

EXAMINING THE TIMING OF MEPP RELEASE AT THE  
AT THE FROG AND  
MOUSE NEUROMUSCULAR JUNCTIONS  
FOR DEVIATIONS FROM RANDOM EXPECTATIONS

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## Summary

The intervals between miniature endplate potentials (MEPPs) were measured from recordings made at the frog and the mouse neuromuscular junctions in normal solutions and in solutions with elevated concentrations of  $\text{Ca}^{2+}$  or  $\text{Sr}^{2+}$ . Data sets with monotonic trends in MEPP frequency were discarded. The data sets studied had between 283 and 5024 MEPPs. The fit to the exponential distribution, which describes a random process, was tested by using Sherman's statistic, a robust method with an added advantage. If the intervals are not distributed exponentially it indicates whether they are more clustered or more diffuse. In 6 of 20 data sets from the frog and in 3 of 7 data sets from the mouse the intervals between MEPPs were not distributed exponentially. In some of the non-random sets the intervals were more concentrated than expected for an exponential distribution, in the remainder they were more diffuse. In 1 frog data set MEPP release appeared to be periodic. In all data sets releases appeared to be independent, judging from the integrated power spectra. One data set may show fractal behavior. Calculations of the approximate entropy gave no indication of an underlying regularity, so there is no evidence for an underlying regularity. The lack of fit to the exponential distribution due to either concentrated or diffuse interval distributions is mimicked by a model in which release is Poisson, but with a mean rate that randomly shifts to a different level.

Key words: quantal release, vesicular exocytosis, miniature endplate currents, fractal, chaos, random, exponential distribution.

## INTRODUCTION

After they discovered miniature endplate potentials (MEPPs) at the neuromuscular junction, Fatt & Katz (1952) measured the intervals between 800 MEPPs. They sorted the intervals into length bins and plotted a histogram of the number in each bin. The largest number of MEPPs fell into the shortest time bin; as the length of the intervals increased the numbers in the bins declined exponentially, like the intervals between emissions from a radioactive source. They concluded that spontaneous release probably is random (Fatt & Katz, 1952). Several groups have supported the interpretation that releases are distributed exponentially (Gage & Hubbard, 1965; Cappell et al., 1988; Yana et al., 1984), and that release is a Poisson process.

Others conclude that there is a periodic component in the timing of the releases, especially in the periods following evoked releases (Erulkar & Rahamimoff, 1978; Rahamimoff et al., 1978; Pawson & Grinnell, 1989). Periodicity might be anticipated because the  $[Ca^{2+}]$  in the motor nerve terminal oscillates, and the frequency of MEPP release should follow the  $[Ca^{2+}]$  in the terminal (Melamed et al., 1993; Rahamimoff & Melamed, 1993).

Some groups examined the fit of the intervals between MEPPs to the exponential distribution with more sensitive statistical tests (Hubbard & Jones, 1973; Cohen et al. 1974a, 1974b, 1974c; Rees, 1974; Bennett & Pettigrew, 1975; Bornstein, 1978; Velussi & Danieli Betto, 1979; Washio & Inouye, 1980). They found that some data sets deviate significantly from the exponential prediction and that releases are not

independent of one another (Cohen, *et al.*, 1974a, 1974b, 1974c; Bennett & Petigrew, 1975; Jonsson, 1981; Bennett & Robinson, 1990). There is increasing speculation that quantal release also is non-independent at central synapses (for example, Perkel & Nicoll, 1993; Murthy *et al.*, 1997).

Nonlinear dynamics shows that deterministic processes can produce data with the surface appearance of randomness (for a physiological introduction see Basingthwaite *et al.*, 1994). Such processes are often loosely termed chaotic or fractal. MEPP release might be chaotic (Glass & Mackey, 1988). Recent measurements on *Xenopus* myocytes and hippocampal synapses in culture suggested that the relative variance of the intervals—the Allan factor—increased as a power-law function of the counting time (Lowen *et al.*, 1997). The interpretation is that spontaneous quantal release exhibits fractal behavior.

Obviously, the conclusions from different groups conflict. To reach a decision among these possibilities, we recorded long sets of MEPP intervals, analyzed only sets without monotonic trends in frequency with the best methods available, and determined confidence limits for all of the measurements. Our data sets rarely displayed periodic or fractal behavior. The intervals were independent of one another. Almost half of the sets deviated from the exponential distribution. The results fit a model in which release is random and that the mean rate of release occasionally randomly changes.

## METHODS

### Data recording

Animals were sacrificed by procedures approved by the Animal Users Committee of the State University of New York at Stony Brook. Sartorius muscles were isolated from double-pithed *Rana pipiens*. The Ringer contained (in mM): 120 NaCl, 2 KCl, 2.5 CaCl<sub>2</sub>, 4 TES (N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid) at pH 7.4. We used other recording solutions, which have been reported to favor non-random releases (reviewed by Van der Kloot and Molgó, 1994). These solutions contained the same constituents except for variations in the concentrations of NaCl and/or CaCl<sub>2</sub>, or the substitution of Sr<sup>2+</sup> for Ca<sup>2+</sup>. In some experiments the [NaCl] was raised to 140 mM to increase the rate of MEPP release (Fatt & Katz, 1952). These changes in the solutions will be specified when the data is reported. The muscles were kept in each solution for at least two hours before recording was begun, except for some of the experiments with elevated NaCl in which additional dissolved salt was added to the solution in the experimental chamber, recording was started after a delay of 30 minutes. Temperature was controlled with a Peltier plate under the experimental chamber, some data sets were recorded below room temperature, to see whether this favored non-random behavior.

The diaphragms were dissected from Sprague Dawley mice after cervical dislocation. Mouse muscles were kept in Tyrode's solution, which contained (in mM): 135 NaCl, 5 KCl, 3 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 1 NaH<sub>2</sub>PO<sub>4</sub>, 5 NaHCO<sub>3</sub>, 11 glucose, gassed with 95% O<sub>2</sub> - 5% CO<sub>2</sub>. Recording was at room temperature.

MEPPs were recorded with an intracellular electrode. The voltage recording was digitized at 1000 Hz and stored on disk. Later the operator used a cursor to locate the time of occurrence of the peak of each MEPP, which is designated as  $t_i$ . The length of each data set is termed  $t_0$ . Each data set contains  $N$  intervals. The intervals between MEPPs are termed  $x_1 \dots x_N$ .

An important decision in planning the experiments was whether or not to also record spontaneous quantal releases with an extracellular electrode positioned near the nerve terminal, hoping to record releases from a highly restricted portion of the terminal. We decide not to do this for two major reasons. At the frog neuromuscular junction, extracellular electrodes commonly record a significant fraction of MEPPs recorded with an intracellular electrode (reviewed by Van der Kloot and Molgó, 1994; Van der Kloot, 1996a, 1996b). Apparently, the equivalent circuit for extracellular recording in this preparation is more complicated than one would expect. The amplitudes of the externally recorded signals usually grade from the maximum value down into the noise. Therefore, to measure the occurrences of spontaneous releases it is necessary to set an arbitrary amplitude threshold, this raises serious concerns about the validity of the data.

## Statistical methods

Some of the statistical methods used were discussed in detail, and examples given of their use, by Van der Kloot *et al.* (1975). The first two tests were used to determine whether the data sets were stationary over time.

*Slope (regression analysis for trends;* Cox & Lewis, 1966). The observation period was divided into a series of 500 ms or 1000 ms bins and the number of MEPPs in each bin was counted. A regression line was calculated by least squares between the time since the beginning of the set and the ln of the number of intervals in each bin (the number zero was given the value 0.01 for the calculations). The reason for using the ln's is discussed by Cox & Lewis (1966). The probability that the slope of the regression line deviated significantly from 0 was determined (Sokal & Rohlf, 1981, pp 469-477).

*μ test for monotonic trends in a Poisson process* (Cox & Lewis, 1966, pp 45-49).

$$\mu = \frac{-t_o / 2 + \sum t_i / N}{t_o (N / 12)^{1/2}}$$

The  $\mu$  statistic is normally distributed with a mean of 0 and a variance of 1. A numerical method was used to determine the probability the values of  $\mu$  calculated from the data sets deviated from the mean by chance. The same method was used for the other statistics that were normally distributed.

*Sherman' statistic* was used to determine whether the intervals between the MEPPs in stationary data sets fit to an exponential distribution (Sherman, 1950; Cox & Lewis, 1966). The mean interval is termed  $\bar{x}$ .

$$\bar{\omega}_n = \sum_{i=1}^N \frac{|X_i - \bar{x}|}{2(n+1)\bar{x}}$$

For an exponential data set the expected mean  $\bar{\omega}_n$  is  $1/e$  and the variance is  $0.059/N - 0.071/N^2 + \theta(1/N^3)$ , where  $\theta$  is a remainder function. Sherman's statistic is especially useful because if there is a significant deviation from  $1/e$ , the direction of the deviation tells something about the nature of the empirical data set. If  $\bar{\omega}_n$  is significantly lower than  $1/e$ , the data contain an excess of intervals near the mean interval length and a deficit of intervals at extremely long or short lengths. Such a set is said to be "concentrated" (Van der Kloot et al., 1975). When  $\bar{\omega}_n$  is significantly greater than  $1/e$  the set has an excess of very long and/or very short intervals, with a deficit of intervals near the mean. Such a set is called "diffuse".

The following tests were used to test for non-independence of events.

Autocorrelation coefficients (Cox & Lewis, 1966). To test for independence the autocorrelation coefficients,  $r_k$ , were calculated

$$r_k = \frac{\sum (x_i - \bar{x}_1) \cdot (x_{i+k} - \bar{x}_2)}{(\sum (x_i - \bar{x}_1)^2 \cdot \sum (x_{i+k} - \bar{x}_2)^2)^{1/2}}$$

where  $\bar{x}_1$  is the mean of the first (N-k) data points and  $\bar{x}_2$  is the mean of the last N-k data points. The summations run from i=1 to N-k, k is the lag between the data points that is being examined. The confidence limits at the x% level are

$$r_x = \frac{t_x}{(t_x^2 + N - 2)^{1/2}}$$

where N is the total number of data points,  $r_x$  is the value of the autocorrelation at the x% level of confidence and t is Student's t. Autocorrelations were calculated for the intervals themselves, to see whether one release alters the probability of succeeding releases. They were also calculated for binned data, in which the number of MEPPs in successive time bins was counted. These autocorrelations would detect periodicity in MEPP release.

*Power spectra.* The power spectrum is the Fourier transform of the autocorrelations. The first 32 autocorrelations were transformed using an FFT algorithm (Press et al., 1989). If the sequence is random, the power in the spectrum should be evenly distributed over the entire frequency range. To test for significant deviations from randomness, the integrated spectrum was calculated by summing the values from 0 to the highest frequency. These summed values were then normalized and plotted as a function of frequency. The expectation for random data is that the points will fall along a line extending from (0,0) to (1, maximum frequency). The goodness of fit of the data points to the line was tested with the Kolmogorov-Smirnov test (Jenkins & Watts, 1968, p. 234-237). This method was used because the points on the spectrum are not independent, so it is not valid to test whether a point that appears to show a peak is statistically different from adjacent points.

*Approximate entropy.* A number of methods have been proposed for detecting an underlying deterministic process in a time series (Aitken et al., 1995). The characteristic of chaotic systems is that over time there is regularity in the data, which on surface examination appears random. Considerable effort has been devoted to developing graphical and computational methods for detecting such regularity. We decided to employ calculations of what is termed approximate entropy (ApEn), a recently developed method for quantifying the regularity in data (Eckmann and Ruelle, 1985; Pincus, 1991; Pincus and Kalman, 1997). This method was selected because detailed explanations of the rationale, the algorithm for the calculation, and a sample computer program with the results of calculations on model data have been published (Pincus et al., 1991). Estimating ApEn requires a series of steps, which will not be repeated here. The number of events in a series of equally spaced time bins is counted. Our calculations were done with the data counted into either 1000 or 2000 bins. Since the number of MEPPs in the sets varies widely, this gave us a considerable range in mean number/bin. Then one must select a value which represents the length of compared runs of data,  $m$ . We will discuss the choice of  $m$  in the section of results. The filtering level,  $r$ , must also be set. If the data is relatively noisy, then  $r$  must be high enough so that the calculations are insensitive to chance variations. Our counts of the number of MEPPs in the time bins has little noise, so we used  $r=0.1$  in the calculations. Increasing  $r$  to 1 did not alter the estimates. Using model data sets in which alternating terms are set to 0 and 1 gives an  $ApEn=0$ . When the values are either 0 or 1 but the sequence is random,  $ApEn=0.69$ . With our data sets, some of the bins contain more than one event, which accounts for values of ApEn above 0.69.

To determine whether the value of ApEn is due to an underlying pattern in the data, we used a Monte Carlo method to estimate the confidence limits for some of the data sets. The position of the bins in the sequence was randomly shuffled 1000 times, using a random number generator that is effectively free of sequential correlation (Press et al., 1989. p 216). The resulting values of ApEn were sorted in ascending order, the 95% confidence limit was taken as the spread between ApEn<sub>25</sub> and ApEn<sub>975</sub>. With other data sets we used 5-10 shuffles, to see whether disrupting the sequence made any marked difference in the values of ApEn from the series.

### **Allan factors**

This method was introduced by Lowen et al. (1997) to detect fractal behavior in sequences of spontaneous quantal releases. A data set with duration L is partitioned into contiguous counting windows, of duration T. The initial T was usually L/1000. The number of MEPPs in each window (k=1.. L/T) is counted, Z<sub>k</sub>(T). The Allan factor for counting time T is then

$$A(T) = \frac{\sum [Z_{k+1}(T) - Z_k(T)]^2}{2 \sum [Z_k(T)]}$$

The calculations are repeated with successively longer values of T, with the upper limit of T being less than L/10. For a Poisson process the predicted value of A(T) for all T's is 1.0. To set confidence limits on the values of A(T), for 1000 repeats the order of the bins was randomly shuffled and A(T) calculated. The confidence limits were estimated from the A(T)'s as described above for ApEn.

## **RESULTS**

Two notable decisions shaped the analysis. First, we analyze only sets without a monotonic trend in MEPP frequency. It has been argued that if every effort is made to keep conditions constant, then every data set should be considered (Van der Kloot et al., 1975). On the other hand, when working with isolated tissue, it is unlikely that homeostasis will always be maintained. Monotonic trends over periods of many minutes are more likely to characterize a pathological than physiological processes. Consequently, each data set was first tested with the slope method for the presence of a monotonic trend. Those exhibiting a trend were rejected. The remaining sets were given the  $\mu$  test; again, those with a trend were rejected. Second, we used Sherman's statistic as the test for the fit to an exponential distribution, rather than the battery of tests used in the past (Van der Kloot et al., 1975). Sherman's statistic is readily calculated, robust and sensitive. As discussed above, if the data deviates from the exponential distribution, the statistic tells whether the intervals are more diffuse or more concentrated.

### **Frog MEPP intervals**

We measured 37 data sets. A significant monotonic trend was found in 17 of these, so they were rejected. The remaining 20 data sets, containing 29,293 intervals are summarized in Table 1. Sherman's statistics that deviate from chance expectations at the 5% level are indicated in bold face (the statistic is two-sided, so the 95% confidence band falls between 0.025 and 0.975). Sherman's statistic shows that in six of the data sets the intervals did not fit an exponential distribution. The direction of the deviations of  $\bar{\omega}_n$  was not uniform. In 4 examples  $\bar{\omega}_n$  was significantly less than the

expected value, so the intervals are concentrated. In 2 examples  $\varpi_n$  was significantly greater than the expected value, so the intervals are diffuse.

[Table 1 near here]

### **Mouse MEPP intervals**

Table 2 summarizes the results of the statistical tests on the intervals between MEPPs recorded at 7 mouse neuromuscular junctions. Two of the mouse data sets showed a monotonic trend in the intervals, so they were not included. In 3 of the sets Sherman's statistic suggests that the deviations from exponential predictions are statistically significant. In 2 of these the deviation is because the intervals are diffuse, while in the others the intervals are clustered.

[Table 2 near here]

### **Periodicity**

The possibility of periodicity was assessed by counting the number of MEPPs occurring in each 1 s period and then calculating the autocorrelations between the numbers in the bins. In 4 of the 20 data sets from the frog two or more of the autocorrelations exceeded the 95% confidence limits. Three of these data sets were recorded in solution containing  $\text{Sr}^{2+}$  (O2,S5,S8) , the fourth was in 12 mM  $\text{Ca}^{2+}$  (T). In  $\text{Sr}^{2+}$  solution bursts of relatively high frequency MEPPs are sometimes observed (Dodge et al., 1969; Cohen et al., 1981).

Fig. 1 shows the further analysis of data set T, which was recorded in 12 mM  $\text{Ca}^{2+}$  solution. Its spectrum has a sharp peak suggesting a period of about 333 s (Fig.

1A). The integrated spectrum clearly deviated markedly from random predictions (Fig. 1B). Fig 1C shows a cumulative plot of the number of MEPPs in 8 s time bins. An almost sinusoidal oscillation in MEPP output can be seen by close examination of this plot. The other three data sets with more than two autocorrelations exceeding the 95% confidence limits were examined in the same manner. Their integrated spectra did not indicate significant deviations from random predictions. Our method also detected a period of about 20 s in one of the data sets not included in Table 1 because it exhibited a trend (N4). These two examples demonstrate that our techniques can detect periodicity in release. Apparently, periodicity is found in a small fraction of the data sets and is probably enhanced by recording under unusual conditions.

[Fig. 1 near here]

In the mouse data sets, the autocorrelations of the events in 1 s time bins and the power spectra did not give any indication of periodic oscillations in frequency (Fig. 2 (old 6)).

[Fig. 2 near here]

## **Independence**

The first step in accessing the independence of spontaneous releases was calculating the autocorrelations of the intervals themselves. We counted the number of the autocorrelations for the first twenty lags that exceed the 95% confidence limits. Four of the 20 frog data sets (A, E, S5, S8) produced autocorrelations of intervals that exceed the 95% confidence limits in 2 or 3 of the first 20 lags. An excess of statistically significant autocorrelations suggests that the events are not independent. It is worth noting that for one of the data sets with 2 and a second with 3

significant autocorrelations the distribution of the intervals were not significantly different from an exponential distribution. The next step was to use the integrated power spectra to evaluate the statistical significance of the distribution of the first 32 autocorrelations, as was done with the binned data discussed above. The integrated spectra did not exceed the 95% confidence limits. An example, for data set y, is shown in Fig. 3. One of the 7 mouse data sets (M1) produced autocorrelations of intervals that exceed the 95% confidence limits in two of the first 20 lags. Again, the integrated spectrum did not exceed the confidence limits. To summarize, in all of the data sets the intervals appear to be independent.

[Fig. 3 near here]

### **Approximate entropy**

The first step was to calculate ApEn for each of the data sets, counted into 1000 bins and using 3 as the value of  $m$ , the length of compared runs of data. For the frog data sets the values of ApEn ranged from 0.79 (F) to 1.73 (a and S2). The magnitude of ApEn increased when the number of MEPPs in the set was larger.

We chose three of the frog data sets for further analysis: A, O2 and T. Table 3 shows the results and the confidence intervals, calculated with  $m=1$ . In example A, the ApEn of the initial, unshuffled data was above the 95% confidence limits, this set will be discussed further below. In the other two sets dealt with comprehensively the value of ApEn was well within the confidence limits. In these three sets, ApEn was calculated for values of  $m$  ranging from 1 to 10. In each set, the value of ApEn went down progressively as  $m$  was increased.

The values of ApEn for the mouse data sets, calculated with  $m=3$ , ranged from 0.41 to 0.48 with  $m=3$ , and from 0.48 to 0.68 when  $m=1$ . The confidence limits for three of the sets are also shown in Table 3.

To estimate of the sensitivity of ApEn to regularity in the data sets, we progressively modified set T, by placing the value 0 in every  $n^{\text{th}}$  bin, if the value was not already zero. The value of ApEn was below the 95% confidence limit when  $n$  was 5 or higher. (With  $n=5$ , 167 of the fifth bins were changed to 0, the remaining 33 bins already contained the value 0). This result, along with similar tests on other sets, suggests that ApEn is not a sensitive test for regularity in MEPP data. Also, recall that set T showed a distinct period in MEPP appearance, which was not indicated in any way by the value of ApEn. This statistic seems to have a low sensitivity for detecting patterns in MEPP appearances.

### **Allan factors**

The Allan factors were calculated for each of the data sets. For those sets in which the value of the Allan factor rose markedly when the bins were lengthened, the confidence limits were calculated by repeating the calculations 1000 times on shuffled data. The curve for the actual data exceeded the confidence limits in only one data set (A) (Fig. 4).

[Fig. 4 near here]

## **DISCUSSION**

Our aim was to analyze comprehensively the timing of MEPP generation, obtaining data sets with many MEPPs and using the most reliable statistical methods

available for their analysis. Many of the data sets showed monotonic trends. A fundamental choice that underlies the data analysis is how to deal with such sets. One possibility is to ignore trends, arguing that they are properties of the physiology of the release system itself (Van der Kloot et al., 1975; Lowen et al., 1997). On the other hand, monotonic trends *in situ* can persist only for limited times, else MEPP release would stop or become so fast that it overwhelms the replenishment system. Therefore, we decide to reject data sets exhibiting a trend, which meant that 41% of the sets were jettisoned. This decision probably accounts for significant differences in interpretation. Some workers have transformed their data to remove trends (Yana et al., 1984), but we thought this might introduce further complications.

### **The exponential distribution**

We used a highly efficient test, Sherman's statistic, which examines all of the intervals, to detect deviations from the exponential distribution. (It is also an easy test to implement). An appreciable fraction of the data sets did not fit the exponential distribution: 30% of the sets from the frog and 41 % of those from the mouse. Some of the sets from the frog were recorded in solutions with elevated  $\text{Ca}^{2+}$ , or with  $\text{Sr}^{2+}$  substituted for  $\text{Ca}^{2+}$ . Deviations from the exponential distribution may be more common in these abnormal solutions, but the total number of sets is too small to justify a firm conclusion on this point.

Sherman's test is especially useful because, if the data do not fit exponential predictions, it indicates whether this is because the data is concentrated or diffuse. From 6 frog sets in which non-exponential distributions were detected, in 4 it was because the intervals are concentrated, and in 2 because the intervals are diffuse. All 7

data sets from the mouse were recorded in normal Tyrode solution. In 2 of the non-exponential sets the intervals were concentrated, in the other 1 they were diffuse.

The conclusion on this point is that in many data sets the intervals do not conform to the exponential distribution, but that the deviations are in one direction, the intervals may be either more concentrated or more diffuse.

### **Periodicity**

One mechanism that would produce a concentration of intervals would be periodicity in the release pattern. We employed a rigorous method for testing for periodicity, examining the integrated power spectra. From all of the frog sets—including these exhibiting a trend—2 appeared to be periodic, in both of these the intervals were also concentrated. One of these sets was recorded in 5 mM  $\text{Sr}^{2+}$  solution, the other was recorded in 12 mM  $\text{Ca}^{2+}$  solution. The infrequent occurrence of periodicity in our data sets is in contrast to some earlier reports in the literature, which found a higher proportion of data sets with periodicity. (Note that periodicity was identified by other workers using methods without the statistical rigor of the integrated spectrum.) Some of these results were at junctions at which the motor nerve had been stimulated tetanically for a period before measurements were begun. The stimulation produces a substantial but transitory increase in the MEPP frequency (Erulkar & Rahamimoff, 1978; Rahamimoff et al., 1978 ; Pawson & Grinnell, 1989). Consequently more MEPPs can be obtained for interval measurement in a relatively short recording period. However, these data sets are almost surely non-stationary, which provides a hurdle for any statistical analysis. This is not the sole explanation for the apparent periodicity however, since periods were also detected in data from resting

preparations, from which release may have been stationary (Pawson & Grinnell, 1989). About 30% of their preparations appear to show periodicity, so it is possible that it occurs in circumstances we have not duplicated.

As mentioned in the Introduction, we might expect periodicity because there appear to be periodic oscillations in the  $[Ca^{2+}]$  in lizard nerve terminals, which would be expected to produce periodic changes in MEPP release (Melamed et al., 1993; Rahamimoff & Melamed, 1993). However, the  $Ca^{2+}$  oscillations are observed in small portions of the extensive motor nerve, and it is by no means clear that oscillations are synchronized along a sufficient length of terminal to produce a detectable period in MEPP frequency.

### **Independence**

In our previous work on MEPP intervals in the frog, we often observed an excess of positive autocorrelations of intervals (Cohen et al., 1974a, 1974b; Van der Kloot et al., 1975). We interpreted the positive autocorrelations as an indication of interactions between releases. Sherman's statistic often indicated that in these sets there was an excess of short intervals. This led us to propose that quantal release is a branching Poisson process, in which the release of a quantum slightly increases the probability of a second release.

In the present paper, we used the integrated spectrum of the autocorrelations of intervals to evaluate independence. By this test, the intervals of all of the accepted data sets are independent. In our earlier work, the data sets were not screened to eliminate monotonic trends. Such trends produce an excess of positive autocorrelations, which would lead to the conclusion that releases are not independent.

## **Chaos and fractal behavior**

Another possible explanation for the deviations from the exponential distribution is that there is an underlying, deterministic process governing MEPP release, in other words, release is chaotic. The independence of the interval durations is one argument against underlying regularity. Another argument is the results of the calculations of approximate entropy, ApEn, whose values in 96% of the data sets were not altered significantly by shuffling the order in which the intervals occurred. Our analysis suggests, however, that ApEn is not an efficient test for regularity in MEPP intervals.

Lowen et al. (1997) proposed that spontaneous quantal release displays fractal behavior. Their data was not screened for monotonic trends, but they argue that their methods are relatively insensitive to trends. They found that the spectrum of the autocorrelations between intervals decreased in a power-law manner with frequency. The Allan factor increases as a power-law function of the counting time. In the present data, in 1 data set from 27, the plot of the Allan factor as a function of counting time exceeded the 95% confidence limits, established by reshuffling the data set. Therefore, by this criterion, our data sets do not exhibit fractal behavior.

## **A model that mimics the results**

The general rule summarizing our results is that in more than half of the sets the intervals are distributed exponentially. Just as expected for a random process. In the remaining sets, the distribution is not exponential, but the deviations from exponential predications are almost equally likely to be due to an excess of short intervals or an excess of long intervals.

A simple model generates set of deviates,  $y$ , with statistical properties like those observed in the data sets. Exponentially distributed random deviates were generated from

$$x_i = -\ln(\text{random} \cdot \lambda)$$

where  $\lambda$  is the rate and random is a random variate between 0 and 1. We modified this model so that the value of  $\lambda$  changes to a new higher or lower value

$$\lambda = (1 + 0.8 \cdot (\text{random} - 0.5))$$

In our model, the changes in the value of  $\lambda$  occur randomly, on average it changes every 50<sup>th</sup> interval. The computer was used to generate sets of 1000 points calculated from this model, and then each set was tested with Sherman's statistic. From 10,000 sets, 5052 fit to the exponential distribution. In 2232 of the sets  $\bar{\omega}_n$  was significantly below the expected value, so in these sets the intervals were concentrated. In the remaining 2716 of the sets  $\bar{\omega}_n$  was significantly above expectation, so the intervals were diffuse.

Manifestly, this model gives results quite like the actual data sets. Therefore, we suggest that the distribution of MEPP intervals can be accounted for by a modest extension of the concept of Fatt & Katz (1952). Namely that releases are random but there also are randomly occurring changes in the mean release rate. Such randomly occurring changes in the release rate might be produced by relatively slight alterations in the  $[Ca^{2+}]$  in parts of the terminal, and are in accord with the truism that physiological parameters are unlikely to be held absolutely constant, but vary about a set point, often in an irregular manner. One of the positive features of our model is

that it does not require consideration of molecular mechanisms for interactions between individual release sites.

Perhaps the most striking data supporting interactions in quantal release was obtained by Barrett et al. (1974). They worked in a solution with decreased  $\text{Ca}^{2+}$  and elevated  $\text{Mg}^{2+}$ , so that evoked quantal output was greatly decreased. They recorded MEPPs and either stimulated the motor nerve as soon as a MEPP was detected or after a delay of many ms. Their analysis suggested that when the nerve was stimulated during MEPP release, evoked output was enhanced. The conclusion was that a spontaneous release enhances the probability of an immediately following evoked release. Data disputing these observations will be presented (Van der Kloot, in preparation).

The methods described above should help in the analysis of intervals between miniatures recorded at synapses in the CNS. However, it would not be a complete surprise if the picture that emerged were somewhat different. The frog motor nerve terminal has hundreds of active zones, each with scores of vesicles available for release. With so many release sites, it is impossible to detect interactions between one site and its immediate neighbors. In synaptic boutons, with far fewer release sites, such interactions might well be revealed.

## References

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example of format:

Aitken PG, Sauer T, Schiff SJ. (1995) Looking for chaos in brain slices. *J. Neurosci. Meth.*, 59: 41-48.

.....

Barrett, E.F., J.N. Barrett, A.R. Martin and R. Rahamimoff. 1974. A note on the interaction of spontaneous and evoked release at the frog neuromuscular junction. *J. Physiol. Lond.* 237: 453-463.

Bennett, M.R. and A.G. Pettigrew. 1975. The formation of synapses in amphibian striated muscle during development. *J. Physiol. Lond.* 252: 203-239.

Barton, S.B. and I.S. Cohen. 1977. Are transmitter release statistics meaningful? *Nature.* 268: 267-268.

Bennett, M.R. and J. Robinson. 1990. Probabilistic secretion of quanta from nerve terminals at synaptic sites on muscle cells: non-uniformity, autoinhibition and the binomial hypothesis. *Proc. R. Soc. Lond. B Biol. Sci.* 239: 329-358.

Bornstein, J.C. 1978. Spontaneous multiquantal release at synapses in guinea-pig hypogastric ganglia: evidence that release can occur in bursts. *J. Physiol. Lond.* 282: 375-398.

Brown, T.H., D.H. Perkel. and M.W. Feldman. 1976. Evoked neurotransmitter release: statistical effects of nonuniformity and nonstationarity. *Proc. Natl. Acad. Sci. USA.* 78:

2913-2917.

Cappell, M.S., D.C. Spray. and M.V.L. Bennett. 1988. Stationary and non-stationary occurrences of miniature end plate potentials are well described as stationary and non-stationary Poisson processes in the mollusc *Navanax inermis*. *Brain Res.* 454: 244-250.

Cohen, I., H. Kita and W. Van der Kloot. 1974a. The intervals between miniature endplate potentials are unlikely to be independently or exponentially distributed. . *J. Physiol. Lond.* 236: 327-339.

Cohen, I., H. Kita and W. Van der Kloot 1974b. Stochastic properties of spontaneous transmitter release at the crayfish neuromuscular junction. *J. Physiol. Lond.* 236, 363-371.

Cohen, I., H. Kita and W. Van der Kloot 1974c. The stochastic properties of spontaneous transmitter release at the frog neuromuscular junction. *J. Physiol. Lond.* 236: 341-361.

Cohen, I.S., W. Van der Kloot and S.B. Barton. 1981. Bursts of miniature end-plate potentials can be released from localized regions of the frog motor nerve terminal. *Brain Res.* 221: 382-6.

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

Dodge, F.A., R. Miledi and R. Rahamimoff. 1969. Strontium and quantal release of transmitter at the neuromuscular junction. . *J. Physiol. Lond.* 200: 267-283.

Eckmann, J.P. and D. Ruelle. 1985. Ergodic theory of chaos and strange attractors. *Rev. Mod. Phys.* 57: 617-656.

Erulkar, S.D. and R. Rahamimoff. 1978. The role of calcium ions in tetanic and post-tetanic increase of miniature end-plate potential frequency. . J. Physiol. Lond. 278: 501-511.

Fatt, P. and B. Katz. 1952. Spontaneous subthreshold activity at motor nerve endings.. J. Physiol. Lond. 117: 109-128.

Gage, P.W. and J. I. Hubbard. 1965. Evidence for a Poisson distribution of miniature end-plate potentials and some implications. Nature. 208: 395-396.

Glass, L. and M.C. Mackey. 1988. From Clocks to Chaos. The Rhythms of Life. Princeton University Press, Princeton.

Hubbard, J.I. and S.F. Jones. 1973. Spontaneous quantal transmitter release: a statistical analysis and some implications. . J. Physiol. Lond. 232: 1-22.

Jenkins, G.H. and D.G. Watts. 1968. Spectral Analysis and its Applications. Holden-Day, San Francisco.

Lowen SB, Cash SS, Poo M-m, Teich MC (1997) Quantal neurotransmitter secretion rate exhibits fractal behavior. Journal of Neuroscience 17(15):5666-5677.

Jonsson, R. 1981. A model for miniature end-plate potentials based on the hypothesis of a release-regulating barrier. Almqvist & Wiksell, Goteborg.

Meiri, H. and R. Rahamimoff. 1978. Clumping and oscillations in evoked transmitter release at the frog neuromuscular junction. J. Physiol. Lond. 278: 513-23.

Melamed, N., P.J. Helm and R. Rahamimoff. 1993. Confocal microscopy reveals coordinated calcium fluctuations and oscillations in synaptic boutons. J. Neurosci. 13: 632-649.

Murthy, V.N., T.J. Stevens, and C.F. Stevens. 1997. Heterogeneous release properties

of visualized individual hippocampal synapses. *Neuron*. 18: 599-612.

Pawson, P.A. and A.D. Grinnell 1989. Oscillation period of MEPP frequency at frog neuromuscular junctions is inversely correlated with release efficacy and independent of acute  $Ca^{2+}$  loading. *Proc. R. Soc. Lond. B Biol. Sci*, 237: 489-499.

Perkel, D.J. and R.A. Nicoll. 1993. Evidence for all-or-none regulation of neurotransmitter release - implications for long-term potentiation. *J. Physiol. Lond.* 471: 481-500.

Pincus, S.M. (1991). Approximate entropy as a measure of system complexity. *Proc. Natl. Acad. Sci. USA*. 88: 2297-2301.

Pincus, S.M., I.M. Gladstone and R.A. Ehrenkranz. 1991. A regularity statistic for medical data analysis. *J. Clin. Monitoring*, 7: 335-345.

Pincus, S. and R.E. Kalman. 1997. Not all (possibly) "random" sequences are created equal. *Proc. Natl. Acad. Sci. USA*. 94: 5513-3518.

Press, W.H., B.P. Flannery, S.A. Teukolsky and W.T. Vetterling. 1989. *Numerical Recipes in Pascal*. Cambridge University Press, Cambridge.

Rahamimoff, R., S.D. Erulkar, A. Lev-Tov and H. Meiri. 1978. Intracellular and extracellular calcium ions in transmitter release at the neuromuscular synapse. *Ann. NY York Acad. Sci.* 307: 583-597.

Rahamimoff, R. and N. Melamed. 1993. Visualization of synaptic structure and function with confocal microscopy: calcium fluctuations and oscillations. *Neurosci. Res.*, 16:173-180.

Rees, D. 1974. The spontaneous release of transmitter from insect nerve terminals as predicted by the negative binomial theorem. *J. Physiol. Lond.* 236:129-142.

Sherman, B. 1950. A random variable related to the spacing of sample values. *Ann. Math. Stat.*, 21: 339-361.

Sokal, R.R. and F.J. Rohlf. 1981. *Biometry*. W.H. Freeman, New York.

Van der Kloot, W. 1996a. Spontaneous and unquantal-evoked endplate currents in normal frogs are indistinguishable. *J. Physiol. Lond.* 492: 155-162.

Van der Kloot, W. 1996b. Localizing quantal currents along frog neuromuscular junctions. *J. Physiol. Lond.* 497: 189-198.

Van der Kloot, W., O.P. Balezina, J. Molgó and L.A. Naves. 1994. The timing of channel opening during miniature endplate currents at the frog and mouse neuromuscular junctions: effects of fasciculin-2, other anti-cholinesterases and vesamicol. *Pfluegers Arch.* 428: 114-126.

Van der Kloot, W., H. Kita and I. Cohen. 1975. The timing of the appearance of miniature end-plate potentials. *Prog. Neurobiol.*, 4, 271-326.

Van der Kloot, W. and J. Molgó. 1994. Quantal acetylcholine release at the vertebrate neuromuscular junction. *Physiol. Rev.* 74: 899-991.

Velussi, C. and D. Danieli Betto, 1979. Effects of calcium, ethanol and hyperosmolarity on the distribution of intervals between MEPP's. *Bollettino - Societa Italiana Biologia Sperimentale*, 55: 1276-82.

Washio, H.M. and S.T. Inouye, S.T. 1980. The statistical analysis of spontaneous transmitter release at individual junctions of cockroach muscle. *J. Exp. Biol.* 87: 195-201.

Yana, K., N. Takeuchi, Y. Takikawa and M. Shimomura. 1984. A method for testing an extended Poisson hypothesis of spontaneous quantal transmitter release at

neuromuscular junctions. Biophys. J. 46: 323-330.

Table 1.

MEPP intervals at the frog neuromuscular junction

File name	Number of intervals	[NaCl] (mM)	Divalent cation (mM)	Temperature (°C)	V <sub>m</sub> (mV)	Mean interval (ms)	Sherman's statistic
A	2175	120	2.5 Ca <sup>2+</sup>	17.2	-91	367	0.11
B2	1497	120	2.5 Ca <sup>2+</sup>	17.1	-85	237	<b>0.00**</b>
C	1300	120	2.5 Ca <sup>2+</sup>	17.8	-86	350	<b>0.00**</b>
E	900	120	2.5 Ca <sup>2+</sup>	8.9	-85	851	0.10
F	420	120	2.5 Ca <sup>2+</sup>	8.8	-86	1138	0.84
G2	649	140	2.5 Ca <sup>2+</sup>	8.8	-80	160	0.04
I	995	140	2.5 Ca <sup>2+</sup>	8.7	-94	994	0.08
J	823	140	2.5 Ca <sup>2+</sup>	8.7	-84	822	0.40
K	1107	120	2.5 Sr <sup>2+</sup>	18.0	-94	419	0.32
L	629	120	2.5 Sr <sup>2+</sup>	18.6	-68	734	0.26
M2	475	120	2.5 Sr <sup>2+</sup>	19.0	-74	864	0.96
O2	1275	140	2.5 Sr <sup>2+</sup>	18.2	-48	69.7	0.04
N	302	120	2.5 Sr <sup>2+</sup>	19.0	-76	1515	<b>0.98*</b>
R	1069	140	2.5 Sr <sup>2+</sup>	19.3	-88	387	0.54
T	1721	120	12 Ca <sup>2+</sup>	19.6	-88	353	<b>1.00**</b>
U2	1097	140	12 Ca <sup>2+</sup>	19.6	-88	55.2	<b>0.00**</b>
X	2000	140	12 Ca <sup>2+</sup>	11.9	-89	246	0.87
A12	2240	140	5.0 Sr <sup>2+</sup>	20.7	-86	71.2	<b>0.00**</b>
S5	5024	140	5.0 Sr <sup>2+</sup>	21.2	-86	111	0.05
S8	3595	140	5.0 Sr <sup>2+</sup>	19.9	-74	166	0.08

Table 2.

MEPP intervals from the mouse diaphragm

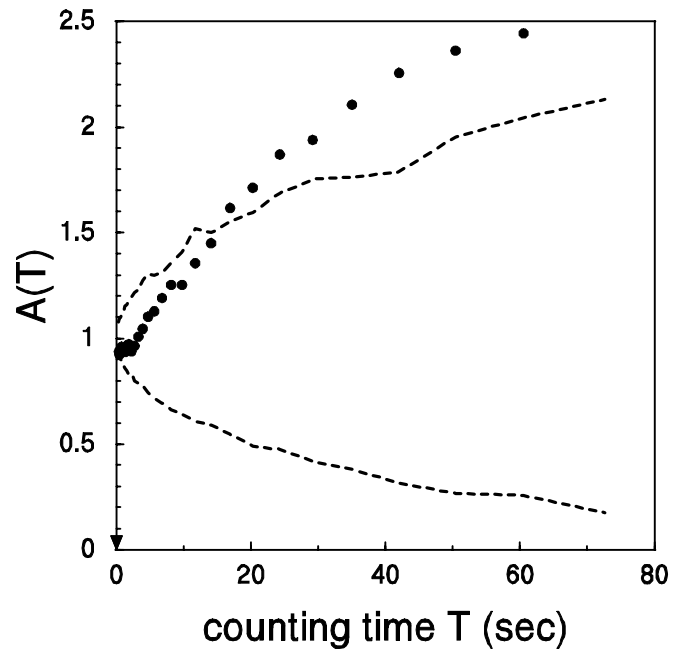
File name	Number of intervals	Mean interval (ms)	Sherman's statistic
B1	283	2129	0.42
M1	426	1563	0.28
A	395	2259	<b>0.98*</b>
B	938	969	0.97
C	912	1314	0.48
BS	863	980	<b>0.98*</b>
A1	283	2568	<b>0.004**</b>

Table 3.

Estimates of ApEn

File name	ApEn	Estimated 95% Confidence limits
Frog		
A	<b>1.73</b>	1.710-1.729
T	1.19	1.148-1.209
O2	1.18	1.159-1.207
Mouse		
BS	0.9720	0.9660-0.9811
A1	0.6264	0.6200-0.6283
A	0.7315	0.7294-0.7397

A data + shuffled (aint=file)





## LEGENDS

Fig. 1. Periodicity in a data set recorded in 12 mM  $\text{Ca}^{2+}$  solution. A. The power spectrum of the data sorted into 8 sec time bins. B. The integrated spectrum, showing how strikingly the data deviates from random predictions. The 95% confidence limits for random data are shown in the dashed lines. A cumulative plot showing the number of MEPPs as a function of time. Explanation in the text.

Fig. 2. The integrated power spectrum shows no sign of periodicity in MEPP releases in this example from the mouse. Data set c. A. The power spectrum. B. The integrated power spectrum. The dashed lines show the 95% confidence limits.

Fig. 3. The integrated power spectrum shows the intervals are independent of one another. Frog data set y. The dashed lines show the 95% confidence limits.

Fig. 4. The example in which the Allan factors exceeded the 95% confidence limits. The dots show the Allan factors. The confidence limits are shown in the dashed lines, they were determined by shuffling the order of the data bins. Frog data set A.



