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Quantal measurement and analysis methods compared for crayfish and *Drosophila* neuromuscular junctions, and rat hippocampus

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Abstract

Quantal content of transmission was estimated for three synaptic systems (crayfish and *Drosophila* neuromuscular junctions, and rat dentate gyrus neurons) with three different methods of measurement: direct counts of released quanta, amplitude measurements of evoked and spontaneous events, and charge measurements of evoked and spontaneous events. At the crayfish neuromuscular junction, comparison of the three methods showed that estimates from charge measurements were closer to estimates from direct counts, since amplitude measurements were more seriously affected by variable latency in evoked release of quantal units. Thus, charge measurements are better for estimating quantal content when direct counts cannot be made, as in crayfish at high frequency of stimulation or in the dentate gyrus neurons. At the *Drosophila* neuromuscular junction, there is almost no latency variation of quantal release in realistic physiological solutions, and the methods based upon amplitudes and charge give similar results. Distributions of evoked synaptic quantal events obtained by direct counts at the crayfish neuromuscular junction were compared to statistical distributions obtained by best fits. Binomial distributions with uniform or non-uniform probabilities of release generally provided good fits to the observations. From best fit distributions, the quantal parameters n (number of release sites) and p (their probability of release) can be calculated. We used two algorithms to estimate n and p: one allows for non-uniform probability of release and uses a modified chi-square (χ^2) criterion, and the second assumes uniform probability of release and derives parameters from maximum likelihood estimation (MLE). The bootstrap estimate of standard errors is used to determine the accuracy of n and p estimates.

Keywords: Dentate gyrus; Neuromuscular; *Drosophila*; Crayfish; Quantal; Presynaptic

1. Introduction

The release of transmitter at the neuromuscular junction has been shown to be quantal in nature (del Castillo and Katz, 1954). At many other synapses, including those of mammalian central neurons, the basic principles of quantal transmission have been confirmed (Redman, 1990; Hessler et al., 1993; Rosenmund et al., 1993). Characterization of transmission, and its alteration by activity or neuromodulators, demands measurement of quantal content of transmission (the number of quantal units generated by a nerve impulse). This is more easily done in some systems than in others. In this paper, we examine some technical problems encountered in measuring quantal content in one of the more easily studied preparations, the crayfish neuromuscular junction. We then determine the applicability and usefulness of the procedures employed for the crayfish in two other synaptic systems of current interest: the neuromuscular junctions of *Drosophila*, and hippocampal synapses of the dentate gyrus in a mammalian brain slice preparation.

The most direct method for determining quantal content is to count the number of quanta released by the nerve impulse. This is feasible at the crayfish neuromuscular junction at low frequencies of stimulation. Such counts can then be used to estimate binomial parameters of transmitter release (Johnson and Wernig, 1971; Wernig, 1972; Smith et al., 1991). In many other preparations, it is not possible to resolve directly all the quantal units evoked by a nerve impulse, unless the preparation is altered experimentally to make this possible. For example, the frog neuromuscular junction is commonly treated with bathing solutions containing high Mg^{2+} concentrations to reduce

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the number of quantal units released. This permits resolution of single evoked quantal events during low-frequency stimulation of the motor neuron, but in a physiologically abnormal preparation. Estimates of quantal content in a normal neuromuscular junction, in which more than a hundred quantal units may appear for each nerve impulse, require a calculation based upon amplitude or charge measurements of the evoked potential or current, and of the individual quantal units, which are often observed as spontaneous events. These are believed to be the same as the quantal elements constituting evoked release (del Castillo and Katz, 1954).

For mammalian central neurons, measurements of charge rather than amplitude have been preferred by some authors (Bekkers and Stevens, 1991). It has been pointed out that measurements of charge are less subject to errors arising from variation in latency (Bekkers and Stevens, 1989), but still numerous reports are based upon the use of amplitude measurements (Malinow and Tsien, 1990; Redman, 1990; Larkman et al., 1991; Liao et al., 1992; Manabe et al., 1992).

The accuracy of the less direct methods of estimating quantal release can be judged at the crayfish neuromuscular junction, in which the results of these methods can be compared with the results from direct quantal counts. For the two other preparations chosen for comparison, direct counts of evoked quanta cannot be made under physiologically 'normal' conditions; therefore, we assessed the utility of the two less direct methods for estimating quantal content. The advantages and disadvantages of the different methods are outlined for each preparation. Since the measurements of quantal release are the starting point for estimates

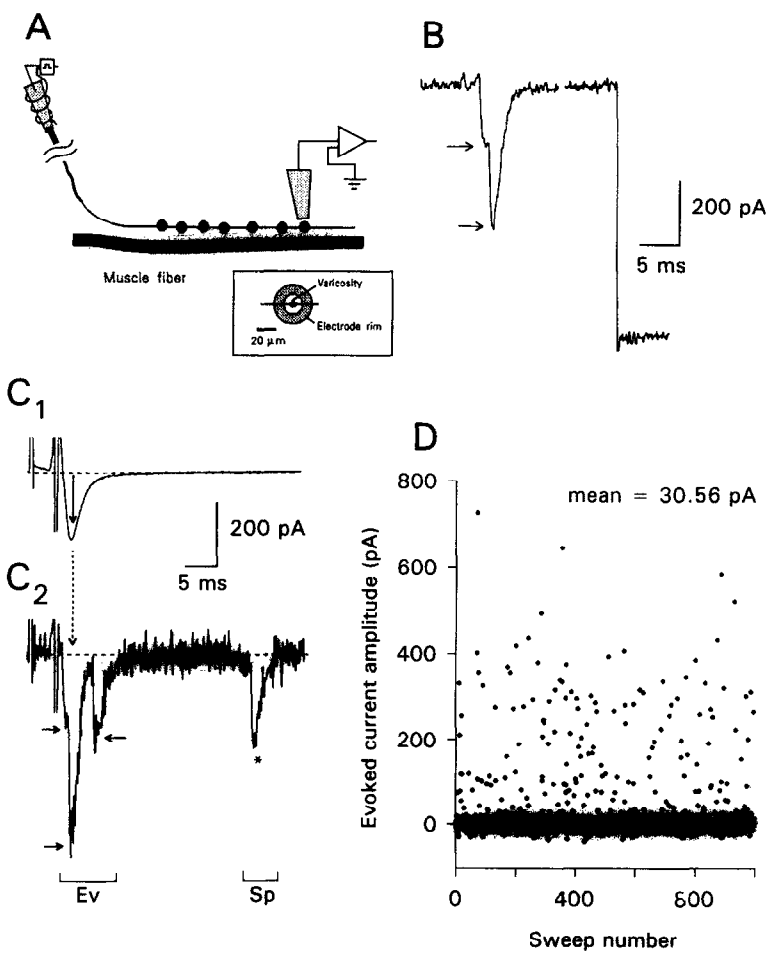

Fig. 1. Quantal events in synaptic transmission at a single varicosity of a crayfish nerve terminal. A: recording method, with focal macro-patch electrode placed over an identified varicosity on nerve terminal of the crayfish opener muscle. A representative electrode placement and varicosity are also sketched in face view, to show complete inclusion of the varicosity within the lumen of the electrode. B: result for a single stimulus which released two quantal units of transmitter (arrows). A test pulse for seal resistance of the recording electrode appears at the end of the record. C: (1) Average of 500 single records to show the time of the peak response. (2) A single sweep of the series, showing evoked release (Ev) of 3 quanta (arrows), and 1 spontaneous event (*). Measurements of charge (time-current integral) were made for the evoked (Ev) and for spontaneous events (Sp). D: a plot of amplitudes of all evoked responses in order of occurrence. A majority of stimuli produced no evoked release. The data were tested for stationarity throughout the experiment.

of probability of release at individual release sites or groups of them, we compare statistical evaluations used to assess the correspondence of observed quantal distributions with binomial and Poisson theoretical distributions.

2. Methods

2.1. Physiological preparations

2.1.1. Crayfish

Freshwater crayfish (*Procambarus clarkii*) used for these experiments measured 5–6 cm in body length (Atchafalaya Biological Supply, Raceland, LA). The 'opener' muscle of the first or second walking leg and its single motor axon were prepared according to standard procedures (Wojtowicz and Atwood, 1986).

Preparations were dissected and maintained in modified Van Harreveld's solution (205 mM NaCl, 5.3 mM KCl, 13.5 mM $CaCl_2 \cdot 2\ H_2O$, 2.45 mM $MgCl_2 \cdot 6\ H_2O$, 0.5 mM HEPES adjusted to pH 7.4). During physiological recordings, preparations were superfused with this solution at 14°C.

The synaptic boutons were visualized with the vital dye 4-Di-2-Asp (Magrassi et al., 1987), which did not affect synaptic transmission at the concentrations and times employed (5 μM, 5-min treatment). With fluorescence microscopy, the lumen of a 'macro-patch' recording electrode (Atwood et al., 1994) could be placed directly over a single isolated bouton (Fig. 1). Stimuli were given at 1, 5 and 10 Hz to the isolated motor axon while recording at a bouton.

2.1.2. Larval Drosophila

Drosophila melanogaster Canton-S strain larvae raised on standard *Drosophila* medium at 25°C were used in all experiments. Larvae were dissected in haemolymph-like solution (HL3) which contained: 70 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2 \cdot 2\ H_2O$, 20 mM $MgCl_2 \cdot 6\ H_2O$, 10 mM $NaHCO_3$, 5 mM trehalose, 115 mM sucrose, 5 mM BES (Stewart et al., 1994). Dissection procedures were standard for this preparation (Atwood et al., 1993; Stewart et al., 1994). The internal organs were carefully removed to expose the body wall muscles and the nervous system.

Electrophysiological data were collected with 'macro-patch' electrode (Mallart, 1993) from type Ib boutons (Atwood et al., 1993) comprising part of the neuromuscular junction of muscle fiber 6 (Crossley, 1978) in abdominal segment 4. The main segmental nerve was stimulated with a suction electrode. The boutons are easily identifiable using Nomarski optics and a 40 × water immersion objective (Jan and Jan, 1976; Atwood et al., 1993; Stewart et al., 1994).

2.1.3. Rat brain slice

Wistar rats (16–30 day old) of both sexes were used. Animals were anaesthetized with halothane, decapitated with a guillotine and their brains quickly removed. The hippocampi were dissected out and cut transversely into 400-μm-thick slices with a tissue chopper. Slices were kept moist and oxygenated at room temperature for at least 1 h before use. Immediately prior to experimentation, slices were transferred to a recording chamber and continuously perfused with artificial cerebrospinal fluid (ACSF) in equilibrium with 95% oxygen and 5% CO_2. The composition of the ACSF was: 124 mM NaCl, 3 mM KCl, 1.25 mM $NaHPO_4$, 2 mM $CaCl_2 \cdot 2\ H_2O$, 1 mM $MgCl_2 \cdot 6\ H_2O$, 26 mM $NaHCO_3$, 10 mM dextrose. The temperature in the chamber was kept at 30–32°C. This temperature is set arbitrarily below the normal body temperature of the rat to assure survival of slices deprived of normal blood circulation.

2.2. Recording synaptic currents

Synaptic currents at the crayfish and larval *Drosophila* neuromuscular junctions were recorded through a macro-patch electrode essentially as described by Dudel (1981), Wojtowicz et al. (1991) and Mallart (1993). Kimax glass (outer diameter: 1.5 mm) was pulled and fire-polished to produce patch tips with inside diameters ranging from 10 to 20 μm. All recordings were obtained using crayfish or larval *Drosophila* physiological solution as the bathing medium and macro-patch electrode recording solution. The patch-clamp amplifier was obtained from Zeitz-Instruments Vertriebs (Augsburg, Germany). Electrode and seal resistance were determined by passing test current pulses through the electrode. Seal resistances ranged from 0.3 to 1.0 MΩ and the electrode resistance ranged from 0.5 to 1.0 MΩ. Seal resistance was monitored throughout the recording; if it changed, the data were not utilized. When synaptic currents were compared between recorded sites, electrode and seal resistances were taken into account as described by Stühmer et al. (1983).

Recordings from dentate gyrus granule neurons were obtained by whole-cell patch recording. A Grass constant-current stimulator, connected to bipolar, tungsten electrodes, was used to stimulate perforant path axons at 0.5 Hz in the middle molecular layer. Stimulation intensity was adjusted to give minimum stable levels of transmission with occasional failures. The patch recording pipettes (outside tip diameter: 1–2 μm) were filled with an intracellular solution containing: 142.5 mM potassium gluconate, 17.5 mM $KMeSO_4$, 8 mM NaCl, 10 mM HEPES, 0.1 mM EGTA, 2 mM MgATP, 0.2 mM GTP, pH 7.3. The osmolarity of the solution was 290–300 mOsm. A similar solution has been used

previously for patch recordings from hippocampal neurons (Baskys and Malenka, 1991). We used a 'blind technique' to obtain whole-cell patch recordings in slices. The procedure consisted of advancing the intracellular patch pipette (usually 5–7 MΩ in resistance) with a motorized micromanipulator in small steps until a cellular membrane in the granule cell layer was encountered. Upon contact, suction was applied to the pipette and a tight seal (> 2 GΩ) established. Rupture of the membrane was produced by additional gentle suction. This was indicated by a sudden drop of input resistance, increase in membrane capacitance and appearance of the membrane potential (usually −70 to −80 mV). In all hippocampal slice experiments described in this study, 10 μM bicuculline methiodide, a $GABA_A$-receptor blocker, was included in the perfusate to eliminate synaptic transmission from hilar neurons and from inhibitory interneurons. Synaptic signals were recorded with an Axopatch-1D amplifier (Axon Instruments) and monitored by a computer. For additional off-line analysis, data were recorded on a Neurodata VCR-based recorder.

2.3. Determination of quantal content

2.3.1. Counts of quantal events

Direct counting of quantal units was possible at the crayfish neuromuscular junction at low stimulation frequencies. For each evoked response, the number of quantal units was determined. For a series of responses, the total number of quantal units was established. Mean quantal content based upon direct counts, m_{co}, was calculated as the total number of quanta divided by the total number of responses.

2.3.2. Peak amplitude measurements

The second determination, used for all three preparations, is to measure the mean peak amplitude of evoked events ($\bar{a}_{pk}$) and spontaneous events ($\bar{q}_{pk}$) and calculate the mean quantal content as $m_{pk} = \bar{a}_{pk}/\bar{q}_{pk}$ (del Castillo and Katz, 1954; Boyd and Martin, 1956).

2.3.3. Charge measurements

The third determination, also used for the three preparations, is to measure charge (pA × ms), by integrating the current trace (Van der Kloot, 1991). The values for mean charge of evoked ($\bar{a}_{ch}$) and spontaneous events ($\bar{q}_{ch}$) then provide an estimate for mean quantal content, calculated as $m_{ch} = \bar{a}_{ch}/\bar{q}_{ch}$. The window of time chosen to measure area of evoked responses was obtained from the averaged trace, starting just prior to the averaged evoked response and ending at the point at which the current returned to baseline. For the mammalian neurons, the end of the time window was set at 90% recovery of the current event, due to a long-lasting 'tail' of slow current. Values for $\bar{a}_{ch}$ were derived from 500 to 1000 trials, and those for $\bar{q}_{ch}$ from at least 30 events.

2.4. Calculation of binomial and Poisson distributions

We tested the fits of data sets to binomial and Poisson distributions, derived from the assumptions outlined in earlier work (Dudel and Kuffler, 1961; Johnson and Wernig, 1971; Wernig, 1975). Binomial distributions have been shown to represent the observed quantal distributions recorded at crayfish neuromuscular junctions (Johnson and Wernig, 1971; Smith et al., 1991; Wojtowicz et al., 1991). For the parameters of non-uniform binomial distributions, we used a model and procedures described in detail by Wojtowicz et al. (1991). The chi-square statistic (χ^2) and a modified Akaike information criterion (AIC) were used to predict the best fitted distribution of events, and quantal parameters n and p. Further estimates of n and p were obtained by MLE as outlined in Smith et al. (1991).

To determine the accuracy of n and p estimates we employed the bootstrap estimate of standard error (Table 2). The bootstrap method consists of drawing many independent (in our case 1000) random samples from the original data set, some appearing zero times, some appearing once, some appearing twice, etc. Calculations of n and p from each bootstrap sample are made and standard errors of n and p are given by empirical standard deviations of the replications. The algorithm and details of the technique are described by Efron and Tibshirani (1993). The bootstrap sampling method used herein is based on an open scheme (Efron and Tibshirani, 1993).

3. Results

3.1. Crayfish neuromuscular junction

3.1.1. Quantal counts

Recordings were obtained from visualized varicosities of the single excitatory axon on the inner surface of the exposed opener muscle (Fig. 1A). It is known that each varicosity has a complement of about 30 individual synapses (Wojtowicz et al., 1994; Atwood et al., 1994), but at low frequencies of stimulation, few quanta are produced, and they can be individually counted without much ambiguity (Fig. 1B,C).

In compound events (more than 1 quantum released by an impulse), each event is usually observed as a deflection in the slope of the current trace. In the crayfish, there is usually some asynchrony in release of individual quantal units (Dudel and Kuffler, 1961); this asynchrony is increased at low temperatures (Zucker, 1973). If the individual evoked events are closely synchronous, discrete events may not be discerned, and

Table 1
Mean quantal content determinations and coefficient of variation (CV) of spontaneous events

	m_{count}	m_{peak} (fixed)	m_{peak} (variable)	m_{charge}	CV_{peak}	CV_{charge}
Crayfish						
Exp. 1 (1 Hz)	0.17	0.21	0.23	0.24	0.51	1.77
Exp. 2 (1 Hz)	0.29	0.21	0.33	0.34	0.48	0.52
Exp. 3 (1 Hz)	0.25	0.14	0.20	0.22	0.41	0.76
(5 Hz)	0.63	0.45	0.58	0.65		
Exp. 4 (2 Hz)	0.66	0.45	0.58	0.69	0.43	0.60
Drosophila						
Exp. 1 (1 Hz)		10.1		8.9	0.39	0.49
Exp. 2 (1 Hz)		7.9		6.9	0.47	0.53
Exp. 3 (1 Hz)		18.5		19.1	0.33	0.51
Rat dentate gyrus						
Exp. 1 (0.5 Hz)		1.5	1.6	1.8	0.55	0.59
Exp. 2 (0.5 Hz)		2.36	2.44	2.82	0.62	0.57
Exp. 3 (0.5 Hz)		2.39	2.41	2.82	0.52	0.57

some events counted as one quantum may contain two. Also, if the evoked response is large, as during high-frequency stimulation of the motor axon, discrete events are often not readily detectable, and direct counts are difficult to obtain with accuracy. In the data sets collected at 1 Hz, a few large evoked events without detectable inflections on rising or falling phases were observed; these responses were counted as single events. Such cases were rare. Thus, at low frequencies, direct counts of quanta provide quite an accurate value for quantal content, on the basis of which the accuracy of the other less direct methods can be judged.

Results obtained from direct quantal counts at low frequencies of stimulation appear in Figs. 1–3, and in Tables 1 and 2.

3.1.2. Estimates based upon amplitude measurements

This method requires measurement of peak evoked responses, and of individual spontaneous quantal events. Measurements of peak amplitudes of evoked responses were obtained in two ways. (a) The average evoked response was obtained, the time of occurrence of its peak was observed, and the maximum values of individual responses were measured as the average of the points occurring ± 300 μs from the time of the average peak (Fig. 1C). An average value for the baseline was taken for the same number of points, and subtracted from the peak value to give the amplitude. (b) The measurement of the peak amplitude was taken as above, but at latency adjusted to match the peak of the individual evoked response. This allowed variation in latency of quantal release to be taken into account more fully.

Amplitude values typically varied widely, with a preponderance of failures at low frequencies (Fig. 1D). Such data sets were tested for stationarity (Wojtowicz et al., 1994) before being accepted.

Values for quantal content obtained from measured amplitudes of evoked and spontaneous events (m_{peak} or m_{pk}) are given in Table 1. Most of the estimates

Table 2
Quantal parameters for crayfish neuromuscular junctions

Distribution				*n*'s and *p*'s		SD
Exp.	Events [a]	Obs. [b]	Theoretical	AIC ($n=$) ($p=$)	MLE ($n=$) ($p=$)	Bootstrap ($\bar{n} \pm$ SE) ($\bar{p} \pm$ SE)
1 (1 Hz)	0	843	846	3	2	2.72 ± 2.7
	1	150	147 Uniform	0.03	0.08	0.07 ± 0.02
	2	7	6			
2 (1 Hz)	0	374	374	–	29	28.18 ± 2
	1	105	109 Poisson	–	0.0001	0.031 ± 0.33
	2	21	16			
	3	0	2			
3 (1Hz)	0	383	383	3	2	2.48 ± 1.79
	1	109	109 Uniform	0.08	0.125	0.11 ± 0.03
	2	8	8			
3 (5 Hz)	0	249	249	7	4	4.18 ± 1.47
	1	189	194 Non-	$p_1 = 0.3$	0.156	0.16 ± 0.0
	2	44	50 uniform	$p_{2-7} = 0.05$		
	3	9	7			
4 (2 Hz)	0	253	254	6	5	6.49 ± 3.42
	1	194	187 Non-	$p_1 = 0.24$	0.124	0.12 ± 0.04
	2	43	51 uniform	$p_{2-6} = 0.07$		
	3	9	7			
	4	1	1			

[a] The number of discrete events, indicated as 0 failures, 1 failure, etc.
[b] Obs., the observed occurrences of each event.

based upon amplitude measurements were lower than those based upon counts (although one experiment is included in which the reverse is true). The estimates derived from measurements at a fixed latency are lower than those based upon measurements made at a variable latency. This arises mainly from variable latency of quantal events (Fig. 1C). For the crayfish, our results show that use of amplitude measurements for quantal content determinations usually leads to under-estimation of the quantal content.

3.1.3. Estimates based upon charge measurements

The time integral of the current below the baseline (in pA × ms) estimates the charge associated with one or more quantal events (Fig. $1C_2$). This method is less susceptible to measurement errors arising from variation in latencies of individual quantal units, as 'late' events (such as the third event in Fig. $1C_2$) are included. In addition, if multiple events which are not clearly discernable for counting occur during the evoked response, the charge measurement takes them into account.

A correction in charge measurements may be necessary for controlling artifactual currents recorded with a macropatch electrode at the neuromuscular junction. Averaging trials which were deemed to be failures in evoked release sometimes yielded a small deviation from the baseline. This spurious current can add or subtract to those measured during the evoked responses depending on the deflection. Adding or subtracting the average of the failures from each trial produced only a slight deviation in mean quantal content.

Table 1 shows that estimates of quantal content derived from charge measurements were invariably larger than those derived from amplitude measurements, and usually closer to the values derived from counts. (Exp. 1 in Table 1, is an exception). Thus, for the crayfish neuromuscular junction, the method based upon charge measurement would provide a better estimate of quantal content than the method based upon amplitude measurements.

This is illustrated in Fig. 2, which presents in more detail the results of the three methods of quantal content determination (Exp. 2 of Table 1). In this case, the amplitude method underestimates the quantal content derived from counts, and the charge method overestimates this value, but the latter method gives a closer estimate of the 'counted' value than the former.

It should be noted that spontaneous events for both amplitude and charge measurements show non-Gaussian distributions (Fig. 2). This type of distribution is commonly seen at the crayfish neuromuscular junction, and elsewhere (Robinson, 1976). Usually the distribution of spontaneous measurements is skewed to the right because of a few large events. Such distributions

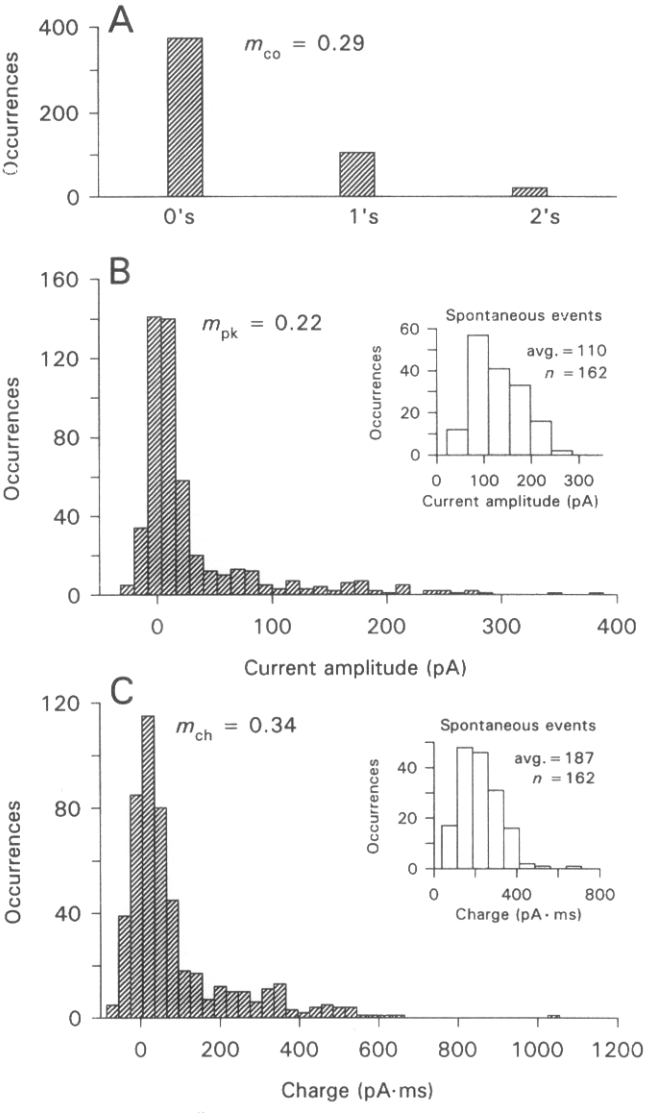

Fig. 2. Comparison of quantal content determinations made at the crayfish neuromuscular junction from: (A) direct quantal counts; (B) peak measurements; (C) charge measurements. In B and C, the inserts show distributions of spontaneously occurring quantal units. Abbreviations: avg, the average value of the spontaneous events; *n*, the number of events measured.

have been described by Robinson (1976) and can be fitted by gamma distributions. It is possible that the skewness results from true 'giant' spontaneous events discussed in detail by Van der Kloot (1991). We included these large spontaneous events in the calculation of the mean value. A more refined statistical treatment that takes account of the non-Gaussian distributions could yield a better value for the quantal content, but would not correct problems associated with variable latency of release. An additional index that can be used which takes into account the variation of the spontaneous events is the coefficient of variation (CV) given as the standard deviation of the events divided by the mean of the events. This index can be

used to compare variation of the measurements of amplitude and charge. The CV values are shown in Table 1 for each of the preparations.

Obtaining a good signal-to-noise ratio is necessary for measuring peak amplitude or charge. Since there is a range of single quantal sizes, some will appear just slightly above the noise level of the recording. One way to insure that single events can be resolved is to plot an amplitude or charge histogram of all the failed events during the time window in which evoked responses normally occur. This will give an indication of the variance of noise during the time of evoked responses. Such a histogram can then be compared to one made using only traces that were marked as having an event. This was done for amplitude and charge measurements in the same sets of data (data not shown). We did not observe any significant difference between the amplitude and the charge mesurements using this approach. There usually is a slight overlap of noise with the smallest of the single evoked events. Another index of signal to noise for quantal detection that may be used is to obtain a ratio of the mean of spontaneous events to the standard deviation of the baseline noise. This ratio can be used to assess whether the amplitude or charge measures provide a better signal-to-noise ratio. In the four experimental crayfish preparations shown in Table 1, this index of signal-to-noise demonstrated that the peak amplitudes gave a larger ratio for Exps. 1 and 2, whereas the charge measure showed a larger ratio for Exps. 3 and 4. The results indicate that one method of measurement is not markedly superior to the other with respect to the signal-to-noise ratio.

3.1.4. Frequency facilitation

As shown by the pioneering work of Dudel and Kuffler (1961), increased frequency of stimulation leads to a higher rate of transmitter release at the crayfish neuromuscular junction. A representative experiment to measure quantal content at different frequencies is given in Fig. 3 (Exp. 2 of Table 1). It was not possible to count quanta accurately at 5 and 10 Hz for this recording site, and the method based upon charge measurement was employed. The value of m_{ch} rose 10-fold when the frequency was increased from 1 to 10 Hz. These data suggest that the calculated value of m_{ch} is closely representative of m_{∞} when both can be compared in the same set of data. We suggest that at higher stimulation frequencies, when direct counts cannot be made, m_{ch} will provide a good representative measure of mean quantal content, as at the lower stimulation frequencies.

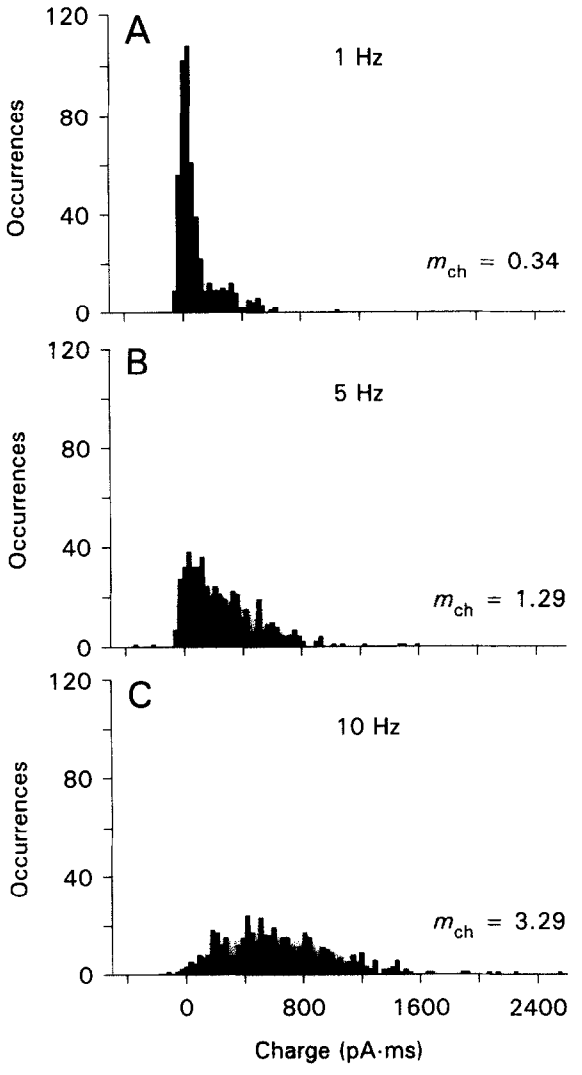

Fig. 3. Changes in quantal content at the crayfish neuromuscular junction, estimated by charge measurements, with increasing frequency of stimulation. Stimulation frequencies were: 1 Hz (A), 5 Hz (B), and 10 Hz (C). As frequency increased, occurrence of failures declined, and multiple quantal events increased, making direct counts of quantal units uncertain or impossible at the higher frequencies.

3.2. Drosophila neuromuscular junction

In physiological solutions with ionic composition close to that of haemolymph (Stewart et al., 1994), currents measured from the larval *Drosophila* neuromuscular junction are large (Fig. 4) and direct counts of evoked quanta are not feasible. However, the responses lend themselves well to amplitude and charge measurements, since there is very little variation in latency of evoked quantal release. To illustrate the latter point, we compared average evoked current with an average of 30 spontaneous currents; the time course is practically identical (Fig. 4B). Therefore, estimates from both amplitude and charge measurements can be expected to give more accurate estimates of quantal content than in the case of the crayfish neuromuscular junction.

It should be noted that previous work on quantal content in this preparation has usually been done under conditions of low calcium and abnormal ion con-

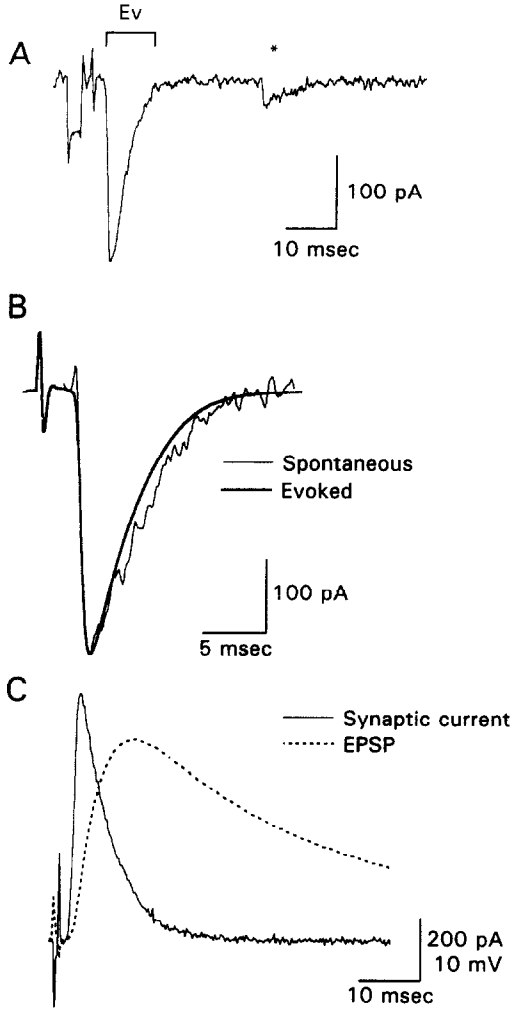

Fig. 4. Recording of evoked and spontaneous synaptic currents at a single varicosity of the neuromuscular junction in larval *Drosophila*. A: results for a single stimulus, showing evoked (Ev) and spontaneous (*) events. B: comparison of time courses of averaged evoked (thick line) and spontaneous (thin line) events, showing identity of time course. The decay time constant of the spontaneous event is 4.4 ms and of the evoked event 3.7 ms. The spontaneous currents were scaled to match the evoked events. C: excitatory post-synaptic potential and simultaneously recorded synaptic current to illustrate that the peak of the current occurs when the voltage displacement from the resting potential is only a small fraction of its final value (here ≈8 mV). The synaptic current trace has been inverted for easier comparison to the EPSP.

tent (Jan and Jan, 1976; Mallart, 1993). Under such conditions there is more variation in quantal latency and a lower quantal content (Mallart, 1993); hence, quantal counts can be obtained.

Because of the relatively large currents at this junction, the excitatory postsynaptic potential is usually 10–30 mV in amplitude (Fig. 4C), and the need for a correction in amplitudes to take account of the loss of driving force for the synaptic current needs to be addressed. Two observations indicated that such a correction was not needed. First, at the time of peak current, the displacement in membrane potential is only a few millivolts (Fig. 4C). Second, we measured current amplitudes with and without voltage clamp (Stewart and Atwood, personal observations), and found that current amplitude did not increase when the membrane potential was clamped at the resting potential; rather, it remained the same size, or decreased slightly.

Evaluations of m in representative experiments are presented in Table 1 and in Fig. 5. Quantal content values were similar, though not identical, for both estimates. At different individual nerve terminal varicosities, the estimated values covered a substantial range, but for each varicosity, estimates of the two methods agreed closely. From present data, there are no grounds for selecting one method over the other for this preparation.

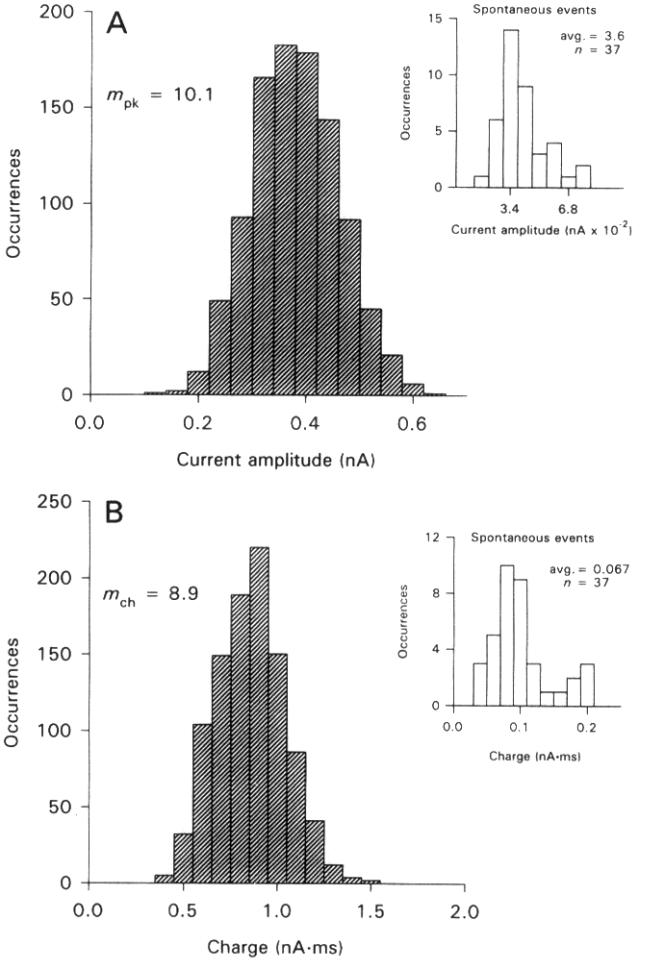

Fig. 5. Comparison of quantal content estimates for evoked release at a single varicosity of the larval *Drosophila* neuromuscular junction, based upon: (A) peak amplitude measurements, and (B) charge measurements. Inserts show measured values for spontaneous events. Abbreviations: avg, the average value of the spontaneous events; n, the number of events measured.

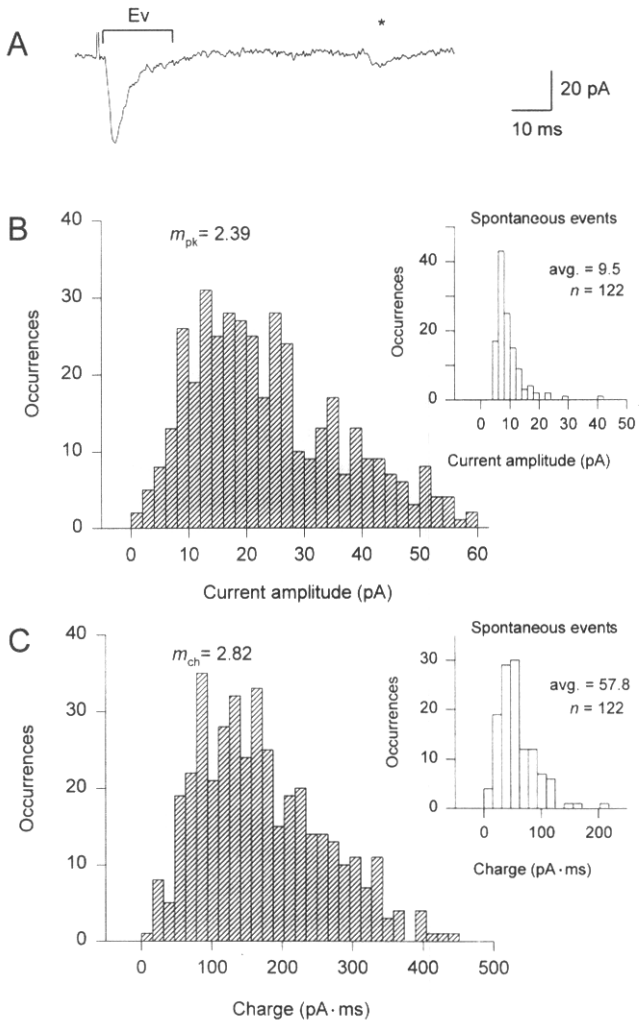

Fig. 6. Comparison of peak amplitude and charge measurements in a dentate gyrus neuron of the rat hippocampus. A: results for a single stimulus, showing evoked (Ev) and spontaneous events (*). B: histograms for evoked and spontaneous events (insert) obtained from peak measurements. C: histograms for evoked and spontaneous events (insert) obtained from charge measurements. The value of m_{pk} is less than that of m_{ch} in this and other experiments. Abbreviations: avg, the average value of the spontaneous events; n, the number of events measured.

3.3. Rat brain neurons

Currents obtained from rat dentate gyrus were analyzed by the methods presented above for the crayfish preparation (Table 1, Fig. 6). Quantal content estimates derived from amplitude values were lower than those derived from charge measurements, as in the crayfish. This supports the view of Bekkers and Stevens who favoured use of charge measurement for mammalian central neurons. In these experiments, several input axons were probably being stimulated, and the quantal events could not be counted individually due to the recording noise. Since there was evidence for latency variation in these recordings, the physiological properties of the synapses are more like those of the crayfish terminals than *Drosophila* terminals. From this, the estimates based upon charge measurements are likely to be closer to values derived from direct counts, if the latter were obtainable.

3.4. Parameters for quantal distributions

Statistical analyses were conducted on direct quantal counts obtained from the crayfish neuromuscular junction. Data from measurements of amplitude or charge present more difficulties in determining the appropriate statistical distribution pattern of the measured responses in frequency histograms.

Comparing the number of observed quantal events to those expected from binomial or Poisson distributions, one can determine the statistical distribution that best represents the data set. Since previous studies have indicated that release at recording sites may have non-uniform temporal or spatial probability (Hatt and Smith, 1976; Wojtowicz et al., 1991; Smith et al., 1991), the present data sets were also compared to theoretical distributions derived using this assumption. The hypothetical models were examined and their calculated distributions are shown in Table 2. Only in one case was the data set best described by a Poisson distribution. The other 4 data sets were best described by the binomial non-uniform or uniform probability model, as judged by the lowest values of the χ^2 (one sample) test.

From the predicted distributions, further analysis was carried out (as described in Methods) to determine the expected number of responding elements or release sites (n) and the probability of release (p). A maximum likelihood procedure was used as well for estimating n and p (Smith et al., 1991). This analysis determined the best fit of quantal parameters for the observed distribution of counted evoked events. The Akaike information criterion (AIC) determined whether the observed distribution of events best fit a uniform or non-uniform binomial model. From the best distribution, n and p values were obtained. The individual p values of the non-uniform distributions in Table 2 showed one divergent value (p_1), with the remaining p values similar to each other.

4. Discussion

4.1. Quantal content determination

The three methods for determining quantal content of transmission have all been used in previous work, but seldom compared for data sets amenable to analysis by all three methods. The crayfish neuromuscular junction provides data for which this can be done

under normal physiological conditions. From the present analysis, it is evident that the commonly used method of determining quantal content from amplitudes of evoked and spontaneous events often produces a value lower than that obtained by counting quanta directly. The major cause of the discrepancy is the variation in latency of evoked quanta (Bekkers and Stevens, 1991). When estimates are based upon measurement of charge rather than amplitude, the values agree more closely with those obtained from direct counts. We conclude that, for synaptic systems in which there is evidence for quantal latency fluctuations in evoked release, but in which individual quantal units cannot be counted, the method based upon charge measurements provides a better estimate of quantal content than methods based upon amplitude measurements. This would hold in particular at higher stimulation frequencies, when latency fluctuation might increase.

The rat dentate gyrus neurons are an example of this type. The evoked current responses show evidence of latency fluctuations and estimates of m_{ch} are larger than those of m_{pk}, as in the crayfish. Since spontaneous events cannot be attributed with certainty to the same synapses participating in evoked release in this preparation, the estimates of quantal content are more uncertain than in the crayfish and *Drosophila* preparations, in which spontaneous events arise at the same recording site as the evoked responses. The CV values for the spontaneous events, in all three preparations, indicate that in the majority of the cases, the variations are larger for the charge measurements than for the amplitude measurements. This suggests that amplitude measurements underestimate the degree of variation in spontaneous events, whereas the charge measurements include not only the amplitude but also the rise and decay phases of the event.

In *Drosophila*, there is very little latency fluctuation in evoked release at haemolymph calcium concentrations and thus no reason to prefer estimates of m_{ch} over those of m_{pk}. If quantal content is reduced in this preparation through low-calcium solutions, latency fluctuation appears (Jan and Jan, 1976; Mallart, 1993), and then the situation resembles that in the crayfish. We emphasize that measurements at abnormally low Ca^{2+} are not likely to represent the normal performance of the synapse (Stewart et al., 1994), though they may be useful for comparing effects of mutations on synaptic transmission (Zhong and Wu, 1991; Mallart, 1993). The variation in amplitude and charge among spontaneous events may be dependent on the anatomical characteristics of the preparation. For example, the variance in the spontaneous events recorded from the dentate neurons is larger than those recorded at the neuromuscular junctions of crayfish and *Drosophila*. This can arise from multiple postsynaptic sites along large dendritic trees of CNS neurons. Another possible explanation for quantal size and shape variation is that receptors may exhibit variable desensitization when events are occurring in a rapid succession at the same synapse (see Dudel et al., 1992).

4.2. Binomial parameters of evoked release

Distribution of quantal release for crayfish data were most often fitted best with binomial distributions, though a few cases occur in which the best fit is a Poisson distribution (Table 2). The best results were obtained using data from direct quantal counts. Attempts to fit amplitude distributions with binomial distributions must contend with several sources of variation, including errors in measurements of evoked release discussed above, and non-Gaussion distributions for spontaneous events. These factors, combined with the range in possible values for n and p, frequently provide many possible solutions which fit equally well the distributions in the histograms.

As illustrated in Table 2, data sets from the crayfish neuromuscular junction are often best fitted with binomial distributions in which the probability of release is non-uniform among the individual release sites (Hatt and Smith, 1976; Smith et al. 1991). However, cases also occur in which a uniform probability gives the best fit. Structural studies provide a basis for non-uniform probability, since some synapses on a varicosity are more complex than others (Wojtowicz et al., 1989, 1994) and structural differences are found at high-output and low-output synapses of a single neuron (Govind et al., 1994). Also, depending on the distances between release sites (i.e., active zones), there can be interactions among sites which affect the probability of release (Cooper, Winslow, Govind, and Atwood, in manuscript). In this regard, crustacean synapses may differ from those of the goldfish Mauthner cell, which are more uniform in structure and for which probability of release appears to be uniform (Korn et al., 1981). Such comparisons emphasize the variability of naturally occurring synaptic systems. Non-uniform probability of release has been recognized as likely for synaptic transmission in hippocampal neurons on the basis of analysis of fluctuations of synaptic currents (Rosenmund et al., 1993; Redman, 1990). A disadvantage of the charge measurement for analysis in central neurons may arise if the recordings exhibit a high rate of spontaneous transmitter release which could result in baseline shifts. This was not a problem in our recordings from dentate gyrus neurons because the rate of occurrence of spontaneous miniature potentials was on average 2 Hz.

Use of uniform and non-uniform probabilities for the same data sets did not lead to a significant difference in the estimated parameters: n changed by 1, and

the estimated standard errors were usually greater than this (Table 2). We present a bootstrap approach for estimating standard errors of quantal parameters based on Efron and Tibshirani (1993). Other approaches to this problem employed either estimates based on the method of moments (Robinson, 1976) or the Fisher information matrix (Smith et al. 1991). These approaches are valid for estimates of continuous functions and can be used with some limitations to calculate standard errors of the parameter p. However, parameter n in quantal models is an integer rather than a continuous function. The bootstrap method can be used in dealing with integer values and is probably the method of choice at this time.

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