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Coding of odours in the anterior olfactory nucleus.

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Abstract
Odorant molecules stimulate olfactory receptor neurones, and axons of these neurones project into the main olfactory bulb where they synapse onto mitral and tufted cells. These project to the primary olfactory cortex including the anterior olfactory nucleus (AON), the piriform cortex, amygdala and the entorhinal cortex. The properties of mitral cells have been investigated extensively, but how odour information is processed in subsequent brain regions is less well known. In the present study, we recorded the electrical activity
of AON neurones in anesthetized rats. Most AON cells fired in bursts of 2-10 spikes separated by very short intervals (<20 ms), in a period linked to the respiratory rhythm.

Simultaneous recordings from adjacent neurones revealed that the rhythms of adjacent cells, while locked to the same underlying rhythm, showed marked differences in phase.

We studied the responses of AON cells to brief high frequency stimulation of the LOT, mimicking brief activation of mitral cells by odour. In different cells, such bursts evoked transient or sustained bursts during stimulation or, more commonly, post-stimulation bursts after inhibition during stimulation. This suggests that, in AON cells, phase shifts occur as a result of post-inhibitory rebound firing, following inhibition by mitral cell input, and we discuss how this supports processing of odour information in the olfactory pathway. Cells were tested for their responsiveness to a social odour (the bedding of a strange male) amongst other simple and complex odours tested. Eleven cells responded strongly and repeatable to bedding odour, and these responses were diverse, including excitation (transient or sustained), inhibition, and activation after odour presentation, indicating that AON neurones respond not only to the type of complex odour but also to temporal features of odour application.

**Abbreviations:** AOB, accessory olfactory bulb; AON, anterior olfactory nucleus; MOB, main olfactory bulb; IoD, index of dispersion; ISI, interspike interval; LOT, lateral olfactory tract;

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Introduction

Olfactory information is transduced when odorant molecules contact the dendrites of olfactory receptor neurones in the olfactory epithelium of the dorsal nasal cavity (Buck & Axel, 1991). These neurones project to the glomerular layer of the main olfactory bulb (MOB) (Mombaerts et al., 1996) where they synapse onto the dendrites of mitral and tufted cells, the output neurones of the olfactory bulb (Shipley & Ennis, 1996; Menini et al., 2004; Lledo et al., 2005). Each mitral cell sends its apical dendrite to a single glomerulus, and this receives inputs from a single class of olfactory receptor neurones (Bargmann, 2006); the mitral cells are thus tuned to respond to odorants that activate a single type of odorant receptor. However, the olfactory sensory neurones are also mechanosensitive (Grosmaitre et al., 2007), and are activated rhythmically by respiratory activity (Duchamp-Viret et al., 2005). Accordingly, the spontaneous spiking activity of many mitral cells follows the respiratory cycle, with peak firing rates occurring typically late in the inspiratory phase in rats (Buonviso et al., 2003; Margrie & Schaefer, 2003). In the presence of an odour that activates (or inhibits) the mitral cell, this cyclic activity is amplified (or attenuated). Thus for each mitral cell, odour information is coded as the amplitude (relative to a basal amplitude) of a signal that oscillates with the respiratory rhythm.

By contrast to mitral cells, tufted cells are more excitable, and respond with a shorter latency to stimulation of olfactory sensory neurones (Griff et al., 2008; Burton & Urban, 2014; Geramita & Urban, 2017; Vaaga & Westbrook, 2017), hence those that show a clear respiratory rhythm are active earlier in the respiratory cycle than mitral cells, and whereas mitral cells show sustained responses to sensory stimulation, the responses of tufted cells adapt.
Axons from the mitral and tufted cells converge to form the lateral olfactory tract (LOT), and collaterals of these axons innervate the anterior olfactory nucleus (AON) an important second-order processing station in the olfactory pathway (Brunjes et al., 2005; Sosulski et al., 2011). Individual cells in the AON receive inputs from mitral cells whose dendrites arise in different glomeruli, and hence they are more broadly tuned than mitral or tufted cells. Transynaptic labelling studies in mice suggest that individual AON cells receive inputs from at least four mitral cells (Miyamichi et al., 2011). In mice, it appears that mitral and tufted cells may project to different regions of the AON (Igarashi et al., 2012).

In urethane-anesthetised rats, mitral cells show complex patterns of spontaneous activity: the mean firing rate is about 6.4 spikes/s, and comprises long bursts (of ~154s) at ~11 spikes/s separated by long silent periods (~ 100s) that are not synchronized amongst even neighbouring mitral cells (Leng et al., 2014). Within bursts, activity in most mitral cells oscillates with a period of 400-600ms, reflecting peak activation during the inspiration phase of respiration, and excitatory responses to odours are typically apparent as an intensification of this respiratory-locked oscillatory discharge. The significance of the long bursting appears to be that cells that alternate between activity and silence can optimally detect both excitatory stimuli (in the silent phase) and inhibitory stimuli (in the active phase).

The AON is a very heterogeneous nucleus. It contains pyramidal glutamatergic cells and a variety of interneurons, many of which are GABA-ergic, but which also contain a considerable diversity of neuropeptides (Kay & Brunjes, 2014). One role of the AON is to assemble the inputs of activated glomeruli into a representation of complex odorants, and one particular role for the AON may be in processing socially relevant
complex odours. Whereas the output cells of the MOB respond selectively to a narrow range of odours, in mice most pyramidal AON neurones can be activated by mixtures of structurally dissimilar components (Lei et al., 2006). The response of an AON neurone to an effective mixture often exceeds the sum of its responses to the components, indicating a nonlinear combinatorial interaction.

Here, we used in vivo electrophysiology to study how inputs from the olfactory bulb are processed in the AON. We studied the spontaneous discharge patterning of AON neurones to identify subpopulations and studied their responsiveness to stimulation of the LOT. To investigate neuronal responses to a social odour, we impregnated air with the odour from the soiled bedding of the cage of a novel adult male rat and applied this odour in an air stream to the nose as well as testing other simple and complex odours.

Material and Methods

Ethical Approval

Procedures conducted in the UK were approved by the local Ethics Committee and the UK Home Office under the Animals Scientific Procedures Act 1986.

Electrophysiology

Single neurones were recorded from 120 adult male Sprague-Dawley rats (250-400 g) anesthetized with urethane (ethyl carbamate, 1.3 g/kg i.p.) using conventional extracellular recording techniques. In some experiments, respiration was monitored using a Pulse Transducer (TN1012/ST, Adinstruments, Oxford, UK). A recording electrode (glass micropipette filled with 0.9% NaCl, 20-40 MΩ) was lowered into the dorsally exposed AON, 1.0-2.5 mm lateral to the midline and 3.8-4.7 mm anterior to bregma. We
recorded neural activity through an amplifier (Axopatch 200b, Molecular Device, Sunnyvale, USA) and processed them by Spike2 software (Cambridge Electronic Design Limited, Cambridge, UK). In initial experiments, the position of the recording electrode was verified by visualizing incorporated neurobiotin in the recorded cell by juxtacellular labelling (Sabatier & Leng, 2008) (Fig. 1A,B). Labelled cells were consistently found in the main, ventrolateral portion of the AON, and were multipolar with oval cell bodies.

To stimulate the LOT, a concentric bipolar stimulating electrode (SNEX-100, Clark Electromedical Instrument, Kent, UK) was positioned on the ipsilateral LOT, via a burr hole in the dorsal surface of the skull, 1.4mm posterior to bregma, 3.2mm lateral to midline, 9.5mm deep (Paxinos & Watson, 2006). Electrical stimulation (1-ms matched biphasic pulses, 1mA peak-to-peak) was generated by a GRASS S88 stimulator with stimulus isolation and constant current units (Grass Products, Warwick, USA) as previously (Leng et al., 2014). In initial experiments the position of the stimulating electrode was confirmed histologically and by verifying that stimulation at this site evoked the expected potential changes in the mitral cell layer of the main olfactory bulb (Leng et al., 2014).

In some cases, double recordings were made with the single recording electrode. When the spike heights were sufficiently different, these were sorted using the LabSpike software generated by (Bhumbra et al., 2004) and available from the CED website (http://www.ced.co.uk/upu.shtml).

Odours were applied through a polythene cannula (0.1mm diameter) placed 3mm in front of the nose of the rat. Beddings were from male rat cages different from the cages of the experimental rats. For each test, 20ml of odour-saturated air was applied to the nose over ~5s using a 50-ml syringe. Some cells were tested with a range of odours,
including heptanal, hexanal, valeraldehyde, lemon, garlic and peppermint, and when
responses were observed, the specificity was checked by a similar application of odour-
free air. For cells that showed a response to bedding odour, activity in 0.5-s bins was
compared 20s before and in the first 10s after bedding was applied.

**Statistical analysis**

Autocorrelation histograms, ISI distributions and peri-stimulus time histograms were
constructed in Spike2. Autocorrelation histograms were constructed from 300s of
spontaneous activity in 100-ms bins, and normalised autocorrelation histograms were
normalised to the total number of spikes. ISI histograms were constructed in 10-ms bins
from at least 300s of stable spontaneous discharge activity. To generate population
averages (consensus ISI distributions), each ISI distribution was normalised to the total
number of ISIs. We measured index of dispersion (IoD) of firing rates in 10-s bins over
300s of spontaneous activity for each cell as the ratio of the variance to the mean.

We used two ways to assess the relationship of spiking activity to respiratory
rhythm. First, we looked at spike activity relative to the respiratory cycle, using the
wavemark function in Spike2 to mark each respiratory cycle at a consistent stage, and
using this as a trigger, constructed a histogram of spike activity relative to this trigger and
used the same trigger to plot the average respiratory waveform. Second, we looked at the
respiratory waveform relative to spike activity, using each recorded spike as a trigger for
the waveform average: this spike-triggered waveform is thus constructed in a way strictly
analogous to the construction of the autocorrelation histogram.

**Results**
Our first objective was to describe the spontaneous firing patterns of AON neurones and test them for responsiveness to bedding odour and for their responsiveness to LOT stimulation. We initially analysed 240 spontaneously active AON cells recorded from 103 rats. Their firing rates ranged from 0.1 to 32.4 spikes s\(^{-1}\), with a mean (SEM) of 5.8 ± 0.37 spikes s\(^{-1}\). Although the spontaneous firing rates were thus generally low, instantaneous frequencies in most cells occasionally exceeded 100 spikes s\(^{-1}\) (Fig. 1C) indicating that the low spontaneous rates did not reflect an inability to discharge at high frequencies. Most cells fired in intermittent short bursts (Fig. 1D) that often were associated with rhythmic changes in the background field potential (Fig. 1E). The firing rates of AON cells were distributed unimodally, and subpopulations could not be distinguished from this alone (Fig. 1F). For AON cells generally, the standard deviation of ISIs was proportional to the mean firing rate, revealing no subpopulations (Fig. 1G).

**Rhythmic and arrhythmic cells**

For each cell, we constructed autocorrelation histograms over 300s or more of stable spontaneous activity (Fig. 2B). This allowed us to identify three large subpopulations. The largest of these, 147 type 1 rhythmic cells (Fig. 2B), fired at 6.8 ± 0.5 spikes s\(^{-1}\) (range 0.18-25.1) and showed cyclic activity characterised by regular peaks in the autocorrelation histogram separated by intervals of a constant duration (mean 0.61 ± 0.008 s; Fig. 2C). This is similar to the periodicity of rhythmic oscillations in mitral cells and corresponds to the mean respiratory rate of urethane-anesthetized rats (85-110 min\(^{-1}\)). Activity mainly comprised bursts of 2-10 spikes separated by ISIs of < 25 ms in each phase of the cycle. About half (70) of the type 1 cells showed virtually no activity between bursts, while the remainder showed sparse spikes between bursts. The ISI
distributions of type 1 cells were all similarly skewed and were unimodal in the range 0-200 ms with a mean mode of 17.1 ± 0.9 ms (median 16), (Fig. 2D,F). Four cells discharged with periods of 300-400 ms, approximately half that of the mean respiratory rhythm (not shown). In some recordings, spikes from two neurones could be readily separated by spike height and waveform. In all cases (10 pairs), the two cells were locked to the same underlying rhythm, but their discharge activity could be either in phase or out of phase with each other (Fig. 2E).

A second subpopulation, 45 type 2 rhythmic cells (Fig. 3) fired at 3.3 ± 06 spikes/s (range 0.1-21.6 spikes/s) and showed cyclic activity with a mean period approximately twice that of type 1 cells. Again, activity mainly comprised bursts of 2-10 spikes separated by ISIs of < 25ms, with sparse spiking between bursts (Fig. 3A). In the typical type 2 cell shown in Figure 3A, we analysed 911 bursts from 1352s of spontaneous activity, defining bursts by the first ISI < 300ms after an ISI of > 500ms. Bursts contained 2-25 spikes (mean (SD) 8(5.1) spikes; burst length 451 (287) ms; period 1.47 (0.46) s; Fig. 3B). There was no frequency adaptation within bursts: the mean (SD) interval between the first two spikes in bursts was 49 (48) ms (n=911) and that between the 15th and 16th spikes was 47 (44) ms (n=96). The ISI distribution was unimodal with a mode of 23ms (Fig. 3C), and the autocorrelation (Fig. 3D) showed peaks at intervals of 1.2 s. The normalized average autocorrelation for all type 2 cells (Fig. 3E), constructed as for type 1 cells, showed no repeated peaks as the periods varied too much between cells. Like type 1 cells, all type 2 cells had ISI distributions that were unimodal in the range 0-300 ms, with a mean mode of 17.2 ± 2.2 ms (median 13.5 ms; Fig. 3F).

A third population of 35 arrhythmic cells fired at 6.0 ± 1.4 spikes/s (range 0.1-32.4) with no apparent oscillatory activity. The ISI distributions again were unimodal in
the range 0-300ms, with a mean mode of 17.5 ± 2.2 ms, but activity in these cells was irregular, with no clear bursting pattern. This group includes slow firing cells where a rhythm may not have been apparent because of the low discharge rate.

Isoperiodic bursting

The discharge rate of many AON cells displayed intermittent long bursts superimposed on a sustained background activity. To quantify this, we measured the index of dispersion (IoD) of firing rates in 10-s bins over 300s of spontaneous activity for each cell. For a cell firing randomly at a constant mean rate, the IoD should equal one regardless of firing rate: of the 248 cells, only 82 had an IoD below 2, while for 80 the IoD exceeded 7.

Some cells displayed repeated, long bursts from which we could measure burst and interburst durations. We analysed 20 such cells (19 type 1 cells and 1 type 2 cell) with a mean IoD of 29.5 ± 4.9). The mean intraburst firing rate was 7.7 ± 1.0 spikes/s (median 6.7, range 1.3-19.8) for bursts of 111 ± 11s (median 92s, range 50-220s). The interburst firing rates were 1.6 ± 0.4 spikes/s (median 0.9, range 0.2-7.5) for intervals of 110 ± 22 s (median 93s, range 43-218s). These are close to the burst and interburst durations of mitral cells in the MOB (bursts, 122 ± 10 s; interburst intervals, 129 ± 11 s), but intraburst firing rates were lower than the mean of 14.3 spikes/s in mitral cells (Leng et al., 2014).

Responses to social odour

We applied the smell of bedding from conspecific male rats to the anesthetized male rats. Of 179 AON cells tested, 14 cells (all type 1) responded strongly and repeatedly to this but not to similar application of clean air (Fig. 4); nine cells were activated (Fig.
4A,B) and four were inhibited (Fig. 4C). In the nine excited cells (Fig. 4B), the mean firing rate increased from $3.9 \pm 1.2$ spikes s$^{-1}$ (in the 10 s before the application) to $11.2 \pm 2.6$ spikes s$^{-1}$ (for 10 s during bedding application) and went back to $5.5 \pm 1.8$ spikes s$^{-1}$ (for 10 s after application). One cell unaffected during odour application was strongly activated after the end of odour application (Fig. 4D), and two inhibited cells showed a strong excitatory rebound after the end of odour application (Fig. 4E). Similar complex responses to either the presentation of an odour or to its removal were seen in other cells in response to odours other than bedding (not shown).

Seven cells activated by bedding odour responded to one or more of the other odours tested (five cells were tested with valderaldehyde of which four responded, four cells were tested with hexanol of which two responded strongly and one weakly).

**Responses to electrical stimulation of the LOT**

Of 26 type 1 cells, ten responded to 1-Hz stimulation of the LOT with clear orthodromic excitation at latencies between 7 and 20 ms (Fig. 5A,B). The other 16 were inhibited, with apparently similar latencies, although determining latency accurately in inhibited cells was generally imprecise.

In response to brief high-frequency stimulation, ten type 1 cells tested displayed virtually complete inhibition during stimulation (Fig. 5C). In six cells, activity swiftly returned to normal after the end of stimulation, and in four there was continued inhibition for several seconds (Fig. 5E). In another 12 cells, stimulation evoked a strong excitatory response. In six cases, this arose as a post-stimulation excitation after inhibition throughout the stimulation (Fig. 5D), in two cases, activation throughout stimulation was followed by a strong inhibition (Fig. 5E,F,H,I). In two cases, activation began during the
stimulation after initial inhibition, and outlasted the stimulation by several seconds (Fig. 5G), and in two cases there was persistent activation in neurones excited throughout stimulation.

Thus all type 1 neurones tested responded strongly to LOT stimulation, in most cases with a short latency, but had mixed inhibitory and excitatory effects, including effects that outlasted stimulation by several seconds.

Figure 6 compares the response of one type 1 cell to bedding with its response to LOT stimulation. This cell was repeatably and strongly activated by bedding odour, weakly activated by garlic, and unresponsive to air or vanilla extract (Fig. 6A). The response to bedding comprised intensified cyclic bursting (Fig. 6B). The same cell was tested for its response to 500 ms of LOT stimulation at between 20 and 200 Hz; it responded to stimulation at 150 and 200 Hz with a strong after-discharge, but showed no response to lower frequencies (Fig. 6C). We then tested it in response to prolonged stimulation at 5, 10 and 20 Hz (Fig. 6D). Stimulation at 10 or 20 Hz was initially ineffective, but progressively revealed a short latency excitation (Fig. 6E,F). This is consistent with a progressive enhancement of synaptic excitation, or with the superposition of constant excitation and a progressively waning synaptic inhibition.

All type 2 cells tested also responded to LOT stimulation, and again the responses varied considerably. The most common responses were inhibitions followed by excitation, and the effects varied over time during continued stimulation (Fig. 7). Of 13 type 2 cells tested with brief high-frequency stimulation (50 Hz, 0.5 s), six showed strong post-stimulus excitation and seven showed strong post-stimulus inhibition.

Comparison with the respiratory cycle.
Thus these initial experiments showed that most of the AON neurones that we encountered showed strong cyclic activity presumed to reflect inputs locked to respiration, but it was clear from the occasional double recordings that different neurones fired with different phase relationships. It was also clear from the studies with LOT stimulation that some AON neurones showed short latency excitation, consistent with a direct excitatory input from the LOT. However, more neurones were inhibited by LOT stimulation, and many showed complex responses to trains of stimulation. This suggesting that their responses were mainly mediated indirectly, presumably involving GABA inputs from neurones within the LOT that were themselves activated by LOT stimulation.

In subsequent experiments we therefore focussed on recording additional type 1 cells, characterised the relationship of their cyclic activity to the respiratory rhythm, and compared this with their responsiveness to LOT stimulation.

We recorded from a further 28 type 1 cells in 17 rats. For each of these we constructed autocorrelation histograms, ISI distributions, and we used two ways to assess the relationship of spiking activity to respiratory rhythm. Nine cells showed cyclic activity beginning in the inspiratory phase of respiration, and nineteen cells showed cyclic activity beginning in the expiratory phase. We could classify 20 of these neurones by their short-latency responses to LOT stimulation; five as being excited by LOT stimulation and 15 as being inhibited. All of the five excited neurones were inspiration neurones (Fig. 8); 13 of the 15 inhibited neurones were expiration neurones (Fig. 9).

**Discussion**

Processed odour information is transmitted from the MOB via the LOT to the AON (Shipley & Ennis, 1996; Menini *et al.*, 2004; Lledo *et al.*, 2005). The AON
distributes the information to the contralateral olfactory bulb and piriform cortex and engages in reciprocal interactions with the ipsilateral bulb and cortex (Mori et al., 1979; Kikuta et al., 2010; Miyamichi et al., 2011; Sosulski et al., 2011).

From the present results, we can draw some inferences about how the AON processes the synaptic input from the MOB. In similar experimental conditions to those used here, the mitral cells and tufted cells of the olfactory bulb display rhythmic spiking activity locked to the phase of respiration. This input to the AON is purely excitatory, as mitral cells and tufted cells are all glutamatergic. Many AON cells are GABAergic, with intrinsic projections within the AON (Kay & Brunjes, 2014). Accordingly, cells in the AON are expected to be excited by LOT stimulation if they receive a direct input from the MOB (primary AON neurones), but to be inhibited if they receive inhibitory inputs from primary recipients. Of course it is to be expected that many AON neurones will receive both direct excitatory inputs and indirect inhibitory inputs.

The first conspicuous feature of these results is that a high proportion of AON neurones display spontaneous cyclic activity tightly locked to the respiratory rhythm. This locking is apparent in the clarity of cycles displayed by the autocorrelation histograms – indeed the cyclicity of AON neurones is more marked than that of mitral cells recorded in similar experimental conditions.

Mitral cells in the anesthetized rat alternate between long periods of activity and silence. During active phases, their mean discharge rate is 11 spikes/s, and, in cells in which a respiratory rhythm is apparent, this typically oscillates between 5 and 20 spikes/s over the respiratory cycle. AON cells are much quieter but display bursts of spikes locked to the respiratory cycle. The bursts generally comprise just a few spikes, but as these bursts are separated by long silent intervals, the periodic activity is much more marked...
than that of mitral cells, which are generally active throughout the respiratory rhythm. The rhythms of type 1 cells were thus more clearly defined than those of mitral cells (Leng et al., 2014) because of the sparsity of spikes between bursts. In effect, the respiratory rhythms of AON cells are more marked because the AON operates as a high-pass filter of the MOB input.

As the respiratory rhythm must reach the AON via the output cells of the olfactory bulb, we conclude that the spontaneous cyclic activity of these AON cells is primarily driven by direct or indirect inputs from output cells of the olfactory bulb whose activity is locked to the same phase of the respiratory cycle. As stated in the introduction, there is evidence that mitral cells in the rat are generally activated late in the inspiration cycle, but not all studies have reported this; for example, Phillips et al., reported that mitral and tufted cells are activated at diverse phases of an artificial respiratory cycle (Phillips et al., 2012). Thus we must consider the possibility that AON neurones that are active during inspiration receive inputs from MOB neurones that are activated during inspiration, while AON neurones active during expiration receive inputs from MOB cells that are activate during expiration. However, if so we would expect to find that AON neurones were activated by LOT stimulation regardless of how their activity was linked to respiration. Instead, we found that AON neurones that were active during inspiration were excited by LOT stimulation, whereas most of those active during expiration were inhibited by LOT stimulation.

Mitral cells typically respond to odours with either inhibition or excitation that is sustained throughout odour application, and in excited cells that display a respiratory rhythm the response involves an intensification of that rhythm (Leng et al. 2014). Mitral cells are also narrowly tuned, responding only to closely-related odours. Here we used a
complex odour – the odour of male rat bedding. We chose this odour as a naturalistic odour of considerable social relevance to rats, of a type that the AON is thought to be particularly important for processing. We had previously tested this odour in our studies of mitral cells but without finding any responsive cells, possibly because we simply were not recording in responsive regions of the bulb (Leng et al., 2014): in mice, the existence of a “specialist glomerulus” narrowly tuned to a compound present in urine has been reported (Lin et al., 2005). In the AON, we found quite a substantial number of cells responsive to bedding odour. The responses were generally complex, including transient “on” responses and marked “off” responses, and responsive cells were typically similarly responsive to valeraldehyde and/or hexanol – the only two ‘simple’ odours that we tested.

A recent fMRI study in rats (Zhao et al., 2017) found no olfactory adaptation to prolonged (200-s) odour application in either the olfactory bulb or the AON, but found strong adaptation in the piriform cortex, and transient responses have recently been reported to be common for odour-responsive neurones in the mouse piriform cortex (Tantirigama et al., 2017). In the AON, we observed complex non-linearities, varying between AON cells, in their response to mitral cell input, including both frequency-dependent non-linearities, which may amplify odour responses, and temporal non-linearities arising presumably from slow activity-dependent effects which in some cells produce “on” and “off” responses to odour stimuli in some cells.

We tested AON cells with brief stimulation of the LOT, which antidromically activates mitral cells and their collaterals to the AON. In some of these experiments we stimulated at 0.5 s for 50 Hz, to mimic the activation during an inhibitory phase of the respiratory cycle as observed during odour application. Such stimulation commonly produced either a burst of activity during stimulation, or an inhibition during stimulation
followed by a burst after stimulation. The latter effect suggests an obvious explanation of how AON cells display rhythmic firing at different phases of the respiration cycle – as a rebound excitation following inhibition.

A feature of the AON was the many cells (the type 2 cells) showed periodic activity with a period apparently twice that of the respiratory rhythm – confirmed in examples recorded in conjunction with recordings of respiratory activity (not shown). This seems likely to occur as a result of prolonged post-activation inhibition that occludes the excitatory input on alternate cycles. If so, the post-activation inhibition is not plausibly accounted for by spike-dependent afterhyperpolarisation intrinsic to type 2 cells, which typically discharged only a few spikes in each burst, but probably arises from aggregate inhibition by quasi-synchronously activated interneurons.

Thus AON neurones represent the rhythmic input from the mitral cells as periodic activation with a varied phase relationship to the original input, depending on the balance between excitation from the mitral cell input, inhibition from intrinsic neurones, and rebound excitation following inhibition, and this is reflected in the diverse phase relationships of periodic activity in adjacent mitral cells.

The spontaneous activity of mitral cells generally oscillates with peaks of activity in the inspiration phase of the respiratory cycle, and in the presence of an odour, MOB cells responsive to that odour discharge more intensely during inspiration. Neurones in the AON however receive inputs derived, directly or indirectly, from many mitral/tufted cells, only a few of which will be activated by a given odour. Considering the aggregate input, the response of the odour responsive cells will be ‘diluted’ by the synchronous, spontaneous discharge of the non-responsive MOB cells. Such a population is simulated in Figure 10. In this simulation, one of eleven cells is activated strongly by
an 'odour' to increase its activity three fold during bursts, but in the aggregate activity of
the population this is imperceptible. The aggregate burst activation increases by less than
10%, within the range of random variation, and it is hard to see how any processing of
this aggregate signal could reliably extract the odour signal. However, it appears that
odour processing in the AON involves ‘phase shifting’ the MOB input by variable
amounts, in addition to a non-linearity of response that yields a pattern of short bursts,
linked to the respiratory cycle, with no activity between bursts. If we look at the aggregate
activity of such a population (Figure 10), the response to odour stimulation of one of the
11 cells is clearly apparent as a 50% increase above a stable background rate. Not only is
the signal: noise ratio protected, but the pattern of response is now readily amenable to
signal extraction by neurones that response non-linearly to this input.

In summary, the processing by AON neurones of the input from the MOB
appears to include four distinct operations. First, there is convergence of inputs, so that
AON cells are broadly tuned. Second, processing acts as a high-pass filter: AON neurones
have a low spontaneous firing rate but because they respond non-linearly to the MOB
input, they retain a marked rhythm in the form of short bursts with a period matching the
respiratory rhythm. Third, this rhythm is phase shifted in many cells, apparently by a
combination of inhibition and post-inhibitory rebound activation. This phase shifting
seems likely to help maintain the signal to noise ratio of odour responses in the population
output.

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T.T. and Ma.L. performed the research; T.T. and G.L. analysed data; and G.L., T.T., C.T.
and M.L. wrote the paper.
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**Figure legends:**

**Figure 1. Juxtacellularly labelled neurone in the AON.**

A, Juxtacellularly labelled neurone (arrowhead) in the rat AON, shown at higher magnification on the lower. AOB; accessory olfactory bulb, MOB; main olfactory bulb, AONd; anterior olfactory nucleus dorsal, AONl; anterior olfactory nucleus lateral. B, Higher magnification of the neurone arrowed in A showing multipolar neurone with oval cell body. C, Extract of voltage trace showing individual spikes. This neurone showed decreasing spike height in spikes clustered at high frequency. D, Voltage record spontaneous activity pattern showing rhythmic discharge of spikes, including short bursts. E, Average action potential from this neurone (average of all spikes fired during 300 s) expanded to show rhythmic oscillations of voltage. F, Distribution of spontaneous firing rates of a sample of 240 AON neurones. G, The SD of ISIs is closely correlated with the
mean ISI ($R^2=0.88$).

**Figure 2. Type 1 neurones in the AON.**

A, Extract of voltage trace of a typical type 1 neurone. B, Autocorrelation of spike activity of the neurone in A showing discharge activity with a period of ~600 ms. C, Normalised autocorrelations from type 1 neurones sorted according to period of rhythm (500-600 ms dotted line, n=67; 600-700 ms solid line, n=50). Means ± SE. D, Consensus ISI distribution for 143 type 1 cells (means ± SE). E, Pair of type 1 neurones, displaying bursts with a period of about 600 ms. Bursts in the smaller cell (one burst circled in dashed line) are out of phase with bursts in the larger cell (circled in dotted line). F, Box plots showing mean and interquartile ranges, median (X) and outliers (circles) for spontaneous firing rates (left and modal ISI (right) for the three groups of AON neurones.

**Figure 3. Type 2 neurones.**

A, The rhythmic discharge cell of a typical type 2 neurone. The cell fired in bursts of 2-20 spikes with a period of 1.5 s. B, ISI distribution of the neurone in A. C, Consensus ISI distribution for 45 type 2 cells (means ± SE). D, Autocorrelation histogram for the neurone in A,B. E, Average normalised autocorrelations for 45 type 2 cells (means ± SE). F, Pair of type 2 neurones, both displaying bursts with a period of about 1 s. Bursts in the smaller cell begin about 200 ms before bursts in the larger cell. G, Cross-correlograms (in 10-ms bins) showing activity in each of the neurones in F relative to spikes in the other, constructed over 300 s of spontaneous activity. Both neurones discharged in bursts in a rhythm with a period of about 1 s, but out of phase by about 200 ms. Bursts in cell 1 (smaller spikes in F) preceded bursts in cell 2 (larger spikes in F). The
dotted line shows timing of spikes in cell 1 relative to spikes in cell 2 and the solid line shows spikes in cell 2 relative to cell A.

**Figure 4. Responses of AON cells to bedding odour.**

A, Example of the response of a type 1 cell to bedding odour (grey bar). B, Averaged responses (± SE) of ten type 1 cells that were excited by bedding odour. C, example of an inhibitory response (one of two cells inhibited). D, Response of a type 2 cell that was excited after the end of odour application. E, Response of the same cell to LOT stimulation – the cell was inhibited during stimulation but showed excitation after stimulation.

**Figure 5. Effects of LOT stimulation on type 1 neurones.**

A, Extract of voltage trace showing spike responses (blue arrows) during a train of stimuli (red arrows) at 50 Hz. B, Post-stimulus time histogram of the response of a type 1 neurone activated by stimulation of the LOT with a latency of about 7 ms. C, A neurone inhibited by LOT stimulation (red bar) at 50 Hz. D, A neurone activated after the end of LOT stimulation (red bar) E, A neurone excited during LOT stimulation (red bar) but inhibited after. F, Expanded voltage trace from E showing that each stimulus pulse (red arrows) is followed by a spike (the first indicated by the blue arrow). G, A neurone progressively excited during LOT stimulation (red bar) with prolonged post-stimulus activation. The top trace shows detected spikes. H, This neurone was synaptically activated by LOT stimulation (red arrows) but each activation was followed by inhibition. I, Expanded voltage trace from H showing spike (blue arrow) following stimulus (red arrow).
Figure 6. Response of a type 1 cell to odours and stimulation of the LOT.

A, This neurone was repeatably activated by bedding odour (blue bars), unaffected by air (yellow bar) or valeraldehyde (val, orange bar) and weakly activated by garlic (green bar). B, Expansion of the response to bedding showing clear rhythmic discharge during odour application. C, Response to trains of stimulation at the frequencies shown. During tests, the cell fell silent and was unresponsive to brief stimulation at 20, 30 and 50 Hz but was activated after stimulation at 150 and 200 Hz. D, Responses to continued lower frequency stimulation. Stimulation at 10 and 20 Hz produced excitation after a delay. E, Voltage record of the response during activation by 20 Hz stimulation – the stimulus artefacts are marked by the red arrowheads. F, Peri-stimulus histograms of responses to stimulation at 10 Hz (green) and 20 Hz (blue, expressed as the probability of a spike occurring in 1-ms bins ($P_{\text{spike}}$). The stimulus time is marked by the red bar.

Figure 7. Response of a type 2 cell to stimulation of the LOT.

A, Extract of spontaneous activity of a type 2 neurone showing intermittent bursts with a period of about 2 s. B, Autocorrelation histogram, typical of type 2 cells. C, ISI distribution, shows the typical unimodal, skewed distribution. D, During 1 Hz stimulation of the LOT (artefacts marked in red in voltage trace) the cell initially was unresponsive. E, With continued stimulation, bursts arose entrained by the stimuli. F, Peri-stimulus time histogram, revealing post stimulus inhibition followed by prolonged excitation. G, The response to a brief train of stimulation at 50 Hz, showing delayed activation that outlasted the simulation.

Figure 8. Type 1 neurones that are activated during inspiration are activated by
LOT stimulation.

A, Extract of recording of respiratory activity in conjunction with recording the spontaneous activity of a type 1 neurone. B, Identification of this neurone as activated by LOT stimulation. The shaded bar covers the stimulus artefact accompanying a stimulus pulse applied to the LOT; the pulse is followed by two spikes from this neurone (arrowed) and a smaller spike (with a negative going waveform) from a second, almost silent neurone. C, ISI distribution from this neurone, typical of type 1 neurones. D, Spike activity relative to respiratory cycle. During 300 s of spontaneous activity, the time of the peak inspiration in each respiratory cycle was marked, and the dotted line shows the average cycle produced from these triggers. The histogram shows the spike activity in 10-ms bins triggered in the same way. E, Respiration relative to spike activity. The solid line shows the conventional autocorrelation histogram for this cell – this is a spike-triggered average of spiking activity. The dotted line shows the directly corresponding spike-triggered average of respiratory activity. Both D and E show, in slightly different ways, that the bursts in this cell were consistently initiated during inspiration.

Figure 9. Type 1 neurones that are activated during expiration are inhibited by LOT stimulation.

A, Extract of recording of respiratory activity in conjunction with recording the spontaneous activity of a type 1 neurone. B, Identification of this neurone as inhibited by LOT stimulation. Peristimulus time histogram of spike activity (in 10-ms bins) of 300 s of activity during 1 Hz LOT stimulation. The shaded bar covers the stimulus artefact accompanying stimulation; the pulse is followed by about 50 ms of inhibition. C, ISI distribution from this neurone, typical of type 1 neurones. D, Spike activity relative to
respiratory cycle. During 300 s of spontaneous activity, the time of the peak inspiration in each respiratory cycle was marked, and the dotted line shows the average cycle produced from these triggers (increased signal corresponds to inspiration). The histogram shows the spike activity in 10-ms bins triggered in the same way. E, Respiration relative to spike activity. The solid line shows the conventional autocorrelation histogram for this cell – this is a spike-triggered average of spiking activity. The dotted line shows the directly corresponding spike-triggered average of respiratory activity. Both D and E show, in slightly different ways, that the bursts in this cell were consistently initiated during expiration.

**Figure 10. The importance of phase-shifts.**

The mitral cells of the MOB fire spontaneously in a rhythm locked to the inspiration phase of the respiratory cycle, and each is narrowly tuned to a specific odour. In the AON, neurones receive convergent inputs from multiple mitral cells, making them more broadly tuned. However, the synchronicity of spontaneous activity rhythms presents a problem illustrated in A. The black traces simulate the activity of eleven ‘cells’ all firing at the same phase of the rhythm. One of these cells (the bottom trace) is activated by an odour in the period marked by the yellow bar. But the aggregate activity (in blue), as seen by a second order neurone, the signal is virtually invisible. The signal is diluted by a factor of 1:11. In B, the same activity of the eleven ‘cells’ is shown, but each cell is phase shifted (red line). Now the signal is clearly apparent in the aggregate activity. The signal is still diluted, but by a much smaller factor of about 1:3. For simple simulation, each ‘cell’ was generated by assuming that the probability of ‘spiking’ was determined by a sinusoidal input.