frequency sampling of the odorant (Figure 7F; Pearson’s linear correlation coefficient $\rho = -0.076$, $p = 0.43$, $n = 109$, testing non-zero correlation), nor was there a significant relationship between the change in coherence and average response duration for a given glomerulus and odorant (Figure 7G; $\rho = -0.154$, $p = 0.17$, $n = 83$). Thus, loss of respiratory patterning of ORN inputs is a robust phenomenon, but one which shows stimulus- and glomerulus-specific variations in magnitude.

**Discussion**

Here, we have characterized in detail the nature of sensory input to the olfactory bulb of awake rats during natural odor sampling, with a particular focus on the temporal structure of this input. These results, together with our earlier reports of ORN inputs in the awake rat (Verhagen et al. 2007; Wesson et al. 2008a), constitute the first description of how mammalian olfactory sensory neurons represent odorant information during natural respiration. Previous studies have characterized ORN response properties *in vivo* by continuously applying odorant to the olfactory epithelium (Duchamp-Viret et al. 1999; Duchamp-Viret et al. 2000), or by measuring electroolfactogram (EOG) signals (which reflect summed receptor potentials from ORNs) from the epithelium in awake or anesthetized animals (Chaput 2000; Chaput and Chalansonnet 1997). The most elementary results of this study are 1) that ORN inputs to the OB are strongly patterned by respiration; 2) that inhalation even in the absence of odorant evokes significant input to many OB glomeruli; 3) that these inputs are temporally diverse, with odorants evoking different temporal patterns of input to different glomeruli and maps of glomerular activation evolving over time; and 4) that changes in odorant sampling behavior can qualitatively change the temporal structure of ORN responses.
These results largely agree with those obtained from anesthetized mice during free respiration and artificial inhalation (Spors et al. 2006). In particular, odorant-evoked presynaptic calcium signals in anesthetized mice are tightly coupled to inhalation, show glomerulus-and odorant-specific temporal dynamics over a range of approximately 200 msec, and show a loss of temporal patterning at high (> 4 Hz) artificial inhalation frequencies (Spors et al. 2006). One difference, however, is the appearance of ORN inputs driven by inhalation alone; we have consistently failed to observe inhalation-driven calcium signals in anesthetized mice, whether freely-breathing or using artificial inhalation (Pírez and Wachowiak 2008; Spors et al. 2006; Wachowiak and Cohen 2001). Nonetheless, experiments in both awake rats and anesthetized mice indicate that primary sensory input to the olfactory system is temporally complex, and that this complexity is shaped both by intrinsic (e.g., odorant-receptor interactions) and extrinsic factors (e.g., respiration).

Extrinsic (respiration-driven) determinants of ORN input dynamics

The fact that ORN activation is strongly coupled to the respiratory cycle is itself not surprising. Indeed, at least in rodents, inhalation is required for odorant molecules to reach the olfactory epithelium and activate ORNs (Wesson et al. 2008a), and EOG signals in freely-breathing rats are – like the presynaptic calcium signals imaged here – phasic and strongly coupled to respiration (Chaput 2000; Chaput and Chalansonnet 1997). Strong respiratory patterning of ORN input is also consistent with the fact that OB projection neurons (M/T cells) often show spiking patterns that are coupled to respiration (Bathellier et al. 2008; Chaput et al. 1992; Khan et al. 2008; Macrides and Chorover 1972; Sobel and Tank 1993). Several features of the inhalation-driven signals measured here, however, are surprising and relevant for understanding information processing dynamics in the olfactory system.
First, inhalation-evoked bursts of ORN input develop over a relatively long time-window. The rise-time \( (t_{10} – t_{90}) \) of the inhalation-evoked calcium signal – while variable (discussed below) – was approximately 85 ms; this value is similar to the rise-time of odorant-evoked EPSPs in M/T cells of anesthetized, freely-breathing rats (Cang and Isaacson 2003; Margrie and Schaefer 2003). We could not reproduce the inhalation-evoked calcium signal waveform using simple trains of isointense electrical stimuli; instead it was necessary to use ramps of increasing stimulus intensity during the train; such a stimulus pattern presumably recruits new subpopulations of afferents over the course of the train. This result suggests that odorant inhalation results in a progressive recruitment of activation of ORNs over a time-window of at least 80 – 100 msec. This time-window may be even longer, as suggested by the fact that the patterned stimulus train effective at evoking realistic rise-times was 280 msec long, with a phase of increasing stimulus intensities lasting 180 msec (see Figure 5). Such recruitment kinetics are consistent with odorant reaching ORNs expressing the same receptor in different regions of the OE or having different odorant sensitivities.

Second, inhalation-evoked bursts of ORN input appear to cease rapidly after inhalation ends. Patterned electrical stimulation having a ‘decay’ phase of only 60 msec after peak stimulus intensities are reached produced response waveforms with decay times similar to that of most inhalation-evoked responses (see Figure 5). Finally, only approximately 10% of glomeruli showed response durations greater than 600 ms (see Figure 4). EOG recordings from freely-breathing rats also show a rapid termination of this signal after the inhalation phase of respiration (Chaput 2000; Chaput and Chalansonnet 1997), suggesting that the decay of the presynaptic calcium signal reflects a true cessation of odorant responsiveness in ORNs. Whether the transient nature of the ORN response is due to rapid clearance of odorant from the receptor site or rapid adaptation of ORNs – as has been demonstrated in vitro (Reisert and Matthews 2001) – remains to be determined.
The relatively slow activation of ORN populations during natural odorant sampling has important consequences for models of synaptic processing in the olfactory bulb. Many of the characteristic features of the olfactory bulb network have been described using single ON shocks to drive postsynaptic activity (reviews: (Schoppa and Urban 2003; Shipley and Ennis 1996; Wachowiak and Shipley 2006)). However, recent studies have revealed that novel response features emerge in this network when afferent inputs begin to approximate those elicited by natural odor sampling. For example, brief trains of ON shocks repeated at frequencies in the range of natural sniffing result in amplification of M/T cell responses, glomerulus-specific synchronization of action potential firing in M/T cells and external tufted cells, increased temporal precision of mitral cell firing, and the emergence of gamma-frequency oscillations in mitral cell membrane potential (Balu et al. 2004; Hayar et al. 2004; Schoppa 2006b; Schoppa and Westbrook 2001). It will thus be interesting to test for the emergence of these and perhaps additional network phenomena using stimulus protocols that evoke more naturalistic, progressively-increasing (i.e., 50 – 100 ms rise-time) inputs to the OB. Likewise, using realistic input dynamics to drive biophysical and network models of the OB (David et al. 2007; Rubin and Cleland 2006) may yield insights into dynamical mechanisms of odor information processing.

We found that inhalation alone evoked detectable ORN input to a large fraction (at least 50%) of dorsal OB glomeruli. This result provides the first direct evidence that ORN input to the olfactory bulb can be driven solely by inhalation in vivo. We found that the magnitude of inhalation-evoked input to most glomeruli was a fraction of that evoked by odorant stimulation; at the same time we found that inhalation evoked large-magnitude inputs to a few glomeruli. These latter glomeruli may receive input from ORNs that are highly sensitive to odorants emitted by the animal. Our results are also consistent with the hypothesis that at least some ORNs are sensitive to mechanical stimulation associated with intranasal airflow: mechanosensitivity to pressures approximating those generated during respiration has
been demonstrated for ORNs in vitro (Grosmaître et al. 2007), and electrophysiological recordings from OB neurons have provided indirect evidence in support of this hypothesis in vivo (Onoda and Mori 1980; Ueki and Domino 1961; Walsh 1956).

The role that ORN inputs driven by inhalation alone play in shaping respiratory patterning of postsynaptic activity in the OB remains controversial. While many studies have reported respiratory patterning of postsynaptic activity in the absence of odorant (Adrian 1942; Chaput et al. 1992; Macrides and Chorover 1972; Rinberg et al. 2006a), others have found no such patterning or observed patterned responses in some OB neurons but not others (Sobel and Tank 1993; Walsh 1956). The fact that different glomeruli appear to receive differing magnitudes of inhalation-driven ORN input may provide one explanation for these conflicting results. In addition, if ORNs are in fact driven by mechanical stimulation, then even slight differences in inhalation parameters – for example, as a result of anesthesia – may lead to large differences in the degree to which inhalation drives ORN inputs. We speculate that, in the awake animal, inhalation-driven afferent input and centrifugal fibers reflecting the respiratory rhythm may interact to shape the temporal patterning of postsynaptic OB neurons in a manner that changes dynamically with sampling behavior.

Intrinsic variability in ORN input dynamics

In addition to the temporal structure imposed on ORN inputs by the respiratory rhythm, we found that ORN inputs showed response dynamics that varied according to the glomerulus they innervated, odorant identity, and – to a lesser degree – odorant concentration. These dynamics occurred mainly as differences in the latency, rise-time, and duration of the burst of ORN input evoked by a single inhalation. Temporally diverse presynaptic calcium signals likely reflect 'intrinsic' variability in the temporal patterns of odorant- and respiration-evoked action potential firing across populations of ORNs converging onto
different glomeruli, rather than temporal shaping of calcium influx by presynaptic inhibition onto the ORN terminal. This conclusion is supported by the fact that, in anesthetized mice, presynaptic inhibition affects the magnitude of ORN inputs but does not contribute significantly to their dynamics, nor does it contribute to the attenuation and loss of respiratory patterning of calcium signals during high-frequency artificial sniffing (Pírez and Wachowiak 2008). Thus, temporally diverse presynaptic calcium signals likely reflect odorant-specific differences in the kinetics of odorant access to the ORNs as well as ligand/receptor interactions.

Dynamic odor representations following a single inhalation may play a role in coding odor information in the awake animal (Schaefer and Margrie 2007). We found that the intrinsic dynamics of ORN input to glomeruli varied reliably across glomeruli and across multiple samples of odorant, consistent with this hypothesis. In addition, rats and mice are capable of performing simple odor discriminations in as little as 160 – 200 ms after an inhalation (Abraham et al. 2004; Rinberg et al. 2006b; Uchida and Mainen 2003; Wesson et al. 2008a), a time at which only a fraction of ORN inputs to glomeruli have become fully-activated (Wesson et al. 2008a). However, for more difficult discriminations, mice and rats take up to 200 ms longer (Abraham et al. 2004; Rinberg et al. 2006b), a time which roughly matches the temporal spread over which odor representations develop in the present study (242 ± 72 ms). Thus, the intrinsic variability in the dynamics of inhalation-evoked ORN inputs to the OB may set an upper limit on the time-window for integration of odor information in the behaving animal.

Sniff frequency and odor information processing in the OB

Odorant sampling frequency had a strong impact on the temporal structure of sensory input to the OB, reducing the degree to which ORN inputs are linked to the respiratory rhythm. While the magnitude
of this effect was variable for different glomeruli, some loss of respiratory patterning occurred for nearly all glomeruli, and was independent of the magnitude of odorant-evoked input to a glomerulus. These results are qualitatively similar to those reported previously in anesthetized mice using artificial inhalation at different frequencies (Spors et al. 2006). As with the temporal dynamics of responses evoked by a single inhalation, the effect of high-frequency sniffing was – to a degree – odorant-specific, with ORN input to the same glomerulus showing different amounts of coherence loss when evoked by different odorants, even when these odorants evoked similar-magnitude responses (e.g., Figures 7A, B, and F). It is unlikely that the frequency-dependent loss of patterning simply reflects inability of the calcium-sensitive dye to follow high-frequency inputs. Instead, this effect likely reflects the kinetics of odorant flow within the nasal cavity and/or the kinetics of receptor/ligand interactions. Limitations on bulk flowrates through the complex nasal cavity may particularly limit the degree to which odorant levels are modulated during high-frequency respiration (Yang et al. 2007); indeed, recent hydrodynamic modeling data suggest that odorant concentrations show significant tonic components during high-frequency sniffing (Zhao and Jiang 2008).

A change in the temporal structure of sensory input from phasic and respiration-locked to predominately tonic may substantially alter the postsynaptic processing of olfactory information. For example, the synapse between ORNs and second-order neurons is subject to strong activity-dependent depression due to feedback presynaptic inhibition and vesicle depletion, and postsynaptic AMPA receptors show rapid desensitization (McGann et al. 2005; Murphy et al. 2004; Wachowiak et al. 2005). Thus, the overall strength of synaptic coupling between ORNs and M/T cells may be suppressed by tonic ORN activation. Second, recurrent inhibition between mitral and granule cells enhances the temporal precision of mitral cell firing when excitatory inputs to mitral cells themselves are temporally patterned (Balu et al. 2004; Schoppa 2006b), but modeling and experimental studies suggest that this precision is
reduced or lost when inputs are temporally dispersed or occur tonically (Balu et al. 2004; David et al. 2007). Thus, M/T cell responses may become largely decoupled from the respiratory cycle – as well as less synchronized with each other - during high-frequency sniffing. Indeed, such a loss of respiratory patterning in M/T cell responses has been observed during high-frequency sniffing in awake, freely-moving rats (Bhalla and Bower 1997; Kay and Laurent 1999; Pager 1985). It will be interesting to test these hypotheses by systematically examining the effect of sampling frequency on both the dynamics and odorant response specificity of M/T cells.

Finally, frequency-dependent changes in the temporal structure of ORN input may alter odor coding strategies in the OB. While respiration-related patterning of OB activity has the potential play an important role in odor coding at low, regular frequencies (i.e., 1 to 3 Hz) (Chaput 1986; Hopfield 1995; Macrides and Chorover 1972; Margrie and Schaefer 2003; Schaefer and Margrie 2007), sniffing behavior in the freely-moving animal is both aperiodic and higher in mean frequency (Bhalla and Bower 1997; Kay and Laurent 1999; Wesson et al. 2008b). The loss of respiratory patterning we observe under these conditions suggests that this respiratory phase-related encoding of odor information might not be possible during active odor-guided behaviors (Kay and Laurent 1999). Instead, alternate strategies may be implemented.
References


Schoppa NE. AMPA/kainate receptors drive rapid output and precise synchrony in olfactory bulb granule cells. *J Neurosci* 26: 12996-13006, 2006a.


Figure legends

Figure 1. Sensory input to olfactory bulb glomeruli is tightly coupled to respiration.
A. Left: Resting fluorescence from the dorsal OB of an awake rat in which ORNs were loaded with calcium-sensitive dye. Right: ‘Sniff-triggered average’ map of inhalation-evoked fluorescence changes evoked by inhalation of the odorant 2-hexanone. The map is the average of 37 ‘sniffs’ in 10 trials. Scale bar, 500 μm.
B. Left: Respiration (‘sniff’, red trace) and ORN presynaptic calcium signals (black traces) from the ten glomeruli shown in (A) during a presentation of 2-hexanone. In most glomeruli, signal activation is linked to respiration and increases in the presence of the odorant. Some glomeruli show responses before odorant presentation. Vertical lines indicate the onset time of each inhalation. Right: Same data but with the calcium signals temporally deconvolved to give estimate temporal patterns of action potential firing during the trial (see Methods).
C. Left: Average calcium signal waveform (left) evoked by odorant inhalation. Black trace shows the mean of all responses evoked by the first sniff after odorant presentation began, averaged across all trials regardless of odorant and glomerulus (data were only included if there was a minimum of 400 ms between the first and second sniffs, N = 837 trials, 5776 sniff responses). Responses were normalized to their peak amplitude and aligned to the start of inhalation before averaging. Gray area indicates the standard deviation of the mean response waveform. Red trace shows the average sniff waveform. Right: Average deconvolved waveform, using the same dataset.

Figure 2. ORN input to some glomeruli is driven by inhalation even in the absence of odorant.
A. Comparison of sniff-evoked optical signals before (red) and during (black) odorant presentation for the ten glomeruli shown in Figure 1. Single black traces show responses to the first inhalation of 2-hexanone
(same data as in Figure 1B). Red traces show sniff-triggered average signals for all sniffs occurring before odorant presentation in all trials in that session (n = 35 trials, 128 sniffs). Also shown are ‘randomly-triggered’ average traces made by random selection of signal epochs during the pre-odor phase (see Results). Shaded areas indicate s.e.m. of the average traces. Asterisks (*) indicate glomeruli for which the sniff-triggered response was significantly greater than the randomly-triggered control (see Results).

B. Histogram and cumulative probability distributions of sniff-triggered response amplitudes of glomeruli before (red) and during (black) odorant presentation. Odorant-evoked response amplitudes were averaged across all sniffs for each odorant, and only the largest response (i.e., the most effective odorant) was used for that glomerulus. Amplitudes are expressed relative to the standard deviation of the randomly-triggered control for that glomerulus. Only data from the three animals with the highest number of tested odorants were included (n = 194 trials). Histogram bin size, 2 s.d..

C. Receiver-operating characteristic curve comparing the distributions of responses (taken from (B)) evoked before (ordinate) and during (abscissa) odorant presentation. Each axis is the fraction of all glomeruli with responses below a particular amplitude cutoff, ‘a’. The dotted lines compare the distributions at an arbitrarily-chosen cutoff value of 0.25 of all odorant-evoked response amplitudes, showing that approximately 90% of glomeruli show pre-odorant responses smaller than this value.

D. Location of inhalation-activated glomeruli in three different animals. Sniff-triggered maps were generated from all sniffs taken before odorant presentation. In each preparation, a cluster of glomeruli in the anterior-medial dorsal OB was most strongly activated by inhalation, although there were glomeruli throughout the dorsal OB that also responded strongly.

Figure 3. Temporal dynamics of ORN inputs differ across glomeruli and between odorants.
A. Sniff-triggered average response maps for three different odorants eliciting input to different but overlapping ensembles of dorsal OB glomeruli. Scale bar, 500 um.

B. Traces showing optical signals in six glomeruli (locations shown in (A)) for one trial for each of the three odorants. The inset in the ethyl butyrate trial shows an expansion of the gray shaded area, showing consistent response dynamics repeated with successive sniffs of odorant.

C. Sniff-triggered average traces for the 6 glomeruli for each odorant, showing that odorant- and glomerulus-specific response dynamics are consistently evoked across sniffs. Shaded areas indicate s.e.m. of each averaged trace. Vertical line indicates the start of inhalation to which each response was aligned prior to averaging.

Figure 4. Summary of ORN input dynamics for all glomeruli and odorants.

A. Histogram and cumulative probability plot of the latency of the optical signal following an inhalation. In this and other panels, the schematic above each histogram illustrates the parameter measured (arrows), using a sample sniff trace (bold) and sample optical response (thin trace). Parameter measurements are based on only the first sniff after the onset of odorant presentation, pooled across animals, sessions, odorants, trials, and glomeruli. Time bin is 10 ms.

B. Histogram and cumulative probability plot of the rise time of the optical signal response ($t_{10}$ to $t_{90}$ of the peak response amplitude). Time bin is 10 ms.

C. Histogram and cumulative probability plot of the duration of the optical signal responses (time during which the response is above 50% of peak amplitude). Time bin is 50 ms.

D. Histogram and cumulative probability plot of the temporal spread of optical signal responses from all glomeruli in a trial. Temporal spread is the time from the onset of the earliest glomerular response in a
trial to the time when all glomeruli have reached 90% of their peak amplitudes. Only trials with at least 5 responsive glomeruli were included. Time bin is 10 ms.

Figure 5. Temporal dynamics of ORN input evoked by patterned electrical stimulation.
A. Traces showing the optical signal imaged from one glomerulus after in vivo electrical stimulation of the olfactory nerve (see Methods). Each trace shows the response to a different stimulus pattern (indicated below). Stimulus protocols consist of a single ON shock (“1”, purple), a train of 3 pulses at 100 Hz (“2”, green), a train of 28 pulses at 100 Hz (“3”, blue), and a train of 28 pulses but with patterned increases in intensity through the train (“4”, red). The arrow indicates the initiation of stimulation. Signals are averages of multiple (4 – 8) trials and are unfiltered. All traces are from the same glomerulus.
B. Comparison of the average shock-evoked responses (n for each protocol given in Text; same color-coding as in (A)) with the average inhalation-evoked odorant response in an awake rat (black with gray shaded s.d., reproduced from Figure 1C). All traces are aligned to the initiation of the calcium response following the stimulation (arrow) and normalized to the same peak amplitude. Pattern 4 best replicates the time course of a sniff-evoked odorant response to odorant. See Results for additional analysis.

Figure 6. Effect of odorant concentration on magnitude and dynamics of ORN inputs
A. Sniff-triggered average maps showing the optical response of the same preparation to four different concentrations of the odorant benzaldehyde. Maps are scaled to their own peak amplitudes (indicated below each map). Increasing odorant concentration recruits ORN input to new glomeruli with a modest increase in maximal response amplitude. Scale bar, 500 μm.
B. Respiration (red) and optical signals (black) recorded from the five glomeruli indicated in (A) while presenting three different concentrations of benzaldehyde. Glomeruli 1 and 2 are recruited by higher
concentrations; glomerulus 3 responses become more reliable, and glomerulus 4 responses appear to increase in duration at higher concentration. The noise in glomerulus 5 is due to its proximity to a blood vessel.

C. Concentration-response functions plotted for glomeruli in four concentration series (2 series in each of two animals). Each plot shows the averaged, normalized amplitude of all activated glomeruli in a series. Responses from individual glomeruli were averaged across multiple (2 – 6, typically 4) trials and normalized to the response at the highest concentration tested, then responses were averaged across all glomeruli in the series. Amplitude is measured from the first response following odorant onset.

Normalized response amplitudes to inhalation alone (“no odor”) are shown at the far left of the x-axis. The benzaldehyde series (top left, blue; N = 17 trials, 5 glomeruli) is the same preparation as from (A) and (B). Other odorants shown are hexanone (red; N = 6 trials, 11 glomeruli); methyl valerate (gray; N = 14 trials, 5 glomeruli); and a four-component mixture (see Methods) (green; N = 14 trials, 13 glomeruli). Note that x-axis units are percent saturated vapor.

D. Odorant concentration versus rise-time for signals imaged in response to the same four odorant concentration series shown in Figure 6C. Each plot indicates a different odorant series, with rise-times averaged across all glomeruli as in (C). Plot colors are the same as in (C). Only glomeruli showing responses with a signal-to-noise ratio > 4 at all concentrations were included.

E. Odorant concentration versus onset latency, averaged and plotted for each concentration series as in (D). Both rise-time and latencies show modest effects of concentration.

Figure 7. Effect of sniff frequency on respiratory patterning of ORN inputs.

A. Optical signals and respiration (“sniff”) measured during two presentations of ethyl butyrate. Right traces show a high-frequency sniffing response to ethyl butyrate (presented for the first time in this case);
left traces show response to low-frequency sampling of ethyl butyrate after habituation to the odorant. Gray area indicates region over which coherence with respiration was measured and includes the time of odorant presentation after the second inhalation of odorant. Bold gray trace shows the simplified sniff waveform used in the coherence measurements (see Results).

B. Optical signals and respiration during two presentations of a different odorant (valeric acid). The behavioral session and glomerulus imaged is the same as in (A). Valeric acid evokes an initial response with a similar amplitude to that evoked by ethyl butyrate, but the response shows less coherence loss during high-frequency sniffing.

C, D. Power spectra (left plots) and coherence functions for the data shown in A and B. The leftmost plot shows the power spectrum of the optical (black) and simplified sniff (gray) waveforms during the low-frequency trials. There are local maxima in both spectra at 1.54 Hz (the mean sniff frequency). The estimated magnitude squared coherence functions between the optical signal and simplified sniff are shown for both low- and high-frequency trials. Points indicate the coherence value at the mean sniff respiration for each trial. Coherence peaks near this frequency in all cases.

E. Scatter plot of coherence values for high versus low-frequency sniffing of the same odorant/glomerulus pair (n = 109 pairs). Coherence values were obtained from a single high-frequency trial and averaged across multiple low-frequency trials. Symbols correspond to the values obtained in the examples of (A) and (B).

F. Change in coherence from low- to high-frequency sniffing trials plotted as a function of initial response amplitude (measured during low-frequency sniffing), using the same data as in (E). Response amplitude is normalized to the maximal response observed in that glomerulus to any odorant, which reflects the intrinsic dynamic range of that glomerulus. There is no clear relationship.
G. Change in coherence as a function of response duration, using a subset (n = 83 pairs) of the data in (E) and (F). There is no clear relationship.
Table 1. *Temporal dynamics of inhalation-evoked optical responses across successive inhalations*

<table>
<thead>
<tr>
<th>Sniff</th>
<th>Duration</th>
<th>Latency</th>
<th>Rise time</th>
<th>Temporal spread</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>436 ± 158 (1879)</td>
<td>139 ± 50 (2591)</td>
<td>88 ± 38 (2591)</td>
<td>229 ± 72 (194)</td>
</tr>
<tr>
<td>2</td>
<td>392 ± 150 (2094)</td>
<td>152 ± 58 (2668)</td>
<td>89 ± 39 (2668)</td>
<td>243 ± 70 (189)</td>
</tr>
<tr>
<td>3</td>
<td>389 ± 146 (2060)</td>
<td>155 ± 58 (2518)</td>
<td>92 ± 39 (2518)</td>
<td>236 ± 68 (187)</td>
</tr>
<tr>
<td>4</td>
<td>394 ± 156 (1958)</td>
<td>159 ± 61 (2356)</td>
<td>94 ± 39 (2356)</td>
<td>252 ± 76 (168)</td>
</tr>
<tr>
<td>5</td>
<td>390 ± 145 (1981)</td>
<td>158 ± 60 (2397)</td>
<td>92 ± 41 (2397)</td>
<td>248 ± 73 (173)</td>
</tr>
<tr>
<td>6</td>
<td>398 ± 373 (1875)</td>
<td>158 ± 60 (2277)</td>
<td>92 ± 41 (2277)</td>
<td>247 ± 72 (172)</td>
</tr>
<tr>
<td>All</td>
<td><strong>392 ± 149 (15172)</strong></td>
<td><strong>154 ± 59 (18865)</strong></td>
<td><strong>91 ± 40 (18865)</strong></td>
<td><strong>242 ± 72 (1382)</strong></td>
</tr>
</tbody>
</table>

Values are means ± s.d. Number in parentheses indicates the number of inhalation-evoked responses for duration, latency, and rise time measurements, and the number of inhalations for temporal spread. See Text for definitions of temporal parameters.
Table 2. **Temporal dynamics of inhalation-evoked optical responses across animals**

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Duration</th>
<th>Latency</th>
<th>Rise time</th>
<th>Temporal spread</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Awake:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>rcr10</td>
<td>360 ± 123</td>
<td>177 ± 44</td>
<td>90 ± 37</td>
<td>191 ± 64</td>
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<td></td>
<td>(1456)</td>
<td>(1738)</td>
<td>(1738)</td>
<td>(120)</td>
</tr>
<tr>
<td>rcr20</td>
<td>361 ± 126</td>
<td>181 ± 47</td>
<td>82 ± 37</td>
<td>224 ± 68</td>
</tr>
<tr>
<td></td>
<td>(2679)</td>
<td>(3306)</td>
<td>(3306)</td>
<td>(239)</td>
</tr>
<tr>
<td>rcr21</td>
<td>401 ± 167</td>
<td>174 ± 50</td>
<td>93 ± 40</td>
<td>241 ± 59</td>
</tr>
<tr>
<td></td>
<td>(3631)</td>
<td>(4817)</td>
<td>(4817)</td>
<td>(365)</td>
</tr>
<tr>
<td>rcr36</td>
<td>406 ± 156</td>
<td>122 ± 61</td>
<td>86 ± 38</td>
<td>241 ± 76</td>
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<tr>
<td></td>
<td>(4459)</td>
<td>(5176)</td>
<td>(5176)</td>
<td>(374)</td>
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<td>rcr38</td>
<td>402 ± 137</td>
<td>138 ± 59</td>
<td>105 ± 43</td>
<td>282 ± 67</td>
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<tr>
<td></td>
<td>(2922)</td>
<td>(3791)</td>
<td>(3791)</td>
<td>(379)</td>
</tr>
<tr>
<td><strong>Anesthetized:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pooled</td>
<td>443 ± 119</td>
<td>-</td>
<td>122 ± 32</td>
<td>264 ± 50</td>
</tr>
<tr>
<td></td>
<td>(596)**</td>
<td></td>
<td>(675)**</td>
<td>(42)*</td>
</tr>
</tbody>
</table>

Values are means ± s.d. Number in parentheses indicates the number of inhalation-evoked responses for duration, latency, and rise time measurements, and the number of inhalations for temporal spread. For pooled anesthetized data, n = 2 animals. Response onset time was not calculated for anesthetized animals due to differences in the respiration waveform between anesthetized and awake animals. See Text for definitions of temporal parameters. Asterisks indicate statistical difference (t-test) between pooled anesthetized and awake measurements; **, p < 0.001; *, p< 0.05.
A

B

C

D

Prep. 1

Prep. 2

Prep. 3
A  
methyl valerate (1% s.v.)  
benzaldehyde (2% s.v.)  
ethyl butyrate (1% s.v.)

B  

sniff

1

2

3

4

5

6

methyl valerate  
benzaldehyde  
ethyl butyrate

C  

average sniff  
N = 3 trials, 17 sniffs  
N = 6 trials, 33 sniffs  
N = 8 trials, 34 sniffs
Carey et al., Figure 4

A. Latency: mean ± s.d. = 154 ± 59 ms
B. Rise time: mean ± s.d. = 84 ± 40 ms
C. Duration: mean ± s.d. = 392 ± 149 ms
D. Temporal spread of responses: mean ± s.d. = 242 ± 72 ms
Carey et al., Figure 5

A

B

average sniff

200 ms
A

benzaldehyde, 0.25% s.v. 0.5% 1% 2%

max = 2.4% ΔF/F  max = 2.6% ΔF/F  max = 2.9% ΔF/F  max = 2.7% ΔF/F

B

0.25% benzaldehyde

0.5% benzaldehyde

2% benzaldehyde

C

D

E

relative amplitude

concentration (% s.v.)

rise time (ms)

concentration (% s.v.)

latency (ms)

References