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Synaptic Plasticity at Crayfish Neuromuscular Junctions

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Introduction

Crustacean neuromuscular junctions have provided some very favorable preparations for the study of synaptic plasticity. The crayfish, in particular, has junctions at which facilitation and depression, both rapid and long-lasting varieties, can be studied in great detail. Since the ability of synapses to adjust their strength of transmission as a consequence of previous activity plays a major role in many theories of learning (Mark, 1974), synaptic plasticity has attracted wide interest among neurobiologists. This chapter will summarize attempts to understand synaptic plasticity in terms of underlying cellular processes.

Short-Term Facilitation

Crustacean neuromuscular junctions have been regarded as "plastic" since the pioneering work of Kees Wiersma and his colleagues. Van Harreveld and Wiersma (1936) and Katz and Kuffler (1946) described a facilitation in crayfish in which the successive postsynaptic responses seen in claw opener muscle fibers grew in response to repetitive stimulation of the single excitatory motor neuron (MN) innervating this muscle. Subsequently, Dudel and Kuffler (1961*a*) showed transmitter release to be quantal at this neuromuscular junction. The number of quanta released by a nerve impulse was a random variable apparently following Poisson statistics. Using a quantal analysis, Dudel and Kuffler (1961*b*) also demonstrated that facilitation was presynaptic in origin, consisting of an increase in the average number of quanta liberated by an impulse in the MN terminals.

By 1970, considerable evidence had accumulated indicating that the Poisson model for transmitter release did not explain events at crustacean neuromuscular junctions (Bittner and Harrison, 1970). A report by Johnson and Wernig (1971) that transmitter release at the crayfish opener neuromuscular junction could be described

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by binomial statistics clarified the statistical anomalies. The binomial model provided a useful framework in which to study the statistical basis of a change in transmitter release. In this model, the average number of quanta, m, released by a presynaptic impulse is governed by a maximum number of releasable quanta, n, and a probability that such a quantum is released, p. Clearly, changes in m could be due to changes in p or n, or both.

I explored this problem by studying the changes in release statistics using paired responses, or steady stimulation at different frequencies. By recording extracellularly from localized regions of neuromuscular contact on the surface of a muscle fiber, one can observe potentials due to the postsynaptic currents evoked by transmitter action. In a favorable recording condition, the single quanta making up these responses can be distinguished and counted directly (Fig. 1). If the statistical process governing transmitter release is bionomial, p and n can be estimated from the average number of quanta released, m, and the variance of this number, σ^2, by $p = 1 - \sigma^2/m$ and $n = m/p$. When p and n were estimated in this fashion, p increased from less than 0.1 for unfacilitated responses to about 0.3 for moderately facilitated responses. Changes in p were often statistically significant. While n could be measured reliably only for facilitated responses, it ranged between 2 and 5, and seemed to be affected little by changes in the degree of facilitation (Table I). The standard errors for p and n in Table I are computed using the equations of Robinson (1976). They correct a minor error in the previously published values (Zucker, 1973).

How can these statistical changes be interpreted? My initial idea was that n was associated with a presynaptic store of releasable quanta and that p was the probability that such releasable quanta would be liberated by an impulse. This idea has

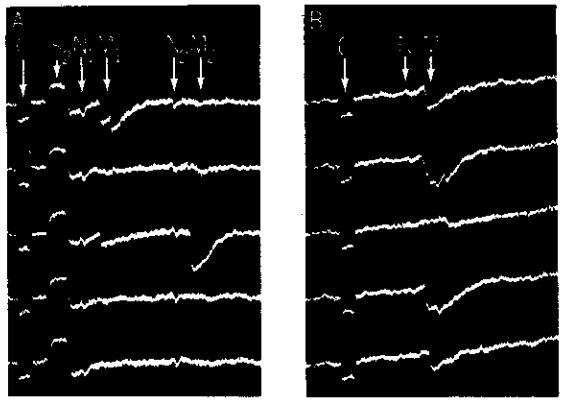

Fig. 1. Extracellular recordings of synaptic currents *(M)* and nerve terminal potentials *(N)* from synaptic sites on the claw opener muscle of crayfish. *C* is a calibration pulse (100μV, 5 msec), while *S* is a stimulus artifact. A: Five paired responses to two stimuli separated by 40 msec. B: Single responses at a frequency of 5 Hz from another preparation. From Zucker (1973).

Table I. Estimates of Statistical Parameters of Transmitter Release in Different States of Facilitation[a]

Site No.	Responses to	T (msec)	N	$m \pm$ S.E.$_m$	$p \pm$ S.E.$_p$	$n \pm$ S.E.$_n$
I	Stimulus 1		548	0.323 ± 0.029	0.039 ± 0.054	8.33 ± 11.6
	Stimulus 2	40	548	0.540 ± 0.028[b]	0.208 ± 0.039[b]	2.60 ± 0.47
II	Stimulus 1		736	0.121 ± 0.013	0.007 ± 0.056	16.76 ± 120
	Stimulus 2	30	736	0.243 ± 0.017[b]	0.108 ± 0.035[b]	2.25 ± 0.72
	10 Hz		594	0.680 ± 0.027[b]	0.357 ± 0.029[b]	1.90 ± 0.14
III	Stimulus 1		218	0.486 ± 0.042	0.218 ± 0.059	2.23 ± 0.57
	Stimulus 2	50	218	0.780 ± 0.049[b]	0.330 ± 0.053	2.37 ± 0.35
IV	Stimulus 1		500	0.334 ± 0.025	0.081 ± 0.051	4.14 ± 2.59
	Stimulus 2	55	500	0.576 ± 0.031[b]	0.165 ± 0.046	3.50 ± 0.95
	5 Hz		710	0.868 ± 0.029[b]	0.298 ± 0.032[b]	2.91 ± 0.29
V	Stimulus 1		431	0.271 ± 0.026	0.039 ± 0.076	7.02 ± 13.6
	Stimulus 2	40	431	0.499 ± 0.032[b]	0.097 ± 0.056	5.16 ± 2.94
VI	Stimulus 1		259	0.224 ± 0.028	0.082 ± 0.065	2.72 ± 2.13
	Stimulus 2	30	259	0.463 ± 0.036[b]	0.260 ± 0.048[b]	1.78 ± 0.29
	5 Hz		715	1.136 ± 0.033[b]	0.332 ± 0.031[b]	3.42 ± 0.31

[a] Abbreviations: T, the separation between first and second stimuli in paired stimulus experiments; N, total number of trials; m, average number of quanta released by each stimulus; p, probability that an active zone releases a quantum; n, number of release sites recorded. Parameter estimates are given ± S.E. Modified from Zucker (1973).
[b] Denotes a significant increase ($P < 0.05$) by t test of this parameter from its value for the unfacilitated first response.

remained popular among many investigators studying the binomial statistical parameters of synaptic transmission at vertebrate neuromuscular junctions and sympathetic ganglia (Bennett and Florin, 1974; McLachlan, 1975*b*; Miyamoto, 1975; Bennett *et al.*, 1976; Branisteanu *et al.*, 1976). This characterization of n and p suggests that facilitation consists of an increase in the effectiveness of an impulse in releasing quanta available for release, p, and not a mobilization of quanta into a releasable store, n.

However, if n is associated with a releasable store, it must be reduced immediately after an impulse by the amount of the store just released, and then recover before the next impulse. Otherwise, if n did not fully recover we would expect to see synaptic depression and a reduction in n due to a depletion of the releasable store. Such a depression and change in n was never observed in our experiments. My estimates of n (and those of Johnson and Wernig, 1971; Wernig, 1972*a*,*b*, 1975) were only about 2–5 at a single extracellular recording site. Clearly, if n were the releasable store it would then drop considerably after the release of a quantum or two, and there is no reason to expect that it would always recover to exactly the same value. Rather, if there were no other restrictions on n, then n should recover randomly to an average value, and fluctuations in n would arise from the statistics of refilling this releasable store from some larger presynaptic depot store. In fact, one would expect the releasable store to recover in a Poisson manner from its depletion

due to release. Then n itself would be a temporally fluctuating Poisson random variable. Vere-Jones (1966) showed that if the releasable store recovers in a Poisson manner, release itself will be Poisson. This contradicts our results.

These problems drove me to consider a different formulation for n and p. A binomial process is one in which some constraint fixes the maximum number of quanta that can be released. We have seen that there are difficulties in conceiving of this as some ill-defined releasable store. Perhaps some other presynaptic structure limits the number of releasable quanta. Anatomical studies of neuromuscular junctions show that synaptic vesicles are clustered at discrete release sites or active zones, which appear to be the points of transmitter release. What if each active zone can release only one quantum in response to a nerve impulse? Then if our microelectrode records from a cluster of n active zones, a response could contain at most n quanta. Vere-Jones (1966) showed that such a system leads to a binomial statistical process for m, where n will correspond to the total number of release sites and p will be the probability that one of them releases a quantum.

Ultrastructural work (Atwood and Morin, 1970) showing that there are about 2–5 active zones along a nerve branch within recording distance of an external microelectrode supports this model. Furthermore, Atwood and Parnas (1968) and Atwood and Johnston (1968) reported that at a number of crustacean neuromuscular junctions the evoked and spontaneous quantal units often fell into two or more distinct populations according to size and shape. This again suggests that a microelectrode at a synaptic site records quantal releases from several distinct release sites. Atwood often found that at most one quantum of each type could be released by a nerve impulse. If n is determined by the anatomically defined number of active zones, it is no wonder that only p changes during facilitation.

We may ask how a change in p comes about. If p is the probability that a release site releases a quantum, p would be influenced by both the probability that a release site is occupied by a releasable quantum, p_{occ}, and the probability that a spike is effective in activating a release site, p_{eff}. Vere-Jones (1966) formulated this situation by observing that a fixed number of release sites will be refilled according to a binomial process with parameters n and p_1, where p_1 is the probability that an unoccupied release site becomes filled between stimuli. Now the probability that a site releases a quantum will be the product of the probabilities that it is occupied, p_{occ}, and that it is activated by an impulse, p_{eff} (p_2 in the notation of Zucker, 1973). The probability that a site is occupied at a given moment depends on the probability that it was refilled since the last impulse p_1 and also on the probability that it was available for refilling at the last impulse, i.e., on whether it was emptied by the last impulse, which depends on p_2. In fact, $p_{occ} = p_1/[1-(1-p_1)\ (1-p_2)]$. Then $p = p_{occ}p_{eff} = p_1p_2/[1-(1-p_1)\ (1-p_2)]$, and release will be binomial, with $m = pn$.

Another way to formulate this is to consider $m = p_{eff}n_{rel}$, where $n_{rel} = p_{occ}n$ is the average number of occupied release sites or of immediately releasable quanta. This number must be less than n and cannot be estimated from a binomial statistical analysis. It may, however, be estimated from the properties of depression at synapses showing a depression due to depletion (see below). It is interesting that at

such synapses the estimate of n_{rel} from depression is less than n measured statistically (Christensen and Martin, 1970).

Is there no way to attribute changes in p to either p_{occ} or p_{eff} from statistical data? Vere-Jones (1966) noted that if release sites are not refilled completely between impulses, then a large release will most likely be followed by a smaller one, and *vice versa*. In fact, an equation for the covariance of successive responses can be solved simultaneously with the above expression for p to get p_1 and p_2. When I measured the covariance of successive responses, it was negligibly different from zero, which means that either p_{occ} or p_{eff} equals 1 and that the other is entirely responsible for facilitation. The absence of depression and the fact that facilitation is maximal immediately after an impulse suggest that p_{eff} is the determining factor, but this is far from proven.

Although we have not explained much about facilitation, the statistical results do suggest some factors that may be at work in limiting quantal release. A necessary requirement for n release sites to generate a binomial with the same parameter n is that each active zone can release only one quantum. Perhaps the rows of vesicles in active zones (Couteaux and Pécot-Dechavassine, 1974) are queuing up for a molecular release site at one point in each zone, or possibly an active zone can accommodate only the membrane surrounding one vesicle (*cf.* Heuser and Reese, 1973). Such possibilities are suggested if we believe that one quantum consists of the release of the contents of one synaptic vesicle. On the other hand, one quantum may consist of the simultaneous release of the contents of those few vesicles attached to the terminal membrane at an active zone. Then variations in quantal effectiveness would be due in part to variations in the number of vesicles contributing to a quantum (Kriebel and Gross, 1974). It is not possible at present to distinguish between these and other intriguing possibilities.

It could also be that our extracellular recordings are made from only one release site. Then n would correspond to the maximum number of quanta releasable from an active zone and might correspond to the number of vesicle attachment sites (Dreyer *et al.*, 1973). However, for the reasons cited above, I think it is unlikely that we are recording from only one release site.

These results differ markedly from those obtained at vertebrate sympathetic ganglia and neuromuscular junctions (Bennett and Florin, 1974; Bennett *et al.*, 1975, 1976; McLachlan, 1975*a*; Branisteanu *et al.*, 1976). There facilitation is frequently accompanied by changes in n, more so than in p. However, crucial differences in method prevent any detailed comparison between crustacean and vertebrate results. The latter analyses all estimated p and n from the variance and mean of the intracellularly recorded epp or epsp amplitude distribution. This method is subject to numerous uncertainties which have not been properly considered in most of these studies.

For example:

1. The quanta composing an epsp sum nonlinearly, but the Martin (1955) correction does not take adequate account of the effect of membrane capacitance on the summation of transient potentials (Martin, 1976).

2. The variance method of statistical analysis must correct the epsp variance for the variance in quantal unit magnitude. It is usually assumed that this can be estimated from the amplitude histogram of mepsp's. However, there is evidence that the evoked quantum and the spontaneously released quantum are not always the same (Highstein and Bennett, 1975), especially under the conditions in which many of the statistical experiments were performed (Dennis and Miledi, 1974; Bennett and Pettigrew, 1975).
3. Souček (1971) has shown that the fluctuations in latency of quantal release times can contribute significantly to the coefficient of variation of an epsp. Considering the known fluctuations in quantal latency, the effective amplitude of noncoincidental summed quanta will be less than 80% of the quantal size. From the theory developed by Williams and Bowen (1974), it can be shown that this leads to a 20% overestimate of p.
4. An intracellular recording from a whole neuromuscular junction is likely to sample from a wider diversity of synaptic sites with different p than an extracellular recording from a single terminal. A spatial nonuniformity in p would result in a serious overestimation of p (Brown *et al.*, 1976). The latter effect could explain, in part, why Wernig's (1975) estimates of p using the variance method were considerably larger than, often double, the estimates based on direct counting of quanta. They may also explain the remarkably large values of p, often approaching unity, that have been reported in vertebrate junctions (Bennett and Florin, 1974; McLachlan, 1975*a,b;* Bennett *et al.*, 1976).

Actually, a nonuniformity in p is likely to be present even at our extracellular recording sites. The resulting underestimation of n explains the occasional observation of more than n quanta in a response. However, Hatt and Smith (1976*b*) have shown that a large variance in p is likely to lead to a departure from binomial statistics that is detectable when quanta can be counted directly and n is small. These effects are likely to be exaggerated in intracellular recordings from many terminals, but will be more difficult to detect when m is large (Brown *et al.*, 1976).

Increases in n estimated in vertebrate experiments as a correlate of facilitation, and changes in calcium and magnesium concentrations, have led some authors to doubt that n could be determined by a fixed number of release sites in their experiments. They propose that n reflects a releasable store of transmitter quanta. This implies either that transmitter release at these synapses is not limited by a fixed number of release sites or that an active zone can release a variable number of quanta. However, changes in estimates of n could be caused entirely by changes in factors which confound estimates of n and p. For example, if all values of p were increased by a certain amount at a population of release sites with nonuniform p, a large, spurious increase in the estimate of n would result, even though the actual number of release sites remains constant (Brown *et al.*, 1976). As the estimate of p (also erroneous) approaches unity, apparent changes in n can occur without any further significant changes in the estimate of p. Thus the estimates of n and p

derived from epsp amplitude fluctuations may bear no simple relationship to the actual values of n and p of the underlying binomial statistical process.

These statistical studies prompted a series of experiments to determine what process might be responsible for increasing p during facilitation. Early studies (Dudel and Kuffler, 1961*a*; Dudel, 1965*b*) suggested that facilitation might be caused by an increase in the amplitude of the electrotonically conducted presynaptic impulse into inexcitable terminals. However, I (Zucker, 1974*a*) found that most MN terminals are locally excitable, and that orthdromic nerve terminal potentials contained prominent negative phases indicating that the terminals were actively invaded. When care was taken to avoid artifacts due to nonspecific muscle currents (Katz and Miledi, 1965), muscle movement, and the averaging of nonsynchronous nerve potentials, then no consistent change in nerve terminal potential was seen to accompany facilitation (see also Ortiz, 1972; Lang and Atwood, 1973) (Fig. 2).

Another possibility was that facilitation was caused by an accumulation of intracellular sodium or extracellular potassium during nerve impulses. These ideas were tested by blocking nerve transmission with tetrodotoxin and by eliciting transmitter release by depolarizing a fine nerve branch or a small patch of nerve membrane (Katz and Miledi, 1967*a,b*). When the parameters of the pulse were adjusted to release as much transmitter as a nerve impulse, it was found that patterns of repeated identical depolarizing pulses caused facilitation of synaptic transmission that was remarkably similar to that caused by similar patterns of repeated nerve impulses (Fig. 3). This confirmed the conclusion that facilitation is not caused by an increase in the presynaptic electrical signal, and also showed that it occurs in the absence of the sodium influx accompanying nerve impulses. That facilitation was not due to an accumulation of extracellular potassium was shown by the fact that increased potassium decreases transmitter release (Zucker, 1974*c*).

Another hypothesis often put forward to account for synaptic facilitation is that it might be caused by hyperpolarizing afterpotentials in nerve terminals following

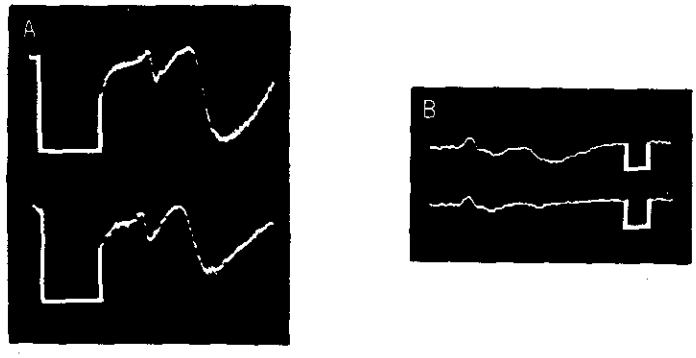

Fig. 2. Facilitation of synaptic transmission with unchanging nerve terminal potentials. Averaged extracellular recordings from two synaptic sites stimulated at 10 Hz (upper traces) and at 5 Hz (lower trace in A) or 1 Hz (lower trace in B). Calibration pulses (retouched): 100 μV, 5 msec (A) or 1 msec (B). From Zucker (1974*a*).

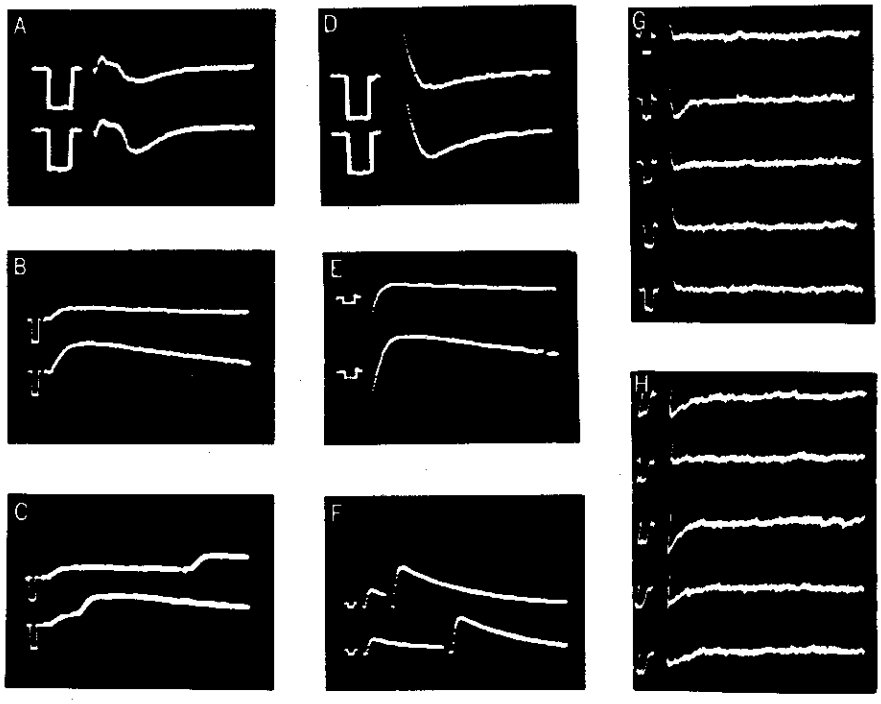

Fig. 3. Facilitated transmitter release evoked by nerve impulses (A–C) and constant nerve-depolarizing pulses (D–H). Averaged responses are recorded extracellularly from a synaptic site in A, D, G, and H, and intracellularly from a muscle fiber in B, C, E, and F. Frequency of stimulation: 2 Hz (upper traces) and 10 Hz (lower traces) in A, B, D, and E. C and F show paired intracellular responses to spikes (C) and pulses (F). On the right is a series of extracellular responses to depolarizing pulses at 1 Hz (G) and 10 Hz (H), showing that the facilitated averaged responses are due to an increase in the average number of quanta released. Calibration pulses: 200 μV, 5 msec. From Zucker (1974*a*).

stimulation. Dudel (1971) has already shown that artificial hyperpolarization of nerve terminals increased the amount of transmitter released by nerve impulses at claw opener synapses.

Since the state of polarization in nerve can be assessed by measuring its excitation threshold to extracellular currents (Wall, 1958), I set about measuring the postactivation recovery of excitability at MN terminals. To my surprise, the brief refractory period was followed by a period of supernormal excitability corresponding to a depolarizing afterpotential in claw opener terminals (see also Dudel, 1973). This supernormal period was larger following a tetanus, present only in terminals, enhanced by steady nerve hyperpolarization and in potassium-free medium, and abolished by enzymes which digest the connective tissue binding nerve to muscle (Figs. 4 and 5). All these properties are consistent with a depolarizing afterpotential generated by a transient accumulation of potassium ions around nerve terminals (Zucker, 1974*c*). However, Dudel (1971) showed that depolarization of MN terminals could only decrease the effectiveness of an impulse in releasing transmitter.

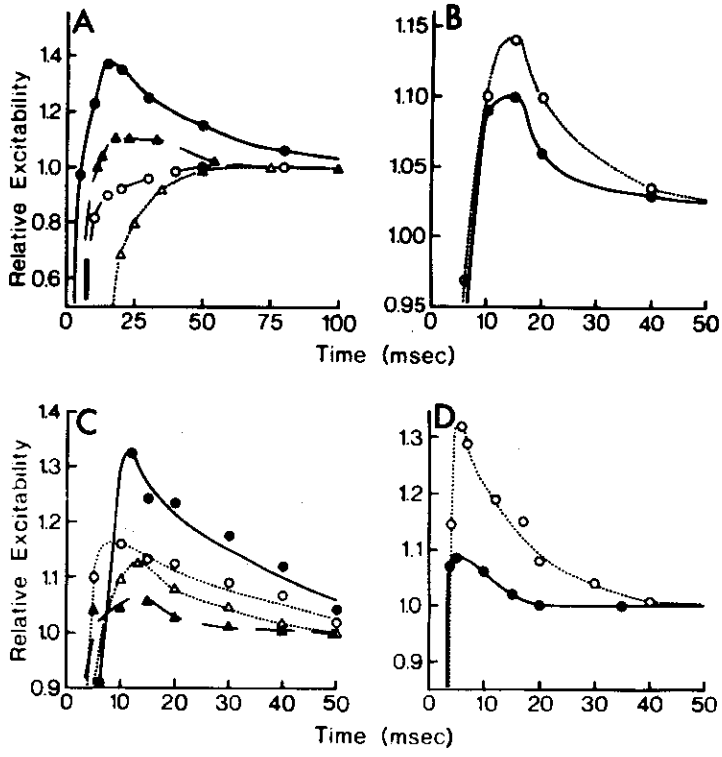

Fig. 4. Postactivation excitability changes in claw opener MN terminals. A: A supernormal excitability is present in all nerve terminals at synaptic sites (●), in some (▲) but not most (○) fine nerve branches, and not in the main axon (Δ). B: It is larger following a tetanus (○) than one impulse (●). C: It is enhanced in potassium-free medium (●) and reduced in high-potassium medium (▲) compared to normal crayfish saline (○, Δ). D: It is reduced by proteolytic enzymes (○, before, and ●, after, treatment). From Zucker (1974*c*).

The hyperpolarization required by the afterpotential hypothesis of facilitation just doesn't seem to be present in crayfish MN terminals.

Next I considered the hypothesis put forward by Katz and Miledi (1968), Rahamimoff (1968), and Miledi and Thies (1971) that facilitation is caused by an intracellular accumulation of calcium or some calcium complex in nerve terminals following stimulation. It is known that calcium enters the nerve terminal during an impulse (Katz and Miledi, 1967*a*,*c*) and that calcium injected intracellularly triggers transmitter release (Miledi, 1973). It was also shown that at the frog neuromuscular junction the facilitating effect of an impulse on transmitter release by subsequent impulses was dependent on calcium being present in the medium, and presumably on the calcium influx accompanying an impulse (Katz and Miledi, 1968). Thus one had only to suppose that some of the calcium entering a nerve during a spike

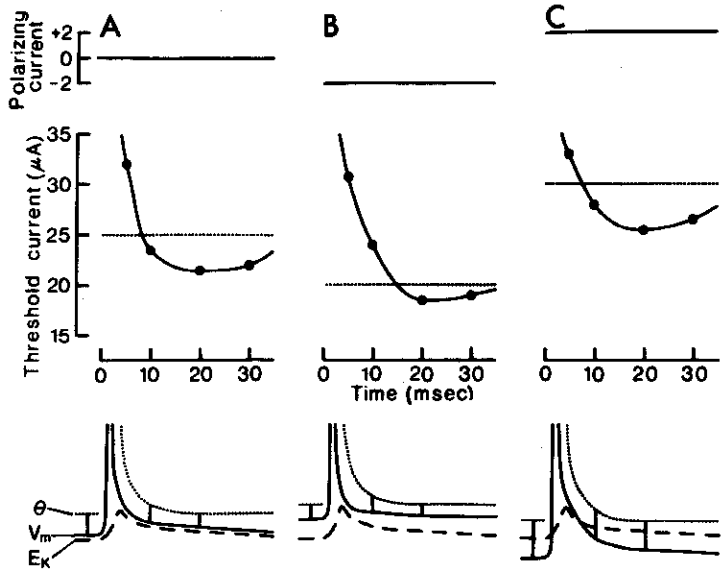

Fig. 5. A: Threshold changes following an impulse in the absence of polarizing current. Depolarization (B) reduces the postactivation period of subnormal threshold; hyperpolarization (C) increases it. Dotted lines on threshold graphs indicate preactivation threshold. Bottom drawings show how a depolarizing afterpotential would affect the extracellular current threshold, which is proportional to the difference between membrane potential (V_m) and the membrane voltage threshold (θ). The afterpotential is probably due to an extracellular potassium accumulation and the consequent change in the potassium equilibrium potential (E_K). From Zucker (1974*c*).

remained present for a time to deduce that facilitation arises as a natural consequence of the other known properties of synaptic transmission.

Specifically, if each spike is accompanied by an influx of calcium of magnitude A, and if at a given time after previous activity an amount B of "active calcium" remained in the nerve terminal, then the peak amount of active calcium achieved following a new nerve impulse would be $A + B$. Then a facilitated response, R', will bear the following simple relation to an unfacilitated response, R: $R'/R = (A + B)^n/A^n$. Here the exponent n allows for a nonlinear relationship between calcium entry, A, and transmitter release. Since A should be proportional to the external calcium concentration, this fits the finding of a nonlinear (power law) relation between transmitter release and external calcium at frog neuromuscular junctions (Dodge and Rahamimoff, 1967).

In this model, the decay of facilitation following an impulse depends on the decay of residual calcium, B. If n is known from the calcium dependence of transmitter release, the equation allows the prediction of the growth of facilitation during repetitive stimulation, and its subsequent decline from the time course of facilitation following an impulse (Linder, 1973; Younkin, 1974).

In crayfish, a linear relation exists between external calcium concentration and transmitter release (Bracho and Orkand, 1970; Ortiz and Bracho, 1972) even when

the calcium is buffered to low levels (Zucker, 1974*b*). With $n = 1$, a linear summation of the facilitating effects of successive impulses is expected. This has not been found (Fig. 6). At highly facilitating synapses, facilitation accumulates more than expected (Zucker, 1974*b*; Linder, 1974; Bittner and Sewell, 1976); however, at synapses showing less facilitation, accumulation is less than linear (Bittner and Sewell, 1976). The latter effect could indicate a saturation of transmitter release at high release levels, as seen before in high calcium concentrations (Rahamimoff, 1968; Zucker, 1974*b*). The former very large growth of facilitation is difficult to explain.

Another difficulty arises from a consideration of the factors which determine the removal of B. In one version of the calcium accumulation model (Katz and Miledi, 1968; Miledi and Thies, 1971), B is removed by nonlinear rate kinetics. One consequence of this idea is that B (and hence facilitation) will decline more rapidly after a tetanus, when B is high, than after one spike. This prediction was not confirmed—facilitation declines with the same time course after a tetanus as after one impulse (Zucker, 1974*b*; Linder, 1974). A second consequence of nonlinear B

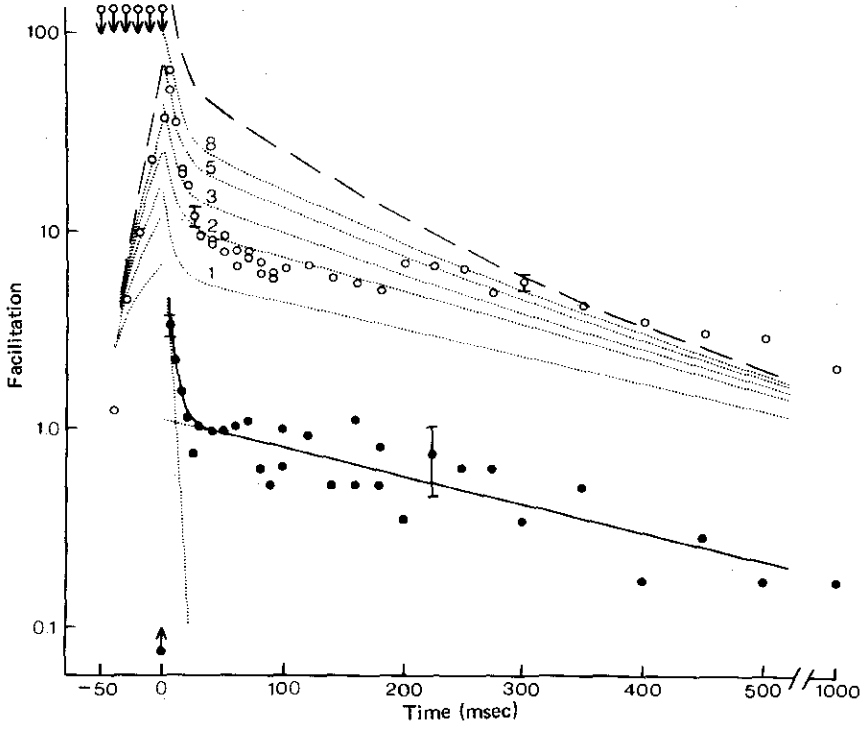

Fig. 6. Time course of facilitation of synaptic transmission following one impulse (●) and during and following a brief tetanus (○). Facilitation is measured as the fractional increase in amplitude of a test response over that of an unfacilitated response. The decay of facilitation can be fitted by a curve which is the sum of two exponentials (note logarithmic ordinate). The dotted lines are the predicted curves for tetanic facilitation and its decay, assuming the values of n indicated. The dashed line is the prediction if facilitation is a multiplicative process. From Zucker (1974*b*).

kinetics is that B becomes nearly independent of external calcium, thus accounting for the calcium independence of posttetanic changes in mepp frequency (Miledi and Thies, 1971). But A is dependent on external calcium, and hence so should facilitation be. However, I found facilitation to be calcium independent (Zucker, 1974*b*), as in the crab (Linder, 1973). These difficulties are not present in a second version of the calcium accumulation model in which B is removed by a series of linear rate processes (Rahamimoff, 1968). Then B and A have the same calcium dependence and facilitation becomes calcium independent (see above equation), and the time course of the removal of B will be independent of the level of facilitation.

Another aspect of the linear summation model is that, as calcium accumulates to cause facilitation, it should also enhance the spontaneous release of transmitter. When a power law governs the calcium dependence of transmitter release, a small residuum of active calcium has little effect on mepp frequency, while its incremental effect on the peak calcium level reached following a spike may cause considerable facilitation (Miledi and Thies, 1971; Barrett and Stevens, 1972). In crayfish, however, a linear calcium dependence of release requires that, if facilitation reaches a level of $R'/R = 5$, then transmitter would be released spontaneously at a rate 4 times greater than the evoked rate of transmitter release for an unfacilitated response! This extraordinary conclusion is emphatically contradicted by the results. Miniature frequency may even deline during facilitation (Dudel and Kuffler, 1961*b*).

One way out of all these contradictions is to suppose that the linear relation between calcium and transmitter release is misleading. Parnas *et al.* (1975) have found evidence for a calcium-dependent tonic release of inhibitory transmitter, which acts both pre- and postsynaptically at crab neuromuscular junctions. They found that the calcium dependence of excitatory transmitter release was partially masked by the calcium dependence of tonic presynaptic inhibition. It follows that increasing calcium not only increased excitatory transmitter release evoked by impulses but also increased the level of tonic presynaptic inhibition. When picrotoxin was added to block inhibition, the calcium dependence of excitatory transmission was seen to be quite nonlinear.

It is unlikely that this effect is confounding the crayfish results. Parnas *et al.* (1975) found that picrotoxin increased the postsynaptic input resistance and the size of ejp's. No such effect was observed at the crayfish junctions (Takeuchi and Takeuchi, 1969). Even if n is greater than 1, there was no value of n which could predict the accumulative properties of facilitation by the above model (Fig. 6). Also, if it is supposed that n calcium ions are needed to form an active complex and that it is this complex, not calcium, which accumulates, then again a linear summation of facilitation is predicted (Magleby, 1973*a*), which is contrary to what is observed.

Another hypothesis is that calcium acts at a different site to trigger facilitation than the site where calcium entering during an impulse acts to evoke release (Balnave and Gage, 1974; Cooke and Quastel, 1973). Then the cumulative effects of facilitation should be multiplicative. This prediction was also not confirmed by the data (Fig. 6).

We need not abandon entirely the idea that calcium accumulation is responsible for facilitation. It may be that residual calcium neither adds to a constant calcium influx in a spike nor conditions the nerve response to that influx. Rather, residual calcium may affect the magnitude of that influx itself, and this effect could be responsible for facilitation. An increase in calcium influx during successive impulses has been observed in *Aplysia* somata (Stinnakre and Tauc, 1973). It remains to be shown whether this increase is in any way related to the accumulation of calcium or a calcium complex.

Long-Term Facilitation

Another type of facilitation seen at crayfish claw opener and other crustacean neuromuscular junctions has been studied by Atwood and his colleagues. Sherman and Atwood (1971*b*) reported that continuous stimulation of a MN at 5–20 Hz led to a progressive increase in synaptic transmission, growing to many times its short-term facilitated amplitude during the course of an hour. This effect persisted for several hours after cessation of stimulation. This long-term facilitation, which is clearly presynaptic in origin (Atwood *et al.*, 1975), appears parametrically similar to tetanic and posttetanic potentiation at vertebrate neuromuscular junctions (Magleby, 1973*a*,*b*; Magleby and Zengel, 1975*a*,*b*).

Long-term facilitation seems to be a consequence of an increase of intracellular sodium during tetanic stimulation. When sodium loading is reduced in low-sodium medium, long-term facilitation is reduced or absent (Sherman and Atwood, 1971*b*; Atwood *et al.*, 1975). Conversely, when sodium loading is enhanced by blocking the sodium pump, long-term facilitation is markedly enhanced and accelerated. None of these effects was observed in vertebrate posttetanic potentiation (Gage and Hubbard, 1966). The latter is highly dependent on calcium entry during a tetanus (Rosenthal, 1969; Weinreich, 1971). In crayfish, long-term facilitation can be elicited in a calcium-free saline or when calcium influx is blocked (Swenarchuk and Atwood, 1975). Thus, unlike vertebrate posttetanic potentiation, crustacean long-term facilitation appears to be due specifically to a sodium accumulation in nerve terminals. It is possible the sodium acts by activating a sodium–calcium exchange pump during the test period (Baker *et al.*, 1969) or by releasing calcium from mitochondria (Alnaes and Rahamimoff, 1975). In that case, the sodium loading may indirectly promote a residual calcium, which may then act in the same manner as in short-term facilitation.

Short-Term Depression

Hatt and Smith (1976*a*) have found a short-lasting depression at walking leg opener junctions of European crayfish *(Astacus leptodactylus)*, using the same sort of stimulus regimen which evokes only long-term facilitation in North American crayfish *(Procambarus clarkii)*. The depression develops only after several minutes

of stimulation of 10–20 Hz, but the effect dissipates within a few seconds if stimulation is halted. A similar depression has been observed at neuromuscular junctions onto fast abdominal extensor muscles in lobsters and crayfish (Parnas, 1972).

Recording extracellularly from synaptic sites, Hatt and Smith (1976*a*) and Parnas (1972) found that failures of transmission were often associated with a sudden loss of the nerve terminal potential. Thus the depression is due, at least in part, to an intermittent presynaptic conduction failure—a block of invasion of MN terminals.

Hatt and Smith (1976*a*,*b*) performed a statistical analysis on the postsynaptic responses in *Astacus* and found that binomial statistics usually provided the best description of the results. When they compared the statistics of depressed responses to undepressed (and facilitated) responses, they found that, even for selected responses in which the nerve terminal potential was present, m and p were reduced, and sometimes so was n. They suggest that the reason p was reduced was that responses which follow those containing conduction failures are essentially responses to a lower effective frequency. Thus facilitation will have decayed, reducing p and therefore m. They tested this hypothesis by comparing responses following a transmission failure to those not following a transmission failure. The former group were larger and had a larger m and p. Therefore, depression is due in part to an intermittent presynaptic conduction failure at some MN terminals, and in part to a defacilitation of responses following such failures.

The reduction in n is harder to explain. The results of Brown *et al.* (1976) show that if p is nonuniform, changes in the distribution of p will probably be associated with similar changes in the estimate of n. Hatt and Smith (1976*b*) have presented evidence for such a nonuniformity in p at this synapse, so the changes in n may be entirely artifactual.

Long-Term Depression

A more persistent form of synaptic depression has been observed at the neuromuscular junctions of the giant MN of crayfish (Kennedy and Takeda, 1965*a*). These synapses show a long-lasting depression extraordinary for neuromuscular junctions (Bruner and Kennedy, 1970) but comparable to that of central synapses known to be responsible for behavioral habituation (Kandel *et al.*, 1970; Zucker, 1972*a*). Recently J. Bruner and I have tried to determine the mechanism responsible for this depression.

The usual model put forward to account for synaptic depression is that there is a depletion of the store of transmitter available for release as a consequence of previous release from this store (Liley and North, 1953; Hubbard, 1970). In this model, recovery from depression reflects the mobilization of transmitter into the releasable store from a depot store—or the rate of refilling of unoccupied release sites, p_1. The initial level of depression, measured as the percentage reduction of the response to an impulse immediately following an undepressed response, can be used

to estimate the fraction of the releasable store liberated by an impulse (p_2 or p_{eff}). We found that, on the average, a single impulse was followed by a 60–70% depression which recovered exponentially with a time constant of about 4.5 min. The exponential recovery suggests that simple first-order kinetics apply to a refilling of the releasable store. From these parameters, one can predict the time course and steady state of depression expected for any frequency of stimulation. We found that the model predicted depression adequately for stimuli repeated once every 5 min or more, but the data diverged markedly from the predictions at frequencies of 1/min or higher (Fig. 7). Tetanic depression was not nearly as large as expected. The same steady-state level was reached at all frequencies between 1/min and 4 Hz.

One possibility is that during repeated stimulation the rate of mobilization (refilling the releasable store) is increased over that following a single impulse (Kusano and Landau, 1975). In that event, recovery from depression following a tetanus would be faster than following one impulse. However, when we compared

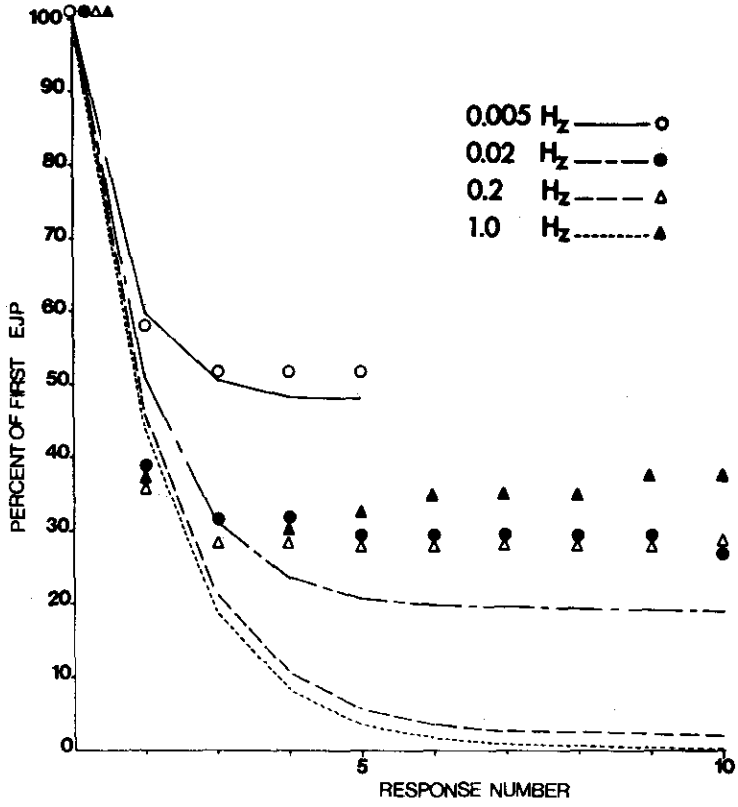

Fig. 7. Successive ejp amplitudes (symbols) in fast flexor muscles to repeated motor giant impulses at the frequencies shown. The lines are predictions of depression based on a depletion model and the recovery from depression following one impulse. From Bruner and Zucker (unpublished).

these recovery rates in a group of seven fibers, we found no difference. We also found that depression immediately following a tetanic response was much less (only 33%) than that following an undepressed response (65%). This result also contradicts the depletion hypothesis.

The hallmark of the depletion hypothesis is that reducing transmitter release alleviates depression by reducing depletion. If the magnesium concentration in the medium is increased sufficiently to reduce transmission to 25%, then the fraction of store released by an impulse should be reduced to one-fourth (Wernig, 1972*b*), and depression should be similarly reduced. In fact, we found that depression was unaffected by this treatment (Fig. 8). This result stands in stark contrast to the behavior of other examples of synaptic depression (e.g., Thies, 1965; Elmqvist and Quastal, 1965; Betz, 1970; Kusana and Landau, 1975).

It still remained possible that the high-magnesium solution interfered with the mobilization process. Then transmitter would have recovered less between stimuli

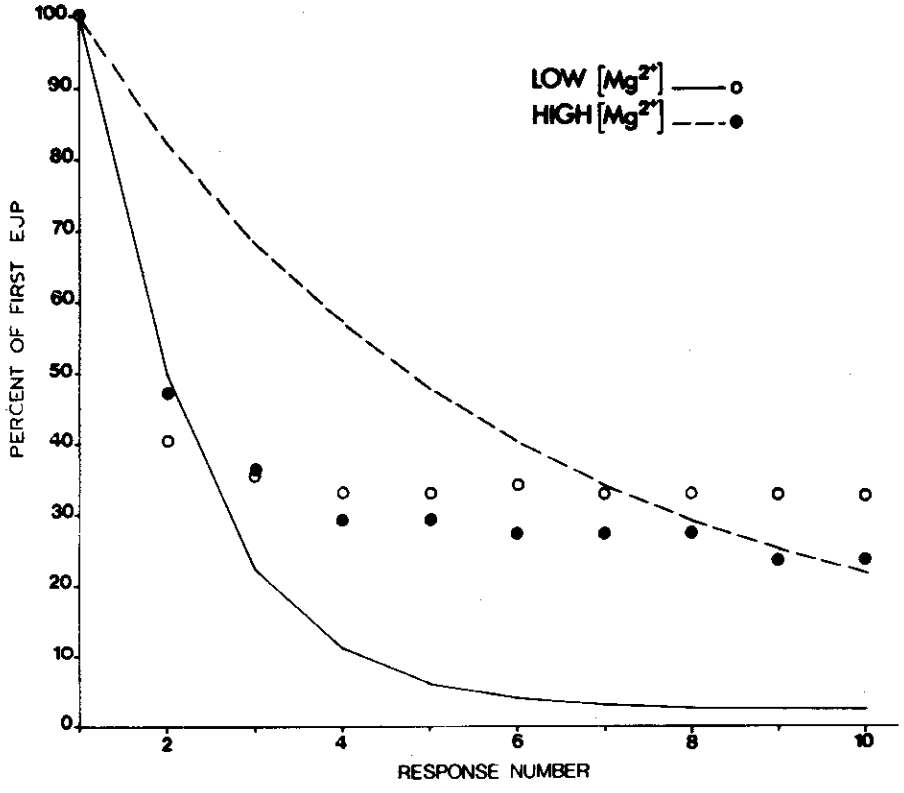

Fig. 8. Successive ejp amplitudes (symbols) to motor giant impulses every 5 sec in high and low magnesium concentrations. The first response in high magnesium was 28% of that in low magnesium. The predictions from the depletion hypothesis are also shown (lines). From Bruner and Zucker (unpublished).

than we had predicted. However, we found no effect of changing the magnesium concentration on the rate of recovery from long-term depression.

In summary, we were unable to confirm any of the predictions of this model and were forced to reject the depletion hypothesis, even in modified form (Betz, 1970; Friesen, 1975), as a satisfactory model for depression at the motor giant neuromuscular junctions. We do not know what may be responsible for this depression. We were unable to record extracellularly from synaptic sites long enough to do a quantal analysis during depression. The absence of distinguishable mjp's in intracellular recordings also precludes a quantal analysis, so we do not even know for sure that the depression arises presynaptically. Clearly, further work is needed to explain this interesting form of synaptic depression.

Other Kinds of Nerve-Induced Modifiability

Besides facilitation and depression, at least two other processes may mediate a modulation of muscle by nerve in crustaceans. Rathmayer and Florey (1974*a*,*b*) found that ejp's recorded from the closer muscles in a variety of crab species were reduced for several minutes following a brief high-frequency burst of impulses in the inhibitor MN to the muscle. This postinhibitory depression is thought to be presynaptic in origin, because the postsynaptic increase in conductance caused by the inhibitor activity lasted only a few seconds after the inhibitory tetanus.

A very different type of nerve–muscle interaction has been discovered by Evans *et al.* (1975). They have found peripheral neurons in lobster which appear to secrete octopamine into the bloodstream. Searching for an effect of the neurohumor, they observed that octopamine elicits a long-lasting contracture of the lobster leg opener muscle and enhances the tension developed during excitatory nerve stimulation. Octopamine has no effect on muscle membrane potential or on the amplitude of ejp's or ijp's. Apparently, octopamine acts at some other point in the muscle, perhaps modifying the chemical reactions that control muscle contraction.

These examples serve to illustrate the variety and subtlety of the long-term control of muscle by nerve and the plastic nature of the usual neuromuscular interaction. We have a long way to go before these processes are understood in biophysical and biochemical terms. At least we have begun the task.

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