Individual and synergistic effects of sniffing frequency and flow rate on olfactory bulb activity

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ABSTRACT

Is faster or stronger sniffing important for the olfactory system? Odorant molecules are captured by sniffing. The features of sniffing constrain both the temporality and intensity of the input to the olfactory structures. In this context, it is clear that variations in both the sniff frequency and flow rate have a major impact on the activation of olfactory structures. However, the question of how frequency and flow rate individually or synergistically impact bulbar output has not been answered. We have addressed this question using multiple experimental approaches. In double-tracheotomized anesthetized rats, we recorded both the bulbar local field potential and mitral/tufted cells activities when the sampling flow rate and frequency were independently controlled. We found that a tradeoff between the sampling frequency and the flow rate could maintain olfactory bulb sampling-related rhythmicity and that only an increase in flow rate could induce a faster odor-evoked response. Local field potential and sniffing were recorded in awake rats. We found that sampling-related rhythmicity was maintained during high-frequency sniffing. Furthermore, we observed that the co-variation between the frequency and flow rate, which was necessary for the tradeoff seen in the anesthetized preparations, also occurred in awake animals. Our study shows that the sampling frequency and flow rate can act either independently or synergistically on bulbar output to shape the neuronal message. The system likely takes advantage of this flexibility to adapt sniffing strategies to animal behavior. Our study provides additional support for the idea that sniffing and olfaction function in an integrated manner.

Keywords: olfaction, sniffing dynamics, LFP, mitral cell
INTRODUCTION

Just as visual perception is dependent on eye movement, olfaction is dependent on the way that odors are sampled (i.e., respiration). Olfactory activity and odor sampling behaviors maintain strong temporal relationships at multiple levels, including the olfactory receptors (Carey et al. 2008; Chaput, 2000), glomerular activation maps (Spors et al. 2002), mitral/tufted (M/T) cells (Chaput et al. 1992; Macrides and Chorover, 1972; Margrie and Schaefer, 2003; Onoda and Mori, 1980; Sobel and Tank, 1993), bulbar local field potential (LFP) (Adrian, 1942; Buonviso et al. 2003) and the piriform cortex (Litaudon et al. 2003; Poo and Isaacson, 2009; Wilson, 1998). Odor sampling behavior is thus a key feature of olfactory perception (Mainland and Sobel, 2006) and sniffing has been proposed to be an olfactory-motor act (Johnson et al., 2003). If this is indeed the case, any variation in the sniffing frequency and/or flow rate should shape olfactory coding. Sniffing has been shown to be highly variable with respect to both the frequency (which ranges from 2 to 12 Hz) and the flow rate (Yougentob et al. 1987). Flow rate has been observed to impact both the olfactory epithelium (Kent et al. 1996; Mozell, 1970; Scott-Johnson et al. 2000) and glomerular activity (Oka et al. 2009). Recently, we described flow rate-induced modifications of bulbar activity (Courtiol et al. 2011). How variation in the sampling frequency impacts bulbar activity remains unclear. At least two factors could be modified by a high frequency. The first factor is the strength of the bulbar response because a high sampling frequency results in a short bulbar activation period, as confirmed by the attenuation of the respiratory pattern of glomerular activation (Verhagen et al. 2007). The second factor that is likely modified by a high frequency is the latency of the bulbar response because the high frequency increases the speed of odorant acquisition (Wesson et al. 2009). While the present study was under review, the effect of sampling frequency on M/T cells activity was described (Carey and Wachowiak, 2011). However, the question of how the frequency and flow rate individually or synergistically impact bulbar output has not yet been addressed.

Our question was then two-fold: does a high sampling frequency modify the strength and/or latency of the bulbar response? If so, can a higher flow rate counterbalance the effects of a high frequency?
To precisely control both of the sampling parameters, we used an anesthetized double-cannulated tracheotomized rat experimental preparation. This preparation allowed flow rate and frequency to be independently controlled, which is not possible in awake animals. We found that a tradeoff between the sampling frequency and the flow rate resulted in the persistence of sampling-related activity in the olfactory bulb (OB) and only an increase in the flow rate could induce M/T cells to respond earlier. Using awake rats, we demonstrated that a similar co-variation between the frequency and flow rate, which was necessary for the tradeoff observed in the anesthetized preparation, occurred in the behaving animal.
MATERIALS AND METHODS

Experiment 1: OB activity recording and control of sampling parameters in anesthetized rats

Preparation and recording

Ten male Wistar rats (200 - 450 g) obtained from Janvier (Le Genest-Saint-Isle, France) were anesthetized with urethane (1.5 g/kg, i.p., with additional supplements as needed) and placed in a stereotaxic apparatus. Anesthesia was maintained by supplemental doses when necessary. LFP oscillations were used to monitor anesthesia depth. The animals were placed on a heating pad to maintain constant body temperature. All surgical procedures were conducted in strict accordance with the European Community Council directive of November 24, 1986 (86/609/EEC) and the guidelines of the French Ethical Committee and French Legislation and they received approval from the Lyon 1 University Ethics Committee (Direction of Veterinary Service # 69387473).

Tracheotomy

When all pain reflexes were abolished, a tracheotomy was performed by inserting the first cannula into the trachea, which allowed the rat to breathe freely. Next, a second cannula was inserted rostrally through the larynx into the post-nasal cavity to allow air to be pushed and pulled through the nasal cavity.

Electrophysiological recordings

The dorsal region of the OB was exposed. Bulbar activity was recorded as a broadband signal (0.1 Hz to 5 kHz) using 16-channel silicon probes (NeuroNexus Technologies, Ann Arbor, MI) and a homemade 16-channel DC amplifier (gain 1,000 ×). 16-channel silicon probes were placed so that we could record both the M/T cell activity from the mitral cell layer and the maximum LFP amplitude in the granular cell layer. The mitral cell layer was located using the following criteria: LFP waveform,
the magnitude of the unit action potentials and the inability to record spikes from the granular cell layer. The granular cell layer was located by LFP waveform as described by Buonviso et al. (2003). Recordings were performed in the whole anteroposterior axis of the OB. Data were digitally sampled at 20 kHz and acquired with a PC using a National Instrument acquisition card (BNC-2111).

**Odors**

Odors (Sigma Aldrich, St Louis, MO and Fluka, Germany) were delivered in a randomized series through a dilution olfactometer (400 ml/min). The odors were 2-heptanone (K07) and isoamyl acetate (ISO). The odors were delivered in front of the animal's nose at the proportion of 18% of the saturated vapor pressure. The time delay between each odor presentation was at least one minute. The recording protocol was as follows: 5 seconds of spontaneous activity, 5 seconds of odor-evoked activity and 5 seconds of post-stimulus activity.

**Imposed nasal airflow**

Airflow was measured using fast response-time airflow sensors. This setup has been extensively described by Roux et al. (2006). We used two sensors; the first, which was placed in front of the tracheal cannula, measured the animal’s own respiration. The second sensor, which was placed at the entrance of the nostril, measured the airflow circulating through the nasal cavity. We thus recorded two airflow signals: the animal’s own respiratory airflow and the imposed nasal airflow.

To simulate respiratory cycles, we used a homemade apparatus that allowed the reproduction of both the inhalation and exhalation phases. For more details, see Courtiol et al. 2011. Briefly, the animal's respiratory signal (collected at the tracheal cannula) was sent to the respiratory signal simulator, which in turn sent a simulated sampling airflow toward the nasal cavity through the nasal cannula. The simulated sampling airflow in the nasal cavity could be controlled precisely in terms of frequency, flow rate and synchronization (with respect to the animal’s own respiration). To study the effects of variations in both the sampling frequency and flow rate, we chose to impose a) six different nasal airflow frequencies, i.e., 1 Hz, basal (which is the respiratory frequency of a urethane-anesthetized rat, mean = 2.3 Hz), 4, 6, 8 and 10 Hz, in a randomized procedure and b) two nasal flow rates of 500
ml/min and 1,000 ml/min. Fig. 1A shows the corresponding flow volumes. A 500 ml/min flow rate coupled with the basal frequency reproduced the bulbar LFP signal in response to ISO that was usually recorded in the anesthetized non-tracheotomized condition (Buonviso et al. 2003). The flow rates and sampling frequencies used corresponded to rat physiological parameters (Yougentob et al. 1987).

**Data processing**

All data processing was performed using OpenElectrophy open-access homemade software (Garcia & Fourcaud-Trocmé, 2009). OpenElectrophy is open-source and is freely available for download at http://neuralensemble.org/trac/OpenElectrophy.

**Respiratory signal**

An important feature of the olfactory signal is its temporal correlation with breathing. Therefore, we developed a method for representing the data as a function of the respiratory phase (Roux et al. 2006). Briefly, the respiratory cycle was first divided into two periods: inspiration and expiration. The time component for these periods was then converted into a circular phase component defined between 0 and 1, where 0 and 1 represent the beginning of inspiration and the end of expiration, respectively. This phase representation of the respiratory cycle was used as a normalized time basis (between 0 and 1) and permitted us to collect and analyze the results in a standardized data format across different recordings. The respiratory phase computation was performed on both the animal’s own respiration and the imposed nasal airflow (i.e., sampling cycles), which allowed us to compare the olfactory activity relative to either of these two signals.

**LFPs**

LFPs were obtained by band-passing the signal at 0-200 Hz.

**Wavelet transform and wavelet ridge extraction for gamma oscillations**

To preserve both time and frequency information, we used a time-frequency representation (TFR) based on a continuous wavelet transform. We have developed an algorithmic procedure (Roux et al.
2007) to extract phase information from the oscillations identified in the signal. Gamma oscillations were easily and clearly discriminated in both the time-frequency representations and the raw signals. A gamma burst was defined as a succession of at least three oscillation cycles. An absolute threshold was defined during the stimulus-epoch for the 35-90 Hz band and for each electrode. This threshold was used to define the time and frequency boxes centered on the points of the maximum signal amplitude. Next, the time and frequency coordinates of all local maxima in the gamma band above this threshold were extracted. From each maximum, we computed the Morlet’s complex wavelet transformation with high time resolution, both forward and backwards, following the line of maximum energy. The computation stopped when the energy fell below the threshold. Therefore, for each maximum detected on the time-frequency map, we obtained with high time resolution a wavelet ridge that was defined by its starting and ending times, instantaneous frequency and instantaneous phase. Thus, each gamma episode was characterized by the coordinates of its maximum (power and frequency) and its wavelet ridge (duration and frequency).

Statistical tests were performed using Excel and StatView software. The level of significance was set at p<0.05 for all statistical tests.

The occurrence of gamma oscillations (defined as the probability of observing at least one gamma burst during odorant stimulation irrespective of the number of gamma bursts) was calculated and compared between the sampling conditions using the Chi\(^2\) test. The average gamma burst duration, frequency, amplitude and recurrence over successive sampling cycles (defined as the total number of oscillatory bursts per total number of sampling cycles) were calculated. These parameters were compared among the sampling conditions using two-way factorial ANOVA with the frequency and flow rate as factors. Post-hoc analyses were performed using the Student Newman-Keuls test.

**LFP-sampling-related modulation**

In addition to gamma oscillations, LFP also presents a slower component linked to the respiratory sampling rate (Buonviso et al. 2003). LFP-sampling-related modulation occurrence was determined
using fast Fourier transformations (FFTs), which are better suited for processing long signals. FFTs were performed during spontaneous and odor-evoked activities. The LFP-sampling-related modulation was analyzed in two different ways. First, the amplitude of the sampling-related modulation was determined. LFP signals were averaged relative to the imposed sampling cycle. Then, the amplitude of the LFP-sampling-related modulation was calculated as the difference between the maxima and the minima of each averaged LFP signal. Second, the cross-correlation coefficient between the LFP signal and the imposed nasal airflow was measured. The cross-correlations between the imposed sampling nasal airflow and the LFP signals were determined according to the following equation:

\[
\frac{\langle (X - \langle x \rangle)(Y - \langle y \rangle) \rangle}{\sigma_x \sigma_y},
\]

where \( x \) denotes the imposed sampling cycle, \( y \) is the LFP signal, \( <> \) is the mean and \( \sigma \) is the standard deviation. Repeated-measures ANOVA with the frequency and flow rate as factors was used for comparisons of the LFP-sampling-related modulation amplitude and the cross-correlogram coefficients. Post-hoc analyses were performed using the Student Newman-Keuls test.

Spikes

**Spike sorting**

Spiking activity was extracted from individual electrodes using a band-pass filter (300-3000 Hz). Multi-unit activity consisted of a few neurons on each electrode. We chose to use only well-discriminated units with a signal / noise ratio \( \geq 5/1 \) and to sort cells according to their spike amplitude. Consequently, the number of units retained for analysis was restricted to 1-3 units per channel. We preferred to use a conservative criterion, which resulted in a limited number of units but was also highly reliable. This procedure has been described and illustrated in detail by Cenier et al. (2009).

**Pattern Classification**

In our preceding reports, the temporal patterns of M/T cell activity were classified according to the variations in the discharge rate with respect to the respiratory cycle (Buonviso et al. 1992; Cenier et al. 2009; Courtiol et al. 2011). Here, the imposed nasal airflow cycle was used as a time reference for
pattern classification. Thus, a pattern was classified as SYNCHRO when its spiking discharge presented synchronized activity relative to the imposed sampling cycle, as NS (non-synchronized pattern) when it presented a uniform distribution of activity along the imposed sampling cycle and as NULL when it exhibited only a few or no spikes. An M/T cell was considered responsive when the frequency rate or activity pattern changed from spontaneous activity to odor-evoked activity. Frequency change was considered as a response when mean and/or maximum frequency values changed by more than 2 SD between two conditions (Buonviso and Chaput, 1990; Chaput et al. 1992).

Measurements and statistics

Statistical tests were performed using Excel and StatView software. The level of significance was set at p<0.05 for all statistical tests.

First, the percentages of odor-responsive M/T cells were calculated for each condition and compared using a Chi² test. Second, the relative proportions of the different M/T cell patterns (SYNCHRO, NS, NULL) were compared among the sampling conditions during odor-evoked activities using a Chi² test. Third, the latencies between the first spike and the first inspiration following odor onset were calculated and compared among the different sampling frequency conditions using two-way factorial ANOVA with the frequency and flow rate as factors. Post-hoc analyses were performed using the Student Newman-Keuls test.

Experiment 2: Respiration recordings in freely moving rats

Preparation and recording

Six male Long Evans rats (Janvier, France) weighing 250-300 g at the start of the experiment were used. Food and water were available ad libitum during the experiment.

Respiration

Respiratory behavior in freely moving rats was measured using whole body plethysmography (Emka TECHNOLOGIES, France) with the goal of disturbing the rats’ behavior as little as possible. This
setup has been described extensively by Hegoburu et al. (2011). Briefly, the plethysmograph is composed of two chambers: a subject chamber and a reference chamber. A differential pressure transducer (Model dpt, Emka TECHNOLOGIES, France) connected to both chambers allows the measurement of pressure differences. This signal reflects the respiratory activity of the rat. The measured signal was amplified, digitally sampled at 1 kHz and acquired with a PC using an acquisition card (MC-1608FS, Measurement Computing, USA).

Protocol

It has been shown that animals increase their sniffing frequency during odor presentation (Wesson et al. 2008a) and they present a slow sniffing frequency at rest. For each rat, the respiration was recorded during thirteen trials. K07 was presented ten times for twenty seconds with an inter-trial interval of four minutes. Using this protocol, we were able to record a large range of sniffing frequencies.

Data processing

All data processing was performed using OpenElectrophy homemade open-access software (Garcia & Fourcaud-Trocmé, 2009). All signals and epochs (i.e., with or without odor) were stored in a SQL database. Measurements were performed as described in detail by Hegoburu et al. (2011).

Respiratory signal

OpenElectrophy can automatically detect 0-crossings of the respiratory signal, which correspond to the point of null airflow in the rising phase (Roux et al. 2006). The negative and positive phases corresponded to inspiration and expiration, respectively (Fig. 5B). Using these phases, we had direct access to the frequency, inspiration time, expiration time, inspiration peak flow rate and expiration peak flow rate. The peak flow rates and times were used to calculate the inspiration and expiration volumes, respectively.

Measurements and Statistics
Statistical tests were performed using Excel and StatView software. The level of significance was set at p<0.05 for all tests. All respiratory cycles where the peak flow rate, volume, or duration value of the inspiration or expiration phase were > the mean +/- 2 SD were discarded from analysis. Comparisons of the peak flow rates and volumes among the different sampling frequency conditions were performed using a factorial ANOVA with sampling frequency as the factor. More precisely, comparisons between the sampling frequency and the peak flow rate were performed for both the inspiration and expiration phases. Post-hoc analyses were performed using the Student Newman-Keuls test.

**Experiment 3: OB recording in freely moving rats**

**Preparation and recording**

**Animal preparation**

Two male Long-Evans rats (Janvier, France) were used; the rats weighed 270 g at the start of the experiment. Food and water were available *ad libitum* during the experiment.

Anesthesia was induced and maintained by intraperitoneal injections of Equithesin (a mixture of chloral hydrate and sodium pentobarbital, 3 ml/kg). A monopolar LFP recording electrode was positioned in the left OB near the mitral cell layer, which was located by its large multiunit mitral cell activity (Martin et al. 2004). A connector was mounted on the skull (Emka TECHNOLOGIES, France). The rats were then allowed to recover for two weeks.

**Respiration recordings**

Respiratory behaviors were acquired and computed as described in the second part of the methods (see above).

**Telemetry recording of OB LFP signals**
To obtain access to both OB activity and sampling behavior, the rats were placed in the plethysmograph chamber. To prevent the possible loss of air-tightness in the plethysmograph as the result of wired recordings, we used radio-frequency acquisition of the LFP signals (RodentPACK, Emka TECHNOLOGIES, France). A transmitter (RodentPACK, Emka TECHNOLOGIES, France) was connected to the head connector for the recording session (Fig. 6A). LFP was acquired at 1 KHz. The transmitter was paired with a specific receiver card and the receiver mainframe provided analog output to our computer via an acquisition card (MC-1608FS, Measurement Computing, USA).

**Data processing**

LFPs

LFP signals were processed as described for anesthetized animals (see above).
Results:

Two experimental preparations were used: a freely moving animal preparation and a double-tracheotomized anesthetized animal preparation. We use the term "sniffing" to refer to the respiratory behavior recorded in freely moving animals and "imposed sampling" refers to the imposed nasal airflow in anesthetized animals.

Experiment 1: Effects of sampling frequency and flow rate variations on bulbar activity

Our aim in experiment 1 was to answer two questions: does a high sampling frequency modify the strength and/or latency of the bulbar response? If so, can a higher flow rate counterbalance the effects of high frequency?

In Experiment 1, ten anesthetized rats were used. As shown in Fig. 1A, OB activity was recorded under six different imposed sampling frequencies: 1 Hz, basal (the animal’s own respiratory frequency under urethane anesthesia, mean=2.3 Hz +/- 0.02), 4 Hz, 6 Hz, 8 Hz and 10 Hz. These frequency conditions were coupled with two flow rate conditions: 500 ml/min and 1,000 ml/min (Fig. 1A). When possible, two odors were used: K07 and ISO. The 243 trials recorded from ten rats were included in the analyses.

Effects of sampling frequency and flow rate on the strength of the bulbar response: flow rate partially counterbalances the decrease in response strength caused by reduced inspiratory duration

The strength of the bulbar response was measured via three different signals in the anesthetized rats: LFP-sampling-related modulation, LFP gamma oscillations and unitary M/T cell activity.

LFP-sampling-related modulation in the anesthetized preparation

During spontaneous activity, the occurrence of LFP-sampling-related modulation significantly decreased when the sampling frequency increased, whereas increased flow rate globally enhanced LFP occurrence (data not shown). During odor presentation, LFP-sampling-related modulation was
universally observed, with a markedly higher power in the sampling-related frequency band in the FFT. We analyzed the amplitude of LFP-sampling-related modulation during odor presentation as a function of the imposed sampling frequency (Fig. 1). As the sampling frequency increased (Fig. 1B, D), the amplitude of LFP-sampling-related modulation significantly decreased (repeated-measures ANOVA, n=228 F (5,90)=21.155, p<0.0001). This decrease was probably the result of a decrease in the sampling volume (Fig. 1A) rather than the result of a possible uncoupling between bulbar activity and imposed sampling at high sampling frequencies. Indeed, as shown in Fig. 1C, regardless of the frequency, the cross-correlation coefficients did not significantly vary as a function of frequency, except in the 1 Hz condition. (Fig. 1C, repeated-measures ANOVA, n=228, F (5,90)=5.292, p<0.001; post-hoc Student Newman-Keuls test, p<0.05). In contrast, an increase in flow rate resulted in an increase in the amplitude of LFP-sampling-related modulation (repeated-measures ANOVA, n=228 F (1,18)=12.861, p<0.01, Fig. 1B). Indeed, a post-hoc test revealed a significant effect (p<0.05) at the basal, 4 Hz, 8 Hz and 10 Hz frequencies and a similar tendency at 6 Hz (p =0.087). An increased flow rate slightly improved the cross-correlation coefficient (repeated measures ANOVA, n=228 F (1,18)=6.703, p<0.05, Fig. 1C).

Using these data, we were able to compare the amplitude of LFP-sampling-related modulation between 2 conditions; for example, we compared the LFP amplitude between the basal / 500 ml/min condition and the 6 Hz / 1,000 ml/min condition. Interestingly, the amplitude of LFP-sampling-related modulation was similar in both conditions (t-test, p>0.05). The same observation was obtained for the 4 Hz / 500 ml/min and 8 Hz / 1,000 ml/min conditions, which shows that a high flow rate can compensate for the effects of high sampling frequency.

Finally, LFP-sampling-related modulation still persisted at high sampling frequencies and was at least partially enhanced by an increased flow rate.

LFP: gamma oscillations in the anesthetized preparation

Using our double-cannulated anesthetized preparation, we next assessed the effects of imposed sampling variations on LFP gamma oscillations. We first looked at the gamma occurrence during the
entire period of odorant stimulation (the gamma occurrence was defined as the probability of observing a gamma burst irrespective of the number of oscillatory bursts occurring during odorant stimulation). The gamma oscillation occurrence did not seem to be strongly affected by variations in the sampling frequency. Indeed, as shown in Figure 2A, we only observed a slight decrease in the gamma occurrence in response to increased sampling frequency and the only significant difference was between 1 Hz (occurrence=68%) and 10 Hz (35%, Chi² (1) = 4.356, p<0.05). In contrast, the occurrence of gamma oscillations was somewhat increased by an increased flow rate (Chi² (1) = 4.31, p<0.05). There was no significant difference between the basal-500 ml/min and the 6 Hz-1,000 ml/min combinations or between the 4 Hz-500 ml/min and 8 Hz-1,000 ml/min combinations (Chi² test, p>0.05). Thus, increased flow rate partly counterbalances the effect of high frequency and this effect was most apparent at the highest sampling frequency. Although the global gamma occurrence was only slightly modified by the sampling frequency, we observed a change in the probability of gamma burst recurrence at each sampling cycle. Indeed, as Figures 2B1 and B2 show, the gamma recurrence significantly decreased when the sampling frequency increased (ANOVA, F (5,137) =27.505, p<0.0001). Notably, the gamma recurrence was higher at 1 Hz than at all other frequencies and higher at the basal frequency than at 6 Hz, 8 Hz or 10 Hz (post-hoc Student Newman-Keuls test, p<0.05). This decrease in the gamma recurrence was not significantly compensated for an increased flow rate (ANOVA, F (1,137) =0.323, p=0.57). Indeed, a comparison of the basal-500 ml/min to the 6 Hz-1,000 ml/min combination or the 4 Hz-500 ml/min to the 8 Hz-1,000 ml/min combination revealed that the gamma recurrence was significantly higher at the low sampling frequencies and was not counterbalanced by increased flow rate.

The decrease in gamma occurrence or recurrence was not caused by a detection bias, which could have been introduced by a decrease in the amplitude of gamma oscillations. Indeed, intrinsic gamma characteristics were affected little or not at all by the frequency and flow rate; neither gamma power nor gamma frequency was affected by the sampling frequency or flow rate (data not shown, ANOVA gamma-power: frequency F (5,137)=0.801, p=0.55; flow rate F (1,137)=0.0001, p=0.992. ANOVA gamma-frequency: frequency F (5,137)=0.57, p=0.72; flow rate F (1,137)=0.7, p=0.4). Only the
gamma duration was affected by the sampling frequency; the duration of gamma bursts was significantly longer at 1 Hz than at basal frequency, 4 Hz or 8 Hz (ANOVA gamma-duration, frequency $F(5,137)=3.970$, $p<0.01$, flow rate $F(1,137)=0.477$, $p=0.49$; post-hoc Student Newman-Keuls test).

Finally, the occurrence of LFP gamma oscillations decreased when the sampling frequency increased and a higher flow rate compensated for this effect to some extent. To gain further insight into the influence of sampling variations, we next looked at the OB unitary level.

Units in the anesthetized experimental preparation: sampling-related patterns of M/T cells persist at high sampling frequencies

In total, 18 M/T cells were recorded, with 29 neuron/odor pairs recorded from ten rats. We measured the percentage of responding cells at each frequency and flow rate condition. Regardless of sampling variation, the responsiveness of M/T cells to odor was stable and ranged from 69% to 84% (data not shown). We observed a slight increase in cell responsiveness to odor when the flow rate was increased. It has been shown by others that M/T units in behaving rodents tend to lose their respiration-related patterning during rapid sampling (Bhalla and Bower, 1997, Kay and Laurent, 1999; Pager, 1985; Rinberg et al. 2006a). Surprisingly, we observed that M/T cells still manifested a sampling-related pattern at high sampling frequencies (Fig. 3B), which confirms the recent results obtained by Carey and Wachowiak (2011). For example, under the 10 Hz and 1,000 ml/min conditions, we observed that 52.4% of cells exhibited sampling-related activity. By plotting M/T cell spike discharges relative to the animal's respiratory cycle, we found that the rhythmicity of M/T cells was effectively linked to the imposed sampling and not to the animal's own respiration (data not shown). In addition to the strong persistence of the sampling-related rhythmicity of M/T cells, we observed that increased sampling frequency led to a progressive decrease in the percentage of cells exhibiting a SYNCRHO pattern from 83.3% in the basal condition to 33.3% in the 10 Hz condition (at 500 mL/min, Fig. 3B). Conversely, an increase in the flow rate (1,000 mL/min) improved these percentages ($\chi^2(1)=6.786$, $p<0.01$). Indeed, at a high flow rate, more than 50% of the SYNCRHO
patterns persisted regardless of the sampling frequency (Fig. 3B). Moreover, there was no significant
difference between the basal-500 ml/min and the 6 Hz-1,000 ml/min combinations or between the 4
Hz-500 ml/min and 8 Hz-1,000 ml/min combinations. Thus, a higher flow rate partially
counterbalanced the influence of a high sampling frequency. We then quantified the phase of the M/T cell discharge according to the sampling cycle, which revealed a cell discharge phase-shift when the sampling frequency increased (Fig. 3A, all cells, Fig. 3C, single cell). This phase-shift was observed in all cells whose SYNCHRO activity persisted over a wide range of frequencies. The phase of the spikes respective to the imposed sampling cycle shifted later in the sampling cycle when the frequency increased. This phase-shift was also observed relative to the LFP-sampling-related modulation (data not shown).

To summarize, when the sampling frequency increased, the probability of SYNCRHO patterns decreased and this tendency could be counterbalanced by a higher flow rate. The sampling frequency also affected the phase of spike discharge relative to the imposed sampling cycle. Next, we asked whether or not the sampling variation also affects M/T cell response latency.

**Effects of sampling frequency and flow rate on the latency of bulbar responses: increased flow rate but not increased sampling frequency decreases the M/T cell response latency**

Wesson et al. (2009) showed that the ORN onset latencies from high-frequency sniffing trials were significantly shorter than those from low frequency trials. Can a shortening of latencies be similarly observed in the M/T cell response? To address this question, we needed to analyze an event with a precise latency. We thus focused our analysis on the latency of the first spike, which is a discrete event. Numerous authors have highlighted the importance of spike latency in odor coding (Cury and Uchida, 2010; Junek et al. 2010). For this purpose, we measured the latency of the first spike relative to the beginning of the first inspiration after odor onset, as described by others (Cury and Uchida 2010; Wesson et al. 2009) (Fig. 4A). Because we were interested in a short-lived event, we restricted the analysis window to 1 second because the minimum sampling frequency was 1 Hz. Surprisingly, we did not detect a significant effect of sampling frequency on first spike latency (Fig. 4B; ANOVA,
n=229, F (5,217)=0.671, p=0.64). In contrast, an increase in the flow rate led to a significant shortening of first spike latency (Fig. 4B1 and B2, ANOVA, n=229, F (1,217) =8.442, p<0.01). The mean of the first spike latency shifted from 179 ms to 119 ms when the flow rate was increased from 500 to 1,000 ml/min (Fig. 4B2). These results show that a higher flow rate, but not a higher sampling frequency decreased the latency of the bulbar response.

To determine the joint effects of sniffing frequency and flow rate, we next looked at how these two parameters evolve in behaving animals and how they change relative to one another.

**Experiment 2: Relationship between sniffing frequency and flow rate in behaving animals**

To precisely analyze the sniffing behavior of freely moving rats, we used a whole-body plethysmograph. Analyses were performed on six rats. Sniffing behavior was well described by Welker (1964) and then by Youngentob et al. (1987). Our aim here was not to describe the different sniffing characteristics as Walker et al. (1997) previously did; rather, our goal was to analyze the relationship between the frequency and flow rate. First, as described by others (Cury and Uchida, 2010; Wesson et al. 2008a), we observed that the distribution of sniffing frequencies was bimodal (Fig. 5A, B) and reflected the prevalence of two respiration modes: a low-frequency mode (1-3 Hz) and a high-frequency mode (6-10 Hz). To determine whether the frequencies and flow rates co-varied, we looked for correlations between the instantaneous sniffing frequency and the peak flow rates during both expiration and inspiration phases. We observed that the sniffing frequency and peak flow rate varied in parallel. Indeed, the correlation between the sniffing frequency and expiration peak flow rate was significant (r=0.446, p<0.0001, n=41717). Similarly, the correlation between the sniffing frequency and inspiration peak flow rate was highly significant (r=0.515, p<0.0001, n=41717). The same observations were obtained when the durations of inspiration and expiration were analyzed as a function of peak flow rate. As the duration decreased (i.e., sniffing frequency increased), the peak flow rate increased (for the expiration phase r=-0.461, p<0.0001; for the inspiration phase r=-0.564, p<0.0001, n=41717; the inspiration and expiration durations were examined independently). Thus, the sniffing frequency and flow rate can co-vary in freely moving animals. To compare these data with our
electrophysiological data, we merged all of the individual frequencies into six classes corresponding to the six sampling frequencies imposed in our anesthetized preparation (+/-0.1 Hz): 1 Hz, 2 Hz, 4 Hz, 6 Hz, 8 Hz and 10 Hz. The results are presented in Figure 5C. The data confirm that an increase in sniffing frequency was associated with a significant increase in both expiratory peak flow rate (Fig. 5C1, ANOVA, F (5, 4051) =191.974, p<0.0001; post-hoc Student Newman-Keuls test, p<0.05) and inspiratory flow rate (Fig. 5C2 ANOVA, F (5, 4051) =314.645, p<0.0001). The inspiratory peak flow rate was different between each sniffing frequency except between 6 Hz and 8 Hz (post-hoc Student Newman-Keuls test, p<0.05). Volume is an important element affected by sniffing flow rate and frequency. We asked whether an increase in flow rate could maintain constancy for volume. As shown in Fig. 5D, as the sniffing frequency increased, the volume significantly decreased during both the expiration (Fig. 5D1, ANOVA, F(5,4051)=273.75, p<0.0001; post-hoc Student Newman-Keuls test, p<0.05) and the inspiration (Fig. 5D2 ANOVA, F (5, 4051)=311.843, p<0.0001) phase. Overall, our results show that when the sniffing frequency increases, there is a concomitant increase in flow rate in the behaving animal. This tradeoff does not allow the sniffing volume to remain constant. We finally asked whether or not the persistence of LFP-sampling-related modulation could be an effect of anesthesia resulting from the imbalance between the peripheral and central influences.

**Experiment 3: LFP-sampling-related modulation persists at a high sampling frequency in the awake experimental preparation**

We recorded LFP in the OB and sniffing activity in two awake rats (Fig. 6A). LFP activity in the OB of awake rats has been well described by numerous authors (Freeman, 1978; Kay, 2005; Martin et al. 2004). Although these authors described a theta rhythm (4-12 Hz) in the OB, none concomitantly recorded the sniffing behavior. We observed that sampling-related modulation was still present in the LFP signal regardless of the animal's sampling frequency (Fig. 6B1, B2). LFP-sampling-related modulation and animal respiration were tightly related and the time-frequency representations of both signals were superimposable (Fig. 6B1, B2). Interestingly, abrupt variations in the sniffing frequency were strictly reflected by the LFP activity. Therefore, even in a preparation where central control was
not depressed, the LFP signals accurately followed the sniffing modulation up to frequencies as high as 10 Hz, as shown in Figure 6B2.

Overall, the results show that LFP-sampling-related modulation persists at high sampling frequencies in anesthetized and awake rats.

DISCUSSION

Sampling is not only a simple vector for odor molecules but also plays a role in olfactory processing (Mainland and Sobel, 2006). Sampling can vary in frequency and flow rate (Yougentob et al. 1987) and the question of how sampling variations affect the activity of the olfactory system is a subject of growing interest. In this study, we asked two major questions: what are the effects of high sampling frequency on OB activity at the levels of M/T cell and network activities? Can sampling flow rate compensate for these effects? We observed a tradeoff effect between sampling frequency and flow rate on some bulbar activity features. This tradeoff allows OB activity to maintain a sampling-related rhythmicity and induces faster odor-evoked responses and it is effectively utilized in awake animals, in which we observed that frequency and flow rate often co-increase. To our knowledge, our study is the first to report that the sampling frequency and flow rate can act either independently or synergistically on bulbar output to shape the neuronal message.

1- Sampling-related activity is maintained at high sampling frequencies

We observed that OB sampling-related modulation persisted at high sampling frequencies, although it was weakened (Fig. 1, 2 and 3). This finding is in agreement with previous studies on olfactory epithelium (Ezeh et al. 1995; Ghatpande and Reisert, 2011), glomerular (Spors et al. 2006) and M/T cell activity (Bhalla and Bower, 1997; Carey and Wachowiak, 2011). The effects of high sampling frequency are probably the result of a decrease in the duration of inspiration, which in turn results in decreased activation of the olfactory epithelium. If this is true, then we can hypothesize that an increased flow rate would compensate for this effect. We observed that increased flow rate
compensated, to some extent, for the effects of high sampling frequency by enhancing LFP-sampling-related modulation, gamma occurrence and synchronized patterns in M/T cells. The only partial compensation of flow rate for the effects of increased frequency is probably because of the loss of volume at higher sniffing frequency (Fig. 1A). Regardless, the increased flow rate allows the OB activity to maintain a sampling-related rhythmicity at high sampling frequencies. This finding was obtained in both the anesthetized and awake preparation (Fig. 5 and Fig. 6) and this persistence is likely to be important in terms of inter-area communication (for review, see Kepecs et al. 2006). Indeed, the sampling rhythm is in the theta band (4-12 Hz) and theta rhythms have been described in systems closely related to or involved in olfactory processing, such as the whiskers (Sobolewski et al. 2011) and the limbic system (Bland, 1986; Buzsaki, 2002; Komisaruk, 1970; Macrides et al. 1982; Vanderwolf, 1969). Moreover, the coherence between OB and hippocampal theta oscillations has been found to be significant only during odor sampling (Kay, 2005). Interestingly, the cerebellum, which is activated by sniffing (Sobel et al. 1998) and has been implicated in breathing control (Colebatch et al. 1991), has been shown to express theta rhythms (Wikgren et al. 2010). Theta rhythms could thus permit the transmission of olfactory information among the olfactory, limbic and cerebellar structures and could thus be the basis for the rapid feedback of the olfactory system on sampling control (Johnson et al. 2003). In this view, the maintenance of a sampling-related rhythm represents a key mechanism.

2- Increased sampling flow rate but not increased sampling frequency shortens the OB response latency

Multiple psychophysical studies have noted the speed of olfactory discrimination in rodents (Abraham et al. 2004; Rinberg et al. 2006b; Uchida and Mainen, 2003), which indicates rapid olfactory processing. We could think that a high sampling frequency would reduce the latency of the bulbar response. Although Wesson et al. (2009) showed that this holds true for neuroreceptor responses in awake animals, we observed that a high sampling frequency did not significantly impact the latency of the M/T cell response. Our observation is in agreement with the studies by Spors et al. (2006) and Carey and Wachowiak (2011). More interestingly, we observed that the M/T cell response latency can
be reduced by increasing the flow rate. Whereas other authors have shown that increasing odor
congestion decreases the M/T cell firing latency (Cang and Isaacson, 2003; Wellis et al. 1989), this
is the first evidence to show that a sampling parameter alone (i.e., flow rate) can impact the M/T cell
response latency. This result may reconcile the data of Wesson et al. with ours because they did not
specify whether or not a high sampling frequency was associated with an increase in flow rate in their
preparation. Because discrimination can be achieved in less than 500 ms (Abraham et al. 2004;
Rinberg et al. 2006b; Uchida and Mainen, 2003), the olfactory system must operate on a rapid
timescale to provide a quick response. We demonstrated that it is possible to shorten the OB response
latency by increasing the sampling flow rate.

We observed a minimal and constant OB response latency regardless of the sampling frequency.
Multiple arguments, which are not mutually exclusive, can be proposed to explain this latency. First,
there is an incompressible time period that corresponds to information transduction in the olfactory
epithelium and/or conduction to the OB (Grosmaître et al. 2006; Kleene, 2008). Second, the shortness
of inspiration at a high sampling frequency might need to be compensated for by the cumulative effect
of several cycles of olfactory epithelium activation. Furthermore, this constant latency is probably
related to the observed phase-shift in the synchronized pattern relative to the imposed sampling cycle.
Because odor onset and odor response latency were fixed, whereas the sampling frequency increased,
the first M/T cell spike was shifted relative to the imposed sampling cycle. This is a possible
explanation, and it does not exclude the alternatives such as a differential odorant deposition as a
function of sampling frequency (Jiang and Zhao, 2010). This observed shift could also be the result of
a modulation of granular inhibition induced by stimulus frequency (Young and Wilson, 1999) or
excitatory centrifugal input (Balu et al. 2007). A phase shift of M/T cell discharge could have
important consequences for downstream area reading, for example by changing the respiratory phase
of piriform cortex cells (Wilson, 1998; Litaudon et al., 2003). The phase shift of the M/T cell
discharge could encode a stimulus parameter, such as a change in sampling frequency or a change in
stimulus concentration because of the reduced inspiration time (Kepecs et al. 2006).
3-Different sniffing strategies right under the nose

The last part of our results (Fig. 5) shows a co-increase between the sampling frequency and flow rate with a correlation of up to 0.5. This result also means that other strategies could exist, such as combinations of low flow rate/high sampling frequency or high flow rate/low frequency. Moreover, previous studies have shown that sniffing varies depending on the task (Kepecs et al. 2007; Wesson et al. 2008b; Youngentob et al. 1987). Thus, an animal has the ability to combine various sniffing parameters differently according to the task and/or environment, which likely confers an important degree of adaptability to the olfactory system. As suggested by Schoenfeld and Cleland (2005, 2006), sampling can improve olfactory capabilities by allowing the optimization of the deposition of odor molecules through the olfactory epithelium. Further studies utilizing behaving animals will be required to gain insight into how the olfactory system can control sniffing and to provide evidence for an olfactory motor-act.

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**Figure legends**

**Figure 1: Although attenuated, the LFP-sampling-related rhythm persists at high sampling frequencies.** A) Imposed sampling conditions. Plots of the imposed sampling volumes as a function of the imposed frequency and flow rate. Six different nasal airflow frequencies of 1 Hz, basal frequency (which is the respiratory frequency of a urethane anesthetized rat, mean = 2.3 Hz), 4, 6, 8 and 10 Hz and two nasal flow rates of 500 ml/min and 1,000 ml/min were imposed in a randomized procedure. B) Plots of the mean amplitude of the LFP-sampling-related modulation (+/- SEM) as a function of the imposed frequency and flow rate. Grey: 500 ml/min flow rate and black: 1,000 ml/min flow rate. The number of trials for each flow rate and for each frequency was 19 (total=228). Statistical analyses were performed using the repeated-measures ANOVA test, which revealed a global effect of the frequency, $p<0.0001$. According to the Newman-Keuls post-hoc test: *, significant difference between 1 Hz and all other frequencies; #, at 1,000 ml/min, there was no difference between basal and 4 Hz, but both were significantly different from 6 Hz, 8 Hz and 10 Hz; ‡, at 500 ml/min, there was no significant difference between basal and 4 Hz, but both were significantly different from 8 Hz and 10 Hz. Global ANOVA effect of flow rate: §§ ($p<0.01$). A post-hoc Newman-Keuls test revealed a significant effect of the flow rate at basal, 4 Hz, 8 Hz and 10 Hz ($§$) frequencies. C) Mean cross-correlation coefficients (+/- SEM) as a function of the imposed frequency and flow rate. The cross-correlation was assessed between the LFP-sampling-related modulation and the imposed nasal airflow. Grey: 500 ml/min flow rate, black: 1,000 ml/min flow rate. The number of trials for each frequency and for each flow rate was 19. Statistical analyses were performed using repeated-measures ANOVA. The ANOVA revealed a global effect of frequency ($p<0.001$). *, significant difference between 1 Hz and all other frequencies when the Newman post-hoc test was applied. Global ANOVA effect of flow rate: § ($p<0.05$). A post-hoc Newman-Keuls test revealed a significant effect of the flow rate at 8 Hz: §. D) Left, Examples of raw data for the LFP recorded from the same channel under six different sampling frequencies at a flow rate of 1,000 ml/min. Note the decrease in the slow LFP modulation amplitude and its persistence across the frequencies. Cross-correlograms from the cross-correlation...
between the imposed sampling signals at 1,000 ml/min are presented on the right and the
corresponding raw data are on the left.

**Figure 2: Gamma oscillations still appear at high sampling frequencies, but their recurrence
over sampling cycles decreases.** The occurrence of gamma oscillations was only slightly affected by
the sampling frequency, but it was more dependent on flow rate. A) A plot of the percentage of trials
where gamma oscillations occurred as a function of the sampling frequency and sampling flow rate is
presented. Yellow: 1 Hz; orange: basal frequency; red: 4 Hz; violet: 6 Hz; blue: 8 Hz and black: 10
Hz. Number of trials where gamma oscillations occurred at 500 ml/min: n=13, 13, 13, 13, 11 and 7 for
1 Hz, basal frequency, 4 Hz, 6 Hz, 8 Hz and 10 Hz, respectively. Number of trials at 1,000 ml/min:
n=15, 16, 13, 11, 12 and 12 for 1 Hz, basal frequency, 4 Hz, 6 Hz, 8 Hz and 10 Hz, respectively.
Statistical test: Chi². § (p<0.05). The difference between the 500 ml/min and 1,000 ml/min flow rate
conditions: * (p<0.05), the difference at 500 ml/min between the 10 Hz and 1 Hz sampling frequency
conditions: * (p<0.05). B1) The recurrence of gamma bursts at each sampling cycle was affected by
variations in the sampling frequency. Plot of the mean (+/- SEM) number of gamma oscillatory bursts
per total number of sampling cycles as a function of the sampling frequency and flow rate. Grey: 500
ml/min flow rate and black: 1,000 ml/min flow rate. Number of trials at 500 ml/min: n=13, 13, 13, 13,
11 and 7 for 1 Hz, basal frequency, 4 Hz, 6 Hz, 8 Hz and 10 Hz, respectively. Number of trials at
1,000 ml/min: n=15, 16, 13, 11, 12 and 12 for 1 Hz, basal, 4 Hz, 6 Hz, 8 Hz and 10 Hz, respectively.
Statistical test: factorial ANOVA. * (p<0.05), Difference between 1 Hz and all other frequencies;
difference between basal and 6 Hz, 8 Hz and 10 Hz: * (p<0.05). B2) Examples of two raw data sets
recorded from the same channel under basal and 6-Hz sampling frequency conditions (at a fixed flow
rate of 1,000 ml/min). Stars indicate the occurrence of gamma oscillations.

**Figure 3: Sampling patterns persist at high sampling frequencies.** A) Representation of the
activities of the whole population of M/T cells relative to the imposed sampling cycle. Dots represent
1/ISI of all units relative to their sampling phase. The sampling cycle is depicted between 0 and 1,
where 0 represents the beginning of inspiration and 0.5 represents the transition between
inspiration/expiration (red bar). The pink line represents the 50th percentile. B) Percentage of
sampling-related M/T cell patterns as a function of the imposed sampling frequency and flow rate
(yellow: 1 Hz, orange: basal frequency, red: 4 Hz, violet: 6 Hz, blue: 8 Hz and black: 10 Hz). Number
of neuron/odor pairs at 500 ml/min: 20, 18, 21, 23, 18 and 18 for 1 Hz, basal frequency, 4 Hz, 6 Hz, 8
Hz and 10 Hz, respectively. Number of neuron/odor pairs at 1,000 ml/min: 20, 20, 19, 20, 19 and 21
for 1 Hz, basal, 4 Hz, 6 Hz, 8 Hz and 10 Hz, respectively. Statistical test: Chi^2, * (p<0.05). C) Example
of the cellular activity occurring during odor presentation showing a phase-shift of the spike discharge
as a function of the sampling frequency. Flow rate: 500 ml/min for all frequencies. The circular
diagram depicts a sampling cycle. The M/T cell spike discharge is presented relative to the sampling
cycle. Examples of M/T cell discharges at 500 ml/min under the six sampling frequencies. Yellow: 1
Hz, orange: basal frequency, red: 4 Hz, violet: 6 Hz, blue: 8 Hz and black: 10 Hz. The dotted lines
represent the total discharge of the cell under each sampling frequency. The solid line represents the
mean direction of the M/T cell spike discharge relative to the circular diagram.

**Figure 4: Sampling stronger but not sampling faster decreases the OB response.** A). First spike
latency is defined as the first spike following the first inspiration after odor onset. B1) Mean (+/SEM)
first spike latencies are presented as a function of sampling frequency and flow rate (grey: 500 ml/min
and black: 1,000 ml/min). Statistical test: factorial ANOVA, §§ (p<0.01). B2) Mean first spike latency
(all frequencies averaged) for flow rates of 500 ml/min (grey) and 1,000 ml/min (black), ANOVA, §§
(p<0.01).

**Figure 5: Sniffing frequency and flow rate significantly co-vary in freely moving rats.** A)
Distribution of the sniffing frequencies. Note the bimodal nature of the distribution. B) Example of the
raw sniffing frequency data recorded by plethysmograph. The black bar corresponds to the point of
null airflow in the rising phase; the negative and positive phases correspond to inspiration and
expiration, respectively. We note that sniffing behavior quickly shifts from a low- to high-frequency
mode and vice versa. C) Box plot representing expiratory (1) and inspiratory (2) peak flow rates as a
function of the sniffing frequency range (+/- 0.1). For both the expiratory and inspiratory phases, n=
266, 1083, 503, 649, 981 and 575 for 1 Hz, 2 Hz, 4 Hz, 6 Hz, 8 Hz and 10 Hz, respectively. Statistical test: factorial ANOVA with post-hoc Student Newman-Keuls test; * (p<0.05). The inspiratory flow rates at 6 Hz and 8 Hz were not significantly different, but both were different from the flow rates at 1 Hz, 2 Hz, 4 Hz and 10 Hz, # (p<0.05). D) Box plot representing the expiratory (1) and inspiratory (2) volumes as a function of the sniffing frequency range (+/- 0.1). For both the expiratory and inspiratory phases, n= 266, 1083, 503, 649, 981 and 575 for 1 Hz, 2 Hz, 4 Hz, 6 Hz, 8 Hz and 10 Hz, respectively. Statistical test: factorial ANOVA with post-hoc Student Newman-Keuls test; * (p<0.05). The inspiratory flow rate under the 4 Hz and 6 Hz conditions and the 6 Hz and 8 Hz conditions were not significantly different, but both were different from the flow rates at the other frequencies, # (p<0.05).

**Figure 6: LFP and sniffing recordings in awake rats.** A) Picture of a rat in the plethysmograph chamber with a telemetry emitter. B1) Example of a sniffing recording and an OB LFP recording in one rat. From top to bottom: sniffing signals measured by the plethysmograph (EMKA technologies); a representation of this raw signal on a time-frequency map; raw data showing LFP bulbar activity and the associated time-frequency representation. In the time-frequency representations, amplitudes are color-coded in arbitrary units. B2) The legend is the same as in B1; an example of a recording in the other rat with a sniffing frequency of up to 10 Hz.