Assessment of the reliability of amplitude histograms from excitatory synapses in rat hippocampal CA1 *in vitro*

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1. Excitatory postsynaptic potentials (EPSPs) were evoked using minimal extracellular stimulation and recorded from pyramidal cells from the CA1 region of slices taken from adult rats and maintained *in vitro*.

2. Segments of data were selected that gave EPSP amplitude frequency histograms that showed approximately equally spaced peaks. Selection was performed either on the basis of stationarity of the EPSP mean and standard deviation, or on the trajectory of a graph of the (coefficient of variation)$^{-2}$ against mean for the EPSP, or, in some cases, by trial and error.

3. For each histogram, we determined the likelihood that peaks of similar sharpness and equality of spacing could have arisen by sampling artifact from a smooth distribution, using a method based on autocorrelation and Monte Carlo simulation.

4. Thirty-three histograms were analysed. For twenty-six of these, the likelihood of sampling artifact was estimated at 1 in 100 or less, and for eleven histograms the likelihood was less than 1 in 1000. For the histogram with the clearest peaks, the likelihood was less than 1 in 350 000. Histograms judged to be reliable by this method could occur when the EPSP mean amplitude was changing.

5. We conclude that random sampling artifact is very unlikely to be the explanation for the peaks in our data histograms. It seems more likely that they are due to a quantal synaptic transmission mechanism with low quantal variability.

6. The autocorrelation method also gives a measure of the mean peak spacing, and hence the mean quantal size, for each histogram. Quantal sizes ranged from 93 to 285 μV, with a mean $\pm$ S.D. of 172 $\pm$ 47 μV.

7. From these quantal sizes and the EPSP mean amplitudes we calculated the mean number of quanta released per trial for each histogram. This ranged from 0·36 to 6·9, with a mean of 3·3 $\pm$ 1·67.

The quantal hypothesis for chemical synaptic transmission proposes that neurotransmitter is released probabilistically from the presynaptic terminal in multimolecular packets of roughly similar size known as quanta (del Castillo & Katz, 1954). There is a considerable body of experimental evidence in favour of quantal release at a limited number of technically favourable synapses, most notably the vertebrate neuromuscular junction (reviewed by Van der Kloot & Molgo, 1994). Probabilistic release of quanta gives rise to trial-to-trial fluctuations in the size of the synaptic response recorded postsynaptically; under ideal conditions, this can result in clear, regularly spaced peaks in the frequency distribution of recorded synaptic amplitudes (e.g. Boyd & Martin, 1956).

The picture is much less clear for mammalian central synapses (Redman, 1990), and many studies have not produced amplitude histograms with clear, equally spaced peaks. In earlier work in the spinal cord, the main reason for this was thought to be the barely adequate signal-to-noise ratio of the recordings (e.g. Jack, Redman & Wong, 1981). An exception is the study of dorsospinal–cerebellar tract cells by Walmsley, Edwards & Tracey (1988) in which obvious peaks and inflections were obtained. In the hippocampus, the study of spontaneous excitatory currents using whole-cell recording has led to the conclusion that the amplitude variability of presumed single-quantal events is so large that histograms of evoked responses would not be expected to show peaks (Bekkers & Stevens, 1990, 1995; Malinow & Tsien, 1990; Manabe, Renner & Nicoll, 1992; Malgaroli & Tsien, 1992; Raastad, Storm & Andersen, 1992; Liu & Tsien, 1995). On the other hand, when working with evoked events, some laboratories have obtained histograms that do show regular peaks (Larkman, Stratford & Jack, 1991; Kullmann & Nicoll, 1992; Larkman, Hannay, Stratford & Jack, 1992; Liao, Jones & Malinow, 1992; Voronin, Kuhnt, Hess, Gusev & Roschin, 1992; Stricker, Redman & Daley, 1994; Stricker,

Field & Redman, 1996), which have been interpreted as evidence for quantal release and which imply relatively low levels of quantal variability, at least for evoked quanta.

In a previous study (Larkman *et al.* 1991), we recorded large numbers of trials of excitatory postsynaptic potentials (EPSPs) from hippocampal CA1 pyramidal cells. These were evoked at relatively high stimulation rates (usually 3 or 5 Hz) in an attempt to minimize the effects of impalement instability. However, at these rates, most EPSPs showed depression, or a progressive reduction in mean peak amplitude over time. When all the available trials were used, the resulting amplitude histograms generally did not show clear peaks. However, we were able to find relatively short epochs of data, usually 500–1000 trials, that did give clear, approximately equally spaced peaks. We interpreted these data as indicating a quantal release process, and during the periods yielding peaky histograms, the quantal variance appeared to be low. During long epochs, however, the peaks became blurred. We have proposed several possible mechanisms for this blurring (see Jack, Kullmann, Larkman, Major & Stratford, 1990), but we suggested that the major cause was likely to be changes in the quantal amplitude over time. Our interpretations were challenged, however. Clements (1991) suggested that the peaks in our amplitude histograms had arisen by sampling error from underlying distributions that were actually smooth. On this view, when we considered longer epochs of data, we increased the sample size and merely corrected the previous sampling error, causing the peaks to become less distinct or disappear. He proposed that our data were therefore consistent with a continuous synaptic amplitude distribution and hence a transmitter release process that was either non-quantal or showed very high levels of quantal variance.

In an attempt to resolve this issue, we have analysed our previous data in more detail and performed further experiments. In this paper we estimate the likelihood that the peaks in our histograms could have arisen by sampling error, making use of autocorrelation (AC) as an objective measure of peakiness and equality of peak spacing in histograms. In a separate paper (Stratford, Jack & Larkman, 1997) we describe the autocorrelation method in detail and compare its ability to distinguish quantal behaviour from sampling error against conventional statistical tests. Finally, we present evidence in a third paper (Larkman, Jack & Stratford, 1997) that is consistent with our interpretation that quantal amplitude can change with time.

METHODS

Experimental procedures

Procedures were generally as described in Larkman *et al.* (1991). Young adult albino rats (Sprague–Dawley, 120–200 g) were deeply anaesthetized by inhalation of halothane (4 ml allowed to evaporate in a transparent-topped container of approximately 10 l capacity) and killed by decapitation using a small-animal guillotine. Transverse hippocampal slices, nominally 400 μm in thickness, were prepared by conventional methods using a vibrating microtome and maintained at ~34 °C in an interface-type chamber. The slices were perfused with artificial cerebrospinal fluid (ACSF) containing (mM): 119 NaCl, 3·5 KCl, 1 NaH_2PO_4, 26 $NaHCO_3$, 11 glucose and either 2·5 $CaCl_2$ and 3 $MgSO_4$, or 4 $CaCl_2$ and 4 $MgSO_4$. For most experiments the ACSF also contained picrotoxin (100 μM) and DL-2-amino-5-phosphonovaleric acid (APV; 50 μM), and in some cases glutamine (1 mM). The ACSF was gassed with a mixture of 95% O_2 and 5% CO_2. Conventional intracellular voltage recordings were made from the CA1 pyramidal cell body layer using microelectrodes containing 2 M potassium methylsulphate.

Small EPSPs were evoked by 'minimal' extracellular stimulation, using a bipolar wire electrode placed in CA1, usually in stratum radiatum. The stimulus intensity was adjusted to produce the smallest EPSP that appeared to be evoked reliably and that was not sensitive to small changes in stimulus intensity. This procedure is likely to have biased our sample away from weak connections that showed a high proportion of apparent failures of transmission. Large numbers of trials (usually 1000–6000) were evoked at stimulation rates between 0·1 and 5 Hz (usually 3 or 5 Hz), filtered at 1 or 2 kHz, digitized at 5 kHz and recorded on computer disk. The EPSP peak amplitude on each trial was measured off line using a computer routine that compared the average voltage during a 1–3 ms period in the baseline with the average voltage during a period of the same duration straddling the EPSP peak. For each EPSP, the periods to be measured were determined from the averaged waveform. Stimulation artifacts were not subtracted, and the measurement periods were chosen to avoid them. The same periods were applied to all the trials recorded for a given EPSP. The recorded trials were grouped into blocks of 50–500 consecutive trials and the mean and standard deviation (S.D.) of the EPSP peak amplitudes were calculated for each block. The block size used depended on the number of trials available and the rate of change of the EPSP mean.

Analysis of histograms

The measured EPSP peak amplitudes were displayed as amplitude frequency histograms, using narrow bins of usually 5 or 10 μV width. For illustrative purposes, the histograms were smoothed in the Fourier domain, but unsmoothed histograms were the starting point for the analysis. Histograms showing apparently quantal peaks were assessed for the likelihood that the peaks could have arisen by finite sampling artifact using an autocorrelation (AC) scoring technique that is described in detail and calibrated in Stratford *et al.* (1997).

A total of forty-five EPSPs of adequate technical quality were recorded. Only five of these gave amplitude histograms showing approximately equally spaced peaks when all the recorded trials were included. For the other cases, epochs of data were selected according to either the stationarity of their mean and S.D., the trajectory of the graph of (coefficient of variation)$^{-2}$ against mean or by trial and error. Results from a sample of thirty-three histograms from twenty-one EPSPs that gave likelihoods of better than 1 in 20 by AC scoring are presented here.

Only histograms with a unimodal convex envelope of peaks were analysed. The AC scoring method is not appropriate for histograms with bimodal or complex envelopes (for example, due to an excess of transmission failures) and any histograms of that type were therefore excluded. One approach that could be adopted for histograms with an apparent excess of failures would be simply to eliminate the failure trials and then assess the peakiness of the non-failure distribution by AC scoring in the normal way. An example of the result of this approach, applied to a histogram with a prominent, but not excessive, failures peak is given in the legend to Fig. 2.

RESULTS

A Stationary EPSP – EPSP 1 (AH 93)

Two hundred and fifty trials were recorded at a stimulation rate of 0·1 Hz, and the averaged EPSP waveform had a peak amplitude of approximately 300 μV (Fig. 1*A*). The EPSP fluctuated in amplitude from trial to trial, including apparent failures (Fig. 1*B*). The EPSP peak amplitude on each trial was measured and the mean and S.D. for successive blocks of fifty trials showed little change over time (Fig. 1*C*). The amplitude histogram for all 250 trials, when binned finely and smoothed, showed four peaks, located at approximately 20, 300, 580 and 890 μV (Fig. 1*D*).

We used the AC method (Stratford *et al.* 1997) to assess the likelihood that this, or a similar, pattern of peaks could have arisen by finite sampling from a smooth distribution. The finely binned histogram was heavily smoothed, essentially until the peaks had disappeared (Fig. 1*E*), and this heavily smoothed version was subtracted from the original to give a difference function (Fig. 1*F*). This was itself smoothed to yield a clear succession of peaks and troughs roughly centred on the zero line. The AC function of the smoothed difference function was calculated and showed a typical 'damped sinusoid' shape (Fig. 1*G*). The first (non-zero lag) peak was located at 285 μV and its amplitude, measured from the base of the preceding valley (see arrows in Fig. 1*G*), was 80·5. This was taken as the 'AC score' for this data histogram.

We fitted a smooth Weibull distribution to the original histogram and used this distribution to generate samples,

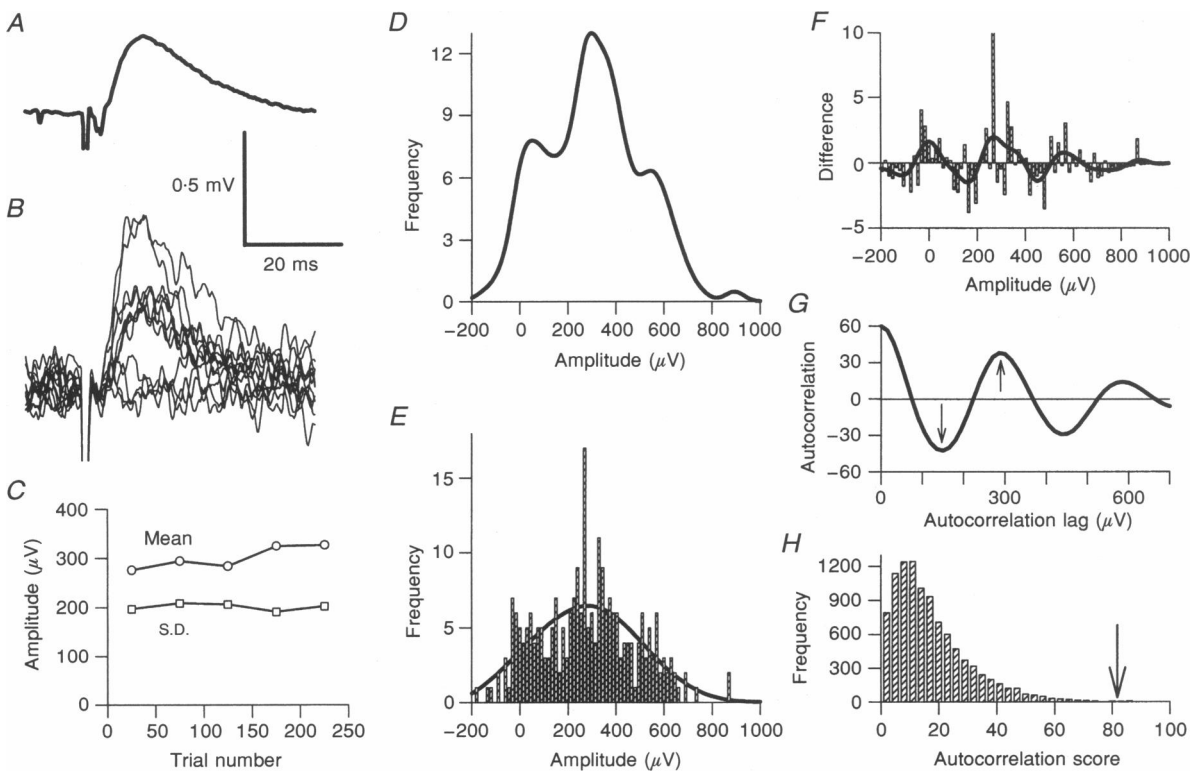

Figure 1. Properties and AC scoring of EPSP 1

A, averaged EPSP waveform, showing mean peak amplitude of approximately 300 μV. *B*, 12 individual trials superimposed, showing apparent quantal fluctuation pattern, with amplitudes of approximately 600 and 300 μV and apparent failures. These trials were from the initial period of recording, and some trials obviously contaminated by spontaneous EPSPs were deleted. *C*, time course of EPSP mean (upper line) and S.D. (lower line) throughout recording, calculated in blocks of 50 trials each. The mean increases slightly but the S.D. is virtually constant. *D*, amplitude frequency histogram for all 250 trials, shown binned finely then smoothed. The histogram shows peaks at approximately 300 μV intervals. *E*, amplitude histogram for all 250 trials binned at 15 μV (bars) and the heavily smoothed version of it (continuous line) that will be subtracted from the histogram. *F*, the difference function obtained by subtracting the smooth distribution from the histogram shown in *E*. The bars show the raw subtraction result and the continuous line the smoothed version. *G*, the AC function of the smoothed difference function shown in *F*. It has a characteristic 'damped sinusoid' shape with the first (non-zero lag) peak at 285 μV. The AC score is taken as the amplitude of this peak measured from the preceding valley, and has a value of 80·5 (indicated by arrows). *H*, distribution of AC scores obtained from 10 000 random samples, each of 250 trials, drawn from a unimodal distribution similar to the continuous line in *E*. The AC score of the EPSP histogram is indicated by the arrow (80·5). Only 22 of the random samples gave scores higher than the data histogram, giving a likelihood of 1 in 454.

each consisting of 250 random numbers. We then calculated the AC score for each of these samples in the same way as for the data histogram. The distribution of scores obtained for 10 000 random samples is shown in Fig. 1*H*, and twenty-two of the random samples gave scores higher than the data score. Thus peaks of the same sharpness and equality of spacing as those in the data were found in only 1 in 454 samples drawn from a smooth distribution of similar shape, and we conclude that it is unlikely that the peaks in the data arose by sampling artifact ($P \approx 0{\cdot}002$).

Although the mean and S.D. of this EPSP were relatively stationary over time, the first few trials showed a particularly clear pattern of amplitude fluctuations. The amplitude histogram for the first twenty-five trials showed very clear peaks, separated by approximately 300 μV (Fig. 2), and individual records from this period showed apparent quantal fluctuations with this spacing (Fig. 1*B*). Should any importance be attached to such small numbers of trials? We repeated the autocorrelation scoring procedure for the histogram from just the first twenty-five trials, and the result was surprising. Only 2 of the 10 000 random samples of each of twenty-five trials that we tested gave scores higher than the data (Fig. 2 inset). Thus it is unlikely that the fluctuation pattern had arisen by chance, and the fact that the first few trials showed a clearer pattern than the data set as a whole suggests that some degree of non-stationarity had occurred to smear the pattern during recording even of this relatively stationary EPSP.

Non-stationary EPSPs

Most of our EPSPs were recorded at higher stimulation rates than the previous example and showed changes in mean amplitude over time. Usually this involved a progressive and often substantial depression, although in a few cases an initial depression was followed by a degree of recovery.

Histograms from stationary periods – EPSP 2 (AH24)

Two thousand trials were evoked at 5 Hz, and the EPSP mean depressed from over 700 μV to about 150 μV (Fig. 3*A*). The depression was most pronounced during the first 1000 trials; during trials 1000–2000 the mean fell only slightly and the S.D. was virtually constant. The amplitude histogram for all 2000 trials was skewed and did not show clear peaks (Fig. 3*B*).

One approach to data of this sort would be to reject the data that were non-stationary by some criterion, and an obvious move in this case would be to discard the first 1000 trials. The histogram for trials 1001–2000 (Fig. 3*C*) shows clear peaks separated by approximately 115 μV. When a heavily smoothed version was subtracted from this histogram, the difference function also showed a regular succession of peaks and troughs, roughly symmetrical about zero (Fig. 3*D*). The AC function of this difference function resembled a damped sinusoid (Fig. 3*E*) with the apex of the first (non-zero lag) peak at 125 μV. The amplitude of this peak was 214·9. Random samples of 1000 trials each were drawn from a smooth Weibull distribution fitted to the data histogram

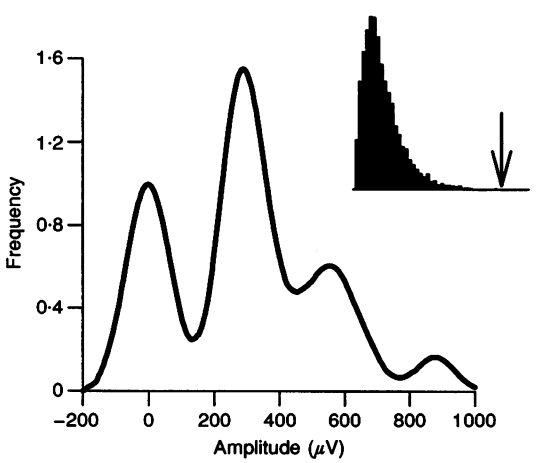

Figure 2. Analysis of the first 25 trials only of EPSP 1

Smoothed amplitude histogram for trials 1–25 of EPSP 1, showing very clear peaks at approximately 300 μV intervals (compare with continuous line in Fig. 1*D*). Inset shows the distribution of AC scores of 10 000 random samples, each of 25 trials, drawn from a unimodal distribution fitted to this histogram. The data histogram gave an AC score of 17·1 (indicated by arrow) which was exceeded by only 2 of the random samples tested (histogram bars too small to be resolved at this scale), giving a likelihood of roughly 1 in 5000. This histogram shows a prominent failures peak, so to ensure that the apparent reliability of the sample was not entirely due to the presence of failures, we repeated the AC analysis after removal of the failures peak. The histogram for the 18 non-failure trials produced an AC function with its first peak in the same location as before, showing that the apparent quantal size of the non-failures was the same as for the full distribution. The AC score was 9·5 and this was exceeded by only 1 in 110 random samples (not shown). Thus even with a very small sample after the elimination of the failures, it could be shown that the peaks were unlikely to have arisen by sampling error.

and their AC scores were calculated in the same way. None of the 10 000 random samples equalled or exceeded the score for the data histogram (Fig. 3*F*). We therefore conclude that peaks of this sharpness and equality of spacing arise only very rarely by random sampling from a smooth distribution and so it is extremely unlikely that the peaks in the data histogram arose by sampling artifact. This histogram is essentially the same as was originally shown in Fig. 1d of Larkman *et al.* (1991; trials 1001–1800 instead of 1001–2000).

Histograms from non-stationary periods

Based on the previous example, a reasonable strategy might be to reject all the data trials that failed to meet some criterion of stationarity over time. However, we discovered, essentially by trial and error, that some periods of data that were obviously non-stationary could still yield histograms with clear peaks.

EPSP 3 (AH3). Six thousand trials were recorded at 5 Hz, and the EPSP mean depressed by a factor of more than three, although the fall in S.D. was much less dramatic (Fig. 4*A*). As in the previous example, most of the depression occurred during the early part of the recording. In our earlier study (Larkman *et al.* 1991) we selected 500 trials for this EPSP (trials 1300–1800), which gave a histogram with clear peaks in spite of coming from a period when the mean was falling rapidly (Fig. 4*B*). Could these peaks have arisen by chance? The AC function for these trials shows a clear succession of peaks, the first of which is at 150 μV and has an amplitude of 52·9 (Fig. 4*C*). Monte Carlo simulations showed that such a score was only produced by 1 in 384 random samples (Fig. 4*C* inset), and so is unlikely to have arisen by chance.

The preceding 1000 trials (Fig. 4*D*) also gave an AC function with a succession of peaks, with the first at 120 μV, which was also unlikely to have arisen by chance (1 in 262; Fig. 4*E* inset). In fact, if the whole period of trials 1 to 1800 was considered, the histogram showed peaks and gave an AC score of 235·7 (first peak location 140 μV) that was not exceeded by any of the 10 000 random samples we tested (Fig. 4*F*).

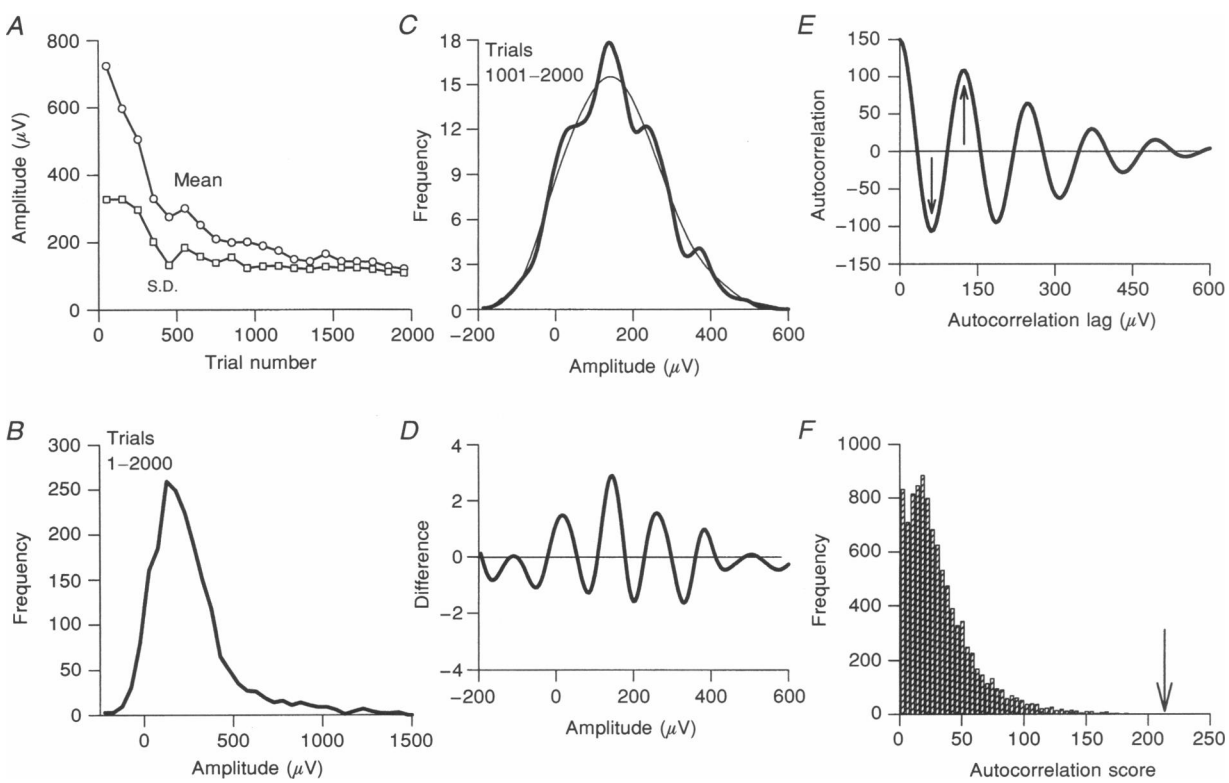

Figure 3. Selection of data and AC scoring for EPSP 2

A, time course of EPSP mean and S.D., showing rapid early depression. *B*, amplitude histogram for all 2000 trials recorded, with no obvious peaks. *C*, thick line shows the lightly smoothed amplitude histogram for trials 1001–2000, with 5 clear peaks separated by 115 μV. Thin line shows heavily smoothed version used as subtractor. *D*, smoothed difference function obtained by subtracting the unimodal distribution shown in *C* from the original finely binned data histogram, then smoothing the difference. It shows clear peaks and troughs that are roughly symmetrical about the zero line. *E*, AC function of the difference function shown in *D*. The first peak is at 125 μV, and its amplitude, measured from the preceding valley, is 214·9 (arrows). This amplitude is taken as the AC score for this data histogram. *F*, distribution of AC scores obtained from 10 000 random samples, each of 1000 trials, drawn from a unimodal Weibull generator distribution fitted to the histogram in *C*. None of the random samples tested exceeded the score for the data histogram (arrow).

One explanation of how histograms such as this with clear and reliable peaks could be generated from non-stationary data is that the change in EPSP mean was due to a change in the number of quanta released, with little change in quantal size. For some, but not all, models of transmitter release, graphs of the (coefficient of variation)$^{-2}$ (CV^{-2}) against mean can give an indication of the cause of the non-stationarity. For this EPSP, the trajectory of this graph was steeper than the diagonal, consistent with a change in the number of quanta released rather than a change in quantal size (Fig. 4*G*).

EPSP 4 (AH46). From the preceding example, it might be hoped that graphs of CV^{-2} against mean might be used to guide the selection of blocks of data during which the quantal size remained relatively constant in spite of changes in the EPSP mean. EPSP 4 was evoked for 2000 trials at 5 Hz and showed considerable depression of both mean and S.D. particularly during the first 600 trials (Fig. 5*A*). The graph of CV^{-2} against mean has a shallow trajectory for the first 600 trials, then becomes steeper (Fig. 5*B*). This suggests that the early depression was due mainly to a reduction in quantal size, but later was due more to a fall in the number of quanta released.

The amplitude histogram for all 2000 trials showed peaks (Fig. 5*C*), and the AC scoring indicated a likelihood of 1 in 114 that they could have arisen by chance (Fig. 5*D* inset). However, if the first 800 trials were discarded, the histogram peaks became sharper (Fig. 5*D*), and the AC scoring for trials 800–2000 suggested greatly improved reliability, with a likelihood now of 1 in 2000 (Fig. 5*D* inset).

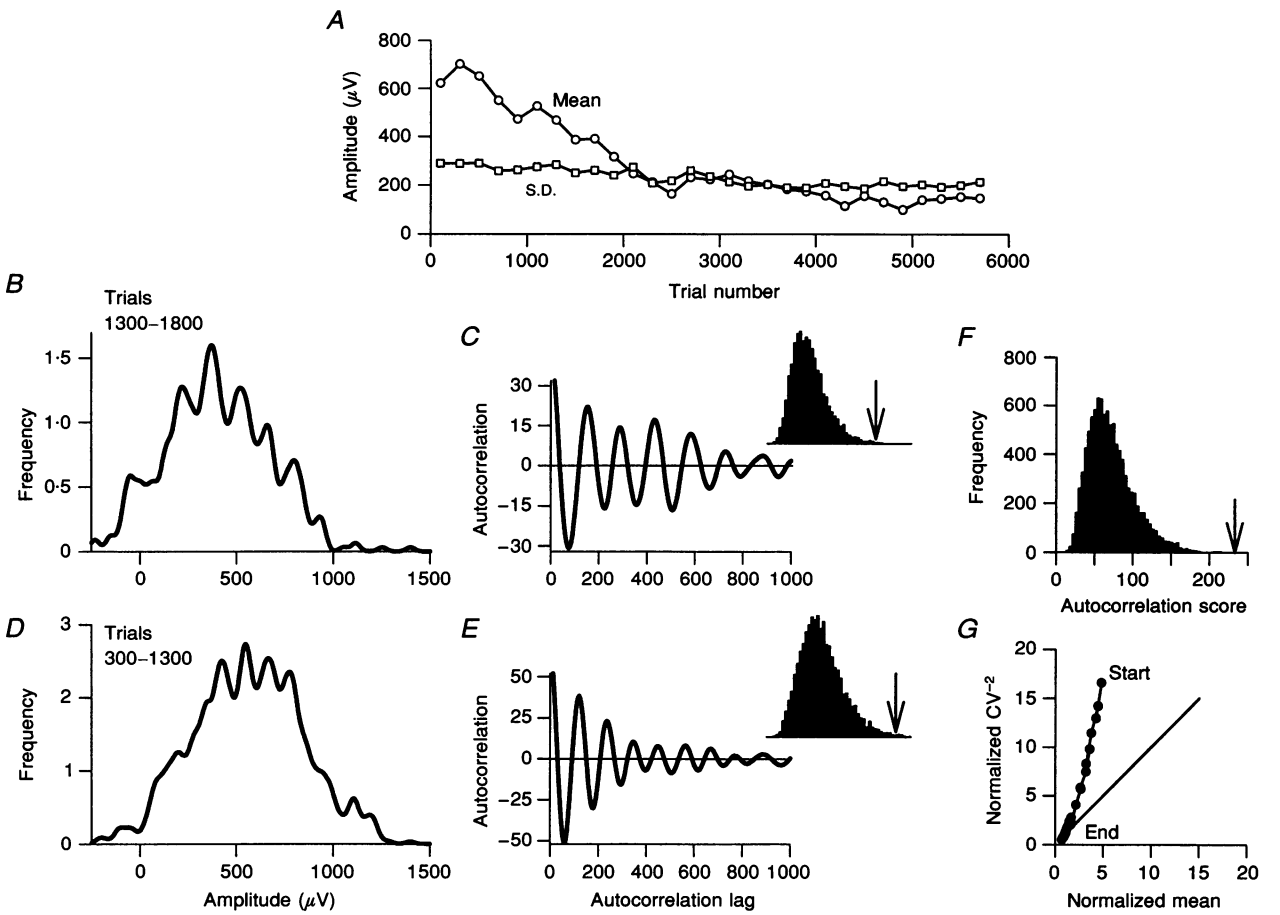

Figure 4. AC scoring for histograms from EPSP 3

A, time course of EPSP mean and S.D. *B*, amplitude histogram for trials 1300–1800. *C*, AC function for trials 1300–1800. The first peak is at 150 μV, and gives an AC score of 52·9. Inset, distribution of AC scores for 10 000 random samples, each of 500 trials, drawn from a smooth distribution fitted to the data histogram in *B*. One in 384 of the random samples scored higher than the data (arrowed). *D*, amplitude histogram for trials 300–1300. *E*, AC function for trials 300–1300. Inset, distribution of AC scores for random samples taken from a unimodal distribution corresponding to trials 300–1300. One in 262 of the random samples scored higher than the data histogram. *F*, distribution of AC scores for random samples taken from a unimodal distribution corresponding to trials 1–1800. None of the 10 000 random samples tested gave scores as high as the data histogram (arrowed). *G*, graph of CV^{-2} against mean. The trajectory is steeper than the diagonal throughout, suggesting that depression is due mainly to changes in the number of quanta released per trial.

EPSP 5 (AH23). Graphs of CV^{-2} against mean proved to be a useful guide for the selection of data likely to yield peaky amplitude histograms when the EPSP mean and S.D. showed a clear trend. However, they were of little use if the mean and S.D. showed irregularities rather than a clear trend. EPSP 5 was evoked for 2000 trials at 5 Hz, and its mean amplitude showed instability over time but only a modest overall depression (Fig. 6*A*). All 2000 trials yielded a histogram with regular but small peaks which the AC scoring procedure (Fig. 6*B*) suggested were of only borderline reliability (likelihood of 1 in 29). By trial and error, we found that if the first 1000 trials were discarded, a much clearer histogram was obtained which gave a likelihood of 1 in 1428 (Fig. 6*C*). It is possible that there was some instability of quantal size during the first 1000 trials that reduced the clarity of the peaks when those trials were included.

EPSP 6 (AH38). Four thousand trials were evoked at 5 Hz, followed by a further 2000 trials at 2 Hz. The EPSP mean depressed at the higher stimulation rate, then showed partial recovery at 2 Hz (Fig. 7*A*). The CV^{-2} graph was complex and difficult to interpret (not shown). One spell of apparent stability during 5 Hz stimulation (trials 2300–3300) produced a histogram with clear peaks (Fig. 7*B* and *C*). The first peak in the AC function was at 140 μV (Fig. 7*D*) and it gave a very high AC score of 561·0. This score was not even approached by any of the 10 000 random samples that we tested initially (Fig. 7*E*), so we went on to test a further 350 000 samples, and none of these gave scores as high as the data histogram.

The subsequent trials evoked at 5 Hz also produced a peaky histogram (trials 3301–4000; Fig. 8*A*), but in this case the peak separation was less and the peak in the AC function was at 115 μV (Fig. 8*B*). This was also very unlikely to have arisen by random sampling (likelihood 1 in 208; Fig. 8*C*). Thus there is clear evidence of a decrease in peak separation, and hence presumably in quantal size, during this period of depression of the EPSP mean. If the two periods of data were combined (trials 2300–4000) the resulting histogram showed peaks that were neither very clear nor equally spaced (Fig. 8*D*). AC scoring indicated that it was not unlikely that such peaks could have arisen by chance (likelihood 1 in 11; Fig. 8*E* and *F*). This EPSP gives a clear illustration of the way in which changes in quantal size can obscure peaks in amplitude histograms and make data selection essential if the underlying quantal behaviour is to be revealed.

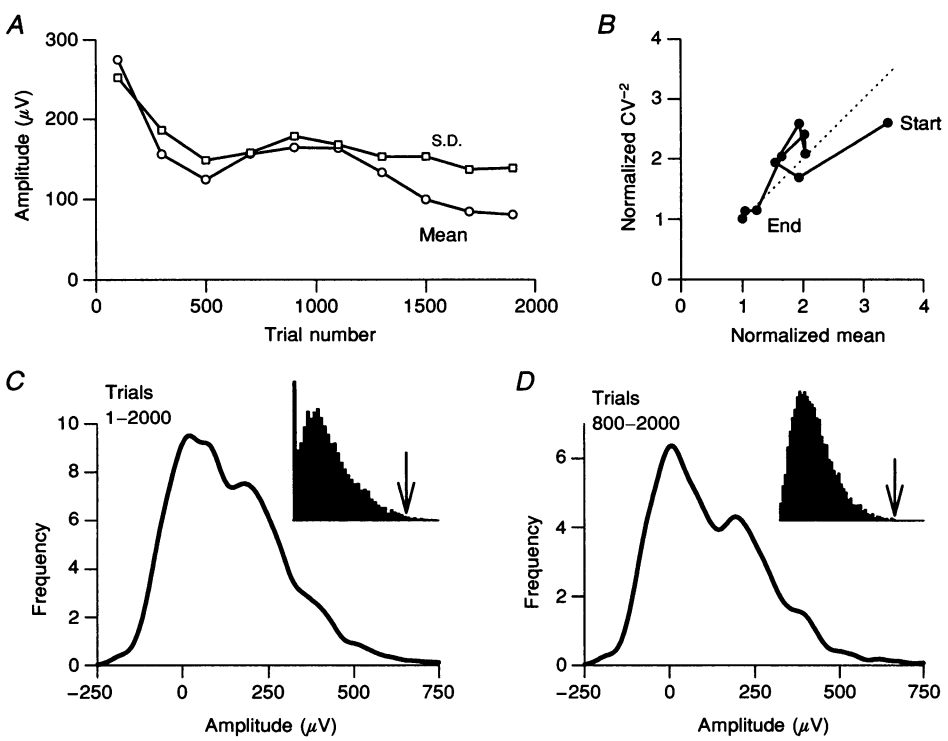

Figure 5. AC scoring for histograms from EPSP 4

A, time course of EPSP mean and S.D., showing initial depression. *B*, graph of CV^{-2} against mean, calculated in blocks of 250 trials. The trajectory is complex, but is less steep than the diagonal for the first 750 or so trials, suggesting a reduction in quantal size during this period. *C*, amplitude histogram for all 2000 trials. This histogram gave an AC score of 540·7. Inset, this score (arrowed) was exceeded by 1 in 114 random samples. *D*, amplitude histogram for trials 800–2000, showing a clearer pattern of peaks than in *C*, and giving an AC score of 487·3. Inset, only 1 in 2000 random samples exceeded the score of the data histogram (arrowed). The exclusion of the first 800 trials has improved the sharpness and reliability of the histogram peaks.

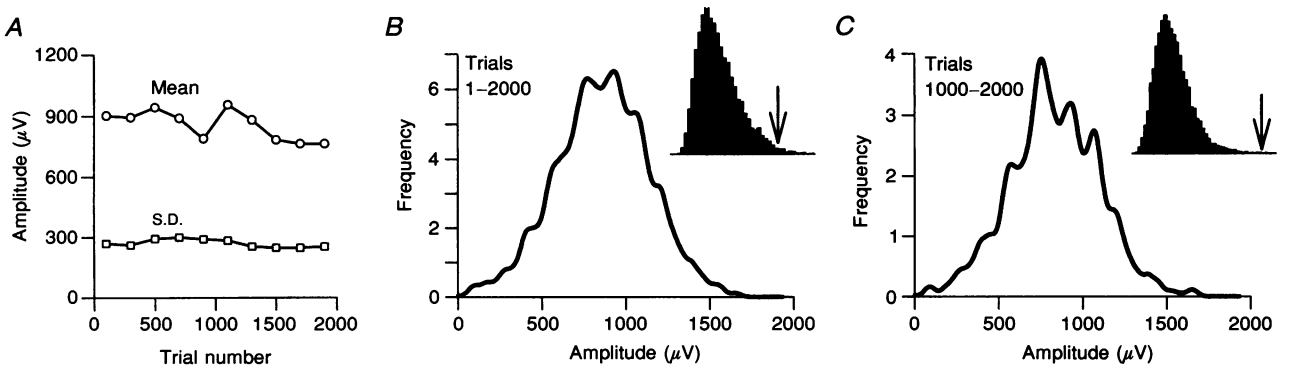

Figure 6. AC scoring for histograms from EPSP 5

A, time course of EPSP mean and S.D., showing instability rather than a clear trend in the mean. *B*, histogram for all 2000 trials, showing a pattern of small peaks and troughs. Inset, random samples from a unimodal distribution of similar shape exceed the AC score for this histogram on 1 in 29 occasions. *C*, histogram for trials 1000–2000. This histogram shows sharper peaks, and, in spite of the reduction in sample size, only 1 in 1428 random samples from a unimodal distribution exceed its AC score (inset). This histogram was shown in Figs 1a–c of Larkman *et al.* (1991).

Summary of other histograms

The results of the AC scoring procedure for all thirty-three histograms are summarized in Table 1. For five EPSPs, inclusion of all the trials recorded resulted in histograms that scored 1 in 20 or better. In the other cases, the exclusion of segments of data led to the finding of histograms with clear peaks, or improved the sharpness of the peaks. For many EPSPs, exclusion of trials close to the beginning and close to the end of the recording period was effective in producing peaky histograms. For some EPSPs,

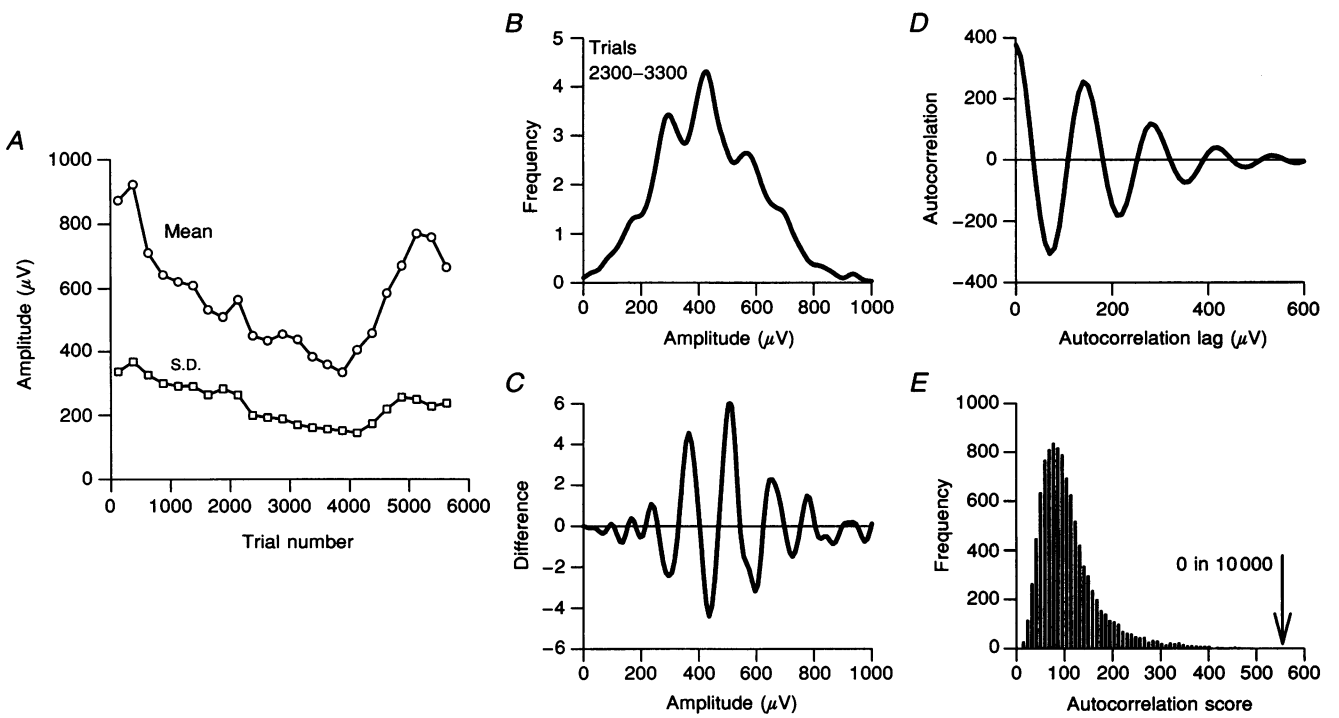

Figure 7. AC scoring for EPSP 6

A, time course of the EPSP mean and S.D. This EPSP was evoked for 4000 trials at 5 Hz, then the stimulation rate was reduced to 2 Hz for a further 2000 trials. The EPSP mean and S.D. showed depression at 5 Hz, then partially recovered at the lower rate. *B*, amplitude histogram for trials 2300–3300. *C*, difference function obtained by subtracting a unimodal distribution from the histogram in *B*. *D*, AC function of the difference function in *C*. This shows a classic damped sinusoid shape. The first peak is located at 140 μV and gave an AC score of 561·0. *E*, distribution of AC scores for 10 000 random samples, each of 1000 trials, taken from a unimodal distribution fitted to the histogram in *B*. None of the random samples approached the score of the data histogram (arrowed).

we analysed two or more histograms where one used a subset of the trials used for the other. In every case, the smaller subset gave a histogram showing a clearer pattern of peaks as judged by eye. However, in some cases, the larger subset was more reliable by AC scoring (e.g. AH3, AH39 and AH80). For seven histograms, none of the 10000 or more random samples that we tested scored higher than the data histogram.

Estimating quantal size by AC

The location of the first peak (which, in our sample, was invariably also the largest peak) in the AC function gives a measure of the mean peak spacing in the histogram. If the peaks have been generated by a quantal transmission process, this spacing will generally correspond to the mean quantal size. The AC scoring method can thus provide estimates of the mean quantal size that do not require any assumptions concerning the statistics of the release process. The AC peak locations for the thirty-three histograms analysed are shown in Table 2, and ranged from 93 to 285 μV (mean, 172 $\pm$ 47 μV).

From the quantal size and the mean EPSP amplitude, it is straightforward to calculate the mean number of quanta released per trial (m) for each histogram, also shown in Table 2. These ranged from 0·36 to 6·9 (mean 3·3 $\pm$ 1·67). Note that only one EPSP (AH46) gave $m < 1$.

DISCUSSION

In this paper, we have addressed the issue of whether it is likely that the approximately equally spaced peaks that we have observed in frequency histograms of evoked EPSP amplitudes have arisen by sampling artifact from underlying distributions that were smooth. We have devised a procedure to quantify the sharpness and equality of spacing of peaks in amplitude distributions, and used this to compare data histograms with random samples drawn from smooth distributions of similar overall shape. We adopted this approach because we felt that conventional statistical tests used to compare distributions, such as the χ^2 or Kolmogorov–Smirnov goodness of fit tests, did not provide sufficient discrimination. This point is considered in detail in a separate paper (Stratford *et al.* 1997), and the conclusion from that study is that AC scoring does indeed offer an important improvement in sensitivity over conventional tests.

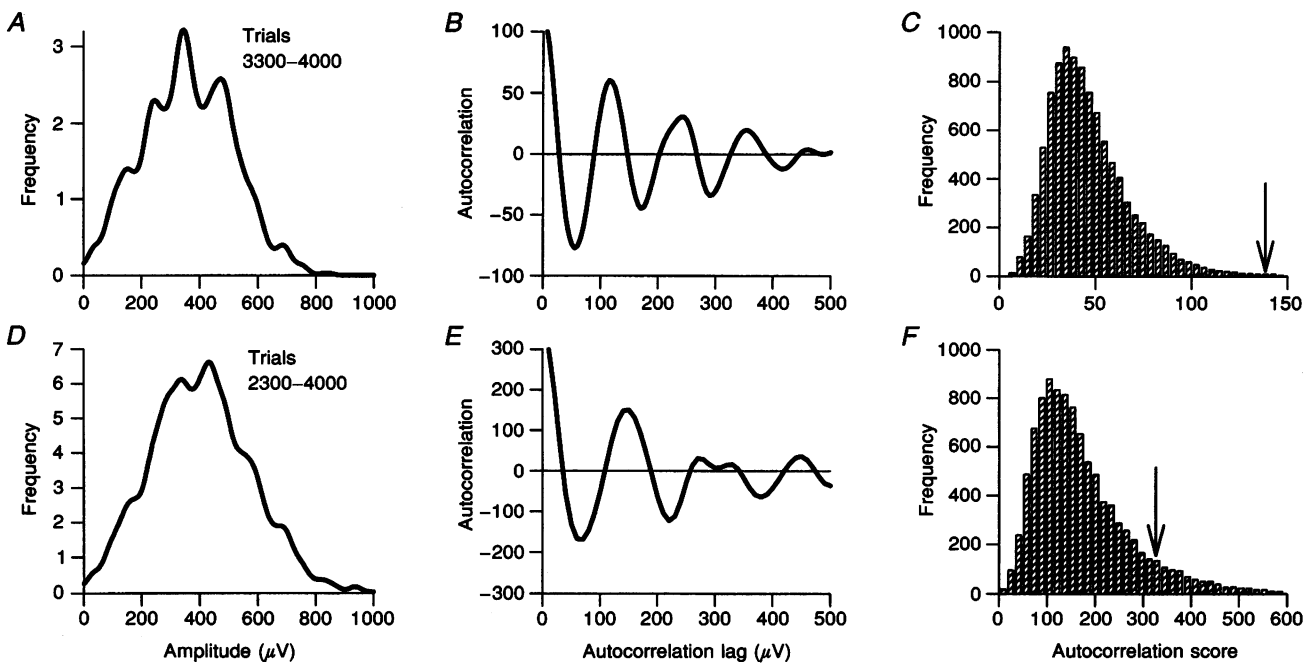

Figure 8. Analysis of EPSP 6 continued

A, amplitude histogram for trials 3301–4000. This shows the same number of peaks as the histogram for the preceding 1000 trials (see Fig. 7*B*), but the peak spacing is reduced. *B*, AC function obtained for the histogram in *A*. The first peak is at 115 μV (compared with 140 μV for the preceding 1000 trials shown in Fig. 7*C*) and gives an AC score of 138·0. *C*, distribution of AC scores for random samples from a unimodal distribution fitted to the histogram in *A*. One in 192 random samples exceeded the score of the data histogram (arrowed). *D*, amplitude histogram for trials 2300–4000, in other words when the histograms in Figs 8*A* and 7*C* are combined. It still shows peaks, but they are less clear than in either of the component histograms separately. *E*, AC function for histogram in *D*, with a first peak at 150 μV and an AC score of 318·8. *F*, distribution of AC scores for random samples from a unimodal distribution fitted to the histogram in *D*. One in 11 of the random samples gave scores higher than the data histogram. Combining the two histograms, each of which was reliable but which had different peak spacings, has resulted in a histogram that must be rejected as unreliable.

Table 1. Summary of histogram AC scoring

EPSP/ histogram no.	Stimulation rate (Hz)	Trials recorded	Trials used for histogram	No. of trials in histogram	Noise S.D. (μV)	AC likelihood	*P*, AC
AH3/A	5	6000	1–1800	1800	76	0 in 10,000	< 0·0001
/B			300–1300	1000	73	1 in 262	0·004
/C			1300–1800	500	76	1 in 384	0·003
/D			4700–5700	1000	87	1 in 81	0·012
AH4	5	2000	800–1300	500	79	1 in 333	0·003
AH6	5	4000	500–1700	1200	175	1 in 41	0·024
AH7	5	1000	100–700	600	57	1 in 142	0·007
AH14	5	2000	900–1500	600	131	1 in 250	0·004
AH15	5	4000	800–2000	1200	155	1 in 769	0·001
AH23/A	5	2000	1–2000	2000	116	1 in 29	0·034
/B			1000–2000	1000	110	1 in 1428	0·001
AH24	5	2000	1000–2000	1000	90	0 in 10,000	< 0·0001
AH29	5	4000	1–4000	4000	188	1 in 322	0·003
AH32	3	2000	1300–1900	600	92	1 in 5,000	0·001
AH33/A	3	4000	1300–3800	2500	71	1 in 2000	0·001
/B			1500–2500	1000	69	0 in 10,000	< 0·0001
AH35	3	2000	800–1300	500	165	1 in 90	0·011
AH38/A	5	4000	2300–3300	1000	82	0 in 350·000	< 0·00001
/B			3301–4000	700	76	1 in 192	0·005
/C	2	2000	4800–6000	1200	91	1 in 40	0·025
AH39/A	5	8000	1400–2800	1400	63	1 in 125	0·008
/B			500–3000	2500	65	0 in 10,000	< 0·0001
AH43	5	6000	2300–3300	1000	98	1 in 110	0·009
AH46/A	5	2000	1–2000	2000	64	1 in 114	0·007
/B			800–2000	1200	64	1 in 2000	0·001
AH47/A	3	6000	600–1600	1000	123	1 in 49	0·020
/B			3700–4700	1000	158	0 in 10,000	< 0·0001
/C			3700–5700	2000	151	1 in 357	0·003
AH55	2	6000	1500–4500	3000	87	1 in 100	0·010
AH80/A	1	4000	1200–1800	600	71	1 in 357	0·002
/B			500–2500	2000	73	0 in 10,000	< 0·0001
AH91	0·1	500	1–500	500	72	1 in 26	0·037
AH93	0·1	250	1–250	250	77	1 in 454	0·002

Summary of results of AC scoring for sample of 33 amplitude histograms. The 2 right-hand columns show the proportion of random samples that gave AC scores higher than the data histograms, expressed as a '1 in' likelihood (AC likelihood) or as a probability (*P*, AC).

Data selection

For all but five of our EPSPs, some degree of data selection was required to produce histograms with clear peaks. In many cases, selection could be performed using external criteria such as non-stationarity of the mean or S.D., or the trajectory of the CV^{-2} graph. However, in some cases the selection was performed solely on the basis of apparent peak sharpness. For these, the reliability of the histogram will have been reduced, and the likelihood obtained by AC scoring should be adjusted by some factor that takes into account the number of extra selections that were considered before the final selection was made. However, the appropriate factor is not easy to determine. One way of exploring the upper limit for the number of selections available is to consider the data set as a whole. In the course of this study, a very large number of trials (approximately 340 000) were recorded of sufficient technical quality to warrant analysis. If we had selected for histograms by sliding through the entire data set in jumps of, say, 100 trials, we could have made about 3400 selection attempts. However, the data were broken up by 118 'break points', caused by recording from a different cell or by a change in stimulus strength, rate, or location, across which a histogram could not span. For a 1000-trial histogram, each break point represents the loss of ten

Table 2. AC peak locations

EPSP/ histogram no.	AC peak location (μV)	Mean EPSP amplitude (μV)	m
AH3/A	135	710	5·3
/B	120	740	6·2
/C	150	582	3·9
/D	130	313	2·4
AH4	125	276	2·2
AH6	170	270	1·6
AH7	93	102	1·1
AH14	215	803	3·7
AH15	190	845	4·5
AH23/A	165	857	5·2
/B	170	830	4·9
AH24	125	149	1·2
AH29	168	875	5·2
AH32	185	917	5·0
AH33/A	150	746	5·0
/B	150	690	4·6
AH35	220	940	4·3
AH38/A	140	440	3·1
/B	115	356	3·1
/C	210	717	3·4
AH39/A	120	828	6·9
/B	120	792	6·6
AH43	170	743	4·4
AH46/A	230	104	0·45
/B	240	86	0·36
AH47/A	190	404	2·1
/B	210	278	1·3
/C	190	259	1·4
AH55	165	794	4·8
AH80/A	145	572	4·0
/B	140	551	3·9
AH91	248	583	2·4
AH93	285	301	1·1

The AC peak location is the location of the first (non-zero lag) peak in the autocorrelation function for each histogram. It is a measure of the dominant peak spacing in the histogram and so provides an estimate of the mean quantal size. When combined with the EPSP mean amplitude for the corresponding period, this allows the mean number of quanta released per trial (m) to be calculated.

possible selections, leaving a maximum of 2220 selections. In fact we found eight histograms of 1000 trials or more that scored 1 in 2500 or better, and seven that scored better than 1 in 10 000. For a 2000-trial histogram, some 2360 selections would be lost, leaving only 1040 possible selections. We recorded four histograms of about 2000 trials that scored better that 1 in 2000. Thus, even on this 'worst-case' view, our data contained histograms of better quality than would be expected to be generated by random sampling. In practice, the number of selections we have actually made was far fewer than calculated above and it is also likely that further peaky histograms await discovery within our data set.

Much of our recorded data did not give amplitude histograms with clear peaks. Stricker *et al.* (1996) list a number of reasons why periods of data might not yield peaky amplitude histograms even if the transmission process is quantal. These include poor signal-to-noise ratio, large quantal variance (either at a single site or between different sites) or non-stationarity of quantal size. We have suggested (Larkman *et al.* 1991) that changes in quantal size over time are an important factor. Here we have shown examples where the exclusion of trials from periods when the CV^{-2} trajectory is shallow, indicating changes in quantal size, leads to improved peakiness. In one example (EPSP 6) we found

histograms taken from subsequent blocks of data during depression of the EPSP mean in which the peak spacing in the second histogram was reduced. This issue is explored in more detail in a separate paper (Larkman *et al.* 1997).

Implications for hippocampal synaptic function

The issue of peaks in synaptic amplitude histograms is an important one, given the current controversy over many aspects of excitatory synaptic transmission in the central nervous system in general and the hippocampus in particular. A number of reports have shown that excitatory spontaneous miniature synaptic events recorded from hippocampal pyramidal neurones show wide variation in size. In some studies, these events could have arisen from any of the excitatory synapses distributed over the dendrites (Manabe *et al.* 1992; Malgaroli & Tsien, 1992). However, in other studies steps were taken to restrict the portion of the dendritic tree from which the miniatures arose (Bekkers & Stevens, 1990; Raastad *et al.* 1992) and in some studies using cells in dispersed culture it is likely that the miniatures were generated at one or only a small number of synapses (Bekkers & Stevens, 1995; Liu & Tsien, 1995). However, these miniatures still showed very large variations in amplitude.

By contrast, several studies of evoked events in acute slices have reported amplitude histograms with several clear peaks (Larkman *et al.* 1991, 1992; Liao *et al.* 1992; Kullmann & Nicoll, 1992; Voronin *et al.* 1992; Kullmann, 1993; Stricker *et al.* 1994, 1996), and several of these studies involved the use of various statistical methods to establish the validity of these peaks. The presence of such peaks is evidence for a quantal release mechanism. In many of these studies, multiple peaks were observed, with no consistent tendency for the peaks to become less clear with increasing quantal content, implying low levels of quantal variability. That probabilistic quantal release does occur at at least some mammalian central synapses has recently been demonstrated directly and convincingly without the need to rely on histogram peaks (Isaacson & Walmsley, 1995). Furthermore, it has recently been reported from the visual cortex that some evoked EPSP amplitude distributions show little more variance than expected from the contaminating noise (Volgushev, Voronin, Chistiakova, Artola & Singer, 1995; Stratford, Tarczy-Hornoch, Martin, Bannister & Jack, 1996). Unless the number of quanta released per trial is very large, which seems unlikely, these results provide strong evidence for low quantal variance, without the need for reliance on the analysis of histogram peaks. Thus there seems little doubt that central excitatory synapses are at least capable of displaying quantal behaviour with low quantal variance. In this study, we have been able to demonstrate that it is extremely unlikely that the peaks we have observed in frequency histograms of evoked synaptic amplitudes arose by sampling artifact. In view of the preceding argument, it seems reasonable to assume that the peaks resulted from some form of quantal transmission process. Some of our apparently reliable histograms showed multiple peaks, so it is likely that the levels of quantal variance associated with this process must have been low.

At the vertebrate neuromuscular junction, quantal variance is of the order of 30% (expressed as CV of the mean quantal size; Fatt & Katz, 1952), and most of this is likely to be due to variations in size and filling between synaptic vesicles. It has been suggested that at some central synapses quantal variance may be lower than this because the number of postsynaptic receptors available is small relative to the number of transmitter molecules released from a vesicle (Jack *et al.* 1981; Jack, Larkman, Major & Stratford, 1994; Tang, Margulis, Shi & Fielding, 1994). Thus most of the receptors become occupied and the quantal size is primarily determined by the number and properties of the receptors present at the synapse. Variations in the number of transmitter molecules released per quantal event would, in this case, have only a minor effect on the quantal size (reviewed by Clements, 1996; Frerking & Wilson, 1996). If something close to postsynaptic receptor saturation does occur, a lower limit for quantal variance at a given release site would be set by the stochastic nature of receptor channel opening, which in turn will depend on the number of channels responding and their open probability. Kullmann (1993) estimated that the opening of fewer than twenty channels could account for the quantal current at CA1 synapses, implying a minimum quantal coefficient of variation of about 15%. However, taking the single-channel conductance to be 8 pS (Jonas & Sakmann, 1992), we have estimated that a quantal EPSP of 130 μV peak amplitude, as measured at the soma, is likely to be due to the opening of roughly 100–150 channels in these cells (Jack *et al.* 1994). This range is very much in line with the estimates of Stricker *et al.* (1996), and suggests that if the open probability of the channels is about 0·7, the lower limit for quantal variability at a given site could be as low as 5–7%. The level of quantal variance shown by an individual release site will depend on many factors, including the number, open probability and kinetics of the functional postsynaptic receptors available, and there is no reason to believe that all central synapses will behave similarly in this respect. It remains to be determined why hippocampal synapses in culture often (Bekkers & Stevens, 1995; Liu & Tsien, 1995), but not always (Tang *et al.* 1994), show high quantal variability.

Our AC method also provides estimates of quantal size that require no assumptions about transmitter release statistics and which are robust to the bin widths and filter settings used (Stratford *et al.* 1997). The AC method tends to overestimate quantal sizes by 5–10%, due to distortion of the outermost histogram peaks during the subtraction of a unimodal distribution (Stratford *et al.* 1997). In our opinion, this drawback is minor. The AC estimates of quantal size were generally close to but slightly higher than those we obtained previously by deconvolution (Larkman *et al.* 1991). One exception was AH46, for which our original deconvolution gave a quantal size of 100 μV but AC gave

240 μV. On re-examination, this appears to be a case of overfitting by our early deconvolution method.

Total charge arriving at the soma can be used as a measure of the size of a synaptic event instead of peak amplitude, and is less sensitive to errors introduced by electrotonic attenuation and poor space clamp. It also permits comparison between sharp-electrode and whole-cell patch-clamp studies. We calculated that a quantal peak amplitude of 130 μV corresponds to a total charge of approximately 80 fC (Larkman *et al.* 1991). The mean peak amplitude of 170 μV from the present study corresponds to ~100 fC. These are very much in line with results from spontaneous miniature events, either quoted (Bekkers & Stevens, 1990, 50–100 fC; Goda & Stevens, 1994, 100 fC) or estimated by measurement from published figures (Manabe *et al.* 1992, 90–120 fC; Raastad *et al.* 1992, 70–120 fC; Oliet, Malenka & Nicoll, 1996, ~100 fC). They are also similar to quantal amplitudes obtained from evoked events (Voronin *et al.* 1992, 120 μV; Liao *et al.* 1992, 50–70 fC; Stevens & Wang, 1994, 50–90 fC; Bolshakov & Siegelbaum, 1995, 80–100 fC; Stricker *et al.* 1996, ~70 fC) but slightly higher than those obtained by Kullmann & Nicoll (1992) and Kullmann (1993) of ~50 fC.

From our quantal size estimates, we calculated m, the mean number of quanta released per trial, which ranged from 0·36 to 6·6, with a mean of 3·3. These are slightly higher than the ranges obtained by Liao *et al.* (1992; range 0·1–5·2, mean 2·2) and Stricker *et al.* (1996; range 0·8–3·1, mean 1·8) and are qualitatively consistent with the multipeaked histograms reported by Kullmann & Nicoll (1992). However, these m values are not in line with other studies concluding that usually a mean of less than one quantum is released per trial (Raastad *et al.* 1992; Stevens & Wang, 1994; Bolshakov & Siegelbaum, 1995). Indeed, one view of hippocampal circuitry is that most axons make only a single contact onto any CA1 pyramid (Raastad *et al.* 1992), and the probability of transmitter release at the majority of these contacts is low, at about 0·06 (Hessler, Shirke & Malinow, 1993). On this basis, most fibres would have $m \approx 0{\cdot}06$. Such an arrangement would have profound implications for neuronal processing, requiring the brain to use designs that produce reliable results from unreliable communication links (Stevens & Wang, 1994). However, it is difficult to reconcile our data with this view of hippocampal connectivity. Although with minimal stimulation one can never be sure that only a single afferent axon is stimulated, it seems unlikely that any of our histograms were the result of stimulating fifty or more axons. These axons would need to generate quanta of similar size for the histograms to show peaks, and such an arrangement would produce histograms with pronounced positive skew, which was not the case. It seems more likely to us that our histograms arose from fibres each contacting the postsynaptic cell via multiple release sites with a moderate probability of release. How can different laboratories produce such disparate data? Some obvious possibilities, such as the age of the animal and the temperature of the preparation, have been explored but rejected (Allen & Stevens, 1994). Another factor may be acceptance and selection of data sets. Our sample is likely to be biased against low m connections. A single release site connection with a release probability of 0·06 will fail to release transmitter on more than 90% of trials, and so could easily be overlooked as we adjusted the location and strength of our stimuli. Conversely, Stevens & Wang (1994) selected in favour of low m connections, since stimuli were only accepted as minimal if they resulted in more than 50% failures. Whatever the explanation, our data strongly point to the existence of at least a subset of connections in CA1 that show low quantal variance and that are neither as weak nor as unreliable as has been proposed.

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