Quantal Unit Populations at the Drosophila Larval Neuromuscular Junction

K. WONG,1 S. KARUNANITHI,2 AND H. L. ATWOOD2
1Department of Statistics and 2Department of Physiology, University of Toronto, Toronto, Ontario M5S 1A8, Canada

Wong, K., S. Karunanithi, and H. L. Atwood. Quantal unit populations at the Drosophila larval neuromuscular junction. J. Neurophysiol. 82: 1497–1511, 1999. Focal extracellular recording at visualized boutons of the Drosophila larval neuromuscular junction was used to determine frequency and time course of the spontaneously occurring quantal events. When simultaneous intracellular recordings from the innervated muscle cell were made, more than one class of quantal event occurred at some of the individual boutons. “True” signals (arising at the bouton within the focal macropatch electrode) were often contaminated by additional signals generated outside the lumen of the focal electrode. Inclusion of these contaminating signals gave spuriously low values for relative amplitude, and spuriously high values for spontaneous quantal emission, for the synapses within the focal electrode. The contaminating signals, which appeared to be conducted along the subsynaptic reticulum surrounding the nerve terminals, generally were characterized by relatively small extracellular signals associated with normal intracellular events in the muscle fiber. From plots of simultaneous extracellular and intracellular recordings, the individual data points were classified according to the angles they subtended with the x axis (extracellular signal axis). Statistical procedures were developed to separate the true signals and contaminants with a high level of confidence. Populations of quantal events were found to be well described by Gaussian mixtures of two or three components, one of which could be characterized as the true signal population. Separation of signals from contaminants provides a basis for improving the estimates of quantal size and spontaneous frequency for the synapses sampled by the focal extracellular electrode.

INTRODUCTION

The Drosophila larval neuromuscular junction, originally brought forward for physiological investigation by Jan and Jan (1976), has proved in the past few years to be increasingly significant for studies of the basic mechanisms of synaptic transmission. The availability of mutants affecting specific components of the neuromuscular synapse (Breadie et al. 1994; Dudai et al. 1976; Jan and Jan 1978; Ranjan et al. 1998), and the facility with which transgenic flies with altered expression of genes important for synaptic structure and function can be produced and used (Davis et al. 1996) have encouraged physiological analysis of the readily accessible larval neuromuscular junction. The growing significance of the preparation for synaptic studies justifies efforts to improve the procedures used to obtain data from it (Stewart et al. 1994). The present study is based on the observation that quantal events recorded at individual varicosities of the neuromuscular junction with focal extracellular electrodes may consist of more than one population. Procedures for dealing with this situation are described. These may be applied to other neuronal or neuromuscular structures in which similar circumstances arise.

Presynaptic performance is often assessed by measuring quantal release from synapse-bearing nerve terminals. For this, an extracellular “macropatch” electrode (Dudel 1981) is placed over a nerve terminal to record spontaneous and nerve-evoked signals (extracellular voltages or currents) caused by release of transmitter and its subsequent opening of ligand-gated channels in the postsynaptic membrane. An estimate of the relative amplitude and time course of the quantal event can be made by analyzing the spontaneously occurring signals (Fatt and Katz 1952), whereas the number of quantal events appearing in response to nerve impulses is often calculated by taking the ratio of the evoked response to the spontaneously occurring quantal event (Cooper et al. 1995; Katz 1966). The macropatch recording method was introduced for the Drosophila preparation by Mallart et al. (1991) and is now being widely used with variation in technique (Davis and Goodman 1998; Davis et al. 1996, 1998; Heckmann et al. 1997). It has also been used extensively for other preparations, including crayfish and frog neuromuscular junctions (Cooper et al. 1996; Parnas et al. 1982; Van der Kloot and Naves 1996a), smooth muscle neuromuscular junctions (Bennett et al. 1993, 1996; Cunnane and Manchanda 1989), and most recently for neurons of the mammalian CNS (e.g., Forti et al. 1997).

While examining spontaneously occurring quantal events in macropatch recordings, we observed that errors in estimating their amplitudes often occur due to contaminating signals arising from sites on the terminal close to but not inside the lumen of the macropatch electrode. The problem is inherent in the relatively low seal resistance of the macropatch electrode. The extraneous signals appear to be conducted along the subsynaptic reticulum (SR) surrounding the nerve terminal (Atwood et al. 1993; Osborne 1967), which can never be effectively sealed at the edge of the recording electrode without damaging the nerve terminal. A similar observation was reported for the frog neuromuscular junction by Van der Kloot and Naves (1996a), who found that extracellular signals could be recorded ≤0.8 mm away from the extracellular electrode. In this report, we provide a demonstration of this effect and a statistical method for distinguishing extralumenal contaminating signals from those arising at the recording site (“true” signals). We show that separation of the contaminating signals provides a more accurate estimate of the amplitude and frequency of the spontaneously occurring quantal events at a defined nerve terminal.
METHODS

Physiological procedures

Canton-S *Drosophila melanogaster* wandering third instar larvae reared on cornmeal medium (at 25°C, 60–70% relative humidity) were dissected and prepared for electrophysiological recordings from muscle 6 of segment 3 as previously described (Stewart et al. 1994). Experiments were conducted at room temperature in hemolymph-like physiological solution (HL3) of the following ionic composition (in mM): 70.0 Na⁺, 5.0 K⁺, 1.0 Ca²⁺, 20.0 Mg²⁺, 10.0 NaHCO₃, 5.0 trehalose, 115.0 sucrose, and 5.0 BES (Stewart et al. 1994).

The organ bath containing the preparation was secured onto the stage of an upright microscope (Nikon, Optiphot-2) equipped with a Nikon ×40 water-immersion lens (N. A. 0.55) and Nomarski optics. Live images of the nerve terminals were viewed on a computer (Apple Power Macintosh 7500/100 using the built-in frame grabber) through a low-light-intensity TV camera (Panasonic, WV-BP310) mounted onto the microscope, enabling accurate placement of the focal macropatch electrode over the chosen bouton. Narishige hydraulic manipulators mounted on the microscope stage were used to maneuver the recording and stimulating electrodes underneath the water immersion lens.

Electrical recordings

Simultaneous intracellular and extracellular recordings of spontaneously occurring quantal events were made (Fig. 1, A and B). Intracellular electrodes (40–60 MΩ), filled with a 2:1 mixture of 3 M potassium acetate to 3 M potassium chloride, were used to record the spontaneous miniature excitatory junctional potential (mEJP). Impalments displaying a resting membrane potential more negative than −70 mV were chosen for analysis. The focal macropatch electrode (tip diameter, 3–5 μm) used to record spontaneous excitatory junction currents extracellularly at selected varicosities was manufactured as previously described (Stewart et al. 1994) and filled with HL3 solution. The tip diameters of these electrodes were made to enclose the selected bouton, minimizing direct pressure on it. After impalement of the muscle fiber by an intracellular electrode, the focal macropatch electrode was placed over the chosen bouton (Kurdyak et al. 1994) (Fig. 1A).

Signals from the focal macropatch and intracellular recording electrodes were amplified using the Axoclamp-2A amplifier (Axon Instruments, Foster City, CA) under bridge mode. The extracellular signal (referred to in this paper as “Ext,” external or extracellular signal, as in Van der Kloot and Naves 1996) was processed further by feeding it into a signal conditioner (Intronix) where it was filtered (low-pass filter: 5.0 kHz; high-pass filter: 0.5 Hz) and further amplified (×100) before being fed into the first input channel of the data-acquisition system. The intracellular mEJP signal was fed straight into the second input channel of the data-acquisition system. The MacLab/4 s data-acquisition system (AD Instruments, Sydney, Australia) was used to record the two electrical signals with the same computer used simultaneously for visualization. Data points were sampled at 40 kHz. Sampling was triggered using the software’s voltage-threshold discriminator set to detect extracellular signals exceeding the amplitude of a specific voltage.

In experiments with two focal macropatch electrodes, the signal from each electrode was amplified by a separate Axoclamp-2A amplifier and handled as described in the preceding text. The MacLab data files were converted to Igor Pro files for analysis with subroutines written for the Igor Pro 3 software analysis package (Wavemetrics). The amplitudes of the signals were measured using methods previously described (Bennett et al. 1996; Feeney et al. 1998; Karunanithi et al. 1995; Sayer et al. 1989). We used t-tests to assess significance (P < 0.05) between groups. When comparisons are made across nonoverlapping groups (for example, between signals and contaminants), the tests are valid assuming our fitted model is correct. In all other cases, t-test results are used as references.

Statistical procedures

At many recording sites, two or more classes of signal were detected in plots of external signals (Ext) against intracellular signals (mEJP). We wished to estimate the mean Ext generated at a primary recording site. Although Exts of relatively large amplitude and/or brief rise time are more likely to have originated at the recording site, point location is not always known with certainty. Thus the problem becomes one of selecting a portion of the data to be classified as “true” signals (those originating at the recording site). Statistical procedures for this purpose are described here.

The analysis proceeds as follows. For a given data set, the bivariate
data were reduced to one dimension by considering only the angles (α) subtended with the extracellular signal axis by the data points (Fig. 1C). The distribution of these angles is assumed to be a mixture of a finite number (m), of Gaussian components. For different values of m, this model is fitted using the maximum likelihood approach. The final value for m is determined using Monte Carlo Likelihood Ratio tests. Finally, each point is classified as a signal or contaminant using Bayes Decision Rule. This above procedure may be applied to the bivariate data although additional constraints on the data may be necessary to adequately fit the model (see Fitting the model).

Data reduction

The data consist of independent observations (Ext, mEJP), i = 1, . . . , N, where N is the number of observations. If the muscle behaves as a passive electrical network, as is known to be the case with small voltage excursions (Jan and Jan 1976), applied currents will evoke a voltage change that increases linearly with current amplitude. Thus the individual signal distributions may be skewed, but true angles should be similar, with some variation due to current duration and other factors. The time course and relative amplitude of the currents at the recording site are represented by the external voltage. However, relationship between Ext and mEJP signals will be different for events generated at different locations with respect to the focal macropatch recording electrode. Thus populations of data points belonging to the true signal category can be distinguished from “contaminating” populations through analysis of their angles, the values of which depend on attenuation of Ext from different sources.

In practice, most data sets exhibit symmetric signal angular distributions that are well fitted by a Gaussian distribution. This justifies the use of a single normal distribution to model the true signal portion of the data. Signals originating from other sources (contaminants) are recorded in attenuated form, but Gaussian mixtures are able to describe the distributions for the angles of these signals.

Normal mixture model

The data consist of angles α, i = 1, . . . , N that are assumed to be independent realizations drawn from a probability density function (finite mixture of normal distributions)

\[ f(\alpha; \theta) = \sum_{j=1}^{m} \pi_j g_j(\alpha) \]  

Here, \( g_j(\alpha) = \frac{1}{\sqrt{2\pi}\sigma_j} \exp\left(-\frac{1}{2\sigma_j^2}(\alpha - \mu_j)^2\right) \)

where \( \theta = (\pi_1, \ldots, \pi_{m-1}, \mu_1, \ldots, \mu_m, \sigma_1^2, \ldots, \sigma_m^2) \), and m, the number of components; \( \pi_i \) is the probability of belonging to the ith component; and \( \mu_i \) and \( \sigma_i^2 \) are the mean and variance of the ith component.

We interpret the first component as the true signal group; points from other sources (contaminants) will usually have smaller currents for a given voltage response, due to greater attenuation of the current (and hence Ext) at the recording site, and therefore larger angles (Fig. 1C, \( \alpha_5 \)).

Fitting the model

We have assumed an m-component Gaussian mixture model defined in Eqs. 1 and 2 that depends on the unknown vector \( \theta \). To estimate \( \theta \), we employ the log-likelihood approach which finds a value \( \hat{\theta} \), which maximizes the log-likelihood function

\[ L(\theta) = \sum_{i=1}^{N} \log f(\alpha_i; \theta) \]  

Because of the large number of unknowns in \( \theta \), L can take on arbitrarily large values that sometimes correspond to models with some single-point components that are unlikely to occur in practice. Practical solutions usually occur at a local maximum

\[ \frac{dL}{d\theta} = 0 \]

The EM algorithm is an iterative procedure that monotonically approaches and converges to a local log-likelihood maximum. Given an initial value of \( \hat{\theta} \), the algorithm updates a new estimate of \( \theta \) at each iteration until the increase in log-likelihood is less than a particular user-specified tolerance level. If the components underlying the data are well separated, the solution \( \hat{\theta} \) is usually insensitive to choice of starting values. Otherwise, several local maxima may exist and care is needed to initialize values that lead to reasonable solutions. A common strategy is to take the largest of the local maxima over a wide range of initial values. [For an extended discussion of the problem of starting values and the general EM algorithm, see McLachlan and Krishnan (1997); and for application in a physiological context, see Kullman (1989).] For our purpose, the algorithm gives an estimate \( \hat{\theta} \) of \( \theta \) that when substituted into Eqs. 1 and 2, gives the fitted model. Assuming a bivariate model involves fitting an additional four parameters for each component and several more local maxima may exist. Often it is then necessary to constrain the data to alleviate the above over-fitting problem. Some authors constrain the mixture component variances to be equal; this is not consistent with the observed data. Dasgupta and Raferty (1998) examine the situation in which the bivariate components are Gaussian but “linear” (long and narrow ellipsoids); this would work well for clearly bimodal data sets (see Fig. 3). We have chosen to reduce the data in a natural way by modeling the distribution of the univariate angles.

Estimating the number of components (m)

For a given number of components m, the procedure outlined in Fitting the model gives an estimate \( \hat{\theta}_m \), and the corresponding fitted log-likelihood \( L(\hat{\theta}_m) \). The larger the latter value, the more likely the data arose from a mixture of m components. However, because of the increased number of parameters, the likelihood will increase as m increases. Whether this increase is statistically significant and not due to chance variation may be determined by applying a Monte Carlo log-likelihood ratio test. This test is applied sequentially pairwise (test \( m = 1 \) vs. \( m = 2 \), then test \( m = 2 \) vs. \( m = 3 \), and so on). The test proceeds as follows: Consider a test of \( m = m_1 \) versus \( m = m_2 \), where \( m_1 < m_2 \).

Let \( \lambda = -2[L(\hat{\theta}_{m_1}) - L(\hat{\theta}_{m_2})] \), where \( L(\hat{\theta}_{m}) \) denotes the fitted log likelihood under the m component model. Let \( \lambda_{obs} \) denote the observed \( \lambda \) corresponding to the observed data. Then the \( P \) value is the probability that \( \lambda \) exceeds \( \lambda_{obs} \) when \( m = m_1 \). If this value is very small, then it is unlikely that such a large difference \( \lambda_{obs} \) would be observed if the data came from a m1 component model. We then would conclude that \( m = m_2 \).

Under certain technical conditions, and for a large enough sample size \( N \), \( \lambda \) has an approximate \( \chi^2 \) (with \( I \) df) distribution when \( m = m_1 \), and the \( P \) value may be evaluated directly. However, not all these conditions are satisfied in the mixture model setting, so we estimate the \( P \) value first by simulating the distribution of \( \lambda \) when \( m = m_1 \) through Monte Carlo sampling as follows. We randomly generate B samples (each consisting of \( N \) angles) from the fitted m1 component model. For each sample, we fit the log-likelihood for each model and compute \( \lambda \). Our \( P \) value is estimated by the proportion of \( \lambda \)s that exceed \( \lambda_{obs} \). We conclude that \( m = m_2 \) if this value is < 0.1. We take...
Let $P_j(a)$ denote the (posterior) probability that an observation with angle $a$ belongs to the $j$th group. In particular, define $P_S = P_1$, the (posterior) probability of belonging to the signal group. Usually, low angles give rise to higher signal probabilities. Using Bayes rule for probabilities (Moore and McCabe 1997)

$$P_j(a) = \frac{\pi_{j}g_j(a)}{\sum_{i=1}^{K} \pi_{i}g_i(a)}$$

The estimate $\hat{\theta}$ corresponding to the fitted model determined in Estimating the number of components (m) consists of individual parameter estimates that can be substituted into Eq. 4 to give an estimate of $P_j(a)$.

The usual and most natural way of classifying an observation is to choose the component it is most likely to come from, that is, the group with the largest fitted (posterior) probability $[\text{largest } P_j(a)]$. Because we are only concerned with distinguishing two groups, signals and contaminants, we may classify an angle $a$ as a signal precisely when $P_S(a) \geq 0.5$. This decision rule is not only intuitive but also minimizes the probability of misclassifying an observation provided the cost of misclassifying a contaminant as a signal. This is a special case of Bayes Decision Rule (Ripley 1996), which classifies an angle $a$ as a signal when $P_j(a) \geq p$ where $p$ is between 0 and 1 and represents the cost of misclassifying a contaminant as a signal relative to the cost of misclassifying a signal as a contaminant (the 2 costs add up to 1).

One also can interpret this rule as only classifying angles as signals if we are $\geq 100p\%$ sure. It is not immediately clear how to choose $p$. Because the goal is to estimate the mean Ext, it may seem desirable to choose $p$ large enough to increase the certainty that all classified signals are actually signals. However, such a rule will tend to misclassify some signals as contaminants, which leads to overestimating the mean Ext. Lower values of $p$ will include more contaminants in the signal group that will underestimate the mean Ext due to low contaminating Ext values. In the absence of information concerning the relative costs of misclassification, we choose $p = 0.5$. However, one may check the impact of such a rule by estimating the mean Ext for several values of $p$ (see Fig. 3).

**RESULTS**

**Drosophila nerve terminals**

The gross structural and ultrastructural features of larval Drosophila nerve terminals have been described by Jan and Jan (1976), Atwood et al. (1993), Jia et al. (1993), Stewart et al. (1996), and Meinertzhagen et al. (1998). The features considered to be significant for extracellular recording of synaptic events with a macropatch electrode are illustrated in Fig. 2. The nerve terminals of the major glutamatergic motor axons gen-

![Fig. 2. Structure of nerve terminals and associated subsynaptic reticulum. A: electron micrograph of a Drosophila Type Ib nerve terminal (NT) on muscle 6, illustrating the underlying subsynaptic reticulum (SR) and the location of synapses (SY) formed between the presynaptic nerve terminals and the granular sarcoplasm of the subsynaptic reticulum. One synapse shows a presynaptic dense body at an active zone (small arrowhead). Typical location of the inner edge of the recording macro-patch electrode is indicated by large arrowheads on either side of the nerve terminal. Scale: 0.5 μm. B: oblique section of the surface of muscle 6, showing Type Is NT and extension of the SR longitudinally away from the nerve terminal. Macropatch recording electrode positioned over the varicosity (inner edges indicated by large arrowheads) would not seal off the longitudinally running subsynaptic reticulum. Scale: 0.5 μm.](image-url)
erally run longitudinally on the surface of the innervated muscle fibers. They comprise a series of varicosities on which most of the individual small synapses occur, and they are embedded in an extensive network of fine postsynaptic processes, referred to as the subsynaptic reticulum, or SR (Osborne 1967). As shown in Fig. 2, there is considerable extracellular space in the SR, which is continuous along the track of the nerve terminal. Extracellular currents generated at individual synapses must flow through the subsynaptic reticulum. A macropatch electrode placed over a varicosity may possibly form a good seal along the lateral edges of the varicosity (Fig. 2A), but there is very little likelihood of a good seal being made over the subsynaptic reticulum where it extends longitudinally from the recorded varicosity (Fig. 2B).

The prominent longitudinal muscles (muscles 6 and 7) are conjointly innervated by two motor axons, one of which (motor neuron RP3) has relatively big varicosities (Type Ib) and the other (a common excitor, motor neuron 6/7b), relatively small varicosities (Type Is). Physiological recordings are usually made from Type Ib varicosities, which are easier to see with Nomarski optics, or (in some previous investigations) from both types at once. In the present study, we selected well-defined individual Type Ib varicosities for recording, to avoid complications arising from simultaneous recording from more than one axon terminal.

Localized synaptic signals

The observations of the present study comprised spontaneously occurring synaptic events, most of which represent single quantal units of transmitter release. We recorded simultaneously with intracellular and extracellular electrodes to observe the transmembrane voltage responses generated by each quantal current (Fig. 1, A and B). Because of the high-input resistance of the postsynaptic muscle fiber and its short length (Jan and Jan 1976), it is practically isopotential, and events originating anywhere on its surface can be recorded with high fidelity at a single intracellular recording site.

As illustrated in Fig. 1, A and B, the intracellular voltage response and the extracellular local signal, Ext (measured as a voltage change at the tip of the macropatch electrode) showed a range of amplitudes. There is also some variation in their total duration. In the example illustrated, the amplitudes of Ext and mEJP covary, as would be expected if the muscle cell behaves as a passive electrical network (Jan and Jan 1976; McLachlan and Martin 1981).

We tested the effects of electrode tip opening on Ext. Electrodes were selected to record from only a single bouton. Results using electrodes with tip diameters that enclosed only the bouton, when compared with those from electrodes that also included all of the SR around that bouton, did not show significant differences in amplitude of the recorded currents and their coefficients of variation, CV (bouton only: Ext = 0.29 mV, CV = 0.58; SR + bouton: Ext = 0.31 mV, CV = 0.52, t-test, P < 0.05, n = 7). Thus minor changes in electrode size relative to bouton size did not appear to have much effect on the recorded Ext.

Mixed populations of signals

Plots of the intracellular and extracellular signals recorded simultaneously from several different recording sites showed that whereas some sites produced a quasilinear relationship between the two signals, others showed clear evidence for two or more discrete populations. Figure 3 illustrates a case in which there appeared to be two classes of signal (shown as I and II). One class (I) was well accounted for by a linear regression line, but the second class (II) diverged strikingly, with little or no dependence of intracellular voltage amplitude on Ext amplitude. Variation about the regression line in the first population (I) is most likely due mainly to variation in time course of the current events. Thus two discernable classes of quantal events were reported by the external electrode at this site.

Origin of contaminating signals

Results like those of Fig. 3 (2 populations with different relationships between Ext and mEJP) suggested that the extra-
cellular signals can be generated at more than one location. It seemed likely that some signals originated at locations along the nerve terminal outside the lumen of the macropatch electrode and could be recorded in attenuated form. The SR (Fig. 2) provides a pathway for current flow between adjacent sites, as illustrated diagrammatically in Fig. 4A. Equivalent electrical circuits for this situation have been presented by other authors, e.g., Bennett et al. (1997). Nearby synapses causing current to flow along the SR could generate a contaminating signal at the external electrode.

A test of this idea was effected by placing two separate macropatch electrodes close together along a nerve terminal for simultaneous recording (Fig. 4, B and C). It was found that signals originating outside the lumen of a macropatch electrode could be recorded within it. On average, they were smaller and often had slower rise times than the signals originating within the lumen of the macropatch electrode (Fig. 4D).

When the second electrode was placed at an equivalent lateral distance from the first, or closer, on the muscle fiber’s surface, no equivalent events were recorded. Thus it is likely that the SR allows contaminating signals to appear at the recording macropatch electrode, but there is little lateral spread of current and little flow through the surface of the muscle fiber.

**Statistical separation of signals from different sources**

The plot of Fig. 3 shows a clear bimodal structure with the exception of a few observations that fall between the two modes. A histogram of the angles (Fig. 5A) describes the angle density. These data were analyzed by developing theoretical curves for two and three components, as described in METHODS, assuming a Gaussian mixture. Both two- and three-component fits (Fig. 5B) clearly show the contaminant peak with a mean angle of 85° and a small standard deviation. However, the three-component model displays a more prominent true signal peak, with mean angle of 48°. This model also provides a much smaller standard deviation for the true signal, corresponding to tight cluster of points about a mean angle. The two-component model fits a mean true signal angle of 54.5° with a large standard deviation, arising from intermediate observations. Thus restricting the model to two Gaussian components results in an awkward fit, as the algorithm tries to overcome the skewness (either in the right signal tail or in the left contaminant tail) present in the data. The three-component model avoids distortion arising from skewness by further differentiating the noncontaminant data into a smaller signal group and a second midregion contaminant class. Because of the Gaussian assumption, the latter class has a mean angle of 60° and a
large standard deviation with tails spread over a large portion of the data range.

Monte Carlo likelihood ratio tests choose the three-component model over the two-component model ($P$ value = 0.04), whereas a four-component model does not significantly improve the fit ($P$ value > 0.15). Based on $p = 0.5$ (Fig. 3), the two-component ($m = 2$; Fig. 6, A–D) model classified most of the intermediate observations as part of the signal group, whereas the three-component model ($m = 3$; Fig. 6, E–H) did the opposite.

**Detailed analysis of signals exhibiting a bimodal distribution**

**Fitting a bimodal estimate to the distribution.** The plot of $P_s$ (the estimated posterior probability that a point belongs to the signal category) as a function of the angle $\alpha$ is shown in Fig. 6A and its theoretical prediction in Fig. 6B. Applied to the data (Fig. 3), the probability values show a relatively clear separation of points that belong to either signals or contaminants with a few intermediate points lying in between. The points lying in the intermediate region can be classified into either of the two categories depending on the cutoff chosen for $P_s$. When a cutoff of $p = 0.5$ is assigned, the resulting classification of data points produces the distributions for signals (●) and contaminants (○) shown in Fig. 7A. Values derived from the analysis for amplitude and frequency of quantal events are summarized in Table 1A. The signal group lies at a mean angle of $54.2 \pm 1.72^\circ$ ($n = 56$), whereas the contaminant group lies at an angle of $84 \pm 0.20^\circ$ ($n = 70$). Thus segregating the population shows that the signals lie at shallower angles than the contaminants. Ext with amplitudes $>0.4$ mV (Fig. 6C) clearly fall into the signal category ($P_s = 1$). Below that value some points fall into the contaminant ($P_s = 0$) group and a few points are intermediate. The amplitude-frequency distributions, the means, and the coefficients of variation (CVs) of Ext belonging to the signal (Fig. 7B) and contaminant (Fig. 7C) groups are shown for an assigned cutoff of $p = 0.5$. The mean amplitude of the signal group was 350% larger than that of the contaminant group and 71% larger than that of the entire data set, or unseparated population ($0.21 \pm 0.02$ mV; $n = 126$). The differences were statistically significant.

The amplitude-frequency distribution, mean, and CV of the mEJPs belonging to the signal category are shown in Fig. 7D.
The mean of the signal group was significantly smaller than those of the contaminant group and the unseparated population (Table 1A).

The CVs of the Exts belonging to the signal and contaminant groups (Fig. 7, B and C) were considerably smaller than that of the unseparated population (Fig. 7). The CV of the mEJPs of the signal group (Fig. 7D) was considerably larger than that of the contaminant group and the unseparated population. After separation there is closer agreement (28% difference) between the CVs of the Exts and mEJPs in the signal group than before separation (43% difference). This suggests that as the true signal group becomes better defined, the relationship between Ext and mEJP becomes more linear.

Exts with rise times ($t_r$) $>$ 2.6 ms fall into the contaminant category (Fig. 6D). When a cutoff of $p = 0.5$ is assigned, the mean $t_r$ of the resulting signal group (1.15 ± 0.06 ms) was found to be 38% faster than for the contaminant group (1.86 ± 0.15 ms) and 25% faster than for the unseparated population (1.54 ± 0.09 ms).

The spontaneous frequency of focally recorded spontaneous quantal events before separation was 0.06 s$^{-1}$. After separation, the spontaneous frequency of the signals was lowered by a factor of 3–0.02 s$^{-1}$ (Table 1A).

In summary, a bimodal fit to the distribution shows that Exts belonging to the signal group are larger and faster than those belonging to the contaminant group. A possible linear relationship between the amplitude of the Ext and the mEJP is also implicated. The frequency of occurrence of Exts from the site of recording is far lower than would be recorded if the signals and contaminants were not separated.

**FITTING A TRIMODAL ESTIMATE TO THE DISTRIBUTION.** This distribution (Fig. 3) also passed the test for fitting a trimodal Gaussian-mixture model to the angular density distribution (Fig.
This estimate further separated the signal group deduced using a bimodal Gaussian-mixture model into a new smaller signal group ($S$, $\blacklozenge$) and a second contaminant group ($C_1$, $\square$; Figs. 6E and 7E). The pure contaminant group ($C_2$, $\circledast$) was still present. The plot of the probability of points belonging to one of the three groups ($P_s$, $P_{C1}$, and $P_{C2}$) as a function of $\alpha$ is shown in Fig. 6E and the theoretical distribution of points belonging to $S$ in Fig. 6F. $S$ was found to lie at a mean angle of $48.9 \pm 0.7^\circ$, $C_1$ at $62.7 \pm 3.3^\circ$, and $C_2$ at $84.9 \pm 0.2^\circ$. The probability of Ext amplitude being in one of the three groups is shown in Fig. 6G. Amplitude-frequency histograms, mean, and CVs are illustrated for the signal group (Fig. 7F) and for both contaminant groups (Fig. 7G: $C_1$, $\square$; $C_2$, $\square$). The mean amplitude of $S$ was 90% larger than $C_1$, 450% larger than $C_2$, and 117% larger than the unseparated population. These differences were all statistically significant. When the mean amplitude of $S$ was compared with the mean of the signal group deduced using a bimodal fit, there was no significant difference between the two. The mean of $C_2$ was similar to the mean of the contaminant group deduced using a bimodal fit.

The mEJP amplitude-frequency histogram, mean, and CV are presented in Fig. 7H. The mean for $S$ was not significantly different to that of $C_1$ but was significantly smaller than that of $C_2$ (Table 1). There were no significant differences between the mean of $S$ and that of the signal group obtained from the bimodal fit nor between the mean of $C_2$ and that of the contaminant group ascertained from the bimodal fit. Thus in this example, mean values obtained with the trinodal fit are not significantly different from those obtained with a bimodal fit. The CVs of the Exts (0.49) and mEJPs (0.48) for $S$ are
almost identical from the trimodal fit, whereas there is a 30% difference between the two using the bimodal fit. This implies that the trimodal fit describes a more linear relationship between the mEJP and Ext amplitudes than does the bimodal fit. The mean \( t_r \) for S (1.24 ± 0.09 ms) was not significantly different from that for C\(_1\) (1.08 ± 0.08 ms) but significantly faster than that for C\(_2\) (1.87 ± 0.16 ms). The \( t_r \) values from bi- and trimodal fits were not significantly different in S or C\(_2\) groups.

In summary, the trimodal fit does not improve the estimates of mean amplitude, but it does reveal the possible existence of an additional source of contaminating signals (C\(_1\)), and it describes a more linear relationship between the amplitude of the mEJP and Ext. Because there are fewer members in the new signal group, the frequency of occurrence of spontaneous events is even lower (0.01 s\(^{-1}\)) than that estimated using a bimodal fit (Table 1).

**Detailed analysis of signals exhibiting a trimodal distribution**

**FITTING A BIMODAL ESTIMATE TO THE DISTRIBUTION.** At some of the recording sites, the data plots exhibited a trimodal rather than a bimodal distribution (Fig. 8A). The angular density plot for the example in Fig. 8A showed two large peaks, with a smaller peak between them (Fig. 8B). To segregate the points into signals and contaminants, we selected a cutoff for \( P_e \) of 0.5. The points belonging to the signal (●) and contaminant (○) groups then could be distinguished in the data (Fig. 8A). The data points for the signal group were found to lie at an angle of 44.4 ± 2.0° and those for the contaminants at 80.4 ± 0.5°.

The mean Ext amplitude of the signals is significantly larger than for contaminants (Fig. 8, C and D) and for the unseparated population (Table 1B). The mean mEJP amplitudes of the signals and contaminants were found to be significantly different (Table 1B). The CVs of the Ext (0.54) and mEJPs (0.53) in the signal category are almost identical, suggesting a linear relationship.

The frequency of occurrence of Exts belonging to the signal group was lower than for the unseparated population (Table 1B).

**FITTING A TRIMODAL ESTIMATE TO THE DISTRIBUTION.** The observed angular density distribution (Fig. 8B) was also well fitted with a trimodal Gaussian mixture model. The signal (S) and pure contaminant group (C\(_1\)) along with a third group (C\(_2\)) were distinguished (Fig. 8E). Points belonging to S reside at a mean angle of 34.6 ± 1.1°, for C\(_1\) at 59.5 ± 1.8°, and for C\(_2\) at 80.4 ± 0.5°.

The mean amplitude of S (Fig. 8F) is significantly larger than those of C\(_1\), C\(_2\) (Fig. 8G), and the unseparated population (Table 1B). However, it was not significantly different from the mean of the signal group estimated with bimodal fit (Fig. 8C). The mean value of C\(_2\) is close to the mean of the contaminants estimated with bimodal fit (Table 1B).

**Analysis of signals with poorly defined distributions**

Figure 9A illustrates a poorly defined data distribution. When the angular density distribution is plotted (Fig. 9B), no

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**TABLE 1: Effect on amplitude and frequency estimates of separating quantal populations**

<table>
<thead>
<tr>
<th>Total Sample</th>
<th>Signals (S)</th>
<th>Contaminants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ext</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean amplitude, mV</td>
<td>0.21 ± 0.02</td>
<td>0.35 ± 0.03</td>
</tr>
<tr>
<td>Frequency, s(^{-1})</td>
<td>0.05</td>
<td>0.07</td>
</tr>
<tr>
<td>mEJP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean amplitude, mV</td>
<td>0.79 ± 0.04</td>
<td>0.52 ± 0.03</td>
</tr>
</tbody>
</table>

Table 1 shows the effect on amplitude and frequency estimates of separating quantal populations. The mean values of the signal and contaminants are significantly different. The CVs of the Ext (0.54) and mEJP (0.53) in the signal category are almost identical, suggesting a linear relationship.

Total sample was unseparated. Values are means ± SD. Ext, extracellularly recorded signal; mEJP, miniature excitatory junction potential. *Significant differences in values (\( t \)-test: \( P < 0.05 \)) before and after separation.
clear peaks could be discerned, unlike the previous two examples. In such situations, it is initially wise to see whether one can simply separate the population into signals and contaminants by applying a bimodal fit. Points lying at angles <78° are allotted to the signal category. At higher angles, a suitable cutoff has to be selected to separate the remaining points into signals and contaminants. When a cutoff of $P_s = 0.5$ is chosen, the resulting separation of the points into signals (●) and contaminants (○) is shown in Fig. 9A. The signal group resides at a mean angle of 74.0 ± 1.21° and the contaminant group at a mean angle of 84.9 ± 0.29°. It is interesting to note that the angles at which the pure contaminants reside fall within the narrow window of 80–85° in all three examples.

The mean amplitude of the signals (Fig. 9C) was significantly larger than those of the contaminants (Fig. 9D) and the unseparated population (0.17 ± 0.02 mV; Table 1C). The mEJP amplitude of the signals (Fig. 9E) was significantly smaller than that of the contaminants but not significantly different from that of the unseparated population (0.94 ± 0.05; Table 1C).

Thus in this poorly defined distribution, the population could be separated into signals and contaminants. After separation, the mean Ext amplitude of the signal group was 33.7% larger than for the unseparated population. Furthermore the close agreement of the CVs of the mEJPs and Exts belonging to the signal group suggests a linear relationship between the two.

**DISCUSSION**

Use of the focal macropatch recording electrode to define events at individual synaptic foci or clusters is becoming...
increasingly widespread for the *Drosophila* neuromuscular junction. Similar recording procedures are used for other preparations, including amphibian neuromuscular junctions, smooth muscle neuromuscular junctions, and mammalian central synapses. Simultaneous whole cell recordings have revealed the lack of correlation between focal and whole cell events at the smooth muscle neuromuscular junction (Cunnane and Manchanda 1989), and evidence for extralumenal events at the frog neuromuscular junction (Van der Kloot and Naves 1996). The present study suggests a mechanism for this type of observation in *Drosophila*, based on the ultrastructure of the pre- and postsynaptic elements, and a procedure for separating the true signals (those arising within the electrode) from contaminants. When this is done, the parameters for true signals are much different than before separation. This will affect measures of synaptic performance, including estimates of relative quantal size and quantal content per synapse, and frequency of spontaneous emission of quanta per synapse.

After the observation that different populations of quantal events can be discerned in the data plots, we examined data from 26 different sites, and found that of these, 3 were clearly unimodal (little evidence of contaminating signals), 9 were bimodal, 5 trimodal, and 9 poorly defined. It is clear that the majority of sites selected for recording produced data that contained a proportion of contaminating signals. Defining the true signals altered the parameters for the recording sites considerably.

**Improved estimates of quantal size and frequency at individual boutons**

It was surprising that the differences between the mean Ext amplitudes of the true and the unseparated populations are quite large in all three examples (Table 1). In these experiments, efforts were made to record Exts from individual varicosities by looking under Nomarski optics for those well separated from others and also by using macropatch electrodes designed to enclose just one varicosity. Such precautions were still not sufficient to eliminate contaminating currents in our recordings because the SR provides a pathway for current flow, allowing currents generated extralumenally to travel over distances of 10 μm in some cases (Fig. 4). This observation...
parallels that of Van der Kloot and Naves (1996) at the frog neuromuscular junction, in which extraluminal signals could be detected at an even greater distance (0.8 mm) away from the recording site. Therefore complete electrical insulation from contaminating sources to record just the signal currents may not be possible in this and similar preparations except in a minority of instances. Contaminating signals are likely to contribute significantly in the estimation of quantal size and frequency in the unseparated population. In the three cases analyzed, the quantal amplitude for the Ext increased by 130–210% after separation.

An even larger proportional change occurs for the frequency of spontaneous release at individual boutons. After separation of the data into signal and contaminant populations, the frequency of spontaneous release of the signal group became much lower than that estimated from the unseparated population (Table 1). Relative frequencies of the true signal events were 20–66% of those for the unseparated population. These values are in agreement with studies in which a focal macro-patch electrode was used to record release from a few release sites at other neuromuscular junctions (frog: Robitaille and Tremblay 1991, <0.05 s⁻¹; guinea-pig vas deferens: Brock and Cunnane 1991; and toad: Karunanithi et al. 1992, 0.3–0.05 s⁻¹).

Coefficient of variation as an addition discriminator

Separation of signals was also assessed for its effect on coefficient of variation (CV). For selecting the best estimate for the mean Ext amplitude, the modal fit that gives the smallest difference between the CVs for the Ext and mEJP provides an additional criterion. Modal estimates that give smaller values of CVs for the signal group are preferred. Application of these criteria produce relationships between Exts and mEJPs converging toward linearity, as illustrated (Fig. 10) for the three examples of the present study. A linear relationship between small junctional currents and EJPs has been previously shown at both frog and mouse neuromuscular junctions (McLachlan and Martin 1981). The smaller the differences in the CVs of the Ext and mEJP in the signal group (Fig. 10A), the more the points diverge from the coordinates of points representing the unseparated populations (●) and approach the 45° line (Fig. 10A, ●, ■, ▲). For the case of the bimodal distribution (BD, ○), the bimodal fit (●) moved the point close to the line, whereas the trimodal fit (■) forced the point to migrate almost onto the line and generated smaller CV values. Thus the best estimate of the mean Ext amplitude of the signal group is given by the trimodal fit (0.45 mV, Table 1). A similar result was obtained for the data showing a trimodal distribution (TD, ○), when bimodal (●) and trimodal (■) fits were applied. The trimodal fit again gave the best estimate of the mean Ext amplitude (0.65 mV). In the case of the poorly defined distribution (PD, ○), the bimodal fit improves the estimate of the mean Ext amplitude (●: 0.22 mV). After separation, the pure contaminants (+) tend to show a greater reduction in CV_{Ext} than CV_{mEJP} (Fig. 10B). Clear-cut data from a recent study on mammalian central synapses (Forti et al. 1997) showed equality of CV when plotted in Fig. 10A (▲), supporting the validity of this approach.

![Graph illustrating the relationship between CVs of the mEJP (CV_{mEJP}) and Ext (CV_{Ext}).](http://jn.physiology.org/)

**FIG. 10.** Relationship between CVs of the mEJP (CV_{mEJP}) and Ext (CV_{Ext}). After separation of signal (A) and contaminant (B) groups, the differences between CV_{mEJP} and CV_{Ext} become reduced. Relationship between CV_{mEJP} and CV_{Ext} before separation (○) is shown for the 3 distributions dealt with in this paper (BD: bimodal distribution; TD: trimodal distribution; PD: poorly defined distribution). CVs following separation using either a bi- or trimodal fit also are shown (●: signal group with bimodal fit; ■: signal group with trimodal fit; ■: contaminant group, C_1, extracted using a trimodal fit; ±: pure contaminants, C_2, extracted using either a bi- or trimodal fit). Forty-five-degree line represents the case where CV_{mEJP} equals CV_{Ext}, →, migration shown of the points following separation. ▲ relationship between the CV of currents from whole cell recordings vs. the CV of currents from loose-seal (bouton) recordings in the work of Forti et al. (1997).

Rise times are less reliable as a separation criterion

For two of the three examples analyzed in the present study, the rise time (t_r) was of relatively small value in differentiating the signal and contaminant groups. It is likely determined by several factors, including the concentration and lifetime of the transmitter in the synaptic cleft, the size of the cleft, and the stochastic nature of channel gating (Adelsberger et al. 1997; Heckmann and Dudel 1997; Heckmann et al. 1996). Passive membrane properties of SR may affect the rise time of the...
contaminating Exts, and the example of Fig. 5 does show a slower rise time for the extraluminal signals. However, in only one of the three examples could a clear differentiation be shown on the basis of rise time (Table 1). This criterion should, however, be tested in larger samples. In the study by Forti et al. (1997), spontaneous miniature postsynaptic currents recorded from individual visualized cultured hippocampal synapses using a macropatch electrode had rise times that were highly conserved, although the amplitudes were variable. In their work, it was reported that contaminants arising from boutons outside the electrode were minimized to <1%. For Drosophila, minimization of evoked contaminating signals may be obtained by reducing Ca\(^{2+}\) in the bathing solution while keeping it high in the recording electrode.

With regard to quantal size, the true signal populations generally indicate a fivefold range in Ext amplitude, with a corresponding range in mEPF amplitude (e.g., Figs. 8E and 9A). This range in quantal size for Drosophila corresponds well with the size ranges reported from other studies, particularly for mammalian central neurons (e.g., Forti et al. 1997; Frerking and Wilson 1996). Various mechanisms have been proposed to explain quantal variability, including variation in synaptic vesicle size and receptor numbers at individual synapses; and although it is known that synapse size varies at the Drosophila neuromuscular junction (Atwood et al. 1993), present information is not sufficient to distinguish critically among available alternatives for this junction.

K. Wong and S. Karunanithi contributed equally to this work.

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Address reprint requests to: H. L. Atwood.

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