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Kepecs A, Uchida N, Mainen ZF. Rapid and precise control of sniffing during olfactory discrimination in rats. *J Neurophysiol* 98: 205–213, 2007. First published April 25, 2007; doi:10.1152/jn.00071.2007. Olfactory perception relies on an active sampling process, sniffing, to rapidly deliver odorants from the environment to the olfactory receptors. The respiration cycle strongly patterns the flow of information into the olfactory systems, but the behavioral significance of particular sniffing patterns is not well understood. Here, we monitored the frequency and timing of nasal respiration in rats performing an odor-mixture–discrimination task that allowed us to test subjects near psychophysical limits and to quantify the precise timing of their behavior. We found that respiration frequencies varied widely from 2 to 12 Hz, but odor discrimination was dependent on 6- to 9-Hz sniffing: rats almost always entered and maintained this frequency band during odor sampling and their accuracy on difficult discrimination dropped when they did not. Moreover, the switch from baseline respiration to sniffing occurred not in response to odor delivery but in anticipation of odor sampling and was executed rapidly, almost always within a single cycle. Interestingly, rats also switched from respiration to rapid sniffing in anticipation of reward delivery, but in a distinct frequency band, 9–12 Hz. These results demonstrate the speed and precision of control over respiration and its significance for olfactory behavioral performance.

INTRODUCTION

Sensory function depends on a combination of feedforward flow of information from sensory organs to the brain and the ensuing readjustment of sense organs by feedback from the brain. A familiar example of this process is the movements we make with our eyes to gather information about the relevant parts of the visual environment, an active process that is integral to vision (Land 2006). In olfaction the active sampling process is sniffing—rapid, repeated bouts of nasal inhalation that generate airflow to convey volatile chemicals from the environment to the olfactory epithelium (Mainland and Sobel 2006). Although understanding how olfactory motor processes dynamically control sensory acquisition is likely to be important to understand olfactory sensory processing, relatively little is known about the nature of this control and its functional significance. Of particular interest is how organisms that rely heavily on the sense of smell for gathering information about the world actively adjust sniffing to alter the flow of olfactory information.

Respiration is proximally controlled by central pattern generator (CPG) nuclei in the brain stem (Feldman and Ellenberger 1988; Mellen et al. 2003; Onimaru and Homma 2003), which integrate a variety of descending inputs from cortical and subcortical structures (Gaytan and Pasaro 1998). Studies in anesthetized animals and in vitro preparations have shown that respiratory patterns can be finely controlled by afferent input (Arata et al. 2000; Morris et al. 2003). Behaviorally, it is also known that sniffing patterns are subject to "bottom-up" regulation by olfactory stimuli (Laing 1983; Sobel et al. 2000; Warren et al. 1994). For instance, a reflex-like process equalizes sniff volume using a subcortical feedback loop in humans (Johnson et al. 2003). Similarly, the frequency and depth of sniffing depend on odor concentration in rats (Youngentob et al. 1987). Much less is known about cognitive control over respiratory centers. In other forms of active sensing, like vision, it is well established that the timing and pattern of eye movements are controlled not only by the visual features of a scene, but also through cognitive processes (Land 2006). For instance, anticipatory visual saccades are able to predict the location of action-relevant information 0.2 s in advance (McLeod 1987; Ripoll et al. 1987). Whether cognitive processes exert temporally precise and active control over sniffing in rodents, as is the case for saccades in primate vision, is not known. However, decerebrate rats do not sniff, and environmental exploration and novel stimuli can initiate sniffing independent of olfactory stimuli (Welker 1964).

Although sniffing clearly subserves olfaction and strongly patterns olfactory processing, the slower respiratory rhythm is sufficient for delivering odorants to olfactory receptors. Therefore it is interesting to observe that under natural conditions sniffing in rats is usually a component of a coordinated rhythmic motor sequence involving nose, head, and whisker movements. Moreover, sniffing can be elicited by reward anticipation in the absence of odors or environmental novelty (Bindra and Campbell 1967; Clarke 1971) as well as by electrical stimulation of brain areas involved in reward processing (Ikemoto and Panksepp 1994). These observations suggest a broader context in which control over sniffing patterns may be relevant for coordinating olfactory processing with other senses and part of an anticipatory brain state.

Two of the principal axes of control over sniffing are the regulation of timing and frequency; we hypothesized that both variables may be under cognitive control in the rat. A single sniff is sufficient to perform elementary olfactory discriminations for both humans (Laing 1983) and rats (Rajan et al. 2006; Uchida and Mainen 2003), but it has not been established how rapidly or precisely an animal can time a sniff in relation to task demands. For rats the frequency of sniffing is typically in the "theta" frequency range (4–12 Hz) during olfactory discrimination (Rajan et al. 2006; Uchida and Mainen 2003; Youngentob et al. 1987) or exploration (Welker 1964), but how accurately sniffing frequency can be regulated and whether olfactory performance depends on the exact sniffing frequency is not known. Respiratory airflow and several other character-

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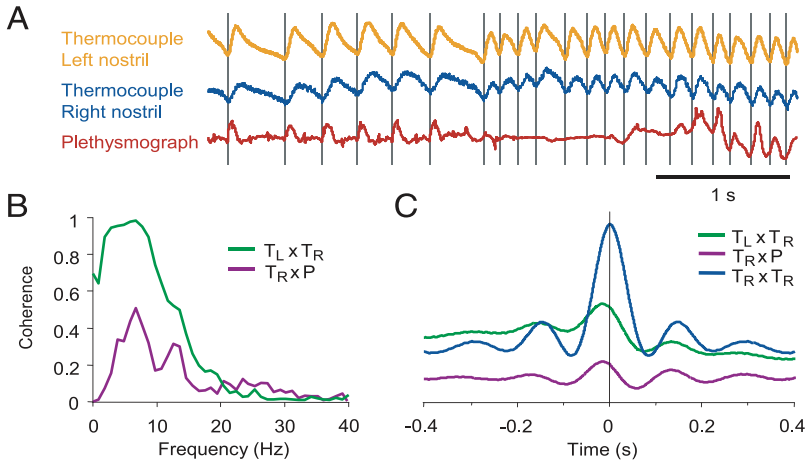

FIG. 1. Nature of the thermocouple signal. *A*: concurrent recordings of 2 thermocouples in the left and right nostrils and a chest plethysmograph (a piezoelectric belt) measuring respiration in a behaving rat. Lines are placed at the inhalation onsets for the left thermocouple. Different measurements mostly agree on a cycle-by-cycle basis with small phase offsets even for high-frequency (>6 Hz) sniffing. Sometimes, however, the plethysmograph signal transiently exhibited dissimilar patterns. *B*: spectral coherence between the 2 thermocouple signals (green) and between one thermocouple and the plethysmograph (purple). *C*: auto- (blue) and cross-correlation between the different respiration signals. Note the small (<30 ms) timing differences between different nostrils and chest expansion are comparable. Although the thermocouples were implanted at the same anterior–posterior (A-P) position bilaterally, small differences could also influence the exact timing of respiration signals.

istics of sniffing covary with its frequency (Walker et al. 1997; Youngentob et al. 1987), so high frequencies could enhance olfactory transduction (Hahn et al. 1993; Kimbell et al. 1997). Alternatively, particular sniffing frequencies within the theta (4- to 12-Hz) range may be favorable for the coordination of olfactory processing with other brain regions (Kay 2005; Kepecs et al. 2006; Komisaruk 1977).

In this study we sought to address two main questions: First, how is sniffing regulated on a fine temporal scale during olfactory discrimination? Second, how does olfactory performance depend on rhythmic sniffing? To answer these questions we quantitatively characterized sniffing patterns and their behavioral regulation during olfactory-discrimination behavior in rats.

METHODS

All procedures involving animals were carried out in accordance with National Institutes of Health standards as approved by the Cold Spring Harbor Laboratory Institutional Animal Care and Use Committee. All statistics reported are means ± SD across the six rats examined unless otherwise noted.

Measurement of respiration

Rats were implanted with a temperature sensor (0.005-in. Teflon-coated thermocouple, Omega, part # 5TC-TT-K-36-36) in one nostril through a hole made in the dorsal skull and secured with dental cement. Thermocouples were inserted into the nasal passage through the nasal bone just rostral to the turbinates. Thermocouples measure the cooling and warming of air arising from breathing (Angyan and Szirmai 1967; Clarke et al. 1970). Although junction potentials and positioning differences make it difficult to interpret the absolute voltage recorded, the timing of inhalations and exhalations can be readily determined from the cooling–warming patterns. Sniffing signals were amplified, filtered between 0.1 and 475 Hz, and digitized at 1,000 Hz.

To establish the fidelity of these timing measurements, in pilot experiments we used a complementary method, a chest plethysmograph (Barrie et al. 1996) to measure respiration. This device is a piezoelectric belt wrapped around the rat's body to measure chest expansions to indicate inhalations and exhalations. Figure 1 shows that the thermocouple and chest plethysmograph signals can agree on a cycle-by-cycle basis with small (<25 ms) differences in time lags confirming the temporal precision of the thermocouple signal. A comparison of the thermocouple and plethysmograph signals also reveals that when respiration is slow the expiration phase is passive. In this range temperature measurements can provide information only about the timing of the inspiration and cannot distinguish passive from active expiration. However, sometimes the thermocouple and the plethysmograph showed transiently dissimilar signals (see Fig. 1*A*). These differences could have arisen from either movement-related artifacts or potentially physiological factors. The thermocouple method was preferred because of its day-to-day stability and its more direct association with olfactory processing (Fig. 2*A*). We tested the variability in the timing of the thermocouple signal resulting from its place-

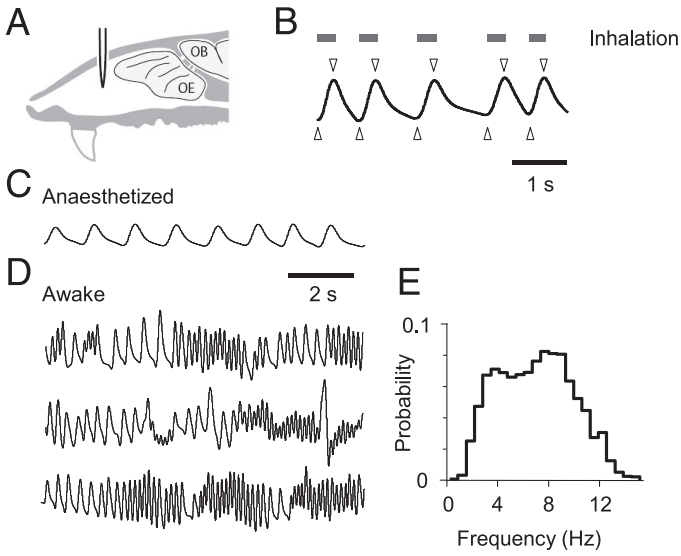

FIG. 2. Respiration frequency has a wide range during behavior. *A*: diagram of the thermocouple placement into the nasal cavity. Sagittal section through the nose is shown with the olfactory epithelium (OE) and the olfactory bulb (OB). *B*: example trace of the thermocouple signal. Rising phase corresponds to cooling, which reflects inhalations. Triangles below and above the trace point to inhalation onsets and offsets, respectively. Bars above mark the inhalation durations. *C*: typical thermocouple signal during anesthesia illustrates the slow and regular breathing pattern (<1 Hz). Scale bar below. *D*: example traces show the rapid and variable respiratory patterns typical in the freely behaving rat. Scale bar above. *E*: histogram of instantaneous respiration frequency (inverse of the respiration cycle duration) across an entire behavioral session shows the wide range of respiratory cycles.

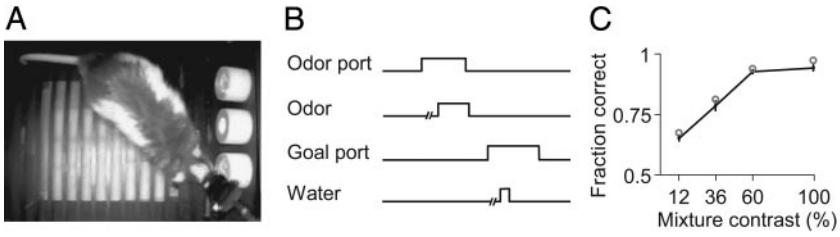

FIG. 3. Two-choice odor-mixture–discrimination task. *A*: picture of a rat performing the olfactory discrimination task. Entry into the center port triggers the delivery of an odor, which is available until the rat stays (or a maximum of 2 s). Based on the identity of the odor rats are trained to respond to the left or right goal port where correct decisions are rewarded with water. *B*: temporal structure of task events. Entry and exit from the ports are recorded using photodetectors. *C*: discrimination accuracy as a function of mixture ratio ($n = 6$ rats). Odor-mixture pairs are pooled and plotted as a function of mixture contrast (e.g., 80/20 and 20/80 ratios yield $|80 - 20| = 60$). Empty circles represent the performance of an individual rat.

ment using paired recordings from both nostrils. These experiments revealed very good correspondence between nostrils on a cycle-by-cycle basis with small (<25 ms) latency differences (Fig. 1*C*).

Odor-discrimination task

Behavioral training and testing were conducted as described previously in Uchida and Mainen (2003). The behavioral box contains a panel of three ports: a central port for odor delivery ("odor port") and two ports on each side ("goal ports") for water delivery (Fig. 3*A*). Entry and exit from the ports are detected based on infrared photo beams located inside each port. Odors are mixed with pure air to produce a 1:20 dilution at a flow rate of 1 L/min using a custom-built olfactometer. The behavioral session is controlled by custom software written in Matlab (The MathWorks) using computer data acquisition hardware (National Instruments) to record the port signals and control the valves of the olfactometer and water delivery.

Male Long–Evans hooded rats were trained to perform olfactory discrimination for water reward. Animals were maintained on a reverse 12-h light/dark cycle and tested during their dark period. They were allowed free access to food but were restricted to water available during the behavioral session and for 30 min after the session.

Rats initiated trials by entering the odor port in the center, which triggered the delivery of an odor with a random delay. Odor-delivery delays followed a uniform random distribution of 0.1–0.2 s for three rats and 0.2–0.5 s for the other three rats. Odor was turned off when rats exited the odor port or after a maximum sampling period of 1 s. Water reward was available for correct choices for ≤2 s after rats left the odor-sampling port. Rats were trained until a performance of 90% correct trials was achieved. Binary odor mixtures of caproic acid and 1-hexanol (1/10 dilution in mineral oil) were used. For three rats eight ratios (100/0, 80/20, 68/32, 56/44, 44/56, 32/68, 20/80, and 0/100; caproic acid/1-hexanol) and for another three rats four or six ratios were used (80/20, 68/32, and sometimes 56/44, etc.). Stimuli were delivered in pseudorandom order within each session.

Analysis methods

The sniffing signals were digitally filtered off-line (0.5–60 Hz) and the timings of inhalation and exhalation onsets were determined as the local maxima (exhalation onset) and minima (inhalation onset) of the temperature signal. The determination of inhalation timing was first done using an automated peak/trough detection algorithm, which was confirmed and adjusted manually when necessary. This preprocessed signal then served as the basis for our analysis. The data set consisted of 12,018 completed trials with good thermocouple signal recorded from six rats (range: 694–3,798 trials/animal).

In principle we could have used spectral analysis to calculate the frequency of the signal (see Fig. 1*B*). However, because the respiration signal is strongly nonsinusoidal, with often unequal durations of exhalation and inhalation, spectral decomposition would show additional frequency components that would have to be disambiguated from the carrier frequency, which is our main interest. In addition because of the rapidly changing nature of the signal it was preferable to analyze the signal in the time domain. Therefore to characterize respiration frequency we first determined the instantaneous frequency defined as the inverse of the cycle duration (beginning at inhalation onset) for the entire duration of each cycle.

To measure how well can the frequencies of different sniffing modes be distinguished, we used receiver-operating characteristic (ROC) analysis. The discriminability index (D) is defined as the area under the ROC curve

$$D = \int_{-\infty}^{\infty} P(s_{odor} = f)P(s_{reward} < f)\mathrm{d}f$$

where s_{odor} and s_{reward} refer to sniffing frequency during odor-sampling and prereward periods, respectively.

We also assessed whether the increase in respiration frequency is the result of a single step change reflecting a binary process using a trial-by-trial analysis of frequency change. If the increase in sniffing frequency constitutes a step change in each trial, then the largest change in sniff cycle duration should also account for the total change in frequency. The total change in frequency was defined to be the difference between a baseline (−1,000 to −300 ms) to odor-sampling (0–300 ms) frequencies. The largest jump in frequency explained the total change in 73 ± 4% of trials (and accounted for 85 ± 1% of the change in the rest of trials) and it explained 90% of the total change in 89 ± 2% of trials (and accounted for 87 ± 0.5% in the rest of trials; means ± SE across rats). Note that because sniff durations vary even within modes (mean cycle-by-cycle change: 0.47 ± 0.2 Hz), reaching the high-frequency sniff mode may require more or less change than total change defined earlier.

RESULTS

Rats were implanted with a thermocouple in one nostril (Fig. 2*A*) to measure the temperature changes between inspired and expired air (Uchida and Mainen 2003). Although nasal temperature cannot provide an exact measurement of respiratory volume or flow rate, it does provide a precise indication about the time of onset of inhalation on a cycle-by-cycle basis (Fig.

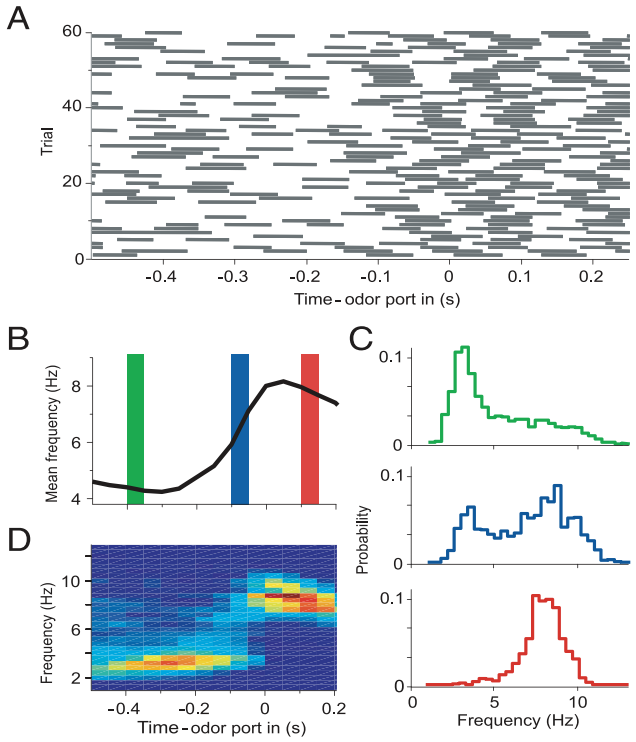

FIG. 4. Anticipatory sniffing mode switch. *A*: sniffing raster. Each row shows a single trial with the timing aligned to entry into the odor port. Gray bars represent inhalation periods; the exhalations in between are not marked. A random selection of 60 trials in one behavioral session is shown. *B*: mean frequency of respiration (instantaneous frequency averaged across trials) locked to the time of entry into to the odor port. Note that the increase in frequency starts before odor port entry. *C*: snapshots of sniffing frequency distributions in different 50-ms time periods. Colors reflect the time intervals indicated in *B*. *Top*: −0.4 to −0.35 s; *middle*: −0.1 to −0.05 s; *bottom*: 0.1 to 0.15 s. Time of odor port entry is 0. *D*: time–frequency representation of respiration locked to odor port entry.

2*B*). Whereas respiration under anesthesia was slow and regular (Fig. 2*C*), awake animals exhibited rapid and complex respiratory patterns (Fig. 2*D*). To analyze sniffing patterns, we first determined the timing and duration of each respiration cycle using a semiautomatic detection algorithm (see METHODS; Fig. 2*B*). During behavior a broad spectrum of respiration frequencies was observed ranging from <2 to >12 Hz (Fig. 2*E*). Sniffing in rats is usually defined as rapid, rhythmic respiration (Clarke et al. 1970; Welker 1964; Youngentob et al. 1987). Because rats, unlike humans, always breathe nasally, some of the observed frequencies presumably correspond to basal respiration whereas others correspond to "sniffing" per se. We will refer to all higher (>5 Hz) frequencies as sniffing from here on.

To understand how respiration is regulated during behavior, we trained rats ($n = 6$) to perform an olfactory-discrimination task (Uchida and Mainen 2003). In this task rats initiate trials with a nose poke into a conical odor port. On entry a computer-controlled olfactometer directs the odor flow into the port (Fig. 3*A*). A binary odor mixture is delivered and the odor with the higher concentration instructs the response to the left or right goal ports where correct decisions are rewarded with water (Fig. 3*B*). Rats achieved high performance (>90%) for easy stimuli (80/20 and 100/0 odor mixtures) in this task, but were challenged by more difficult discriminations (Fig. 3*C*) as previously reported (Uchida and Mainen 2003).

As rats entered the odor-sampling port they increased their sniffing rate. This change in sniffing pattern can be seen in a raster representation of inhalation durations in Fig. 4*A*. For each trial the sequence of inhalation periods is marked with a gray bar and aligned to the time of entry into the odor port. For further analysis we determined the instantaneous frequency of respiration for each sniff cycle. On average the rate of respiration systematically increased before rats entered the odor-sampling port (Fig. 4*B*). However, this mean frequency was not representative of individual trials, where respiration appeared to be restricted into distinct frequency bands: a low-frequency (2–4 Hz) mode before entry and a high-frequency (6–9 Hz) mode during odor sampling. This can be seen by examining the distribution of sniffing frequencies at different time points relative to odor port entry (Fig. 4*C*). The distribution of respiration frequencies from 150 ms before until the time of entry into the odor port was bimodal in all rats ($P < 0.01$, Hartigan's dip statistic). Figure 4*D* illustrates the full temporal evolution of respiration frequency aligned to odor port entry. This spectral representation reveals two separate respiration modes. During the odor-sampling period all rats displayed high-frequency respiration or sniffing at 7.5 ± 0.4 Hz. Rats spent an average of three sniffs (range: 1–8) at the odor-sampling port. Because of the delay in odor onset, on average two full sniffs (range: 0–6) were actually spent sampling odors.

Sniffing frequency changes could reflect the shortening of the duration of inhalations, exhalations, or both. Therefore we separately analyzed changes in inhalation and exhalation subcycles. For every time point in each trial we assigned the instantaneous inhalation and exhalation durations separately; during inhalation no exhalation durations were assigned and vice versa. By averaging this representation across trials, we could examine variations in inhalation and exhalation periods at a high temporal resolution. As Fig. 5 shows, the median duration of inhalations changed little, whereas exhalation periods shortened substantially. Consequently, the inhalation–exhalation cycle became more symmetric during the odor-sampling period. Although the overall increase in sniffing

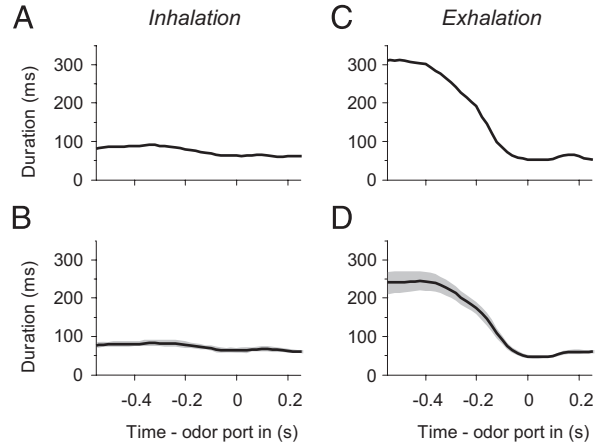

FIG. 5. Sniffing frequency increase reflects shortening of exhalations. *A*: time course of inhalation duration (median) for one rat. *B*: mean time course of inhalation duration across 6 rats. Shading represents SE across trials. *C*: time course of exhalation duration (median) for one rat. *D*: mean time course of exhalation duration across 6 rats. Shading represents SE across rats.

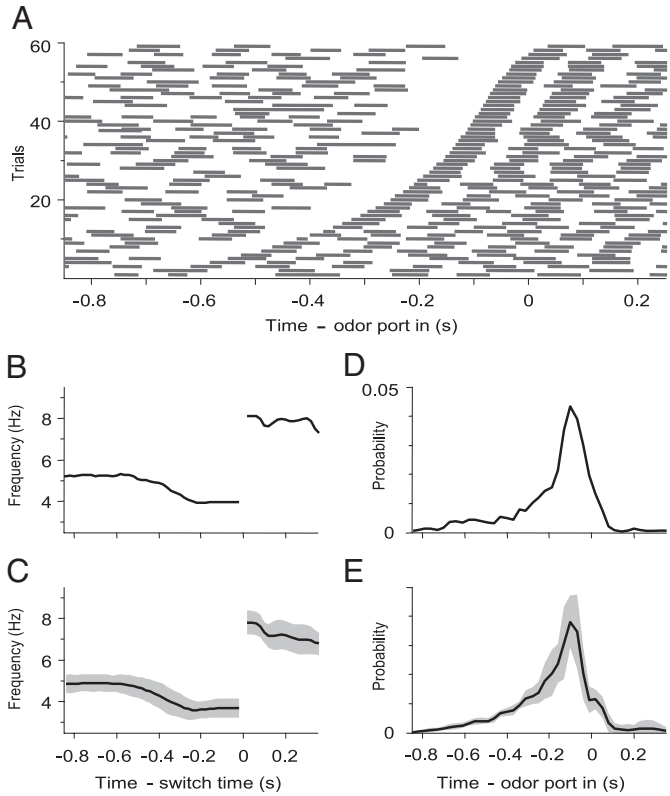

FIG. 6. Rapid sniffing mode switch occurs at variable times. *A*: sniffing raster. Each row shows a single trial with the timing aligned to the entry into the odor port. Gray bars represent inhalation periods. Trials are sorted according to the largest increase in sniffing frequency from −1 s before odor port entry until the exit. Sixty trials were selected that reflect the temporal distribution of frequency jumps for one rat. Trials are uniformly spaced based on their percentile rank of the frequency jump time. *B*: mean sniffing frequency aligned to the sniff cycle where the largest increase occurred for one rat. *C*: average of sniffing frequency across rats ($n = 6$). Shading indicates SE. *D*: distribution of putative mode switch times across trials for one rat. Note that the peak occurs around −150 to −50 ms before odor port entry. *E*: average distribution of putative mode switch times across rats ($n = 6$). Shading indicates SE.

frequency was mostly attributed to the truncation of exhalation periods or equivalently the rate at which inhalations occurred, this was accompanied by a small but significant decrease in the mean inhalation duration as well ($P < 0.01$, two-sample Kolmogorov–Smirnov test between −0.5 and −0.4 s and 0.1 and 0.2 s aligned to odor port entry).

Inspection of the time–frequency histogram of sniffing in Fig. 4*D* suggests that rats switch between modes rapidly, perhaps in a single respiratory cycle. To quantify this suggestion, we turned to a trial-by-trial analysis. For each trial we found the sniff cycle where the largest increase in frequency occurred from 1 s before entry into the odor port until leaving it. The mean size of the largest jump was 4.2 ± 0.3 Hz. This was significantly different from the average change in sniffing frequency from cycle to cycle (0.47 ± 0.2 Hz, excluding the largest one) as well as from the second largest jump (1.5 ± 1.2 Hz) in each trial. Therefore we were able to assign for each trial a time point at which the frequency jump presumably occurred. Figure 6*A* shows a sniffing raster with trials sorted according to the presumed time of mode switch. Aligning each trial to the largest jump in sniff frequency showed that sniffing frequency did not increase before or after this jump on average (Fig. 6, *B* and *C*). The mean frequencies before and after the switch correspond to the sniffing modes evident in Fig. 4*D*.

The timing of the jump from low-frequency respiration to the faster odor sampling mode varied from trial to trial. The likelihood of a sniffing mode switch began to increase as early as 500 ms before the odor-port entry and peaked around 150–50 ms before (Fig. 6, *D* and *E*). If the anticipatory increase in mean sniffing frequency (Fig. 4*B*) is the result of rapid yet temporally variable switches between sniffing modes on individual trials, then the distribution of switch times (Fig. 6, *D* and *E*) should account for the shape of frequency increase. Indeed, the mean time course of the sniffing frequency change matched well the distribution of switch timings. Moreover, the size of the putative jumps accounts for the total change in frequency in the majority of trials (see METHODS). Taken together, the results of the trial-by-trial analysis confirm that the timing of sniffing frequency change is somewhat variable but occurs abruptly in individual trials and demonstrates that switches between sniffing modes can take place in a single respiration cycle.

Such rapid and stereotyped control of sniffing pattern raises the question of whether behavioral performance actually depends on sniffing frequency. Whereas in most trials rats sniffed within the 6- to 9-Hz mode, there was some variation. This allowed us to calculate the choice accuracy for each rat as a function of the sniffing frequency during odor sampling. Odor-mixture stimuli were classified as easy (100/0 and 80/20 ratio mixtures; 94% accuracy) or difficult (68/32 and 56/44 mixtures; 72% accuracy). Because there was variation in absolute frequencies, accuracy was calculated as a function of the normalized frequency for each rat. Figure 7*A* shows that choice accuracy slightly improved with increasing sniffing frequency during odor sampling. Whether the effects of frequency on

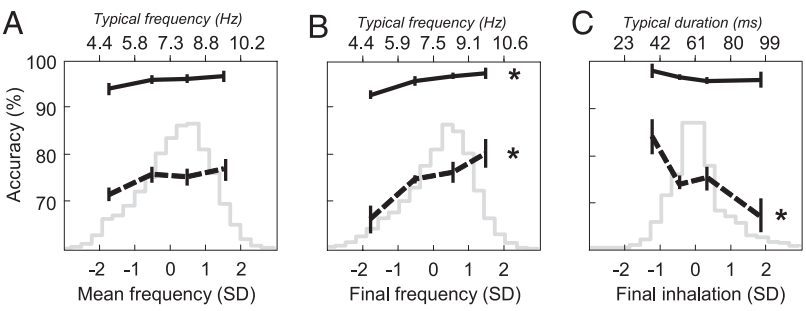

FIG. 7. Sniffing frequency and behavioral performance. Choice accuracy is plotted as a function of sniffing frequency. Solid lines show easy discriminations; stimuli with 100/0 and 80/20 mixture ratios were pooled. Dashed lines are difficult discriminations with 68/32 and 56/44 mixture ratios. Error bars represent SE across rats ($n = 6$). Asterisks marks significant differences in performance at the population level ($P < 0.05$, Kruskal–Wallis test across means). Frequency/duration distributions pooled across rats are plotted in gray. To take into account the variations in absolute frequencies between rats, the analysis was done by first normalizing the data for each rat; therefore frequency and duration are shown in units of SD. Corresponding across-rat median values are shown on the *top*. *A*: mean frequency during the odor-sampling period. *B*: instantaneous frequency of the last completed sniff during odor sampling. *C*: duration of inhalation for the last completed sniff.

performance are statistically significant were examined in two ways: across trials in individual animals and across means at the population level. The impact of sniffing frequency on accuracy was significant for easy (but not difficult) discriminations in three of six rats ($P < 0.05$, Kruskal–Wallis nonparametric ANOVA across trials) but this trend was not significant at the population level ($P > 0.5$, Kruskal–Wallis test across rats). Perhaps it is not the mean odor-sampling frequency but only the last sniff before responding that is important for discrimination performance. Indeed, discrimination accuracy increased more substantially with instantaneous frequency when only final sniffs were considered (Fig. 7*B*; $P < 0.05$, Kruskal–Wallis test across trials in two rats for easy and three rats for difficult mixtures and at the population level $P < 0.05$, Kruskal–Wallis test across means for both easy and difficult mixtures).

What could account for the correlation between sniffing frequency and performance? It is possible that frequency influences the process of odorant capture and therefore accuracy should depend even more strongly on inhalation duration. Indeed, when only the last inhalation period was considered accuracy decreased as the inhalation duration increased (Fig. 7*C*; $P < 0.05$, Kruskal–Wallis test across trials in one of six rats for all stimuli and $P < 0.05$, Kruskal–Wallis test across means for difficult stimuli). At face value it seems surprising that performance drops when there is more time available both for odorants to reach the olfactory epithelium, as well as for olfactory processing. Perhaps inhalation duration is a correlate of a more relevant variable such as flow rate (Walker et al. 1997) and shorter inhalations influence performance indirectly through higher flow rates. On the other hand, performance showed a stronger dependency on the final sniff duration (i.e., instantaneous frequency, Fig. 7*B*) than on the final inhalation duration alone ($P < 0.05$, Friedman's test, six of six rats).

After odor sampling, rats tended to exhibit one long exhalation during their movement to the goal ports (Fig. 8, *A* and *B*). However, respiration did not remain at low-frequency breathing rates but instead switched to a high-frequency pattern before reaching the port. Similar to the change in sniffing pattern in anticipation of odor sampling described earlier, we analyzed this phenomenon in relation to the time of entry into the goal port. Figure 8*C* shows that respiration frequency was higher on average at the goal port than at the odor port. After odor sampling there was a low-frequency (2–4 Hz) mode corresponding to a sniff with a very long exhalation phase followed by a higher (9–12 Hz) mode around the time of entry into the goal port (Fig. 8*D*). Respiration frequency distributions were bimodal between 100 and 200 ms before entry into the goal port for each rat ($P < 0.01$, Hartigan's dip statistic). The abrupt switch into this high-frequency mode can be seen in the spectral representation of sniffing (Fig. 8*E*). The mean frequency of this sniffing mode was 10 ± 0.5 Hz. Note that whereas this prereward sniffing mode is at a higher frequency than sniffing during odor sampling, the amplitude of the thermocouple signal is smaller (Fig. 8*A*), suggesting lower airflow rates (Walker et al. 1997). This anticipatory sniffing mode was not associated with licking by visual inspection. Shortly after goal port entry, respiration frequency dropped again and was in the breathing range (2–4 Hz) while rats were licking rhythmically around 10 Hz (data not shown).

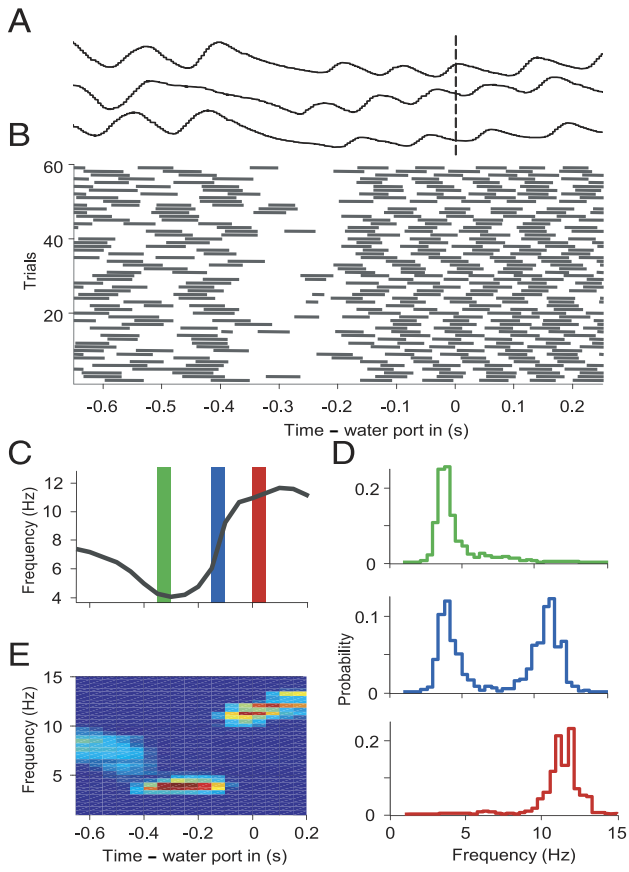

FIG. 8. Rapid anticipatory sniffing mode switch before reward. *A*: example thermocouple recordings aligned to entry into a water port (dashed line). Same timescale as *B*. Rising phases correspond to cooling, which reflect inhalation. *B*: sniffing raster. Each row shows a single trial with the timing aligned to the entry into the goal port. Gray bars represent inhalation periods; intervening exhalations are not marked. A random selection of 60 trials in one behavioral session is shown. Note the long exhalation after odor sampling. For correct trials water is available about 0.1–-0.2 s after entry into the goal port. *C*: mean frequency of respiration (instantaneous frequency averaged across trials) locked to the time of entry into the goal port. *D*: snapshots of sniffing frequency distributions in different 50-ms time periods. Colors reflect the time intervals indicated in *B*. *Top*: −0.35 to −0.3 s; *middle*: −0.15 to −0.1 s; *bottom*: 0.0 to 0.05 s. Time of goal port entry is 0. *E*: time–frequency representation of respiration locked to goal port entry. Note the difference between the sniffing frequency during odor-sampling period (*left*) and the prereward period (*right*).

Both high-frequency (>5 Hz) respiration patterns described earlier (Figs. 4 and 8) may be considered "sniffing" if defined as rapid breathing or polypnea (Welker 1964). However, sniffing during the prereward period had a systematically higher frequency compared with that of odor sampling (difference of 2.1 ± 1 Hz). Even though these distributions overlap on a trial-by-trial basis, the frequency of sniffing during these two periods was mostly distinct (Fig. 9, *B* and *C*). To quantify this, we calculated the discriminability index, which measures how well an optimal observer could distinguish the two sniffing modes trial by trial based on their frequency. This analysis confirmed that sniffing modes are distinguishable with high accuracy ($D = 0.86 \pm 0.09$, means ± SE). In addition, the frequencies of these different sniffing epochs were generally uncorrelated within trials ($r = 0.08$ and 0.10 at $P < 0.01$ for two of six rats and $P > 0.1$ for four of six rats).

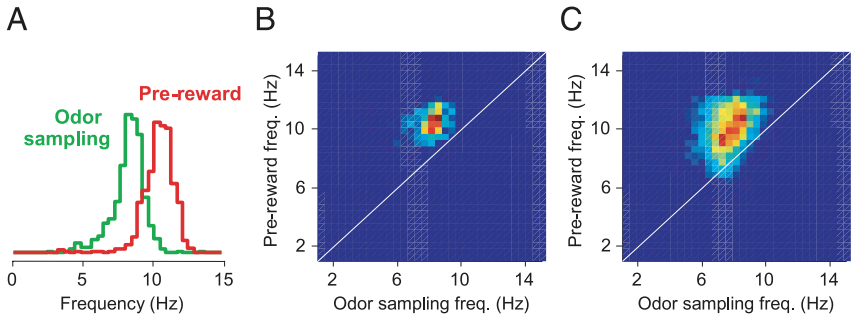

FIG. 9. Two distinct sniffing modes occur in different behavioral epochs. *A*: sniffing frequency distributions during the odor-sampling and prereward periods are largely distinct. Data are from one rat. *B*: 2D histogram of the odor-sampling and prereward period sniffing frequencies shows very little correlation ($r = 0.08$ for this rat) and the prereward frequency is almost always higher. Same rat as in *A*. *C*: average of normalized 2D histograms across 6 rats. Because of quantitative differences in sniffing frequencies between rats, the distribution is wider and exhibits an artifactual correlation between frequency modes that is not present in individual rats.

DISCUSSION

Olfactory processing in mammals begins with the act of inhalation: a sniff. The central importance of sniffing to olfaction may be compared with the integral role of eye movements in vision. As a first step toward understanding the behavioral control of sniffing, we examined sniffing patterns in rats performing an olfactory-discrimination task (Uchida and Mainen 2003). The well-controlled and stereotyped nature of behavior in our task allowed us to relate sniffing patterns precisely to behavioral timing and performance. We obtained three principal results. First, we found two distinct high-frequency respiration modes, one for odor sampling and one associated with reward anticipation, which we term type I and type II sniffing. Second, we established that switches between modes occur in a single respiratory cycle. Third, we determined that behavioral performance is degraded when rats do not enter type I sniffing during odor sampling.

Multiple sniffing modes

Respiration frequency does not vary continuously but rather has different preferred frequency bands. Before odor sampling, respiration frequency was around 2–4 Hz, and shortly before entry into the odor port rats switched to a theta frequency mode (6–9 Hz) and remained in this frequency mode throughout odor presentation. After exiting the odor port, a long exhalation followed during the movement to the goal ports. Immediately before reaching the goal ports rats switched to an even higher-frequency (9–12 Hz) sniffing mode.

Rats are obligate nose-breathers and therefore not every act of respiration should be considered sniffing per se, although it is also clear that even low-frequency respiration is adequate for smelling. Traditionally, sniffing has been defined as a rapid and shallow breathing or polypnea (Clarke et al. 1970; Welker 1964; Youngentob et al. 1987). Here we found that this general description can be further refined by considering its precise respiration frequency and the behavioral epoch in which sniffing occurs. During odor sampling we observed a relatively separate frequency band ranging from 6 to 9 Hz, which we term type I sniffing. In our task, this mode occurred almost without fail during odor sampling and therefore it is most likely related to the acquisition of olfactory information. Importantly, this sniffing mode was not triggered by the odor stimulus, but by the animals' anticipation of entry into the odor-sampling port.

More curiously, there is a second, higher-frequency (9–12 Hz) respiration mode, type II sniffing, that occurred during the prereward period, commencing before entry into a goal port. Because water was delivered after entry into the goal ports for all correct discriminations (with the valve clicks providing the earliest and most prominent cue) there is no apparent functional role for olfaction during this second sniffing mode. Also, the amplitude changes in the thermocouple signal were smaller than those during type I sniffing, indicating lower nasal flow rates (Walker et al. 1997). If this type of sniffing does not directly serve odor acquisition, what behavioral function could it be related to? Several lines of evidence suggest that sniffing is associated with reward anticipation. First, in classical conditioning as rats learn to predict reward based on a sensory cue, sniffing becomes part of the conditioned response (Bindra and Campbell 1967; Freeman et al. 1983). Similarly, in fixed-ratio conditioning for sucrose or brain-stimulation reward, sniffing rates increase in anticipation of reward delivery (Clarke 1971; Waranch and Terman 1975). Finally, brain regions whose activation is intrinsically rewarding, such as the lateral hypothalamus (Olds and Milner 1954), when electrically stimulated elicit exploratory behaviors as well as high-frequency sniffing (Ikemoto and Panksepp 1994). Thus sniffing appears to be a correlate of an anticipatory behavioral state in rats. The odor-discrimination task allowed us to separate odor-sampling and reward-anticipation periods, revealing that the reward-anticipatory sniffing mode (type II) covers a largely distinct and higher-frequency range compared with the odor-sampling mode (type I; Fig. 9). The behavioral stereotypy in the task that allowed us to make these observations may also have contributed to the low variability in sniffing frequencies. However, the two different frequency bands during different epochs cannot be solely attributed to stereotypy and were not predicted by previous observations. On the other hand, the distinct behavioral and neural demands of other olfactory behaviors may reveal additional sniffing modes.

The properties of the reward-anticipatory sniffing mode call into question whether it can be properly termed sniffing, if sniffing is defined as nasal inhalation for the purpose of smelling. Because all respiration in rats occurs nasally, one possibility is that this respiration mode is related to hyperventilation in humans, a behavior linked to certain emotional states. On the other hand, the nonfunctional nature of this sniffing mode may be a reflection of the artificial spatial separation of exploration from appetitive reward in our task. For instance, during foraging for food, the anticipation of an impending discovery might bring about this higher-frequency sniffing mode. More rapid sampling in turn would enhance the spatial resolution of olfactory perception and improve the ability of the animal to tag particular odors to specific spatial locations in the neighborhood of the food source. More ethological studies will be required to resolve these issues.

Rapid switching between sniffing modes

Changes between different respiration modes occurred very rapidly. Trial-by-trial examination showed that rats usually increased respiration frequency from basal breathing (2–4 Hz) to type I sniffing frequencies (6–9 Hz) in a single cycle. The timing of the switch was variable across trials, but occurred in anticipation of odor-port entry and odor delivery rather than as a reaction to olfactory stimuli. This result implies that forebrain control of brain stem respiratory CPGs is rapid and precise during behavior, similar to the peripheral control of respiration (Feldman et al. 2003). One possibility is that there are different CPGs underlying these distinct sniffing modes, which may constitute discrete networks that are either within the same region or in different brain regions (Feldman and Del Negro 2006; Janczewski and Feldman 2006; Onimaru and Homma 2003) Alternatively, it may be that the same pre-Bötzinger complex that is responsible for normal breathing can be rapidly reconfigured to generate multiple motor patterns (Arata et al. 2000; Lieske et al. 2000), perhaps by neuromodulation (Marder and Calabrese 1996). Either way, afferent feedback from higher brain areas to the respiratory center must be able to transform network output quite rapidly, within a single respiration cycle, and do so in an anticipatory manner.

Relation to rhythmic whisking and hippocampal oscillations

The rapid and precise control of sniffing frequency observed here may be relevant for understanding other active senses and, in particular, the rhythmic control of facial whiskers in rodents. Whisking subserves tactile sensory acquisition and is also characterized by rhythmic motion in the "theta" frequency range (Berg and Kleinfeld 2003; Carvell and Simons 1995; Knutsen et al. 2005). At present, the significance of particular whisking frequency modes is not well understood (Ahissar et al. 2000; Harvey et al. 2001; O'Connor et al. 2002), but because the sniffing and whisking rhythms can couple during exploratory behaviors (Welker 1964; our unpublished data), the distinct sniffing modes observed here raise the possibility of corresponding whisking modes.

The observation of different frequency modes within the theta range (4–12 Hz) has interesting precedents in the hippocampus. Theta oscillations in the hippocampus are subdivided into type 1 or type 2 based on frequency (6–12 and 4–6 Hz, respectively) and pharmacological profile (Buzsáki 2002). Both sniffing modes we observed fall within the range of type 1 hippocampal theta and therefore it will be interesting to examine whether both modes could couple to hippocampal oscillations, as previously reported for sniffing during odor sampling (Macrides et al. 1982).

Behavioral correlates of sniffing frequency

The task used allowed us to rigorously test for the first time the possibility that performance during olfactory-mixture discrimination is correlated with sniffing patterns. We took advantage of the fact that the odor-mixture–discrimination task challenged the animals' abilities; rats performed at about 60% for the most difficult stimuli. Previous research has shown that a single sniff is sufficient for maximal performance in this task and taking multiple sniffs does not improve the accuracy of rats (Rajan et al. 2006; Uchida and Mainen 2003). Here we found a significant effect of sniffing frequency on performance, with accuracy increasing at higher sniffing frequencies. This effect was primarily explained by a minority of trials with frequencies below the 6- to 9-Hz range and there was little effect within the 6- to 9-Hz range (Fig. 7). Therefore the impact of sniffing frequency on behavioral performance mostly depended on whether rats switched from respiration to type I sniffing, within the theta frequency range, and not on the precise frequency itself. The correlation between performance and sniffing frequency was somewhat larger when only the last sniff was considered. Therefore the behavioral impact of frequency, or in this case inverse sniff duration, cannot be a result of changes in the number of sniffs taken. Furthermore, this finding indicates that the last sniff contributes disproportionately to the decision made by rats, which may be relevant for understanding why taking multiple sniffs does not improve the performance of rats in our task (Uchida and Mainen 2003).

Two types of factors, physical and neural, could contribute to the correlation between sniff frequency and performance. Physical factors center on the possible effects of frequency on the process of odorant capture. Because odor concentrations in this study were well above threshold (around 0.1–5% of saturated vapor at 1 L/min flow rate), it is not clear why odorant capture would be a limiting step for discrimination. Although relatively little is known about how sniffing parameters affect odorant capture, simulations of fluid flow suggest that higher inspiratory flow rates direct more air toward the olfactory portion of the nasal cavity (Kimbell et al. 1997; Wilson and Sullivan 1999). Our technique did not measure flow rates directly, but it has been shown previously that frequency and flow rate are correlated (Walker et al. 1997). Indeed, performance was better for shorter inhalation durations. However, performance was actually better correlated with the duration of the full respiration cycle than with the inhalation period alone, suggesting a process unrelated to odorant capture.

We consider it likely that sniffing frequency and performance are not causally connected but are both correlated with a third variable such as the level of attention or engagement. Presumably the odorant information necessary to perform discriminations was conveyed to olfactory receptors just as well when rats sniffed at lower frequencies and the onset of sniffing preceded odor signals, suggesting that sniffing is a correlate of an anticipatory or attentional process. A related idea is that higher sniffing frequencies or frequencies within a particular mode facilitate neural processing. The intrinsic time constants of neural circuits involved in the processing of odor signals in the olfactory bulb and piriform cortex may be tuned to 6- to 9-Hz or theta-band signals (Buonviso et al. 2006). In addition, theta oscillations occur not only in olfactory areas but also in the hippocampus, amygdala, striatum, and neocortex and, under some behavioral conditions, sniffing can synchronize with oscillations in different brain areas (Fontanini and Bower 2006; Kay 2005; Macrides et al. 1982). Coordination of these oscillations or phase coupling of neuronal populations at preferred frequencies is a mechanism that has been proposed to facilitate sensorimotor integration (Hyman et al. 2005; Kay 2005; Kepecs et al. 2006; Komisaruk 1977; Siapas et al. 2005). Therefore degraded performance at lower respiration frequencies might result from the failure of sniffing to couple properly to central frequencies.

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