CHARACTERIZATION OF AUTONOMIC RELEASE SITES USING THE TIME-FREQUENCY ANALYSIS OF JUNCTION POTENTIALS IN SMOOTH MUSCLE

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ABSTRACT

The determination of probability of neurotransmitter release from neuronal release sites and their electrical characterization is an issue of central interest in neurophysiology. For autonomic nerves, this can be done by analysing the inflexions in the rising phases of the evoked junction potentials (EJPs) recorded from smooth muscle. Since these inflexions contain time-varying frequency information, we have applied recent methods of time-frequency analysis based upon wavelet transforms on EJPs to characterize autonomic neuronal function. We find that these methods allow accurate and convenient characterization of individual release sites, and that their probability of release falls between 0.002 and 0.003. These results are compared with those reported earlier using analogue filtering techniques. The present method is advantageous as regards automation, accuracy and suppression of noise.

1. INTRODUCTION

Synaptic junction potentials are transient changes of transmembrane potential following signal transmission from nerves. The rising phases of these potentials contain physiological information about the biophysics of the nerve terminals, such as the probability of release of chemical neurotransmitter from neuronal release sites [1]. This parameter is a crucial determinant of neuronal function since changes in release probabilities can affect the efficiency of signal transmission at synapses. Specifically at the junctions made by peripheral autonomic nerves with smooth muscle, the rising phases of the excitatory junction potentials (EJPs) recorded intracellularly, vary considerably from one EJP to the next. They are inflected to different extents at different time instants within a narrow latency band from the instant of nerve stimulation. The problem is to characterize the inflexions in terms of frequency and time instant, in order to get the probability of transmitter release from a release site.

Fig. 1 illustrates 3 EJPs showing inflexions at two closely spaced latencies following stimulation (S). These transient inflexions in slope of the rising phases reflect the activity of individual presynaptic release sites [3], and thus help characterize the functioning of these sites. Such indirect electrical characterization is the only available method of assessing autonomic nerve function, since the release sites (varicosities) are too small (diameter 1 μ m) to be visualized or explored directly.

Electrical characterization or "fingerprinting" of autonomic varicosities has been done previously by analogue signal processing, employing a first time derivative of the rising phases of EJPs. This gives sharp peaks ("discrete events" or DE's) in the resulting dV/dt records, corresponding to the slope and amplitude of the inflections. A DE of a particular configuration (amplitude and time



Figure 1: Showing EJPs (9,10,12) recorded at the 9th, 10th and 12th stimulation respectively from the start of recording. S is the stimulus artifact and denotes the instant of stimulation. The arrows indicate inflexions in the rising phases (absent in 12). In this and subsequent figures EJPs commence from a resting potential of \approx -70 mV and the x-axis indicates time in ms.

course) is held to represent a particular release site [3]. In trains of several hundred EJPs, any particular DE is repeated (i.e., can be visually matched with another DE on the basis of amplitude and time course) on an average only once in 100-1000 events. This suggests that the probability of release from a particular site is very low (0.01-0.001). This is in stark contrast with the situation at the neuromuscular junction in skeletal muscle, where the probability of release is much higher, and closer to unity [3]. However estimation of the exact probability of release from autonomic varicosities is largely dependent on subjective factors, e.g. the visual accuracy of the match between DE's.

Since the evaluation of neuronal function in this way involves an accurate analysis of the time-frequency information present in the rising phase of the EJP, the newer methods of time-frequency analysis may offer a novel and more accurate approach to this problem. In this paper, we report the results of employing a combination of the wavelet transform, instantaneous frequency characterization and clustering techniques in the analysis of EJPs, in order to fingerprint varicosities. Our analyses are performed on EJPs recorded in our laboratory from a smooth muscle organ that is extensively explored in electrophysiological recordings, the vas deferens of the guinea-pig.

Our results show that probability of transmitter release assessed by this method agrees with that reported from discrete event analysis at its best resolution. Furthermore, the present technique overcomes the disadvantages of subjectivity and technical uncertainty of previously employed methods.

2. ELECTROPHYSIOLOGICAL RECORDING AND DATA COLLECTION

To record EJPs, male Hartley guinea-pigs were stunned and exsanguinated, and the vasa deferentia were dissected along with the innervating branch of the hypogastric nerve. The vas was pinned out on the silicone rubber base of a Perspex organ bath in which the recordings were carried out. The tissue was continuously superfused with physiological Krebs solution at 2/3 ml per min. Intracellular recordings of the EJPs were obtained as previously described [9] by the use of glass high impedance (20-60 M Ω) microelectrodes filled with 3 M KCl. EJPs were evoked by stimulating the hypogastric nerve using rectangular voltage pulses (amplitude 2-10 V, width 0.01-0.1 ms) delivered through bipolar Ag/AgCl electrodes at 0.7 Hz. Signals were fed to an intracellular electrometer (IE201, Warner Instruments, USA) through its high-impedance headstage $(10^{11}\Omega)$, and recorded on a DAT recorder (Biologic 1204, France, bandwidth d.c.-22 kHz). The data was sampled at 1 kHz using SCAN (Synaptic Current Analysis software, kindly provided by Dr. J. Dempster, University of Strathclyde, Glasgow) driving an A/D card (PCL 209, Dynalog Microsystems, India) installed on a PC- AT 80486 compatible.

3. DE-NOISING AND INFLEXION DETECTION

To detect and characterize the inflexions in the rising phases of EJPs, we carried out time-frequency analysis to accomplish the following : suppression of noise, detection of the position of the inflexion using a scheme proposed by Mallat [7], and characterization of inflexions in terms of instantaneous frequency.

3.1. De-noising

The experimentally recorded data is contaminated by considerable noise picked up by the high impedance microelectrodes. Noise can lead to the false detection of inflexions in the rising phase. Due to inadequate knowledge about the origin and statistical characteristics of noise, the popular de-noising algorithms failed to work. From the standpoint of suppressing this noise having a non-stationary character, a wavelet transform representation of the signal is advantageous as it permits selective smoothing at each level of the transform through controlled regularisation [5]. This method is a filtering scheme in the wavelet domain, which uses the entire wavelet transform. Selective smoothing is applied at each level of the wavelet transform and then the de-noised version of the signal is reconstructed. A typical signal would often have a transform in which the essential features of the signal are captured in a single coefficient that is markedly different in value from its neighbors. So a sudden change in the wavelet coefficient magnitude is likely to be a signal feature and not a creation of noise, and performing blurring across such a sudden change would distort the signal features. Hence gradient-based switches are used to prevent the blurring of important signal features. Also a cost function is minimized which smoothes the signal at each scale by reducing power in its gradient. This algorithm was implemented using the Daub-6 wavelet.

3.2. Detecting exact time instants of inflexions

The Wavelet Transform decomposes a signal into a family of functions $(\psi(at-b))_{(a,b)\in L^2(R)}$ which are the translates and dilates of a unique function $\psi(t)$ called the basic wavelet. Mathematically,

$$W_f(a,b) = a^{-\frac{1}{2}} \int f(t)\psi(\frac{t-b}{a})dt = a^{-\frac{1}{2}} \int f(t)\psi_{ab}(t)dt$$
(1)

Following Mallat [8] the basic wavelet $\psi(x)$ is taken to be a quadratic spline, also specified as the derivative of a smoothing function $\theta(x)$, a cubic spline.

$$\psi(x) = \frac{d\theta(x)}{dx} \tag{2}$$

The wavelet transform of f(x) at scale s and position a, computed with respect to wavelet $\psi(x)$, is defined by

$$W_f(a,s) = f * \psi(a,s) \tag{3}$$

It can be seen that [8]

$$W_f(a,s) = f * \left(s\frac{d\theta}{dx}\right)(x) = s\frac{d}{dx}(f*\theta)(x)$$
(4)

Thus the wavelet transform at scale 2^{j} , $W_{f}(d, 2^{j})$, is proportional to the derivative of the original signal smoothed at scale 2^{j} . The maxima of the absolute value of the wavelet transform correspond to the sharply varying points of $f * \theta_s(x)$, which are essentially the points of inflexion in the original signal [7]. The wavelet $\psi(x)$ is characterized by discrete filters H and G [7]. In the implementation the related discrete filters H_p and G_p , were derived by putting 2^p zeroes between each of the coefficients of the filters H and G, where p is the scale 2^{j} . Thus downsampling was not done and hence the data length was held constant, at each scale 2^{j} . The wavelet transform was obtained and corresponding modulus maxima (local maxima of the dvadic wavelet transform modulus) were also found. The inflexions in the signal are seen as maxima in all the scales in the transform. However for the inflexions of interest in EJPs the maxima peak at the 4^{th} scale (see Fig. 2), hence maxima at this scale have been used to obtain the exact time instant of occurrence of inflexions.

3.3. Instantaneous Frequency (f_i) Characterization

Since the wavelet transform only specifies the band in which the frequency content is present, to characterize the inflexions in terms of frequency, one needs to find out the corresponding f_i 's.

We follow [4] in defining f_i . For a signal of interest $s(\tau)$ at time $\tau = t$, let the analytic signal corresponding to s(t) be

$$z(t) = s(t) + j\mathcal{H}[s(t)] = a(t)e^{j\varphi t}$$
(5)

where \mathcal{H} is the discrete time Hilbert transform operator. Then the instantaneous frequency of $s(\tau)$ at time $t = \tau$, is defined as

$$f_i(t) = \frac{1}{2\pi} \frac{d\varphi(t)}{dt}$$
(6)

We interpret the above definition as

$$f_i(t) = \lim_{\delta t \to 0} \frac{1}{4\pi\delta t} (\arg[z(t+\delta t)] - \arg[z(t-\delta t)])$$
(7)

The concept of instantaneous frequency can be extended to the discrete time case by using the central finite difference of the phase



Figure 2: a: experimentally recorded EJP; b: de-noised version of EJP in a, showing retention of features of interest (inflexions in rising phase); c: dyadic wavelet transform at 4 scales; d: corresponding modulus maxima. Note the maxima at the fourth scale (*) corresponding to the 3 inflexions (*) in the EJP.

of the discrete time analytic signal. The discrete instantaneous frequency of the discrete time sequence s(m) at m = n is thus given by

$$f_i(n) = \frac{f_s}{4\pi} (\arg[z(n+1)] - \arg[z(n-1)]) \mod 2\pi$$
 (8)

where

$$z(n) = s(n) + j\mathcal{H}[s(n)]$$
(9)

 \mathcal{H} being the discrete time Hilbert transform of s(m).

4. RESULTS

Analysis has been performed on several sets of data, each containing between 300-1000 EJPs (1024 data points each) in individual files generated using SCAN. The EJPs in each file were recorded from a single smooth muscle cell and evoked in a continuous train of stimuli. The result of applying the wavelet transform to a sample EJP after de-noising is shown in Fig. 2. Modulus maxima at each scale 2^{j} of the wavelet transform were obtained (Fig. 2c) and the maxima were computed (Fig. 2d). The time instants of their occurrence were found by tracing back the instants to the first scale. f_i 's corresponding to these maxima were obtained for several series of EJPs. The time instants and peak amplitudes of the modulus maxima and the corresponding f_i 's were stored in a look up table to search for EJPs having identical or near identical



Figure 3: Plot giving the relative comparison of different inflexions found out from the signals recorded from a single cell. The dots denote an inflexion whose x-coordinate corresponds to the time instant of inflexion after stimulation(ms), y-coordinate gives the relative frequency characterization of each inflexion(i.e the amplitude of its maxima at fourth scale). The arrows point to the identical maxima.

frequency characterization at the same latency after stimulation. The automated search was carried out by the K-means clustering method.

Fig. 3 shows a plot of relative amplitude of maxima at 4th scale vs latency after stimulation for all 700 EJPs evoked in a single cell (J301) at 0.7 Hz. In this cell, the rising phases of the EJPs had one or more of three distinct latencies following stimulation, as evident from the clustering of points at \approx 30, 70 and 100 ms. Each symbol represents an inflexion in an EJP unique in terms of f_i and latency, and therefore, by previously established induction [3], fingerprints a particular release site. Overlap of two or more symbols (arrows) indicates that a particular release site has activated more than once. From these overlaps and the look-up table it is possible to identify those EJPs during which the same release site was active.

Examples are provided in Figs. 4 & 5 of pairs of EJPs recorded in the cell J301 for which an identical f_i was found at the same latency (* and # in Fig. 3). In Fig. 4 EJP 47 is inflected at the first two latencies, and EJP 346 at the first and third. Note that the rising phases at the *first* latency are precisely superimposed, as reflected in the indenticality of the modulus maxima. No other EJP recorded in this cell had an identical f_i (17 Hz) at this latency. Fig. 5 shows two other EJPs, the 324^{th} which has only the first two latencies and the 337^{th} , which has all three. Amongst these, there was only one identical f_i (21 Hz) occurring at the same instant.

The simple overall probability of activation of each of the sites characterized in Figs. 4 & 5 during the period of observation can therefore be estimated as 2/700. However, while the site characterized in Fig. 5 reactivates after just 13 stimuli, the site characterized in Fig. 4 reactivates after 299 stimuli. Such differences in conditional probability of release are similar to those reported earlier [3]. Similar analyses performed on EJPs in different cells yielded release probabilities of 0.002 - 0.003.



Figure 4: 346 and 47th EJP having identical inflexion. Modulus maxima shown are at fourth scale.

5. DISCUSSION

Techniques of time-frequency analysis have not been applied to the analysis of cellular synaptic potentials before, although they have been used to characterize surface bioelectric signals such as the EMG and ECG [6].

We have explored the use of time-frequency analysis techniques on intracellular junction potentials to assess the probability of neurotransmitter release from autonomic release sites. We find that this approach offers a convenient method to "fingerprint" release sites by characterizing inflexions in the rising phases of the EJPs in terms of time instant of occurrence (latency) and instantaneous frequency. Exact "matches" of latency and f_i in long series of EJPs can be reliably detected, thus aiding the functional assessment of release sites.

The reliability of the previously used analogue method to characterize release sites (i.e. DE analysis, see Introduction) was questionable because of uncertainties inherent in the technique. For example, DE's are obtained after analogue filtering whose characteristics are not accurately defined in the literature [3]. Furthermore, simple visual matching of DE's has the limitations of subjective assessment. Owing to these uncertainties, conclusions about transmitter release probabilities from varicosities had to be revised from an earlier estimate of as high as 0.5 [2] to as low as 0.001 in subsequent work [3]. The method explored here does not suffer from the uncertainties of analogue processing and subjective matching, and therefore offers a more rigorous way of assessing release probabilities. It may be noted that the probabilities inferred in the present work fall in the lower range of probabilities estimated from recent DE analysis.

Certain chemicals are thought to alter neuronal function by specifically altering transmitter release probability. The present method of analysis therefore offers a tool to assess the effects of such chemicals on various kinds of neurons. Work is currently in progress on applying this technique to the effects on EJPs of a substance (1-heptanol) that is suspected to possess such effects [9].



Figure 5: 324 and 337th EJP having identical inflexion. Modulus maxima shown are at fourth scale.

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