Rapid and precise control of sniffing during olfactory discrimination in rats

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Abstract

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 2-12 Hz, but odor discrimination was dependent on 6-9 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 acquisition of olfactory information.
Introduction

Sensory function depends on a combination of feed-forward 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 in order 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). While 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 brainstem (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 feed-back loop in humans (Johnson et al. 2003). Similarly, the frequency and depth of sniffing depends 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 0.2s in advance (McLeod 1987; Ripoll et al. 1987). Whether cognitive processes are exert temporally precise and active control over sniffing in rodents, as 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).

While sniffing clearly subserves olfaction and strongly patterns olfactory processing, under natural conditions it is usually a component of a coordinated rhythmic movement sequence involving whisker and head movements. Moreover, sniffing can be elicited by reward anticipation in 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, and 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 characteristics of sniffing covary with its frequency (Walker et al. 1997; Yougentob 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 (4-12Hz) 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.
Materials and 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 mean ± SD across the 6 rats examined unless otherwise noted.

Measurement of respiration

Rats were implanted with a temperature sensor (0.005” 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 due to breathing (Angyan and Szirmai 1967; Clarke et al. 1970). While 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 0.1-475Hz and digitized at 1000Hz.

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 in order 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 (<25ms) 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 only provide information 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 Figure 1A). These differences could have been due to either movement-
related artifacts or potentially physiological factors. The thermocouple method was preferred due
to its day to day stability and its more direct association with olfactory processing (Figure 2A).
We tested the variability in the timing of the thermocouple signal due to its placement 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 (Figure 1C).

**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 (Figure 3A). Entry and exit
from the ports is 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
(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 hr 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 minutes 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 3 rats, and 0.2-0.5 s for the other 3 rats. Odor was turned off when rats exited the
odor port or after a maximum sampling period of 1 second. Water reward was available for correct choices for up to 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 3 rats eight ratios (100/0, 80/20, 68/32, 56/44, 44/56, 32/68, 20/80 and 0/100; caproic acid/1-hexanol), for another 3 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 12018 completed trials with good thermocouple signal recorded from 6 rats (range:694-3798 trials / animal).

In principle we could have used spectral analysis to calculate the frequency of the signal (see Figure 1B). But since the respiration signal is strongly non-sinusoidal 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 distinguishable are the frequencies of different sniffing modes, we used receiver-operating characteristic (ROC) analysis. The discriminability index is defined as the area under the ROC curve:

\[ D = \int_{-\infty}^{\infty} P(s_{\text{odor}} = f)P(s_{\text{reward}} < f)df \]

where \( s_{\text{odor}} \) and \( s_{\text{reward}} \) refer to sniffing frequency during odor-sampling and pre-reward 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 (-1000 – 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; mean ± SEM 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 above.
Results

Rats were implanted with a thermocouple in one nostril (Figure 2A) 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 (Figure 2B). While respiration under anesthesia was slow and regular (Figure 2C), awake animals exhibited rapid and complex respiratory patterns (Figure 2D). To analyze sniffing patterns, we first determined the timing and duration of each respiration cycle using a semi-automatic detection algorithm (see Methods; Fig. 2B). During behavior a broad spectrum of respiration frequencies was observed ranging from below 2Hz to above 12Hz (Figure 2E). Sniffing in rats is usually defined as rapid, rhythmic respiration (Clarke et al. 1970; Welker 1964; Yougentob et al. 1987). Because rats, unlike humans, always breathe nasally, some of the observed frequencies presumably correspond to basal respiration while others correspond to “sniffing” per se. We will refer to all higher (>5Hz) 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. Upon entry a computer-controlled olfactometer directs the odor flow into the port (Figure 3A). 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 (Figure 3B). 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 (Figure 3C) 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 Figure 4A. For
each trial the sequence of inhalation periods are 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 (Figure 4B). 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 (Figure 4C). The distribution of
respiration frequencies from 150 ms prior until the time of entry into the odor port was bimodal
in all rats ($P < 0.01$, Hartigan’s dip statistic). Figure 4D 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 3 sniffs (range: 1-8) at the odor sampling
port. Because of the delay in the odor onset, on average 2 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 sub-
cycles. 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 Figure 5 shows, the median duration of
inhalations changed little while exhalation periods shortened substantially. Consequently the inhalation-exhalation cycle became more symmetric during the odor-sampling period. While the overall increase in sniffing frequency was mostly due 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 to -0.4 s and 0.1 to 0.2 s aligned to odor port entry).

Inspection of the time-frequency histogram of sniffing in Figure 4D 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 second 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 6A 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 (Figure 6B, C). The mean frequencies before and after the switch correspond to the sniffing modes evident in Figure 4D.

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 prior to the odor-port entry and peaked around 150–50 ms before (Figure 6D, E). If the anticipatory increase in mean sniffing frequency (Figure 4B) is the result of rapid yet temporally variable switches between sniffing modes on individual trials then the distribution of switch
times (Figure 6D, 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 whether behavioral performance actually depends on sniffing frequency. While in most trials rats sniffed within the 6–9 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 function of the normalized frequency for each rat. Figure 7A shows that choice accuracy slightly improved with increasing sniffing frequency during odor sampling. Whether the effects of frequency on 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 3 of 6 rats ($P < 0.05$, Kruskal Wallis non-parametric analysis of variance 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 (Figure 7B; $P < 0.05$, Kruskal Wallis test across trials in 2 rats for easy and 3 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 on 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 (Figure 7C; $P < 0.05$, Kruskal Wallis test across trials 1/6 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 for odorants to reach the olfactory epithelium and 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 dependence on the final sniff duration (i.e. instantaneous frequency, Figure 7B) than on the final inhalation duration alone ($P < 0.05$, Friedman's test, 6/6 rats).

After odor sampling, rats tended to exhibit one long exhalation during their movement to the goal ports (Figure 8A, 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 above, we analyzed this phenomenon in relation to the time of entry into the goal port. Figure 8C 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 (Figure 8D).
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 (Figure 8E). The mean frequency of this sniffing mode was $10 \pm 0.5$ Hz. Note, that while this pre-reward sniffing mode is at a higher frequency than sniffing during odor-sampling, the amplitude of the thermocouple signal is smaller (Fig 8A), 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).

Both high frequency (>5 Hz) respiration patterns described above (Figure 4 and 8) may be considered “sniffing” if defined as rapid breathing or polypnea (Welker 1964). However, sniffing during the pre-reward period had a systematically higher frequency compared to odor-sampling (difference of $2.1 \pm 1$ Hz). Even though these distributions overlap, on a trial by trial basis the frequency of sniffing during these two periods was mostly distinct (Figure 9B, C). To quantify this, we calculated the discriminability index, which measures how well an optimal observer could distinguish trial-by-trial the two sniffing modes based on their frequency. This analysis confirmed that sniffing modes are distinguishable with high accuracy ($D = 0.86 \pm 0.09$, mean ± SEM). 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 2/6 rats, and $P > 0.1$ for 4/6 rats).
Discussion

Olfactory processing in mammals begins with the act of inhalation: a sniff. The central importance of sniffing to olfaction may be compared to the integral role of eye movements in vision. As a first step towards 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 2–4Hz, 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; Youngetob 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, which occurred during the pre-reward period, commencing prior to 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 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 to the odor sampling mode (type I; Figure 9). The behavioral stereotypy in the task that allowed us to make these observations may have also contributed to the low variability in sniffing frequencies. However, the two different frequency bands during different epochs cannot be solely due 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. Since all respiration in rats occurs nasally, one possibility is that this respiration mode is related to hyperventilation in humans, a behavior which is linked to certain emotional states. On the other hand, the non-functional 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 brainstem 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 via 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 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 since 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 (Buzsaki 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 due to a minority of trials with frequencies below the 6–9 Hz range and there was little effect within the 6-9 Hz range (Figure 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 due to 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 (Rajan et al. 2006; Uchida and Mainen 2003).

Two types of factors, physical and neural, could contribute to correlations 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. While relatively little is known about how snifing
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 could 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. 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 6–9 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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Figure Legends

Figure 1: Nature of the thermocouple signal
(A) Concurrent recordings of two 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. The different measurements mostly agree on a cycle-by-cycle basis with small phase offsets even for high frequency >6Hz sniffing. Sometimes, however, the plethysmograph signal transiently exhibited dissimilar patterns.
(B) Spectral coherence between the two 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. While the thermocouples were implanted at the same A-P position bilaterally, small differences could also impact the exact timing of respiration signals.

Figure 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. The 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 <1Hz. 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.

**Figure 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.

**Figure 4: Anticipatory sniffing mode switch**
(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, the exhalations in between are not marked. A random selection of 60 trials in one behavioral session is shown.

(B) The mean frequency of respiration (instantaneous frequency averaged across trials) locked to the time of entry into 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. The 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.

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**Figure 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.

(C) Time course of exhalation duration (median) for one rat. Shading represents S.E.M across trials.

(D) Mean time course of exhalation duration across 6 rats. Shading represents S.E.M. across rats.

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**Figure 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. 60 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) The average of sniffing frequency across rats (n=6). Shading indicates S.E.M.

(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) The average distribution of putative mode switch times across rats (n=6). Shading indicates S.E.M.

**Figure 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 pooled. Dashed lines are difficult discriminations with 68/32 and 56/44 mixture ratios. Error bars represent S.E.M across rats (n=6). Asterisks marks significant differences in performance at the population level (P < 0.05, Kruskal Wallis test across means). The 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 standard deviation. The 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.
Figure 8: Rapid anticipatory sniffing mode switch before reward

(A) Example thermocouple recordings aligned to entry into a water port (dashed line). Same time scale 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 following odor sampling. For correct trials water is available approx. 0.1-0.2 s after entry into the goal port.

(C) The 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. The 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 pre-reward period (right).

Figure 9: Two distinct sniffing modes occur in different behavioral epochs

(A) Sniffing frequency distributions during the odor-sampling and pre-reward periods are largely distinct. Data are from one rat.

(B) 2D histogram of the odor-sampling and pre-reward period sniffing frequencies shows very little correlation ($r = 0.08$ for this rat) and the pre-reward frequency is almost always higher. Same rat as in (A).
(C) Average of normalized 2D histogram across 6 rats. Due to 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.
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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. The 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 <1Hz. 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.
Figure 3
Figure 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. (C) Time course of exhalation duration (median) for one rat. Shading represents S.E.M across trials. (D) Mean time course of exhalation duration across 6 rats. Shading represents S.E.M. across rats.
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. 60 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) The average of sniffing frequency across rats (n=6). Shading indicates S.E.M. (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) The average distribution of putative mode switch times across rats (n=6). Shading indicates S.E.M.
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Figure 8
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