Why Sniff Fast? The Relationship Between Sniff Frequency, Odor Discrimination, and Receptor Neuron Activation in the Rat

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INTRODUCTION

Sniffing is a voluntary inhalation of air through the nose, typically expressed in the context of odor sampling. Most mammals studied—including humans (Johnson et al. 2003; Laing 1982; Porter et al. 2007; Sobel et al. 2000), dogs (Steen et al. 1996; Thesen et al. 1993), rats (Welker 1964; Youngentob et al. 1987), mice (Sorwell et al. 2008; Wesson et al. 2008b; Youngentob 2005), hamsters (Macrides 1975), rabbits (Freeman et al. 1983; Karpov 1980; Pager 1985) and even semi-aquatic shrews (Catania et al. 2008)—alter their sniffing behavior when investigating odors. Furthermore, rhythmic odor sampling behaviors analogous to sniffing are shown by terrestrial and aquatic mammalian vertebrates and invertebrates (Budick and Dickinson 2006; Goldman and Patek 2002; Nevitt 1991; Schmitt and Ache 1979). The contributions of these rhythmic odor sampling behaviors to the coding of odor information is poorly understood.

Studies in rodents have particularly found that sniffing is dramatically altered when performing operant odor-guided tasks. These studies have reported robust and precisely controlled bouts of high-frequency (4–10 Hz) sniffing when sampling odors. Given this, high-frequency sniffing is thought to play an important role in odor information processing. Here, we asked what role rapid sampling behavior plays in odor coding and odor discrimination by monitoring sniffing during performance of discrimination tasks under different paradigms and across different levels of difficulty and by imaging olfactory receptor neuron (ORN) input to the olfactory bulb (OB) during behavior. To eliminate confounds of locomotion and object approach, all experiments were performed in head-fixed rats. Rats showed individual differences in sniffing strategies that emerged during discrimination learning, with some rats showing brief bouts of rapid sniffing on odorant onset and others showing little or no change in sniff frequency. All rats performed with high accuracy, indicating that rapid sniffing is not necessary for odor discrimination. Sniffing strategies remained unchanged even when task difficulty was increased. In the imaging experiments, rapid sniff bouts did not alter the magnitude of odorant-evoked inputs compared with trials in which rapid sniffing was not expressed. Furthermore, rapid sniff bouts typically began before detectable activation of ORNs and ended immediately afterward. Thus rapid sniffing did not enable multiple samples of an odorant before decision-making. These results suggest that the major functional contribution of rapid sniffing to odor discrimination performance is to enable the animal to acquire the stimulus more quickly once it is available rather than to directly influence the low-level neural processes underlying odor perception.

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Mozell et al. 1991; Schmitt and Ache 1979; Youngetob et al. 1987), generating more distinct patterns of sensory neuron activation (Schoenfeld and Cleland 2006; Verhagen et al. 2007; Young and Wilson 1999; Youngetob et al. 1987), or controlling the magnitude of sensory input from background odorants (Verhagen et al. 2007). In addition, sniffing imposes a temporal structure on ORN input that shapes responses of postsynaptic neurons and may optimize the central processing of odor information (Kay 2005; Kepcs et al. 2006; Macrides 1975; Schaefer and Margrie 2007; Uchida et al. 2006). High-frequency sniffing of odorant may also alter the nature of postsynaptic processing in ways that facilitate odor identification, for example, by increasing the temporal precision or odorant specificity of olfactory bulb (OB) output neurons (Wachowiak and Shipley 2006; Young and Wilson 1999) or enabling the iterative encoding of odorant identity across multiple sniff cycles (Ambros-Ingerson et al. 1990). Only a few of these hypotheses have been tested directly in behaving animals (Kay 2005; Verhagen et al. 2007).

In this study, we attempted to achieve a better understanding of the role that rapid sniffing plays in odor discrimination by monitoring sniffing in rats performing discriminations in a head-fixed paradigm that eliminated confounds of movement and object approach and allowed for precise control over stimulus presentation. In a subset of these animals, we also imaged ORN input to the OB during task performance and asked whether rapid sniffing altered odorant-evoked inputs to OB glomeruli. We found that individual differences in sniffing strategies emerged during task learning but that even rats showing little or no change in sniff frequency performed odor discriminations accurately. Furthermore, we found that sniffing strategies remained unchanged even as task difficulty was increased to the point of discrimination failure, indicating that rats do not alter sniffing strategy to improve performance and implying that sniffing strategy does not substantially alter discriminability or detection threshold. Finally, we found that rapid sniffing nearly always preceded odorant-evoked ORN input to the OB and ceased as soon as inputs were activated. These results limit the role that rapid sniffing may play in directly shaping the low-level neural processes underlying odor discrimination and suggest instead that the main consequence of rapid sniffing is to increase the speed of odorant acquisition. Thus this behavior is likely strongly tied to internal factors such as anticipation and motivational state.

METHODS

Data were acquired from 12 adult female Long-Evans rats (Charles River Labs, Wilmington, MA) outfitted with a head bolt for restraint (Katz et al. 2001) and an intranasal cannula for chronic measurement of respiration. A different dataset from a subset of these animals (n = 7) has been published in earlier studies (Carey et al. 2009; Verhagen et al. 2007; Wesson et al. 2008a); all surgical and recording procedures are as described in those studies. These methods are briefly outlined again below.

Surgical procedures

Each rat was implanted with an intranasal (sniff) cannula and a stainless steel headbolt in a single surgical procedure using aseptic techniques under general anesthesia induced by isoflurane. Additionally, bupivicaine (~300 μl of a 1% solution; Sigma-Aldrich, St. Louis, MO) was injected into the epidermis overlying the frontonasal bone for local anesthesia. A midline incision was made, and the skull was cleaned using 3% H2O2. The headbolt was adhered to the skull with 0–80 stainless steel screws (Small Parts, Miramar, FL) and dental cement. As shown in Fig. 1A, a small hole was drilled unilaterally through the right nasal bone for cannula placement (position: 0 mm ANT frontal/nasal fissure, 1 mm LAT). A hollow cannula (C313G, Plastics One, Roanoke, VA) was cut to extend 2.3 mm from the pedestal, lowered into the hole, and fixed in place with dental cement. Rats were given carprofen (Rimadyl, 5 mg/kg, Pfizer) as an analgesic immediately before and for 4 days following surgery.

Head-fixed lick/no-lick tasks

All rats were initially trained on a simple lick/no-lick task structure. Behavioral training began 1–2 wk after head bolt and intranasal cannula surgery. Rats were water deprived to ~85% of baseline body weight [average deprived weight: 264.7 ± 4.9 (SD) g] and gradually habituated to restraint. During this phase of training, rats were allowed to lick for a small water reward (~20 μl) from a spout placed in front of their mouth at a 5- to 10-s intertrial interval (ITI).

After acclimation to restraint, one cohort of rats (n = 7; rats 1–7) was shaped to discriminate odorants in an “uncued odor” paradigm. In this paradigm (Fig. 2A), rats discriminated a rewarded odorant (CS+) from an unrewarded odorant (CS−) by licking the lick spout in response to the CS+ and refraining from licking to the CS−. In the final discrimination paradigm, odorants were presented for 4–5.5 s with an ITI varying randomly from 15 to 24 s. Notably, odorant onset was accompanied by auditory (solenoide valve opening) and somatosensory (change in air flow from the odor delivery tube) cues. Incorrect licking (false alarms) at any time during presentation of the CS− was punished with a 7-s increase in the following ITI. After the full training sequence, rats were tested in a single daily session (range: 50–140 trials; 30–60 min). A separate cohort of three rats (rats 8–10) was trained in a “cued odor” paradigm, with the same task structure and methods as outlined above. In this task, a 1-s, 7-kHz tone was presented starting 2 s before the odorant onset in each trial. The tone was presented starting in the first session of CS+ training and was presented throughout all further training and data collection.

Following training and data collection in the uncued-odor paradigm, some rats were used in further experiments to assess effects of perceptual difficulty and odor occurrence on sniffing behavior. In one experiment, we tested whether similar mixtures of odorants might affect sniffing behavior (rats 6 and 7). In this binary ratio mixture task (Abraham et al. 2004; Uchida and Mainen 2003), the compositions of the CS+ (0.025% isoamyl acetate) and CS− (0.0025% ethyl butyrate, matched in concentration to the experimenters perceived intensity) varied in their overlap across five different phases (phase 1 = most dissimilar, phase 5 = identical). In the first phase, the CS+ was a 100:0 mixture (isoamyl acetate:ethyl butyrate) and the CS− was 0:100 (ethyl butyrate:isoamyl acetate). Phase 2 was 80:20 (CS+) versus 20:80 (CS−). Phase 3 was 68:32 (CS+) versus 32:68 (CS−). Phase 4 was 56:44 (CS+) versus 44:56 (CS−). Finally, in phase 5, the mixtures were 50:50 compositions of both the CS+ and CS−. Testing occurred in order from phase 1 to phase 5, on which a recovery session was administered wherein the rats returned to the original phase 1 discrimination. Each daily session consisted of 60 trials of one phase, followed by 60 trials of the following phase, resulting in 120 trials/phase/animal. Finally, we tested the effects of odor concentration on sniffing frequencies by testing animals (rats 1–3) on four sequential dilutions of the CS+ (1.25% isoamyl acetate) and CS− (1.25% methyl valerate). Dilutions (expressed in log scale) occurred in the following order (matched to perceived intensity at low dilutions): 1) 1 × 10−2 CS+ versus 1 × 10−2 CS−, 2) 1 × 10−4 CS+ versus 1 × 10−3 CS−, 3) 1 × 10−6 CS+ versus 1 × 10−5 CS−, and 4) 1 × 10−8 CS+ versus 1 × 10−7 CS−. Finally, all animals were...
returned to the original $1 \times 10^{-2}$ CS+ versus CS− discrimination for recovery testing.

We also trained a separate cohort of rats (rats 11 and 12), which were experimentally naïve to odor presentations, to discriminate auditory cues using a similar paradigm as outlined above. Briefly, rats were conditioned to lick to a CS+ cue (7-kHz tone, 2-s duration) by rewarding licks with a small water reward. Rats were conditioned to lick for the CS+ but not a CS− cue (white noise, 2 s). Auditory stimuli were presented at a variable 15- to 20-s ITI, and false alarms resulted in a 7-s ITI increase.

Optical recordings

ORN input to the dorsal OB of three of the rats (rats 7–9) was imaged by loading ORNs with calcium-sensitive dye as described previously (Verhagen et al. 2007; Wesson et al. 2008a). After behavioral shaping and 1 day before imaging, an optical window was placed over the dorsal surface of both OBs by thinning the frontal bone and applying a thin coat of ethyl-2-cyanoacrylate glue. Optical signals were recorded for no more than six daily behavioral sessions. Signals were collected using an Olympus epifluorescence illumination turret (BX51) and full light from a 150-W Xenon arc lamp (Opti-Quip) and appropriate filter sets (Verhagen et al. 2007). The imaged area covered a region of $\sim3$ (ant-post) $\times 1.5$ mm (med-lat) over one OB. Images were acquired using a 256 $\times$ 256 pixel CCD camera and digitized at 25 Hz along with respiration and behavioral (licking) signals using an integrated hardware/software package (NeuroCCD SM-256 and NeuroPlex, RedShirtImaging).

Olfactometry

Odor control in all olfactory tasks was achieved using a custom, computer-controlled flow-dilution olfactometer that allowed precise control of odorant concentration, identity, and onset timing in concert with the behavioral paradigm (Verhagen et al. 2007). In this design, odorant was continuously flowing to an odor delivery tube placed in front of the animal’s nose but was removed by a vacuum before exiting the tube; turning off this vacuum via a solenoid valve allowed rapid flow of odorant to the animal. In all training paradigms, the CS+ and CS− were presented via separate flow-dilution channels joined via a short (~30 cm) final common path. Control blocks in which the same odorant was presented in each channel were periodically used to confirm that line contamination or nonolfactory cues were not affecting discrimination performance. Saturated vapor of pure liquid odorant was generated in a nitrogen stream and diluted in air for a final flow rate of 2 l/min. Both air and nitrogen were medical grade and filtered through a hydrocarbon filter cartridge before use. Besides the odor threshold testing experiments, concentrations are reported as percent dilution of saturated vapor (% s.v.), and ranged from 0.5 to 1.5%. For liquid dilutions, odorants were diluted in light mineral oil and thoroughly mixed. Binary odorant mixtures were created in liquid phase. Linearity, stability, and onset timing of odorant delivery were verified with a photoionization detector (MiniRae 2000, RAE Systems, San Jose, CA). All odors were single monomolecular hydrocarbon compounds (Sigma Aldrich).

Data analysis

Sniffing and behavioral performance data were extracted using custom software written in LabVIEW (National Instruments, Austin, TX). We focused our quantitative analysis of sniffing behavior to measures of sniff frequency, which was determined by detecting the peak of each inhalation off-line (Fig. 1B). The intranasal pressure signal was first band-pass filtered (0.1–100 Hz), and the threshold for...
peak detection was set manually by visual inspection of each sniff trace. Robustness and accuracy of the peak detection was verified by visual inspection for each recording. The time point of each detected sniff peak was recorded relative to odorant onset for each trial (unless otherwise stated). To analyze sniff frequency within and across trials, each trial was divided into 50-ms time bins, and the instantaneous sniff frequency was calculated for each sniff based on the interval between a sniff and the one preceding it. This value was assigned to each trial within a session and, when specified, across sessions and animals.

Comparisons were made using ANOVA or t-test (see RESULTS for specific tests).

In a subset of data, we also analyzed sniff amplitude during the odor discrimination task. Sniff amplitude was calculated as the difference between the baseline (resting) intranasal pressure signal and the peak of the inhalation. Each amplitude value was extracted along with its corresponding time point. Values were z-normalized within a session and placed into 100-ms time bins relative to the time of odorant onset, and all values within a bin were averaged to produce a plot of sniff amplitude versus time. Because of the unequal display of sniffs across time in any given trial, statistical comparisons were made using unpaired t-test.

Initial processing of optical signals was performed as described previously (Wesson et al. 2008a). Briefly, optical signals were first processed to remove widespread intrinsic signals and movement artifacts, divided by the resting fluorescence to correct for differences in labeling intensity across glomeruli, and then regions of interest (ROIs) representing one or a few glomeruli were chosen, and signals were spatially averaged across each ROI. ROIs were chosen from sniff-triggered average response maps by visual inspection and were chosen to be, on average, slightly smaller than the half-width of the underlying optical signal focus. Signals digitized at 25 Hz were up-sampled to 100 Hz to match the respiration signal. Thus the temporal precision of measurements based on the optical signals was 10 ms but only included temporal frequencies <12.5 Hz. Thresholding of odorant-evoked responses in individual ROIs was performed based on amplitude relative to preodor noise, which was defined as the SD of the preodor optical signal, measured across all trials for a given ROI.

Signal onset times and amplitude measurements were made using a custom algorithm that fit the optical signals to a double-sigmoid function. The algorithm is described in detail in Wesson et al. (2008a). The general process was to first denoise the optical signal with a combination of band-pass filtering (2nd-order Butterworth, 0.4–8 Hz) and wavelet decomposition-based denoising (4th-order Daubechies wavelet decomposition) and to define the onset time (latency) of the response based on the time of the peak in the product of the second and third derivatives of the optical signal. Starting at this time, the denoised optical signal was fit to a rising sigmoid function. Response amplitude was defined as the peak of this fitted function.

FIG. 2. Sniffing behavior during odor discrimination learning and performance. A: the head-fixed lick/no-lick operant task training sequence. B: sniff frequency plots relative to trial start (i.e., valve click cue for phase 1; odor presentation and valve click for phases 2 and 3) from 3 rats while performing in phases 1 (black), 2 (green), and 3 (blue) of training on the lick/no-lick task. Each trace represents sniff frequency over time averaged across all trials in a single behavioral session after reaching criterion performance. Time bins in this and all subsequent plots, 50 ms. C: sniff frequency plot showing inter-animal variability in sniff strategies that emerged during phase 4 of training. Data are from the same rats as in B. Plots show behavior in the 1st session in which rats performed the odor discrimination to >80% correct response accuracy. D: sniff frequency plots averaged across 4 sessions of phase 4 performance for each rat (all sessions >80% correct responses). E: pseudocolor sniff frequency plot taken from 1 rat (rat 2) throughout 8 consecutive sessions of lick/no-lick task performance. Each row is a separate session. Training phase and performance accuracy (% correct) are indicated at left. Rapid sniff bouts emerge around acquisition of phase 4 (row 4) and is preserved throughout later sessions. Time bins, 100 ms. F: sample sniff traces from each rat in both phase 1 (top) and phase 4 (bottom) of odor discrimination training: [star], moment the animal licked for a reward. Sniff trace displayed with inhalation as an upward deflection. Traces filtered between 0.5 and 50 Hz.
All analyses were performed using custom software written in Matlab (Mathworks, Natick, MA) or LabVIEW. Statistical tests were performed with Matlab. All values are reported as mean ± SD unless otherwise stated.

RESULTS

We monitored respiratory behavior in head-fixed rats performing operant tasks by measuring intranasal pressure transients via a chronically implanted cannula inserted into the dorsal recess of one nasal cavity (Fig. 1A). Pressure transients associated with inhalations are herein referred to as sniffs without attempting to differentiate between active sniffing and passive respiration (Kepecs et al. 2007; Macrides 1975; Welker 1964; Youngentob et al. 1987). We used a variety of task variants to test hypotheses about the role that sniffing plays in odor perception, focusing in particular on the frequency of sniffing around the time of odorant sampling and decision-making by the animal. The basic paradigm, outlined in Fig. 2A, was a lick/nolick discrimination task, either cued or not cued by a tone, in which the animal was trained to lick for a rewarded odorant (CS+) and to refrain from licking for an unrewarded odorant (CS−) (Verhagen et al. 2007).

Emergence of sniffing strategies during odor discrimination tasks

We first characterized how sniffing strategies emerged in the course of learning the lick/no-lick odor discrimination task. We measured sniffing from a cohort of three rats (rats 1–3) as they progressed through the four training phases of the uncued odor discrimination paradigm (Fig. 2A; see METHODS). All rats were naïve to olfactory conditioning at the beginning of training. During the initial training phase (phase 1, ITI conditioning), head-fixed rats were shaped to lick in response to an auditory cue (a solenoid valve click) on every trial (~8-s ITI) in exchange for a small water reward. Rats took from 9 to 10 sessions (55–131 trials per session) to reach response criterion on this task (licking to >80% of all trials). On the 11th session, the three rats licked to an average of 92.1 ± 6.4% of trials. The rats showed low and stable sniff frequencies ranging from 1 to 3 Hz (Fig. 2B, black), consistent with previous reports in awake, quiescent rats (Walker et al. 1997; Welker 1964). In the next phase (phase 2, CS+ odor conditioning), the rats were conditioned to lick to the presentation of odorant in exchange for a water reward (CS+, 1% s.v. isooamyl acetate). After two sessions on phase 2 (159–206 total trials per rat), rats licked to 88.5 ± 9.4% of trials. Despite the presumed active sampling of odorant in this phase, sniffing frequencies remained low and stable (Fig. 2B, green trace). Next, in phase 3 (odor detection), the rats were conditioned to lick to the CS+ odorant but not to presentation of a clean air puff as a nonrewarded stimulus (CS−). Rats reached performance criterion (86.1 ± 4.9% correct) in four to five sessions of phase 3 training (497–601 total trials). Again, sniff frequency remained low and stable throughout the trial (Fig. 2B, blue trace). We tested for brief increases in sniff frequency by taking the peak instantaneous sniff frequency in any one time bin (50 ms) in the 1 s after trial start/orodant onset and comparing this to the peak frequency in a window between 3 and 4 s before trial start. Even using this analysis, which is biased toward finding the highest-frequency sniffs across trials, we failed to find significant increases in peak sniff frequency during any of the three phases of training (P > 0.05, unpaired t-test for each rat in each phase).

Finally, animals were shaped on the final training phase (phase 4; odor discrimination), in which the CS− was replaced with an odorant (1% s.v. methyl valerate). All three rats reached the >80% performance criterion on the fifth session of phase 4 training (465–528 total trials per rat; 88.2 ± 6.8% correct performance). At this stage, two of the three rats continued to show low-frequency, stable sniffing throughout the discrimination trials, whereas distinct sniffing behavior emerged in the third rat (rat 2; Fig. 2C). This rat displayed a brief bout of high-frequency sniffing at the time of odorant delivery, with a significant peak in sniff frequency after odorant onset of 5.8 ± 2.6 Hz [vs. 2.3 ± 1.7 Hz preodor; unpaired t(238) = −12.2, P < 0.0001]. The other two rats (rats 1 and 3) showed no significant change in sniff frequency at the time of odorant onset (Fig. 2C, unpaired t-test for each rat). These sniffing patterns were maintained in each animal through the next four sessions of odor discrimination task performance (Fig. 2D), with all rats on average performing with high accuracy (90.5 ± 5.7% correct responses, 305 total trials per rat). Thus individualized sniffing strategies can emerge during learning of odor-guided tasks, and these distinct strategies are maintained after the task is learned. For rat 2, the brief increase in sniff frequency at the time of odorant onset emerged over the course of the second and third sessions of training on the phase 4 discrimination task (Fig. 2E). Qualitatively, the timing of the sniff frequency increase became more precise and restricted to the time just after odorant delivery with successive training sessions. We also found that the rat that showed an increase in sniff frequency during this task also showed shorter latencies to lick for a reward during CS+ trials [rat 2; F(2, 9) = 5.533, P = 0.006]—609 ± 187 ms (median ± SD) in comparison to the other two rats [1,425 ± 1,016 (rat 1) and 1,578 ± 1,159 ms (rat 3; 4 sessions/rat).

Inspection of the intranasal pressure signals in these animals showed that sniffing changed in waveform and frequency at the time of odorant onset. This can be seen in Fig. 2F, which shows examples of sniffing records taken from each rat during criterion performance in phases 1 and 4 of the task. In these examples, it is clear that the high-frequency sniff bout shown during odor discrimination by rat 2 consists of several very short, small-amplitude inhalations beginning immediately after odorant onset and preceding licking for the reward (Fig. 2E, right). Such brief sniffs were occasionally expressed in the other two rats (e.g., rat 1 trace), but in general, these animals showed little change in sniff waveform around the time of odorant presentation. This short bout of rapid inhalations seems qualitatively different from high-frequency sniffing exhibited during active exploration of novel stimuli, which is sustained for several seconds and includes larger-amplitude sniffs with a clear exhalation component (Macrides 1975; Verhagen et al. 2007; Welker 1964; Wesson et al. 2008a).

Sniffing during performance of learned lick/no-lick odor discriminations

To better describe sniffing behavior of head-fixed rats during performance in the lick/no-lick odor discrimination task, we collected sniffing data from an additional four rats (rats 4–7; n = 7 total; see METHODS for odorants). These rats were shaped
using the same training sequence outlined in Fig. 2A, and sniffing was recorded in four consecutive sessions per rat (58–148 trials/session) following the first session after reaching >80% correct performance. Sniffing behavior during training was not measured in these four rats; the progression of training through the four phases was similar to that described above. We combined these measurements with those from the initial three rats described above to produce a dataset of seven rats for this paradigm, recorded over four sessions each, for a total of 3,937 odor discrimination trials. The average performance across all of these trials was 89.8 ± 5.6% correct.

Data from these additional animals showed more cases of individualized sniffing strategies (Fig. 3A). Comparing averaged peak sniff frequency data across four behavioral sessions from each of the seven rats, three of the seven showed statistically significant increases in sniff frequency on the time of odorant presentation, with average peak frequency values of 5.7 ± 3.2 [rat 2; unpaired t(6) = −5.298, P = 0.002], 8.2 ± 2.1 [rat 6; unpaired t(6) = −3.37, P = 0.015], and 5.0 ± 3.1 Hz [rat 7; unpaired t(6) = −7.51, P < 0.0001]. Additionally, each animal’s average preodor sniff frequencies also varied [F(1,27) = 11.16, P = 0.0063, 1 preodor average peak value/session/rat; Fig. 3A]. Interestingly, there was a strong relationship between average peak sniff frequency and average time to lick for a reward on CS+ trials (Fig. 3A, right; Pearson’s r = −0.82). Nonetheless, for all seven animals, lick latencies exceeded (often by several hundred milliseconds) odor discrimination times as measured by head withdrawal in freely moving rodents (Abraham et al. 2004; Kelliher et al. 2003; Slotnick 2007; Uchida and Mainen 2003) or by expression of exploratory sniffing behavior in head-fixed rats (Wesson et al. 2008b). Thus at least in this paradigm, lick latency more likely reflects motivational state than the time required for the formation of distinct odor percepts.

Figure 3B shows cumulative sniff histograms and sample traces from two rats: one showing little change in sniffing on odor onset and one showing a brief increase in sniff frequency. Notably, the brief bout of high-frequency sniffing shown by rat 6 in this example is qualitatively similar to that shown by rat 2 (Fig. 2E), consisting of a few short inhalations lasting only several hundred milliseconds in duration. The sniff histogram (Fig. 3B) indicates that these sniff bouts were precisely timed to occur just after odorant onset. Individual differences in odorant sampling were also apparent in the amplitude of individual sniffs; in general, the rapid sniff bouts expressed in some animals were associated with a reduction in inhalation amplitude; rats showing little or no modulation in sniff frequency also showed little modulation in sniff amplitude at the time of odorant onset (Fig. 3C). Figure 3D shows that sniffing behavior at the time of odorant onset did not depend on stimulus valence. In particular, peak sniff frequencies during the first second of odorant presentation did not differ between CS+ and CS− trials in any of the seven animals (P > 0.05, t as above)

FIG. 3. Variability in sniffing strategies during odor discriminations. A: sniff frequency plots of individual rats (n = 7) performing the lick/no-lick 2-odor discrimination. Traces represent the average of 4 behavioral sessions per rat at >80% correct response accuracy, plotted as in Fig. 1. These traces include data from the 3 rats shown in Fig. 1. At right are peak sniff frequencies during odor sampling and lick latencies for each rat. Peak sniff frequencies were determined from each animal’s average sniff trace. Median lick latencies relative to odorant onset were calculated from CS+ trials. There was a strong relationship between sniff frequency and lick latencies. B: sniff count histograms and sample sniff traces from rat 5 (top) and rat 6 (bottom) during odor discrimination. Rat 6 frequently showed a rapid sniff bout on odor presentation, whereas rat 5 maintained resting sniff frequencies. C: sniff amplitude plots for rats 5 and 6 during the odor discrimination. Peak inhalation amplitudes for each sniff were placed in 100-ms time bins and averaged across all trials in a given session. Amplitude data were z-normalized before averaging. Each plot shows data from 1 session (~100 trials) from each rat. Rat 5 shows a slight decrease in sniff amplitude for the duration of odorant presentation, whereas rat 6 shows a larger, brief decrease corresponding to the bout of high-frequency sniffing. D: sniff frequency traces averaged across all 7 rats plotted separately for CS+ (rewarded odorant, solid line) and CS− (nonrewarded odorant, dashed line) trials. Cumulative lick probability (from CS− trials) relative to the time of odorant onset is also shown. Sniff frequency, on average, diverges after the time of licking for the reward.
Effects of stimulus expectation on sniffing behavior

The lack of changes in sniff frequency seen in four of the seven rats tested in the first cohort suggests that high-frequency sniffing is not necessary for the accurate performance of two-choice odor discrimination tasks. This result is in contrast to earlier work in freely moving rats, in which high-frequency sniffing occurs reliably during odorant sampling (Kepecs et al. 2007; Youngentob et al. 1987). It is possible that nose-poking into an odor sampling port, a method used in the previous studies, enhances sniff frequency because of anticipatory factors (Wesson et al. 2008b). We thus addressed the hypothesis that anticipation of a potentially rewarding stimulus alters sniff frequency (Kepecs et al. 2007; Wesson et al. 2008b). We trained a new cohort of three rats (rats 8–10) to perform the task of odor discrimination (Kepecs et al. 2007; Wesson et al. 2008b).

We used two of the rats from the previous odor discrimination paradigm (rats 6 and 7), which were among the four that showed significant increases in sniff frequency on odorant presentation in the initial discrimination tests (Fig. 3A).

First, baseline sniffing behavior was established for these rats using the amyl acetate:ethyl butyrate odorant pair, with the CS+ and CS− being completely dissimilar (i.e., 100:0 A:B vs. 0:100 A:B). This easy phase was used for three full sessions (range, 97–147 trials/session). On the fourth session, the CS+ and CS− odorants were made progressively more similar by changing the ratio to 80:20 versus 20:80 (phase 2) and to 68:32 versus 32:68 (phase 3) in a single session, with each ratio presented for a block of 60 trials. On the fifth session, difficulty was increased further from the phase 3 ratio to 56:44 versus 44:56 (phase 4) and to 50:50 versus 50:50 (phase 5). In a final block of 60 trials in a fifth session, the phases 2 stimuli (80:20 vs. 20:80) were presented again to ensure that the rats were still motivated to perform the task. Task performance and sniffing behavior is shown for each stage of this paradigm for each rat in Fig. 5A. As expected, discrimination performance was high for the initial phase (average accuracy: 87.5 ± 3.5% correct), dropped as odorant similarity increased, and was at chance for the 50:50 discrimination (accuracy: 51 ± 5.6% correct). Performance returned to high levels for the final easy block (Fig. 5A). The loss of performance accuracy indicates that acoustic cues or potential contamination of odorant delivery lines did not contribute to discrimination performance. Aside from the expected high-frequency, exploratory sniffing for the first one
Effects of task difficulty on sniffing behavior. A: sniffing behavior (pseudocolor plots), peak sniff frequency (black traces), and behavioral performance (red traces) of 2 rats discriminating odors in a binary ratio mixture paradigm. Sniffing behavior is shown across 5 consecutive sessions. Each row in the pseudocolor plot shows a block of trials at a particular phase of difficulty (indicated to the left of each plot). Sessions 1–5 include 97–147 trials per row; sessions 4 and 5 include 59–68 trials per row. The 2 rats differed in their initial sniff strategies, and such strategies were maintained throughout all phases of the task, despite decreased behavioral performance. The peak frequency plot indicates the peak frequency during the 1st 1 s of odorant presentation, averaged across all trials in a block. Time bins = 100 ms. B: sniffing behavior, peak sniff frequency, and performance in 3 rats discriminating odors as odorant concentration is decreased. Performance is shown across 5 consecutive sessions. Each row in the pseudocolor plot is a different block at a particular concentration. Session 1 includes 88–130 trials per row; sessions 2–5 include 60–74 trials per row. Colored circles indicate relative concentration of odorant in its liquid phase, ranging from pure odorant (top row, filled circle) to $10^{-3}$ dilution (next-to-bottom row). In the last block, rats were retested on the next-to-highest concentration. All odorants were presented at 1.25% s.v. Sniff frequency data were calculated and plotted as in A. Sniffing behavior remains unchanged as odorant concentration decreases but drops significantly when animals can no longer perform the discrimination task. Time bins = 100 ms. C: sniff amplitude plots during odorant discrimination at the highest concentration (filled green circle, 1.25% s.v. of pure ($1 \times 10^0$ dilution) odorant) and at near-threshold concentration (partial circle, 1.25% s.v. of $1 \times 10^{-3}$ dilution odorant), shown for each of the 3 rats. For each rat, data are taken from 100–120 trials at each concentration. There is no clear change in sniff amplitudes during odor discriminations at the different concentrations.

to three trials after introduction of a different odorant mixture (Verhagen et al. 2007), there was no clear change in sniffing behavior as odorant similarity increased and performance decreased. Both of the rats continued to exhibit brief increases in sniff frequency just after odorant onset, in a qualitatively similar manner throughout all phases. Neither the peak nor the average sniff frequency during the first 1 s of odorant presentation differed between phase 1 and phase 5 (unpaired t-test, $P > 0.05$), and there was no significant correlation between performance accuracy and peak sniff frequency for either animal (Pearson’s $r < 0.5$ in both animals). These results suggest that task difficulty—at least in the course of one behavioral session—is not sufficient to alter the sniffing behavior of rats performing an odor discrimination task.

In a second experiment, we increased task difficulty by lowering odorant concentration. Previous reports in rats (Doty et al. 1988; Slotnick and Nigroshi 1974) and mice (Bodyak and Slotnick 1999; Clevenger and Restrepo 2006; Kellinger et al. 2003; Sorwell et al. 2008) have shown that lowering stimulus concentration affects odor discrimination performance. Therefore we gradually decreased the concentration (i.e., dilution of liquid phase) of the CS+ (isomyl acetate) and the CS- odorants (methyl valerate) by 2 log units each behavioral session (see METHODS). All liquid odorants were presented at 1.25% s.v. Each day, rats (rats 1–3, the same as those in Fig. 2) were tested on one dilution for the first half of a session and the next lower dilution for the second half of the session (~60 trials/dilution). Each subsequent session followed the same sequence (with the first half of one session using the same concentration as the second half of the previous session), until the rats finally reached chance performance ($\pm 60\%$ correct responses; see METHODS). For the pure odorant and $10^{-2}$ dilutions, rats performed with high accuracy, showing a mean performance of $95.2 \pm 3.5\%$ correct. Performance dropped
when concentration was lowered to the $10^{-4}$ (79.8 ± 5.9% correct) and $10^{-6}$ dilutions (71.8 ± 4.4% correct) and reached near-chance levels at the $10^{-8}$ dilution (53.3 ± 2.1% correct; Fig. 5B).

We found that each rat roughly maintained its sniffing strategy as odorant concentration decreased (Fig. 5B). For each rat, peak sniff frequency did not change significantly between the $10^{-2}$, $10^{-4}$, and $10^{-6}$ dilutions, despite the drop in performance accuracy (unpaired $t$-test in comparison to phase 1 peak frequencies, $P > 0.05$). Interestingly, at the $10^{-8}$ dilution, at which discrimination failed (Fig. 5B), each rat showed a significant decrease in peak sniff frequency in comparison to frequency during the $10^{-7}$ odor discrimination [Fig. 5B, rows 3 and 4 vs. 9; rat 1, unpaired $t$(169) = 5.57, $P < 0.0001$; rat 2, unpaired $t$(138) = 8.96, $P < 0.0001$; and rat 3, unpaired $t$(190) = 6.59, $P < 0.0001$]. Both sniff frequency and discrimination accuracy returned to their original levels during the recovery block of $10^{-2}$ dilution trials (Fig. 5B, rows 3 and 4 vs. 10). Thus sniff frequency does not change as task difficulty is increased by lowering odorant concentration. Instead, rats maintain their original sniff frequency to the point at which they can no longer perform the discrimination, after which sniff frequency decreases. Finally, because previous work in humans (Johnson et al. 2003) and rats (Youngentob et al. 1987) has found that sniff amplitude increases when odorant concentration decreases, we also measured sniff amplitudes during performance of this task at the strongest concentration and at the near-threshold concentration ($10^{-6}$ dilution trials). We found no clear change in sniff amplitude around the time of odorant onset across this concentration range (Fig. 5C).

**Sniffing behavior during nonolfactory discriminations**

Given that individualized sniffing patterns within animals were generally unaffected by manipulating task difficulty, it is possible that such patterns might be a general phenomenon of task engagement and not necessarily be driven by odor discrimination performance. Thus in a final behavioral experiment, we monitored sniffing behavior during performance of a structurally identical, yet nonolfactory lick/no lick discrimination task. We trained two additional rats (rats 11 and 12) to perform a head-fixed auditory discrimination using the same lick/no lick paradigm and shaping regimen as for the noncued odor discrimination. Importantly, these rats were naïve to experimental olfactory conditioning. In this case, the CS+ was a 7-kHz tone and the CS− was white noise, with each presented for 2 s. After reaching criterion performance (>80% correct), sniffing was monitored in four consecutive sessions per animal. Mean performance across these sessions was 88.5 ± 2.6% correct.

Interestingly, we found that sniffing was also modulated in the auditory discrimination task (Fig. 6). One rat showed a significant increase in peak sniff frequency during the first 1 s of cue onset of 5.1 ± 2.4 Hz [rat 11; unpaired $t$(858) = -17.421, $P < .0001$], whereas the other failed to show a significant change (rat 12; peak frequency 3.0 ± 2.3 Hz, unpaired $t$-test, $P > 0.05$). The brief bouts of high-frequency sniffing after tone onset (most obvious in rat 11) occurred immediately around the time of lick in most trials (Fig. 6A). Qualitatively, these rapid sniff bouts were similar to those expressed during odor discriminations, consisting of a few small-amplitude, short-duration inhalations (Fig. 6B). Thus, although surveyed in only two animals, these results show that discrimination task performance alone, independent of olfactory demands, is capable of eliciting significant and precisely controlled changes in sniff frequency. These findings agree with previous work from our laboratory (Wesson et al. 2008b) and others (Harrison 1979), showing that nonolfactory sensory stimuli are capable of modulating sniffing.

**Calcium imaging of glomerular responses during rapid sniffing**

Although variable in expression from animal to animal, high-frequency sniff bouts had distinctive characteristic features: they occurred near the time of odorant presentation, they were short-lived, consisting of, at most, five high-frequency sniffs lasting in total no more than ~600 ms, and the individual sniffs in such a bout were often smaller in amplitude than sniffs occurring at low frequency and had little or no exhalation phase. This sniffing behavior was qualitatively distinct from that exhibited in response to novel odors, which consists of sustained sniffing at 4–6 Hz lasting several seconds (Verhagen et al. 2007; Wesson et al. 2008a). We asked what functional role these distinctive sniff bouts might play in odor discrimination by imaging odorant-evoked ORN input to the OB in a subset of the animals showing this behavior (see METHODS and
Verhagen et al. 2007). We focused on several hypotheses: First, that changes in sniff frequency may enhance the responsiveness of ORNs by increasing odorant access. Second, that the sniff bouts enable a greater number of odorant samples to be processed in a short time. Finally, that high-frequency sniff bouts enable a more rapid detection of odorant than do low-frequency sniffs. To test these hypotheses, we took advantage of the variability in expression of the rapid sniff bouts—both within and across animals—to compare odorant-evoked ORN inputs to glomeruli in the presence and absence of this mode of odor sampling (Fig. 6A). Analyses were done for the three imaged animals (rats 7–9) that most frequently showed rapid sniff bouts; each of these rats showed high-frequency bouts in >20% of trials (max: 36%, rat 7).

To address whether rapid sniff bouts enhance ORN responsiveness, we compared the amplitudes of the first sniff-evoked optical signal after odorant presentation (Fig. 7A, black circles) in trials in which the odorant was sampled with a rapid sniff bout [defined as having an inter-sniff interval (ISI) preceding the 1st response (t1) of <250 ms; Fig. 7A, right] with trials in which it was sampled with a low-frequency sniff (t1 > 400 ms; Fig. 7A, left). The first odorant-evoked response was conservatively defined as a sniff that evoked optical signals exceeding 2 SD of the mean preodor noise (see Methods) in at least two glomeruli. Peak response amplitudes from glomeruli with responses above this threshold were measured in all trials and normalized to the maximum response measured in a given session. This dataset included response magnitudes ranging from 0.56 to 5.15% ΔF/F. Pairwise comparisons were made between response values for the same glomerulus-odorant pair imaged during rapid sniffing versus nonrapid sniffing trials in the same session. In cases where a glomerulus responded to the same odorant in more than one trial, response amplitudes were averaged before normalization. As shown in Fig. 6B, the amplitude of odorant-evoked glomerular responses were similar regardless of the sniff frequency immediately preceding the

Fig. 7. Olfactory receptor neuron responses imaged during discrimination task performance. A: example sniff traces (sniff) and optical signals representing calcium influx into ORNs (Ca\(^{2+}\)) imaged from 1 glomerulus in a rat performing the 2-odor discrimination task. The 2 sample traces show data evoked by the same odor from different trials in the same rat and behavioral session—1 in which the rat shows no change in sniff frequency (left) and 1 in which the rat samples the odorant with a short, rapid sniff bout (right). Each vertical line indicates the time of inhalation onset. The 1st sniff that evokes an odorant response (the stim-sniff) is indicated by a dashed vertical line. t1, interval between the stim-sniff and the preceding sniff; t2, interval between the stim-sniff and the following sniff. Black horizontal bar, latency from odorant onset to onset of the odor-evoked optical signal; black circle, peak amplitude of the odorant-evoked response. B: comparison of normalized response amplitudes for odorant-glomerulus pairs imaged during rapid (t1 < 250 ms) and slow (t1 > 400 ms) sniffing trials. Black line, the diagonal; open circles, individual odorant-glomerulus pairs; filled circles, mean amplitudes averaged across the low, middle, and high tertiles of the population. Data from rats 7–9. C: histogram of normalized sniff counts from each rat aligned to the stim-sniff; i.e., the sniff that evoked detectable olfactory receptor neuron (ORN) input to the OB (A, dashed line). Note that sniff probability increases before the stim-sniff and decreases immediately afterward. Data are taken from 83 trials with rapid sniff bout (t1 < 250 ms) and normalized to the maximum sniff count within each animal. Same animals as in A. D: probability that a rapid sniff (inter-sniff interval < 250 ms) follows any other rapid sniff during the intertrial interval (gray, preodor) or follows the stim-sniff (black). Probabilities calculated from same trials as in C. E: mean optical signal onset latencies relative to odorant onset, for rapid (t1 < 2.5 Hz, gray) vs. slow (t1 > 400 ms, black) sniffing at odorant onset. Onset latency indicates the time to arrival of odorant-evoked sensory input at the OB. Latencies are significantly shorter for rapid than for slow sniffing of odorant. Horizontal dashed lines = SE.
response (t1 < 250 ms vs. t1 > 400 ms; paired t-test, P > 0.05; n = 146 responses). Binning this data into equal thirds based on glomerular response amplitudes showed that this lack of effect was apparent for weakly, moderately, and strongly activated glomeruli (Fig. 7B; paired t-test between slow and fast-sniff data within each amplitude range, P > 0.05). Likewise, relative amplitudes of input to all activated glomeruli (normalized and pooled across sessions) were highly correlated for responses evoked by rapid and nonrapid sniffing (Pearson’s r = 0.90). This analysis suggests that rapid sniff bouts alter neither initial ORN responsiveness nor overall patterns of glomerular activation.

Next, to address whether rapid sniff bouts enable increased sampling of odorant, we analyzed the timing of the odorant-evoked ORN input signal relative to that of the sniff bout. In the example in Fig. 6A from rat 9 (right), it is clear that the sniff bout occurs before the onset of the first optical signal indicating ORN activation and seems to end as soon as ORN activation occurs. To examine the consistency of this trend across trials and animals, we performed two analyses. In the first, we constructed sniff histograms after aligning each trial according to the time of the sniff that immediately preceded the earliest optical signal (stim-sniff; Fig. 7A, vertical dashed line). For this analysis, any trial showing a high-frequency sniff (t1 < 250 ms) before the first response was included. Display of sniffing data in this way indicated that, in all three animals, high-frequency sniff bouts preceded ORN activation and terminated rapidly afterward.

For the second analysis, we measured the probability that a high-frequency sniff (t1 < 250 ms) occurred after ORN activation. This ISI (t2; see Fig. 7A) was defined as the interval between the stim-sniff and the sniff immediately following it. Of >300 total trials from rats 7–9, we found 83 trials in which t1 was <250 ms (i.e., 83 trials wherein rats showed a rapid sniff bout). Of these 83, only in 11 was the t2 also <250 ms. Thus there was only a 13.3% chance that rats continued a rapid sniff bout after the first glomerular response (Fig. 7D). Might this low probability simply reflect the fact that rats often show short bouts of sniffing, even in the absence of odorant sampling? To control for this possibility, in the same trials, we analyzed all sniffs that occurred from 4 s to 500 ms before odorant onset whose preceding ISI was <250 ms (>4 Hz) and measured the probability that the following sniff also occurred with an ISI <250 ms. This probability (52%, n = 202 fast ISIs) was four times that for the sniff bouts occurring around the time of the first glomerular response. Together these two analyses suggest two important features of the high-frequency sniffing associated with odor sampling. First, these sniff bouts precede the activation of ORNs by the odorant; second, they are terminated immediately after ORN activation. These findings argue that high-frequency sniffing on stimulus onset—at least in the context of this task—does not result in increased sampling or prolonged processing of the odorant.

Finally, we asked whether high-frequency sniff bouts enable a more rapid detection of the odorant. To explore this, we measured the latency between odorant onset and the onset of the first optical response in any glomerulus (Fig. 7A, black horizontal line) and compared these latencies in high-frequency versus low-frequency sniffing trials (i.e., t1 < 250 ms vs. t1 > 400 ms). The onset latencies from high-frequency sniffing trials were significantly shorter (376.4 ± 106 ms, n = 83; Fig. 7E) than those from low-frequency trials [702.6 ± 603 ms, n = 152; unpaired t(192) = −3.97, P < 0.0001]. These findings show that rapid sniff bouts are initiated more closely to the time of odorant onset and effectively serve to shorten the time it takes for the animal to detect the odorant once it becomes available.

**DISCUSSION**

The precise and reliable expression of high-frequency (6–10 Hz) sniffing during odorant sampling and the modulation of sniffing parameters by odorant features have led to the idea that rapid sniffing plays an important functional role in odor detection and discrimination (Kepecs et al. 2007; Macrides et al. 1982; Rajan et al. 2006; Uchida and Mainen 2003; Youngentob et al. 1987). Here, we explored the role of one form of rapid sniffing by monitoring respiration in head-fixed rats as they perform odor discrimination tasks and by simultaneously imaging sensory input to the OB. We found that rats can accurately discriminate odors without using rapid sniffing; we also found that sniffing strategies—once established during the initial learning of the task—remained consistent even as task difficulty increased and performance decreased. Imaging sensory input to the OB showed no evidence that initially sampling an odorant with rapid sniffing alters the magnitude or pattern of olfactory inputs and instead showed that rapid sniffing precedes odorant-evoked activation of the sensory neurons and ends immediately afterward. The main conclusion suggested by these results is that, at least in the context of the simple, learned two-odor discrimination task used here, rapid sniffing facilitates stimulus acquisition but is not directly involved in odor information processing.

**Sniffing strategies during odor discrimination tasks**

We monitored sniffing behavior in head-fixed rats performing a lick/no-lick odor discrimination task, a paradigm that enabled us to simultaneously image sensory input to the OB but that also differs considerably from that of previous studies characterizing rodent sniffing behavior. Not surprisingly, we found several substantial differences between head-fixed and unrestrained rats in sniffing strategies associated with odor discrimination. The major difference was the reliability with which rapid sniff bouts at the time of odorant onset were expressed. For example, the majority of head-fixed rats did not show detectable increases in sniff frequency before odor discrimination, whereas such increases seem to be reliably expressed in freely moving rats (Kepecs et al. 2007; Macrides et al. 1982; Rajan et al. 2006; Uchida and Mainen 2003; Youngentob et al. 1987). The peak frequency of rapid sniff bouts was also lower in head-fixed than in freely moving rats (Kepecs et al. 2007). We also found significant differences in the sniffing strategies expressed by different head-fixed rats; although the sniffing strategy was robust within a subject; individual differences in sniffing behavior have not been reported in freely moving rats. Individualized stimulus sampling strategies have, however, been described in other sensory systems including human saccadic eye movement (Castelhano and Henderson 2008) and rodent vibrissal movements (Carvell and Simons 1995).

One possible explanation for the differences in sniffing behavior is the absence of confounds related to movement and
nose-poke in the head-fixed paradigm. For example, respiration frequency increases during locomotion (Bramble and Carrier 1983) and during an animal’s approach to an object. A second possibility is that, because rapid sniffing typically precedes odor sampling in freely moving paradigms (Kepecs et al. 2007; Rajan et al. 2006; Wesson et al. 2008b), differences in anticipation of the stimulus might account for the different sniffing behaviors. However, cueing odor presentation with a tone still failed to elicit rapid sniffing in the head-fixed rats, making this explanation unlikely. A third possibility is that differences in motivational state contribute to differences in the expression of rapid sniffing; increases in motivation driven by classical conditioning are known to increase sniff frequency (Clarke 1971; Clarke and Trowill 1971). Consistent with this idea, odor discrimination performance in freely moving rats performing a nose-poke-based task has been positively correlated with sniff frequency at the time of odor sampling (Kepecs et al. 2007). Likewise, in our head-fixed paradigm, we observed a strong correlation between the frequency of odor sampling and latency to lick for CS+ trials.

On the other hand, there were many similarities in the sniffing behavior of head-fixed and unrestrained rats. Although a minority of head-fixed rats showed rapid sniffing associated with odor discriminations, these bouts were similar to those seen in unrestrained animals performing operant go/no-go or two-alternative forced choice tasks in that they consisted of brief (several hundred milliseconds) bouts of rapid sniffing ranging in frequency from 4 to 10 Hz and beginning at or just before the time of odor sampling (Kepecs et al. 2007; Macrides et al. 1982; Rajan et al. 2006; Wesson et al. 2008b). We also found that the few rats that did show rapid sniffing in our paradigm did so with a temporal precision similar to that reported for freely moving rats (Kepecs et al. 2007). In addition, baseline sniffing in head-fixed rats was similar in frequency to that of unrestrained but quiescent rats (Walker et al. 1997) (Macrides 1975; Randall and Brown 1994), and head-fixed rats readily respond to novel stimuli with the same high-frequency, exploratory sniffing behavior seen in unrestrained rodents (Freeman et al. 1983; Macrides 1975; Verhagen et al. 2007; Welker 1964; Wesson et al. 2008b). Thus, although some aspects of sniffing behavior are altered in head-fixed animals, it does not seem that head fixation significantly impairs either baseline respiration or the spontaneous expression or temporal precision of rapid sniffing.

Head fixation may nonetheless introduce some confounds in sniffing behavior, especially given that sniffing is coordinated with small head movements in freely moving rats (Welker 1964). One possibility is that sniffing may be generally suppressed in head-fixed rats. Thus the head-fixed paradigm may not be useful for describing an animal’s natural sniffing behavior. This paradigm is useful, however, in providing a highly constrained setting for evaluating the contribution of sniffing to the performance of odor-guided tasks, as we discuss in more detail below.

Odor discriminations independent of rapid sniffing

An important result of this study is that rats can perform even difficult discriminations (e.g., discrimination of near-threshold odors and binary-mixture discriminations) without altering the sniffing strategy used for easier discriminations and, in at least some animals (e.g., rat 7; Fig. 4), without exhibiting rapid sniffing. The fact that discrimination performance is correlated with sniff frequency in freely moving rats (Kepecs et al. 2007) and that sniffing parameters change with odorant concentration in an odor detection task (Youngentob et al. 1987) has led to the hypothesis that sniffing facilitates odor-guided behaviors, presumably through low-level mechanisms such as changes in the access of odorant to sensory neurons (Mozell 1970; Schoenfeld and Cl electrodes; Youngetob et al. 1987). This hypothesis predicts that sniffing behavior should change with the demands of an odor discrimination task; however, both in terms of sniff frequency and inhalation amplitude, we did not observe such changes. Instead, the constancy of sniffing strategies was striking: once a particular strategy emerged during the initial learning of the task, this strategy remained stable through multiple sessions involving hundreds of trials and during the learning of new odorant pairs. Sniff frequency remained consistent even as the similarity between two odorants was increased to the point at which they were no longer discriminated or as concentration was reduced. In the concentration experiments, the reduced performance in the 10^-4 and 10^-6 dilution sessions represent a total of 240 trials per rat; prior studies suggest that it is unlikely that performance accuracy would have increased with more trials (Bodyak and Slotnick 1999). However, the possibility that changes in sniffing behavior resulting in improved performance may have emerged over additional sessions cannot be ruled out. It was also striking that inhalation amplitude did not change as odorant concentration was decreased over a range of 6 log units. This result is inconsistent with the hypothesis that mammals actively adjust sniff strength to achieve a degree of intensity invariance in odor perception (Johnson et al. 2003; Mainland and Sibel 2006; Youngetob et al. 1987). This and related hypotheses need to be tested more thoroughly in rodents using a variety of behavioral paradigms and with direct measures of intranasal airflow.

The only situation in which rats did alter their sniffing strategy was when odorant concentration was lowered to a point at which rats could no longer discriminate the CS+ from CS-. Importantly, although the above hypothesis would predict that sniff frequency might increase as the task reached perceptual limits, we instead found that sniff frequency decreased in the three animals tested. In contrast, sniff frequency was unchanged when the animals were performing at chance levels in the binary ratio mixture experiment. This difference may be attributable to differences in saliency of the stimulus in these two paradigms: saliency remained unchanged in the binary ratio mixture experiment, whereas saliency (as reflected in stimulus intensity) decreased markedly as odorant concentration was reduced to threshold levels. Overall, the fact that rats did not alter their sniffing strategy in an attempt to increase discrimination performance suggests that sniffing behavior, in particular frequency, does not directly contribute to the ability of the olfactory system to discriminate one odorant from another.

Sniffing and low-level olfactory processing

We also tested the hypothesis that sniffing behavior shapes the low-level processes underlying odor discrimination by imaging ORN input to the OB during task performance. We took advantage of the trial-to-trial variability in sniffing behavior to ask whether odorants sampled during a rapid sniff bout
evoked ORN responses that differed from those evoked by odorant sampling during low-frequency sniffing. Although rapid sniff bouts serve to increase the volume of inspired air during sampling and thus may deliver more odorant to ORNs (Youngentob et al. 1987), we found no change in the magnitude of ORN input evoked when an odorant was sampled during a bout of rapid sniffing. Likewise, rapid sniffing caused no significant change in the relative pattern of ORN inputs across glomeruli. In an earlier study examining the role of a different form of high-frequency sniffing, we found a similar lack of effect on ORN responses to odorant onset (Verhagen et al. 2007). In fact, sustained high-frequency sniffing of odorant causes an attenuation of the magnitude of inputs, presumably caused by rapid adaptation of ORN responses (Verhagen et al. 2007).

Another hypothesized role of rapid sniffing is to allow for multiple samples of odorant to be used to process odor information. Multiple sniffs of odorant could allow for iterative tuning of postsynaptic odor representations (Ambros-Ingeron et al. 1990) or for enhanced temporal precision and response specificity of mitral/tufted cell spiking caused by activity-dependent inhibition (Arevian et al. 2008; Hayar et al. 2004; Wachowiak and Shipley 2006; Young and Wilson 1999).

However, we found that the majority of rapid sniff bouts occurred before ORNs were activated by an odorant and ended immediately after odorant-evoked ORN activity arrived at the OB. Thus, at least for the discrimination paradigm used here, our data suggest that rapid sniffing does not enable multiple odorant samples to be processed centrally. In fact, our data suggest that rapid sniffing of familiar odors is actively terminated by the animal as soon as odorant is detected. The cessation of a sniff bouts immediately after ORN activation suggests that olfactory input is capable of influencing odor sampling behavior extremely rapidly: cycle-by-cycle control of rapid sniffing in the frequency range of 4–10 Hz suggests a sensorimotor control loop that requires well under 200 ms. Thus these data add to an extensive body of literature suggesting that information processing underlying a diverse range of odor-guided behaviors occurs rapidly and, as some have reported, requires only a single sniff of odorant (Abraham et al. 2004; Goldberg and Moulton 1987; Karpov 1980; Rajan et al. 2006; Rinberg et al. 2006; Uchida and Mainen 2003; Wesson et al. 2008a).

Why sniff fast?

In general, this study fails to support the idea that the specialized high-frequency sniffing behavior typically seen during odor discrimination tasks influences odor coding or enhances low-level information processing. What, then, is the functional role of rapid sniffing during odor discriminations? Our data suggest that the primary consequence of rapid sniffing is to expedite odorant delivery to ORNs. Thus rapid sniffing is an indicator of an animal’s search for the stimulus rather than its need to enhance information processing. This (perhaps disappointingly) simple explanation of sniffing behavior is consistent with our finding that stimulus salience (e.g., odorant concentration) and motivational state (as reflected in lick latencies) were more strongly correlated with sniff frequency than task difficulty. This explanation is also consistent with results in freely moving rats, in which the instantaneous frequency of the last sniff of odorant was most strongly correlated with performance on a discrimination task (Kepecs et al. 2007).

Using a slightly different paradigm, a classic study by Youngentob et al. (1987) found that rats performing an odor detection task increased both the number and amplitude of rapid sniffs as odorant concentration neared perceptual threshold, a result that is qualitatively different from what we observed in this study. The Youngentob et al. (1987) study differs from this and other studies (Kepecs et al. 2007; Uchida and Mainen 2003), however, in that odorant was not actively delivered to the animal by bulk flow but instead could only be acquired through active sniffing from a still-air reservoir. Thus the increase in sniff number and amplitude as odorant concentration decreased may reflect increased difficulty in obtaining the stimulus. If so, the results of that study are likely consistent with a role for rapid sniffing in stimulus acquisition rather than a direct effect on odor representations.

Given that, at least in the head-fixed paradigm, rapid sniffing is only rarely expressed, we might also ask the question, why not sniff fast? There are numerous potential costs to rapid sniffing, including increased energy expenditure, decreased air exchange (i.e., hypoventilation), and the potential for inhaling aversive or harmful substances. These potential costs to rapid sniffing may explain the brief duration of rapid sniff bouts during odorant sampling and may also be important in establishing individual sniffing strategies. We speculate that animals express a diverse range of sniffing strategies depending on both external factors reflecting task demands and on internal factors reflecting behavioral state. Additional variants in odor-guided behavioral paradigms will likely show additional diversity in odorant sampling strategies, with different strategies likely playing different roles in facilitating odor-guided behaviors. In general, however, sniffing seems best considered as part of a general sensorimotor repertoire that is coordinated with other (nonolfactory) systems such as respiration, locomotion, and whisking (Komisaruk 1970).

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