Chem. Senses 28: 671–679, 2003 DOI: 10.1093/chemse/bjg059

Estimation of the Individual Firing Frequencies of Two Neurons Recorded with a Single Electrode

Nicolas Meunier[1], Frédéric Marion-Poll[1], Petr Lansky[2] and Jean Pierre Rospars[1,3]

[1]Unité de Phytopharmacie et Médiateurs chimiques, INRA, F-78026 Versailles Cedex, France, [2]Institute of Physiology, Academy of Sciences, CZ-142 20 Prague 4, Czech Republic and [3]Unité BIA, INRA, F-78352 Jouy-en-Josas, France

Correspondence to be sent to: Nicolas Meunier, Station de Phytopharmacie et Médiateurs Chimiques, INRA Centre de Versailles, F-78026 Versailles Cedex, France

Abstract

When monitoring neurons with a single extracellular electrode, it is common to record action potentials from different neurons. A recurring problem with such recordings is to identify which neuron is active. Sorting spikes into separate classes is possible if each neuron discharge spikes differing by their shapes and sizes. However, this approach is not applicable when the spikes are indistinguishable. In this paper, we develop a method for estimating the respective firing frequencies of two neurons, producing indistinguishable spikes. It is based on the fact that, when a neuron fires a spike, there is an interval of time during which the probability of generating a second spike is very low. If a spike occurs during this 'silent period', it is likely to be generated from another neuron and the number of occurrences of such 'doublets' can be used to estimate the respective frequencies of two spike trains. We demonstrate here that a simple relation holds between the frequency of doublets d, the respective frequencies of the two neurons A and B, f_A and f_B, and a chosen value Δ shorter than the silent period, $d = 2f_Af_B\Delta$. This relation holds for a wide class of firing processes. We used this method to analyze responses from *Drosophila* taste sensilla. We first checked if the method was consistent with results obtained with stimuli that elicit responses of two taste neurons firing *distinguishable* spikes. We then applied this method to the study of a pair of taste neurons involved in the coding for salt taste in *Drosophila melanogaster*.

Key words: *Drosophila*, interspike intervals, mathematical model, spikes sorting

Introduction

Extracellular recordings are a common practice in neurophysiology and often represent the only way to measure the electrical activity of neurons. Arthropod sensilla represent such a situation because neurons are tightly packed and intimately embedded within a layer of epithelial cells just below the cuticula. Most insect chemosensory sensilla house two to four neurons whose dendrites extrude in an hair-like structure covered with cuticle (Stocker, 1994; Steinbrecht, 1996). Given the layout of the neurons within the layer of epithelial cells, when a single electrode is brought into contact with the sensillum liquor, it will record electrical activities originating from this small group of neurons (Hodgson *et al.*, 1955). In order to fully exploit these recordings, each spike should be assigned to its neuron of origin. This is sometimes possible when each neuron fires spikes with a distinct amplitude and/or shape (Stitt *et al.*, 1998). However, when neurons discharge indistinguishable spikes, no method has yet been proposed to estimate the firing frequencies of each neuron. A similar problem is faced when recording from several channels present under the same patch-clamp electrode. Methods have been developed to analyze such multi-channel recordings to get single-channel information (Jackson, 1985; Colquhoun and Hawkes, 1995), but they are of limited value here because they are based on the study of tens of thousands events. More recently, methods has been developed based on multi recording sites (Pouzat *et al.*, 2002; Takahashi *et al.*, 2003) but they are also based on the study of a number of events much higher than data usually obtained when stimulating chemoreceptor neurons.

In order to circumvent these limitations, we propose to take advantage of the firing properties of the neurons. Firing of a neuron under given stimulation conditions can be characterized by the histogram of its interspike intervals (ISIs). The smallest ISIs on the left-hand side of the histogram can be used to define the silent period of the neuron, i.e. the longest interval during which the probability of having a

'natural' spike (i.e. in the absence of artificial electrical stimulation) is negligible. This silent period is usually much longer than the refractory period, especially under moderate stimulation. By this definition, ISIs shorter than the silent period are very rare in single-neuron recordings. Conversely, in recordings from two neurons, such ISIs can be frequent, with the leading spike coming from one neuron and the following spike from the other neuron. Here we call 'doublets' two spikes separated by a chosen time interval Δ shorter than the silent period. The presence of doublets in recordings has been used to show that a stimulus triggers the response of more than one neuron when their spikes are indistinguishable (Perkel *et al.*, 1967). In the following we show that the frequency of doublets can be used to estimate the individual neuron frequencies in a two-neuron recording containing indistinguishable spikes.

We used this method of doublets to analyze experimental data obtained from insect taste sensilla which house four taste neurons. Previous studies showed that stimulation with sugars elicited the activity of two neurons in taste sensilla of *Drosophila*, one, S, sensitive to sugars and the other, W, to water (Fujishiro *et al.*, 1984). The spikes from these two neurons are very different in amplitude, and easy to sort manually. Using three sugars eliciting different ratio of firing frequencies in S and W neurons, we first verified that the firing activities predicted with our theoretical method were consistent with the experimental data. Then, we recorded on the same preparation, the responses to salts. Previous studies have shown that salts (NaCl, KCl) stimulate two taste neurons (L1 and L2) firing indistinguishable spikes (Meunier *et al.*, 2000) and the respective sensitivities of these two neurons remained unknown. The use of our new method allows to discriminate the activities of these two neurons across a range of salt concentrations and to obtain for the first time dose–response plots for these tarsal taste neurons.

Materials and methods

Chemicals

Glucose, fructose, sucrose, NaCl and KCl were obtained from Sigma Chemical Co. (France). Solutions were prepared in advance and stored at –15°C. Glucose, fructose and sucrose solutions were prepared as dilutions in 1 mM KCl and kept at 4°C for <1 week.

Flies

Stocks (*Drosophila melanogaster*, Canton-S) were maintained at 25°C on a standard corn-meal agar medium. Flies were kept for 1 day on fresh medium after emergence prior to electrophysiological experiments.

Electrophysiology

Taste cell recording technique

A decapitated fly was secured to a flat support with insect pins and tape, and electrically grounded via a glass capillary filled with Ringer's solution inserted into the abdomen. All stimulations were performed on tarsal sensilla 5b and 4s (Meunier *et al.*, 2003). Consecutive stimuli were applied at least 1 min apart to avoid adaptation. Each stimulation was performed by covering the tip of a sensillum for <2 s with a recording electrode containing both an electrolyte (1 mM KCl) and the stimulus (Hodgson *et al.*, 1955). The recording electrode (a glass capillary with a tip diameter of 20 μm) was connected to a TastePROBE amplifier (Marion-Poll and Van der Pers, 1996). The electric signals were amplified and filtered (CyberAmp 320, Axon Instrument, USA; gain: 1000; eighth-order Bessel pass-band filter: 1–2800 Hz). Contacting a taste hair with the stimulus electrode triggered the data acquisition and storage (sampling rate 10 kHz, 12 bits; Data Translation DT2821), under the control of a custom-made software Awave (Marion-Poll, 1996). Sensilla used in this study are located symmetrically and present on both legs. Thus, we could record from four homologous sensilla per preparation. This sampling procedure introduced pseudo-replicates as noted below. However, only two sensilla 5b and one sensillum 4s were recorded on average on a single fly and care was taken to sample data from at least four different flies.

Stimulation with sugars and salts

A first set of data was obtained by stimulating sensilla 5b or 4s (Meunier *et al.*, 2003) with 100 mM glucose, fructose and sucrose, or with increasing concentrations of sucrose (10, 20, 30, 50 and 100 mM). In these recordings ($n \geq 16$ observations per data point), the spikes clearly fell into two amplitude classes, which allowed to sort them manually and to estimate their respective firing frequencies. A second set of data was recorded from sensilla 5b and 4s to a series of dilutions (100, 200, 400, 700, 1000 mM) of either NaCl or KCl (four different flies for each compound; $n \geq 15$ recordings per data point). In these recordings, spike amplitudes could not be separated into different classes, although earlier observations on the proboscis suggested that two cells are concurrently active (Siddiqi and Rodrigues, 1980).

Data analysis and spikes sorting

Action potentials frequency was determined by counting spikes during the 0.2–1 s interval after stimulation. We excluded from the analysis the first 200 ms, in order to keep only the tonic part of the response (Meunier *et al.*, 2000) and to meet one of the conditions imposed by the method (see Discussion). No more than two taste neurons were concurrently active in our recordings. When spikes could be differentiated by their amplitude and shape (i.e. responses to sugars), spikes were sorted manually (see Figure 1). For all recordings, we calculated two parameters, the total frequency f and the frequency of doublets d. Both those operations and the manual sorting were done using interactive procedures of custom-made software dbWave (available at http://quasimodo.versailles.inra.fr/fred/awave/awave1.htm).

Results

Mathematical method

Presentation of the problem

We consider two neurons, A and B, recorded simultaneously, so that only the pooled process resulting from the superposition of the two spike trains can be observed and we wish to estimate their respective frequencies f_A and f_B . Even if the action potentials fired by neurons A and B are indistinguishable by their shape and size, f_A and f_B can be estimated, provided the overall frequency f and the frequency of the doublets d, are known. Two spikes are considered as a doublet if they are separated by a chosen time interval Δ shorter than a period we call the silent period. By silent period, we mean a time interval following a spike during which the probability of occurrence of a second spike from the same neuron is small. In case the silent periods of the neurons are different, the smallest must be taken. This interval Δ does not need to be chosen equal to the absolute or relative refractory period; although it can be taken shorter, it is usually more useful to take it longer (see Discussion). The only condition is that Δ remains less than the shortest ISIs of the most active neuron in the experimental conditions considered. Then one of the spikes in the doublet comes from neuron A and the other from neuron B.

The basic relationship between these quantities is

$$d = 2f_A f_B \Delta \tag{1}$$

For a given recording the frequency of doublets increases with higher values of Δ. The second equation necessary for identification of individual frequencies is based on the fact that the frequency f of the pooled process is the sum of the component frequencies, so

$$f = f_A + f_B \tag{2}$$

A general proof of equation (1) is given in Appendix. In practice, frequency f can be estimated from the total number of spikes N during the observation period T,

$$f = N/T \tag{3}$$

Similarly, from the number N_d of doublets in the observation period T, their frequency is

$$d = N_d/T \tag{4}$$

Heuristic proof

A simple proof can be given in the special case of a couple of Poissonian neurons, i.e. neurons for which the probability of emitting a spike within any time interval dt has probability $f_i dt$ (i = A or B), where dt is sufficiently short. Then, during the observation period, T the number of spikes fired by neuron A is statistically equal to $f_A T$. Each of these spikes can be followed by a spike from neuron B within time Δ with probability $f_B\Delta$. Thus the number of doublets in which the leading spike is from neuron A and the second from neuron B is $f_A T f_B\Delta$. In the same way, the number of spikes fired by neuron B is $f_B T$ and each of them can be followed by a coincident spike emitted by neuron A with probability $f_A\Delta$. Thus the number of doublets in which the leading spike is from neuron B and the second from neuron A is $f_B T f_A\Delta$. The total number of doublets N_d is the sum of the two types of doublets, so

$$N_d = 2f_A f_B \Delta T \tag{5}$$

from which equation (1) is obtained from equation (4), dividing both sides by T.

Method for estimating frequencies

Utilizing equation (2), equation (1) can be written as

$$d = -2\Delta f_A^2 + 2\Delta f f_A \tag{6}$$

The graph of function $d(f_A)$ is a parabola starting from $d = 0$ at $f_A = 0$ (only neuron B is firing), rising to a maximum for $f_A = f/2$ (both neurons are equally active),

$$d_{max} = \Delta f^2/2 \tag{7}$$

and returning to $d = 0$ at $f_A = f$ (only neuron A is active) (Figure 2). Equations (1) and (2) have two solutions, one on the rising branch and the other on the decreasing branch of the parabola,

$$f_A = \frac{1}{2}\left(f + \sqrt{f^2 - \frac{2d}{\Delta}}\right) \tag{8}$$

$$f_B = \frac{1}{2}\left(f - \sqrt{f^2 - \frac{2d}{\Delta}}\right) \tag{9}$$

in which we chose to call A the most active neuron and B the least active one.

Experimental validation of the method

We stimulated terminal tarsal sensilla of *Drosophila* with sucrose, fructose and glucose at 100 mM. As it is the case of most sensilla on the proboscis (Fujishiro *et al.*, 1984), sugars elicit the responses of two neurons with different spike amplitudes on tarsal taste sensilla (Meunier *et al.*, 2000). Small spikes originate from the S cell, which is sensitive to sugar while large spikes originate from the W cell (Figure 1A). The sugars stimulated the S neuron at different frequencies while the W cell was less affected. This situation allows a comparison between experimental data and predicted values for different ratios of the firing discharges from both neurons and for different values of the overall frequency f (Table 1). We also compared the experimental and estimated value of f_A according to (8) for Δ varying

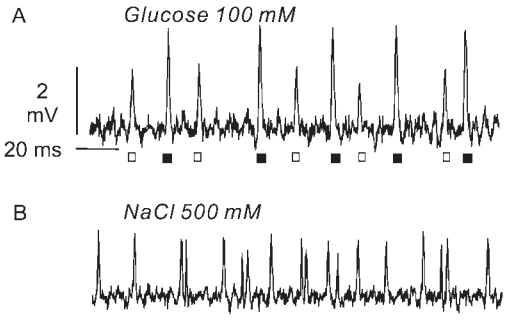

Figure 1 Representative samples of extracellular recordings from a tarsal taste sensillum of *Drosophila melanogaster* where two neurons are active. **(A)** Response to glucose. Spikes exhibit different amplitudes and are easy to sort: (filled square) spike from the taste neuron responding to water; (open square) spike from the taste neuron responding to sugars. **(B)** Response of the same sensillum to NaCl, in which two neurons are firing but their amplitude and shape are similar, making classical sorting methods useless.

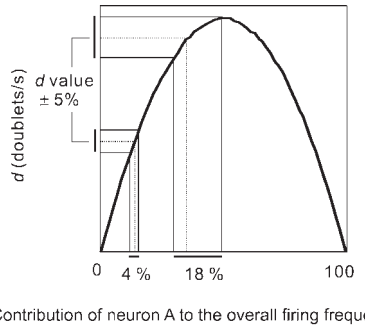

Figure 2 Frequency of doublets (d) as a function of the relative firing frequency of neuron A vs. neuron B according to equation (1). The maximum value of d (d_{max}) is reached when neurons A and B discharge at the same rate ($f/2$). Since the experimental values of d and f are easy to observe, we can use equation (1) to estimate the ratio between the firing of neuron A and B. The error on the estimation of the firing frequency is much higher if d is close to d_{max}, i.e. if neurons A and B fire at almost the same rate. If we assume for example a 5% error in the experimental value of d, our estimate of the firing ratio of neurons A and B will vary from 4% to 18%.

between 1 and 15 ms (Figure 3A). For some values of Δ, equation (6) had no solution. This happened when experimental values of d were higher than d_{max}. In this case, if d did not exceed d_{max} by more than 10%, we chose a value of f_A equal to $f/2$ (corresponding to d_{max}). Because in the plot f_A vs. Δ, the differences for the small values of Δ are difficult to interpret, we also compared the theoretical and experimental values of d for Δ varying between 1 and 15 ms (Figure 3B). There was a good fit between experimental and theoretical values of f_A for Δ between 5 and 9 ms. For $\Delta < 5$ ms, theoretical values were higher than experimental one and this was the reverse for $\Delta > 9$ ms. The best fit was obtained for $\Delta = 6$ ms and we chose this value in the analysis of the second set of experimental data (Table 1).

By using a Δ value of 6 ms, we extended the previous experiment to determine the response of the S cell over a series of concentrations of sucrose (Figure 4). Both estimated and experimental values were found to be in good agreement. We obtained a value of d slightly greater than d_{max} for the recordings corresponding to the concentration 20 mM ($d = 7.73$; $d_{max} = 7.43$ doublets/s), thus we estimated frequencies of the W and S cell to be half the total frequency (24.6 spikes/s).

Table 1 Comparison between measured and estimated frequencies of W- and S-neuron responses to different sugars (Δ = 6ms)

Stimulus	f[a]	d[b]	f_A[c]	f_B[d]
Sucrose 100 mM				
Experimental values	86	16	66	20
Calculated values[e]	–	15.7	65	21
Error (%)		2	1	5
Glucose 100 mM				
Experimental values	50	7.23	30	20
Calculated values[e]	–	7.20	30	20
Error (%)		0.4	0	0
Fructose 100 mM				
Experimental values	38	$4.40 > d_{max}$	19	19
Calculated values[e]	–	$4.32 = d_{max}$	~19	~19
Error (%)		2	0	0

[a]Frequency (spikes/s) of both neurons A and B together.
[b]Frequency (spikes/s) of doublets in the record A + B.
[c]Frequency (spikes/s) of the most active neuron A (S neuron in this case).
[d]Frequency (spikes/s) of the least active neuron $f_B = f - f_A$ (W neuron in this case).
[e]Theoretical d and estimated f_A, f_B.

Application to responses to salts on *Drosophila* tarsal sensilla

The sensitivity to salts in *Drosophila* involves two taste neurons named L1 and L2 that are present in all sensilla responding to salts. These two neurons fire indistinguishable spikes on tarsal sensilla (Figure 1B) and thus no data exists yet on their relative frequencies (Meunier *et al.*, 2000). On the proboscis they can be sorted manually (Siddiqi and Rodrigues, 1980) and L1 neuron is known to be activated at low concentration of salts whereas L2 is activated at higher concentration (Singh, 1997). We used the method presented above to estimate the respective firing of L1 and L2 neurons in response to increasing concentration of NaCl and KCl in terminal tarsal sensilla (Figure 5). Both neurons were activated by the two kinds of salts without reaching saturation of their activity below 1 molar of salt. We found that L1 neuron activity was always much higher than that of L2, and that L1 was more sensitive to KCl.

Discussion

In this paper, we develop an approach for estimating the respective frequencies of two neurons firing indistinguish-

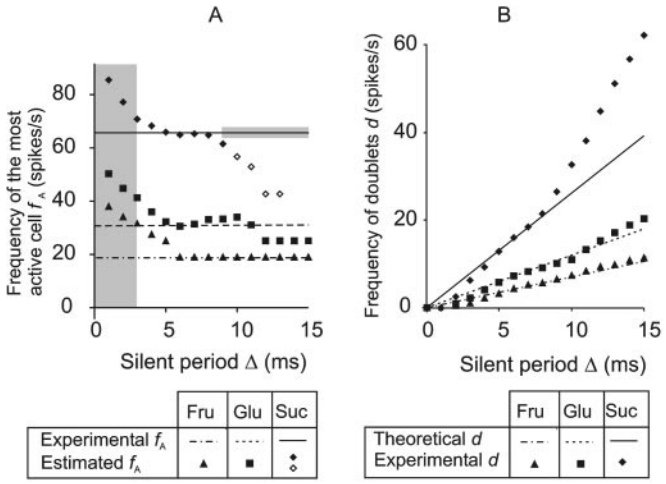

Figure 3 Providing a correct estimate of the silent period is critical to the method. **(A)** Comparison between experimental and estimated values of the firing frequency of the most active neuron as a function of the silent period Δ under different stimulation conditions. For each value of Δ, f_A was calculated according to equation (8). When experimental values of d were higher than d_{max}, equation (7) has no solution as for the last two data points of the sucrose curve. However if d did not exceed d_{max} by more than 10%, we chose a value of f_A equal to $f/2$ (corresponding to d_{max}). It allows to keep data resulting from experimental value of d close to d_{max}. Gray areas show forbidden values of the silent period. The forbidden area on the left side comes from the difficulty to discriminate superimposed spikes and thus missing to count them as doublets. It does not depend on f but on the duration of spikes. On the right side, forbidden values depend on f according to equation (10). Such values of Δ should be avoided and the corresponding estimated data points are indicated as open symbol. **(B)** Comparison between theoretical and experimental values of the frequency of doublets d as a function of the silent period Δ. For each value of Δ, we calculated the theoretical value of d from relation (1) using the manually sorted frequencies of neuron W and S. (See Table 1 for experimental values of frequencies f_A and f_B).

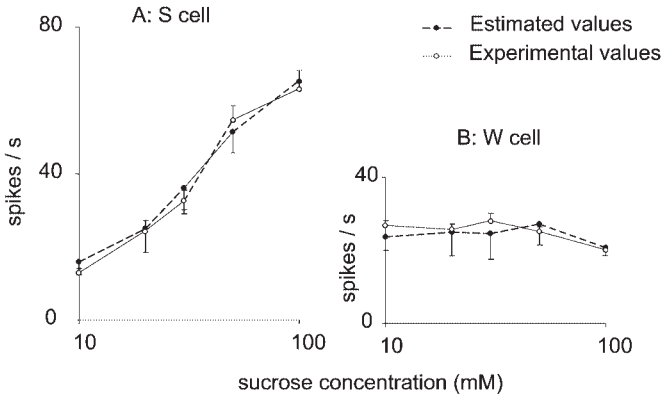

Figure 4 Estimated and experimental frequencies of neuron W and S stimulated by increasing concentration of sucrose. Error bars mean ± SEM for experimental values ($n \geq 16$). Error bars for estimated frequencies were determined from SEM on d as shown in Figure 2.

able spikes based on the occurrence of 'doublets'. A doublet corresponds to an interspike interval shorter than a chosen value Δ that must be less than the smallest silence period of

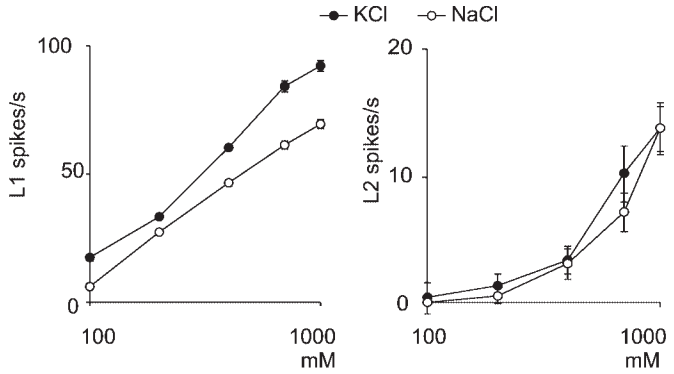

Figure 5 Estimated frequencies of neurons L1 and L2 in tarsal taste sensilla of *D. melanogaster* stimulated by increasing concentrations of salts. Error bars ($n \geq 15$) were determined from SEM on d as shown in Figure 2; on the left, bars are the same size or smaller than the circles.

the two neurons considered. We showed that a simple relationship exists between the discharge rate of the two neurons firing independently, the number of doublets and the total number of spikes observed. We tested the validity of this method on spike trains originating from neurons firing spikes of different amplitudes. Then, we used this method to discriminate the firing activities from two neurons delivering spikes of similar amplitudes in the presence of salts. This method was designed to analyze time series where only two neurons are active. It is not applicable in other situations, except if the other neurons exhibit different spike shapes, so that they can be removed from the time series prior to analysis.

Conditions of application of the method

The proof of relation (1) given in the Appendix is based on two assumptions. The first assumption is that spike generation in each of the component processes follows a renewal process, i.e. a process in which the intervals (ISIs) between events are stationary, independent (not serially correlated) and identically distributed random variables. The second assumption is that the individual processes are mutually independent, i.e. that firing in one of the neurons does not modify the probability of firing in the other. These conditions must be met by the pair of neurons under study otherwise formula (1) may lead to wrong results. They may be difficult to prove although gross deviations from them will be easily observable, e.g. from abnormal regularities in the recording (thus recordings including erratic bursting activities should be avoided).

Sources of errors and limitation of the method

If the conditions of the method are fulfilled, errors in estimation of the component frequencies f_A and f_B can arise from three possible causes, as apparent from equations (1) and (2). The first source concerning the choice of Δ higher than the silent period is discussed in the next section. The two

other sources concern the reliability of the estimates of the firing frequency f and of the frequency of doublets d. They depend on the observation period T which must be as long as possible within the limits of experimental constraints and the requirement of stationarity. For this reason we chose to analyze data coming from taste neurons during the tonic part of the response, i.e. the interval 0.2–1 s after the stimulation (Meunier *et al.*, 2000).

Errors on the estimation of the frequency of doublets d had two different effects. The first effect, illustrated in Figure 2, is that the error on f_A depends on the magnitude of d. The same small relative error on d yields a larger relative error on f_A if d is near its maximum value d_{max} [corresponding to an equal frequency of discharge from both neurons, equation (7)] than if d is relatively small and far from d_{max}. If d is close to d_{max}, it is thus advisable to use different recording conditions (e.g. changing the concentration of stimulus). The second effect arises when the two neurons fire at approximately the same rate. Then the measured value of d may happen to be slightly greater or smaller than d_{max}. If it is smaller, a unique solution f_A is found, but if it is greater no solution exists. This means that two random fluctuations of equal magnitude around d lead to different situations depending on the direction of the fluctuation. In practice if d is close to d_{max} (e.g. do not differ by more than 10%, see Figure 3), d can be safely replaced by d_{max}. If the difference is greater some caution must be exerted because either the conditions of application of the method are not fulfilled or the value of Δ has not be well chosen (see below).

It should be stressed that this method does not allow to assign an estimated frequency either to neuron A or to B. If one is concerned by the identity of the neurons, other sources of information must be used. For example, when stimulating the same neurons with a series of closely related stimuli, we expect to find a graduated effect on the firing activities. If the two neurons have different dose–response curves, it is then possible to resolve the activity of cell A and cell B without ambiguity. We used that approach to identify the cell responding to bitter compounds in *Drosophila* (Meunier *et al.*, 2003).

Choice of the interval of time Δ

We have investigated quantitatively the validity of the method by estimating f_A and d for a range of Δs from recordings where the spikes from the two neurons could be distinguished. We then compared the predicted values of f_A and d to their experimentally observed counterparts. Figure 3 shows that predicted values depart from the experimental ones when Δ is too small or too large, and that the range of acceptable values depends on the firing rate. This is a positive feature of the method proposed that, in general, the result obtained does not depend on a strict choice of Δ. How can we restrict the range of potentially good Δ values?

For small values of Δ, on the left-hand side of plots in Figure 3, the difference between experimental and estimated values can be explained by our inability to differentiate two superimposed spikes. In the case of responses to sugars, spikes from the W neuron last 3 ms and are much bigger than those of the S neuron (Figure 1A). Therefore it is easy to miss a spike coming from the S neuron if they are occurring close to each other and mistakes on counting doublets are more frequent when Δ is small (only a few ms). To avoid this difficulty, Δ should be greater than 3 ms. This type of error is critical when spikes are dissimilar, because it is easy to miss superposition of a small spike over a large spike. This source of errors may be of less concern when neurons discharge indistinguishable spikes. However, values of Δ less than the duration of the spikes studied should be avoided.

For large values of Δ, the difference on the right side comes from the fact that the selected Δ is greater than the silent period. Therefore some doublets come from the same neuron, which violates one of the assumptions of the model. The values of Δ must be smaller than the minimum ISI of the fastest firing neuron and thus significantly smaller than the mean ISI (inverse of the firing rate, which is not known) to take into account statistical fluctuations. We observed this difference only for the stimulation with sucrose which elicits a strong response of the taste neurons, $f = 86$ spikes/s, which is the upper limit of f_A (the other neuron being silent, $f_B = 0$). In this case, experimental and estimated values start to diverge at $\Delta = 9$ ms, i.e. at 75% of the mean ISI of the pooled process. We can generalize this observation to set a maximum value of Δ, Δ_{max} for a mean frequency f at

$$\Delta_{max} = 0.75/f \tag{10}$$

On Figure 3A this limit is indicated on the right-hand side of the sucrose line. For glucose and fructose, it does not appear on the graph because f is too small. Those left and right limits are only 'rules of thumb' to investigate a range of potentially good values of Δ. When frequency increases, this range narrows down and reaches a limit of 4 ms for spike duration of 3 ms. According to equation (10), this limit corresponds to an overall frequency f of 190 spikes/s. Thus the method should not be applied to estimate the firing rate of neurons with an overall frequency higher than that.

In summary, the choice of Δ is a compromise between the number of doublets counted, which must be as large as possible (and this calls for Δ as long as possible), and the number of doublets coming from the same neuron, which must be negligible. Doublets obtained with a long Δ minimize the inherent error coming from superposition of spikes and the corresponding failure to detect some doublets. The optimal value of Δ will thus be close to the shortest silent period of the two neurons considered. For neurons firing at a much lower rate than the maximum, this value will be longer than the refractory period. It will be close to the relative refractory period (the time interval following a spike

that require an electrical stimulation to evoke another spike from the same neuron) only for neurons firing at very high rate. But in that case it would be better not to use our method, since Δ would be probably to small to minimize the error coming from superposition of spikes.

Estimation of the respective frequency of L1 and L2 cells on response to salts

Whereas sensilla on the proboscis respond to NaCl at a threshold of 1 mM (Fujishiro *et al.*, 1984), we found that tarsal sensilla are not activated below 100 mM. On the proboscis, the L1 neuron was defined on the proboscis as responding to low concentrations of salts (Singh, 1997); it reaches a maximal response at around 100 mM with a frequency of 70 Hz. At this concentration, the L2 neuron starts being activated and reaches 30 Hz for 1 M NaCl (Siddiqi and Rodrigues, 1980). Our estimations of the respective frequencies of the two neurons activated by salts on tarsae are consistent with those values. Even if the range of salts concentration activating both types of sensilla are different, we can assign the most active neuron on tarsae to L1 (Figure 5). The least active neuron can be assigned to L2. This result is coherent with previous data because, the level of activation of L2 neuron by salts on tarsae is (i) very similar to the level elicited on L2 neurons of the proboscis in response to salts (Siddiqi and Rodrigues, 1980), and (ii) consistent with the level of activation of L2 neuron by bitter compounds on tarsae (Meunier *et al.*, 2003).

Appendix: general proof of equation (1)

The aim of this section is to prove that relation (1) holds true for any renewal firing process, not only for Poissonian processes as shown in the Result section. First we determine the cumulative distribution function (cdf) of the intervals in the pooled process. Then from the value of this function at D we deduce the probability to have an interval shorter than Δ, which is the probability of a doublet. Finally from the relation between the individual and pooled firing frequencies we obtain equation (1).

1. Frequency of spikes in the pooled process

For a given value of Δ, which is assumed to be less or equal to the shortest silent period of both neurons, we can write for individual frequencies

$$f_i = 1/(\mu_i + \Delta) \tag{A1}$$

where $m_i + \Delta$ is the mean ISI of neuron i and i = A, B. Using equation (2) it can be shown that the frequency of spikes in the pooled process is

$$f = \frac{\mu_A + \mu_B + 2\Delta}{(\mu_A + \Delta)(\mu_B + \Delta)} \tag{A1}$$

2. Distribution of interspike intervals in the pooled process

In this section, we determine the cdf, denoted $F(t)$, of the ISIs in the pooled process, i.e. the probability of an interval of length smaller than t.

Let us denote $u(t)$ the probability density function (pdf) of the time interval, τ, to the next action potential from a randomly selected instant of time (forward recurrence time) in the pooled process. The corresponding cdf is denoted by $U(t)$. The relationship between the forward recurrence time and the length of intervals between events, i.e. spikes in the pooled process, is given by the (standard) formula (Cox and Lewis, 1966)

$$u(t) = [1 - F(t)]/\mu \tag{A3}$$

where μ is the mean ISI in the pooled process. So, $F(t)$ is given by

$$F(t) = 1 - \mu u(t) \tag{A4}$$

therefore for evaluation of F we need to know u.

The same definitions and relationships hold for each of the two superimposed processes. We denote $F_i(t)$ the cdf of the ISIs in the ith process and $u_i(t)$ the pdf of the forward recurrence time τ_i, i = A, B. Relation (A3) now reads

$$u_i(t) = \frac{1 - F_i(t)}{\mu_i + \Delta} \tag{A5}$$

It follows from these definitions that the probability of forward recurrence time longer than t is

$$\text{Prob}(\tau_i \geq t) = \int_t^\infty u_i(s)ds \tag{A6}$$

and by using (A5) in (A6) it gives

$$\text{Prob}(\tau_i \geq t) = \int_t^\infty \frac{1 - F_i(s)}{\mu_i + \Delta} ds \tag{A7}$$

This quantity is used now for evaluating $U(t)$.

The forward recurrence time for the pooled process is the minimum of the recurrence times of the components, $\tau = \min(\tau_A, \tau_B)$ and thus $\text{Prob}(\tau \geq t) = \text{Prob}(\tau_A \geq t \text{ and } \tau_B \geq t)$. The cdf of τ is $U(t) = 1 - \text{Prob}(\tau \geq t)$, therefore

$$U(t) = 1 - \text{Prob}(\tau_A \geq t)\ \text{Prob}(\tau_B \geq t) \tag{A8}$$

where multiplication follows from the independence of the components. Using (A7), we obtain

$$U(t) = 1 - \int_t^\infty \frac{1 - F_A(s)}{\mu_A + \Delta} ds \int_t^\infty \frac{1 - F_B(s)}{\mu_B + \Delta} ds \tag{A9}$$

The pdf $u(t)$ is the derivative of $U(t)$,

$$u(t) = \frac{1-F_B(t)}{\mu_B+\Delta} - \int_t^\infty \frac{1-F_A(s)}{\mu_A+\Delta}ds + \frac{1-F_A(t)}{\mu_A+\Delta}\int_t^\infty \frac{1-F_B(t)}{\mu_B+\Delta}ds \quad \text{(A10)}$$

Finally, utilizing $u(t)$ given by equation (A12), equation (A6) becomes

$$F(t) = 1-\mu\left(\frac{1-F_B(t)}{\mu_B+\Delta} - \int_t^\infty \frac{1-F_A(s)}{\mu_A+\Delta}ds + \frac{1-F_A(t)}{\mu_A+\Delta}\int_t^\infty \frac{1-F_B(t)}{\mu_B+\Delta}ds\right) \quad \text{(A11)}$$

3. Probability of doublets

Now, we derive the probability $F(\Delta)$ of having an interval shorter than Δ in the pooled process. Because of the silent period properties, the probability of an interval shorter than Δ in the component process i is zero, $F_i(\Delta) = 0$. So, using $t = \Delta$ in (A11) gives

$$F(\Delta) = 1-\mu\left(\frac{1}{\mu_B+\Delta}\int_\Delta^\infty \frac{1-F_A(s)}{\mu_A+\Delta}ds + \frac{1}{\mu_A+\Delta}\int_\Delta^\infty \frac{1-F_B(s)}{\mu_B+\Delta}ds\right) \quad \text{(A12)}$$

which changes by using equation (A3) to

$$F(\Delta) = 1-\frac{1}{\mu_A+\mu_B+2\Delta}\left\{\int_\Delta^\infty [1-F_A(s)]ds + \int_\Delta^\infty [1-F_B(s)]ds\right\} \quad \text{(A13)}$$

From the standard formula for the mean, m, of a positive random variable

$$m = \int_0^\infty [1-F_i(s)]ds$$

and from the fact that $F_i(\Delta) = 0$, we have

$$\mu_i = \int_\Delta^\infty [1-F_i(s)]ds \quad \text{(A14)}$$

Thus, using (A14) in (A13) gives

$$F(\Delta) = \frac{2\Delta}{\mu_A+\mu_B+2\Delta} \quad \text{(A15)}$$

Now, replacing the denominator of (A15) by its expression derived from (A2), we find

$$F(\Delta) = 2\Delta/[f(\mu_A+\Delta)(\mu_B+\Delta)] \quad \text{(A16)}$$

and using relationship (A1) we obtain

$$F(\Delta) = 2f_Af_B\Delta/f \quad \text{(A17)}$$

Taking into account that,

$$F(\Delta) = N_d/N = d/f \quad \text{(A18)}$$

we obtain equation (1).

Acknowledgements

This work was supported by the French Ministry of Education and Research and by the French Ministry of Foreign Affairs through a Lavoisier program grant delivered by EGIDE, by joint cooperation project Barrande No. 972SL between France and the Czech Republic, by NATO linkage grant LST CLG 976786 and by Grant Agency of the Czech Republic (309/02/0168).

References

Colquhoun, D. and **Hawkes, A.G.** (1995) Single-channel Recording. Plenum Press, New York.

Cox, D.R. and **Lewis, P.A.W.** (1966) Statistical Analysis of Series of Events. Methuen, London.

Fujishiro, N., **Kijima, H.** and **Morita, H.** (1984) *Impulse frequency and action potential amplitude in the labellar chemosensory neurones of* Drosophila melanogaster. J. Insect Physiol., 30, 317–325.

Hodgson, E.S., **Lettvin, J.Y.** and **Roeder, K.D.** (1955) *Physiology of a primary chemoreceptor unit*. Science, 22, 417–418.

Jackson, M.B. (1985) *Stochastic behavior of a many-channel membrane system*. Biophys. J., 47, 129–137.

Marion-Poll, F. (1996) *Display and analysis of electrophysiological data under MS-Windows*. Entomol. Exp. Appl., 80, 113–115.

Marion-Poll, F. and **Van der Pers, J.** (1996) *Un-filtered recordings from insect taste sensilla*. Entomol. Exp. Appl., 80, 116–119.

Meunier, N., Ferveur, J.F. and **Marion-Poll, F.** (2000) *Sex-specific non-pheromonal taste receptors in Drosophila*. Curr. Biol., 10, 1583–1586.

Meunier, N., **Marion-Poll, F., Rospars, J.P.** and **Tanimura, T.** (2003) *Peripheral coding of bitter taste in Drosophila*. J. Neurobiol., 56, 139–152.

Perkel, D.H., **Gerstein, G.L.** and **Moore, G.P.** (1967) *Neuronal spike trains and stochastic point processes. II. Simultaneous spike trains.* Biophys. J., 7, 419–440.

Pouzat, C., **Mazor, O.** and **Laurent, G.** (2002) *Using noise signature to optimize spike-sorting and to assess neuronal classification quality.* J. Neurosci. Methods, 122, 43–57.

Siddiqi, O. and **Rodrigues, V.** (1980) *Genetic analysis of a complex chemoreceptor*. Basic Life Sci., 16, 347–359.

Singh, R.N. (1997) *Neurobiology of the gustatory systems of* Drosophila *and some terrestrial insects*. Microsc. Res. Tech., 39, 547–563.

Steinbrecht, R.A. (1996) *Structure and function of insect olfactory sensilla*. Ciba Found. Symp., 200, 158–174; discussion 174–157.

Stitt, J.P., **Gaumond, R.P., Frazier, J.L.** and **Hanson, F.E.** (1998) *Action potential classifiers: a functional comparison of template matching,*

principal components analysis and an artificial neural network. Chem Senses, 23, 531–539.

Stocker, R.F. (1994) *The organization of the chemosensory system in* Drosophila melanogaster*: a review*. Cell Tissue Res., 275, 3–26.

Takahashi, S., **Anzai, Y.** and **Sakurai, Y.** (2003) *A new approach to spike sorting for multi-neuronal activities recorded with a tetrode—how ICA can be practical*. Neurosci. Res., 46, 265–272.

Accepted August 4, 2003