J Appl Physiol 96: 1681–1690, 2004;
10.1152/japplphysiol.01177.2003.

5-HT offsets homeostasis of synaptic transmission during short-term facilitation

G. M. Sparks and R. L. Cooper

Department of Biology, University of Kentucky, Lexington, Kentucky 40506-0225

Submitted 3 November 2003; accepted in final form 30 December 2003

Sparks, G. M., and R. L. Cooper. 5-HT offsets homeostasis of synaptic transmission during short-term facilitation. *J Appl Physiol* 96: 1681–1690, 2004; 10.1152/japplphysiol.01177.2003.—In this study, we approach the topic of vesicle recruitment and recycling by perturbing neurotransmission at the crayfish neuromuscular junction with altered electrical activity and the presence of the neuromodulator serotonin (5-HT). After induction of short-term facilitation (STF) with stimulus pulse trains (40 Hz, 20 pulses), the amount of synaptic transmission can be maintained at a relatively constant level, producing a plateau in the amplitude of the excitatory postsynaptic potentials (EPSPs) throughout the remaining stimuli within a train of a few hundred milliseconds. With an increase in the frequency of the stimuli within a train (60 Hz, 20 pulses), an altered plateau of larger EPSP amplitudes occurs. This suggests that differential rates of vesicle recruitment can be rapidly reached and maintained. Exposure of nerve terminals to 5-HT further enhances the EPSP amplitudes to yet a higher plateau level. The effect of 5-HT is more pronounced for 40-Hz pulse trains than for 60-Hz trains. This suggests that 5-HT can recruit vesicles into the readily releasable pool (RRP) and that the recruitment is limited at higher stimulation frequencies. The attainment of a larger amplitude in the plateaus of the EPSPs at 60 Hz compared with 40 Hz also suggests that the rapid induction of STF enhances the entry of vesicles into the RRP. By direct quantal counts, mean quantal content increases linearly during STF, and 5-HT offsets the linear release. We propose that 5-HT and electrically induced recruitment of vesicles from a reserve pool to the RRP may share similar recruitment mechanisms.

synapse; calcium; serotonin; quantal; vesicle

REPETITIVE STIMULATION OF presynaptic nerve terminals enhances the amplitude of subsequent excitatory postsynaptic potentials (EPSPs) in a process known as short-term facilitation (STF) (7, 38, 45–48). EPSPs may reach a plateau in their amplitude at a particular frequency given a sufficient number of pulses within the stimulus train, and this plateau may persist for some time. The eventual depression of EPSP responses depends on several factors associated with any given synaptic connection, which we do not address here. The persistence of a plateau in EPSP amplitude marks an equilibrium and steady state among synaptic processes. If the frequency of stimulation increases within a pulse train, a new plateau and equilibrium can be observed. Depending on the type of preparation, the electrochemical driving gradient may be sufficient to produce larger EPSP responses during this plateau period. Such is the case for the neuromuscular junction (NMJ) preparations of the crayfish presented in this study.

The intracellular calcium concentration ($[Ca^{2+}]_i$) increases within the presynaptic terminal during the stimulation train, and presumably the continual influx, buffering, and exchange mechanisms reach an equilibrium that produces a steady state of transmitter release (9, 16, 25, 28–31, 33, 66, 67). In crustacean NMJs, increasing the frequency of stimulation leads to a new equilibrium with a higher $[Ca^{2+}]_i$, and the EPSPs reach the new plateau more quickly than with lower stimulation frequencies (15). As the stimulation frequency is increased, the increase in the maximum plateau response becomes less pronounced. This process is likely regulated by a $[Ca^{2+}]_i$-induced enhancement of a Na^+/Ca^{2+} exchanger, which can result in a steady-state process that regulates $[Ca^{2+}]_i$ effectively during repetitive stimulation. The number of vesicles that can be released at the active zones (AZs) might also be limited. Such an anatomic limitation could explain why higher output terminals associated with phasic motor nerves do not facilitate as much as lower output tonic motor nerve terminals (9), as well as why there are differences in the synaptic efficacy of low- and high-output tonic terminals (16). In this study, we address what effect a faster induction of STF has on the plateau of the EPSP amplitudes to address whether an equilibrium in vesicle recycling is one of the mechanisms that could account for the phenomena.

Neuromodulators that offset synaptic function offer an approach to experimentally address the mechanisms underlying differences in facilitation caused by changes in stimulation frequency and inherent differences among synapses. The neuromodulator serotonin (5-HT), which is endogenous to the hemolymph of crustaceans, greatly enhances transmitter release (24) presynaptically at NMJs by increasing the probability of vesicular fusion (54). In crayfish motoneurons, 5-HT mediates its rapid effect through the inositol 1,4,5-trisphosphate (IP_3) second messenger system (23, 19). Dudel (27) showed that when nerve terminals are stimulated directly while sodium channels are blocked by tetrodotoxin, synaptic release may be enhanced because of increased docking of vesicles and/or promotion of the release of vesicles on a rise in $[Ca^{2+}]_i$ by locally stimulating the voltage-gated calcium channels. Quantal content rose with an increase in the stimulus current. With the addition of 5-HT, the increase in quantal content was even greater for a given stimulus current.

In this report, we show that the equilibrium of vesicle docking and release during the steady plateau phase produced by short trains of stimulation is not limited by the availability of synaptic vesicles, because exposure to 5-HT further enhances vesicular release.

METHODS

All experiments were performed using the first and second walking legs of freshly obtained crayfish, *Procambarus clarkii*, measuring 6–10 cm in body length (Atchafalaya Biological Supply, Raceland, LA). Animals were housed individually in an aquatic facility and fed

Address for reprint requests and other correspondence: R. L. Cooper, 101 T. H. Morgan School of Biological Sciences, Univ. of Kentucky, Lexington, KY 40506-0225 (E-mail: RLCOOP1@pop.uky.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

 8750-7587/04 $5.00 Copyright © 2004 the American Physiological Society

dried fish food and chicken egg shells. Dissected preparations were maintained in crayfish saline, a modified Van Harreveld's solution (in mM: 205 NaCl; 5.3 KCl; 13.5 $CaCl_2 \cdot 2H_2O$; 2.45 $MgCl_2 \cdot 6H_2O$; 5 HEPES, adjusted to pH 7.4). Crayfish were induced to autotomize the first or second walking leg by forceful pinching at the merus segment.

Physiology. To elicit an evoked response, the excitatory axon was selectively stimulated by placing a branch of the leg nerve (from the merus segment) into a suction electrode connected to a Grass stimulator (32). The STF was induced by providing a train of 20 pulses, at 10-s intervals, to the excitatory nerve. The frequency of stimulation within the train varied from 40 to 60 Hz to compare with earlier observations in facilitation measures (16). Intracellular EPSP recordings were performed by standard procedures (12, 16). The 5-HT (100 nM) containing saline was made in crayfish saline from frozen stock of 1 mM 5-HT. This concentration has been used for standardization to earlier published reports on this same preparation (54, 55, 57) and for the crayfish leg extensor preparation (9). When exposing the preparation to 5-HT, the entire bathing medium was rapidly exchanged (<30 s). All chemicals were obtained from Sigma Chemical (St. Louis, MO). Electrical signals were recorded online to a Power Mac 9500 via a MacLab/4s interface.

Field excitatory postsynaptic potentials. In addition, synaptic field potentials were also measured with focal macropatch electrodes to assess presynaptic vesicular events. The varicosities on the living terminals were visualized with vital fluorescent dye 4-Di-2-ASP (Molecular Probes) (12, 41). It was demonstrated in an earlier report that 4-Di-2-ASP had no effect on transmission within the concentration (5 μM) used in this study (12). The synaptic potentials were obtained via the loose-patch technique by lightly placing a 10- to 20-μm fire-polished glass electrode directly over a spatially isolated varicosity along the nerve terminal. The evoked field excitatory postsynaptic potentials (fEPSPs) and field miniature excitatory postsynaptic potentials were recorded and analyzed to determine the mean quantal content (m) (11, 13, 20). Direct counts of the number of evoked quantal events and failures in evoked release were used as an index of altering synaptic function. If only one single event occurred after the spike, it was counted as one; when double events occurred, they were counted as two, and so on.

Analysis. To index facilitation, the ratio in the peak amplitude of the EPSP for one of the preceding pulses from the last one within the stimulus train was used. A unitary value of 1 was subtracted from the ratio to provide a facilitation index. The subtraction of 1 ensures that, if no facilitation is present (i.e., the amplitudes of the responses are the same), the facilitation index will be zero. In addition, the amplitudes of the plateau EPSPs were compared within preparations at different stimulation frequencies by procedures previously described (16).

Statistics employed were either the Student's t-test or a Wilcoxon's paired rank sum test (a nonparametric test).

RESULTS

Because the opener muscle is regionally divided into three muscle fiber phenotypes (43), we consistently used the third to fifth muscle bundles from the most distal aspect in these studies (the arrow in Fig. 1 points to this region). STF was induced by a stimulus train of 20 pulses at either 40 or 60 Hz. Even though the EPSP amplitudes reach a plateau around the 15th stimulus of the stimulation train at 40 Hz, the responses are not saturated, because either an exposure of 5-HT or higher stimulation frequency to 60 Hz further enhances the EPSP amplitudes. This suggests that the number of docked vesicles and the recycling rate are not limiting factors in the responses reaching a plateau at 40-Hz stimulus trains. Incubation of the preparation for 5 min with 5-HT promotes the docking of vesicles such that even the first few EPSPs within each train are enhanced in amplitude. This enhancement is maintained throughout the train for both the 40- and 60-Hz pulse trains. Because the plateau of the EPSP amplitudes at 60 Hz can also be offset by 5-HT, this suggests that a homeostasis in the handling of the calcium efflux and recycling of vesicles that occurs during saline exposure can be raised to a new level after exposure to 5-HT. In a few preparations, an 80-Hz stimulation was given and the responses still showed an enhancement in amplitude but to a lesser degree (data not shown), suggesting some saturation of release and/or possibly a decrease in the ion driving gradient.

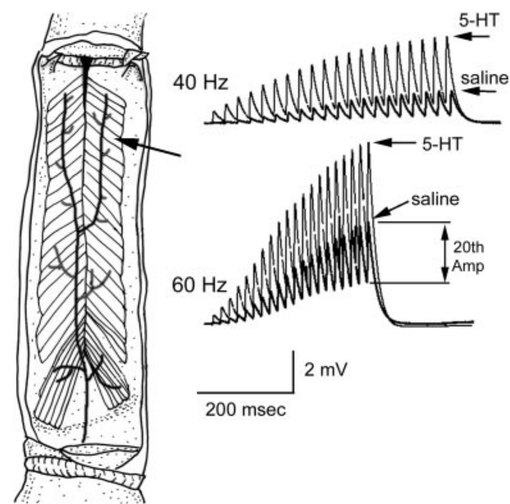

Fig. 1. Schematic of the opener muscle in the crayfish walking leg. Shown are representative excitatory postsynaptic potential (EPSP) responses to a train of 20 stimulation pulses given at 40 and 60 Hz before and during exposure to serotonin (5-HT; 100 nM). The amplitude of the EPSPs is measured from the trough preceding the EPSP of interest to peak response, as shown for the 20th pulse during saline exposure. Note that the EPSP amplitudes are greater throughout the entire stimulus train when exposed to 5-HT compared with saline. Amp, amplitude.

Changes induced by increasing stimulation frequency or exposure to 5-HT were quantified as an average of the responses over a given period of time. As shown in Fig. 2, the EPSP amplitudes showed some fluctuation over time. To standardize the measurements, an average value for the period of 250–500 s was used to obtain a mean amplitude within the saline or 5-HT exposure for either the 40- or 60-Hz stimulation. By this time, the EPSP responses ramped up to a plateau level. The preparations were allowed to recover for 5 min during the period before the bath was exchanged with 5-HT. Recordings were resumed after an additional 5 min after the exchange of the bath. The amplitude of the EPSPs returned to baseline values observed in saline at 40-Hz stimulation before bath exchange with 5-HT.

A mean value was used for further calculating a percent change among the EPSP amplitudes for the conditions within each given preparation. Determining an average of the percent change for every fifth stimulus within the trains among the preparations showed that the effect of 5-HT exposure was more pronounced for the 40-Hz stimulation paradigm than for the 60-Hz paradigm (Fig. 3*A*). However, because of the large variation in the percent changes between preparations, the mean differences were not significant by a parametric analysis. If the trends in the percent differences are compared within each preparation by a rank summed Wilcoxon's nonparametric

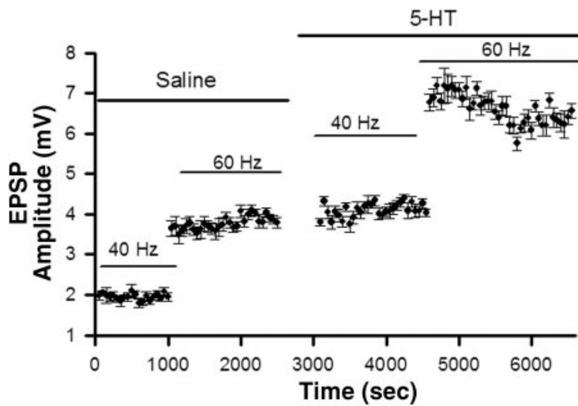

Fig. 2. Mean amplitude of EPSP during the 10th pulse in a 20-pulse train for 10 consecutive trials was used to show the effects over time due to the increase in stimulation frequency and/or exposure of 5-HT (100 nM). The effect of increasing the stimulation frequency from 40 to 60 Hz is relatively rapid. Means and SE are shown for each 50 s. On completion of the 60-Hz stimulation in saline, the preparation was allowed to recover for 5 min. The responses were tested to ensure that baseline conditions resumed for 40-Hz stimulation before the bathing medium was exchanged with 5-HT solution. The bathing medium was exchanged 3 times with the 5-HT containing saline. The preparation was then left to soak for 5 min before data collection in the 40-Hz stimulation paradigm with 5-HT exposure. The break in the time axis illustrates this recovery and incubation time before addition of the 5-HT-containing bath.

analysis, there is a significant trend showing that 5-HT exposure always produced a more pronounced effect at a 40-Hz stimulation paradigm than for a 60-Hz paradigm (Fig. 3*A*, $P < 0.05$ nonparametric as indicated by an asterisk). This is illustrated when comparing the responses within each preparation for both the 10th and 20th EPSPs within the stimulus train (Fig. 4) ($n = 5$, $P < 0.05$ Wilcoxon's rank sum).

The standard facilitation index was calculated by measures of the 5th, 10th, and 15th EPSPs in relation to the 20th EPSP to determine whether 5-HT offset STF during 40- and 60-Hz stimulations. No significant differences were present (Fig. 3*B*). However, the amplitude of the EPSPs began to reach a plateau by the 10th event, and by the 15th event very little facilitation continued to occur throughout the rest of the pulse train (Fig. 3*B*).

Plots of the individual responses in the percent change induced by 5-HT for each preparation (Fig. 4) showed that the preparations that demonstrated the largest percent change at 40 Hz also showed the largest change at 60 Hz for the 20th event (Fig. 4*B*). The preparations that produced large changes to 5-HT for the 10th events at 40 Hz did not always show the largest responses at 60 Hz. Perhaps this difference can be partly explained that in some preparations the responses have not yet reached the plateau in the amplitude of the EPSPs by the 10th event. The enhancement in the amplitude of the EPSPs by STF is presynaptic within these preparations, but the effects induced by 5-HT are likely due to a composite of pre- and postsynaptic modifications. Direct quantal counts allow one to assess the presynaptic contribution of STF and the actions of 5-HT. The glutamate released by fusion of the presynaptic vesicles results in a rapid inward current in the muscle fibers that produces a field potential, which was monitored by a focal macropatch electrode placed directly over a visualized varicosity on the nerve terminal. The potentials are quantal in nature and provide a means of counting the number of presynaptic vesicular events.

Single stimuli may evoke one or more quantal responses (a singlet, doublet, etc.), or none at all (a failure) (Fig. 5*A*). Highlighting the period after each of the 10 stimuli within each train for each experimental condition produced 40,000 traces for direct visual assessment. The observed quantal occurrences are presented in Table 1 for the 1,000 trials in each condition. There is an increase in the number of single and multiple evoked events occurring throughout the train for both 40 and 60 Hz. During exposure to 5-HT, a decrease in the number of failures occurred overall for each of the stimuli throughout the trains for both stimulation frequencies.

It is possible, in some cases, that the true number of quantal events may have been underestimated when two events occurred simultaneously and were not detected as a multiple release. This potential underestimation is not likely in the recordings presented in this study because the probability of an evoked event was relatively low. As shown in Fig. 5*B*, two evoked events followed the ninth stimulus, and one after the tenth (see enlarged inset). If counts were underestimated, the ensemble averages of the 1,000 trials would measure all the

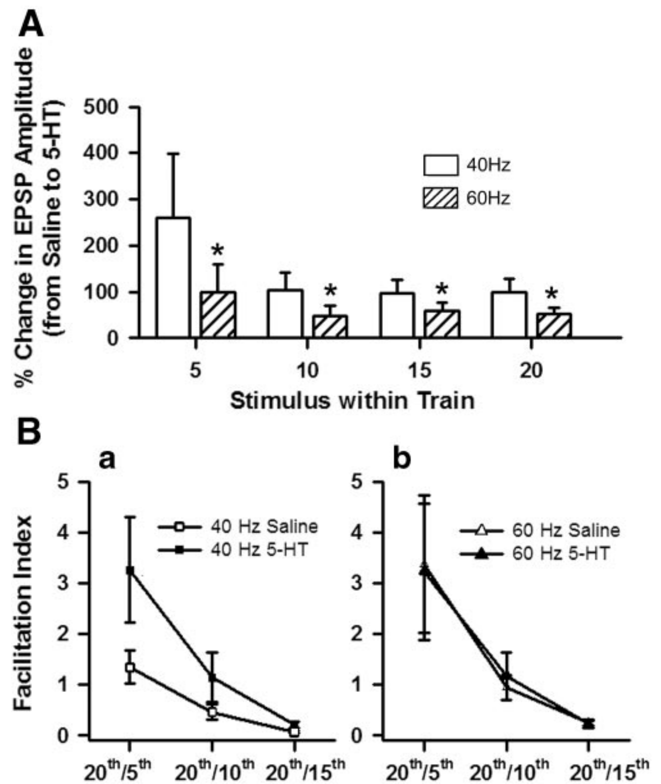

Fig. 3. Effect of 5-HT increasing the EPSP amplitudes within the stimulus train was measured by examining the overall average in the percent change for the 5th, 10th, 15th, and 20th events (*A*). Means and SE were determined by the average for the percent changes calculated for each preparation ($n = 5$) during the 250- to 500-s period for each of the paradigms shown in Fig. 2. The percent change in the response induced by 5-HT was determined for both 40- and 60-Hz trials. *B*: the facilitation index was calculated for the 5th, 10th, and 15th EPSPs in relation to the 20th EPSP for 40 Hz (*a*) and 60 Hz (*b*) in the absence and presence of 5-HT. The mean and SE for the facilitation index are shown for the 5 preparations. No significant differences were detected before and during 5-HT exposure. The amplitude of the EPSPs began to reach a plateau by the 10th event, and by the 15th event very little facilitation remained.

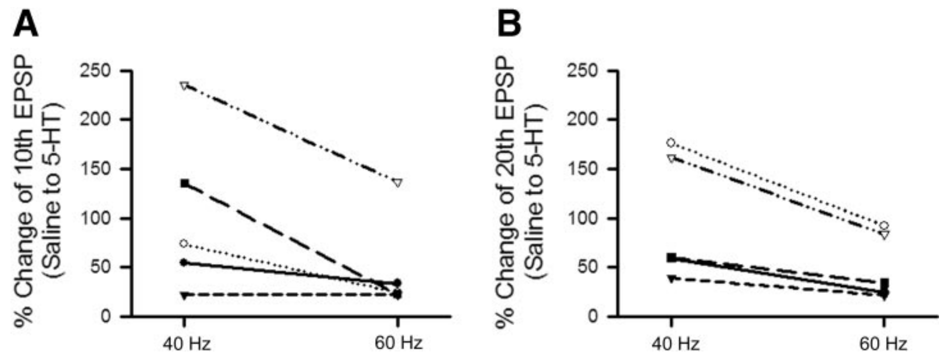

Fig. 4. Percent change in the 10th (*A*) and 20th (*B*) EPSP amplitudes for each preparation induced by 5-HT during the 40- and 60-Hz stimulation indicated that a smaller change occurred for the 60-Hz paradigm. In addition, the preparations that showed the largest change at 40 Hz also showed the largest change at 60 Hz during the 5-HT exposure for the 20th event.

responses. The overall trends of the ensemble averages paralleled the discrete quantal measures (Fig. 5, *C* and *D*).

The index of m was used to quantitatively compare the effects of 5-HT and STF on vesicular events. As shown in Fig. 6, m increases linearly throughout the 40- and 60-Hz stimulus trains. Application of 5-HT (100 nM) enhances the m throughout the stimulus train in a fairly uniform manner. A linear least squares fit of the data is superimposed on the scatter plots with the slopes reported.

Estimates of the quantal parameters, such as the probability of vesicular release and the number of release sites, using the standard binomial and Poisson distribution analysis are not sufficient because the process of counting single quantal events occurring at one site at any one time is inherently flawed (60). We did not choose to report estimates of the number of release sites and the probability of release at a site because the number of failures decreases throughout the stimulus train, implying an increase in the average probability of release from the terminal. Because 5-HT also decreases the number of failures, the probability of overall release is increased.

DISCUSSION

We have demonstrated that there is a steady state in the amount of synaptic transmission after STF has been initiated and maintained within a stimulus train. In addition, the steady state can be augmented either by increasing the frequency of nerve stimulation or by addition of 5-HT. These results support the notion that release is not saturated during the steady state of release after STF induction, because the terminal can release more transmitter, implying that synaptic area for docking is robust. Thus synaptic area is not a limiting factor for vesicular fusion at 40 Hz used in this study of the tonic motor nerve terminals. However, the size and complexity of the synapses are likely important for the initial amount and maintenance of synaptic transmission. Within short stimulus trains (10 pulses at 40 and 60 Hz), the m of synaptic transmission increased in a linear fashion. The presence of 5-HT (100 nM) induced an increase in m to the same degree throughout the stimulation train, offsetting the linear effect of electrical stimulation. Thus a pool of vesicles is recruited for release in parallel with the ones utilized for STF within the train. This general phenomenon was present for both the 40- and 60-Hz stimulus trains. A similar result to 5-HT was also reported for the opener muscle in lobster and thought to be independent of calcium (35). Because the first response within a stimulus train is enhanced after 5-HT incubation without evoked stimulation, 5-HT's action is independent of induced electrical activity.

The extent and rate at which calcium influx reaches a threshold level to induce vesicular fusion are likely related to the morphology of the AZ and synaptic structure. In addition, the proximity of AZs on a synapse, as well as their size (i.e., length), is correlated to synaptic efficacy (49, 59, 61, 62). The calcium dynamics for variations in spacing of AZs on crustacean synapses have been computationally investigated and correlated with synaptic efficacy (11, 14). The density and location of the Na^+/Ca^{2+} exchangers within nerve terminals as a whole remain unknown. A relationship may exist between the density of the exchanger, synaptic strength, and the degree of facilitation among a variety of synapses. Ohnuma et al. (44) showed a cooperativity of calcium removal from motor nerve terminals in lobster such that, with a higher stimulation rate, calcium was removed more quickly. It is interesting to note that about at the 10th event within the 40- and the 60-Hz trains, the EPSP amplitudes started to reach a plateau. If the Na^+/Ca^{2+} exchanger was able to extrude faster at 60 Hz owing to the increase in entry of calcium and residual calcium buildup, then one would expect that the plateau in the EPSP amplitudes would have been reached earlier within the train. There is likely a trade-off in the kinetics of the Na^+/Ca^{2+} exchanger for the rapid entry of sodium as well as calcium during the depolarization of the membrane. At this time we are not sure how much the entry of sodium influences the Na^+/Ca^{2+} exchanger within the nerve terminal. Thus the Na^+/Ca^{2+} exchanger might well be able to work faster with higher $[Ca^{2+}]_i$, but there are likely other factors that need to be also considered.

The results of this study, as well as those of Dudel (26, 27), suggest that the neuromodulator 5-HT alters the equilibrium of vesicle fusion and possibly $[Ca^{2+}]_i$ dynamics. Glusman and Kravitz (35) did demonstrate that, in a calcium-free bath, along with EGTA and high $MgCl_2$, 5-HT could still cause spontaneous release of transmitter as well as an enhancement in the EPSP amplitude for lobster NMJs. Thus they suggest that 5-HT works in the absence of extracellular calcium entry and that possible internal stores of calcium maybe released; however, they do caution that it is difficult to be sure that all extracellular calcium is removed from within the synaptic cleft. Dixon and Atwood (22) had also confirmed that 5-HT produced an effect with low or zero extracellular calcium in the crayfish opener but added that 5-HT's effect depended on extracellular sodium. One does need to be concerned with low or zero extracellular calcium conditions because, in various preparations, synaptic transmission is enhanced with low calcium by yet-unknown mechanisms (36, 58, 65). It was shown that 5-HT did cause an

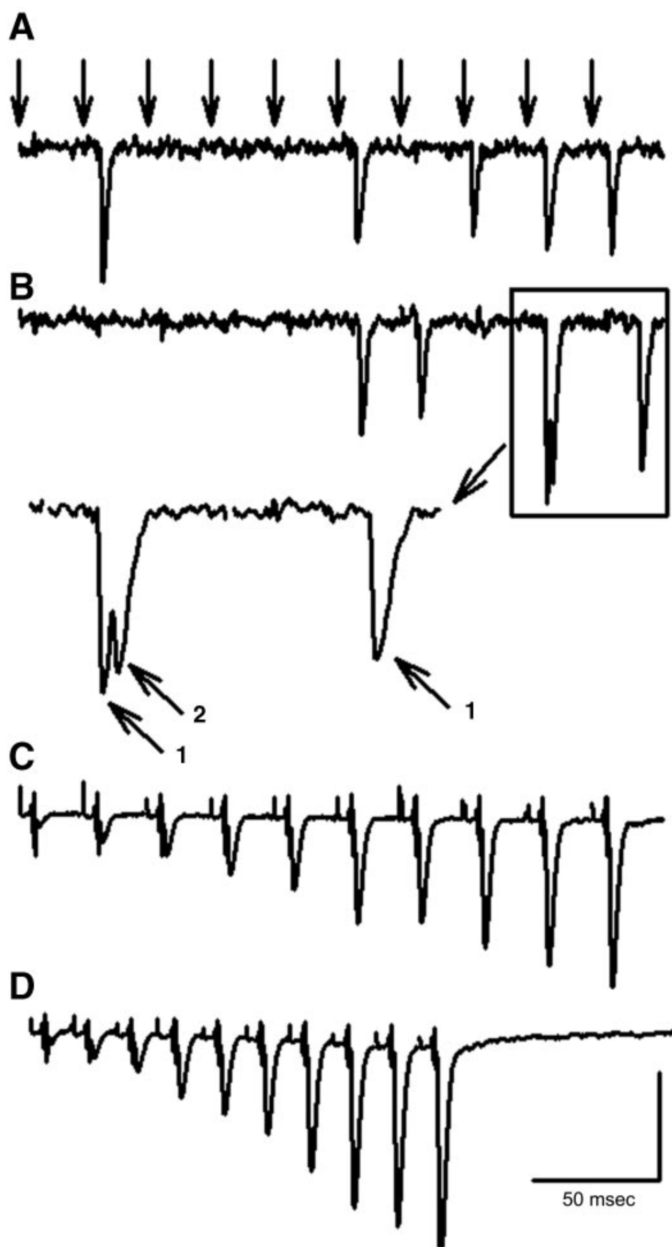

Fig. 5. Representative single traces of evoked field excitatory postsynaptic potentials (fEPSPs) are shown for a 10-pulse stimulus train (*A*). Arrows indicate when each evoked stimulus occurred. These are seen as stimulus artifacts in the original traces, but they have been removed here because they are only artifacts and can be noted by the small gaps (1 ms). In *train 1*, evoked fEPSPs occur for the 2nd, 6th, and 8th through 10th stimuli. These are all noted as single quantal events (*A*). In *train 2* (*B*), the fEPSPs occur in the 6th, 7th, 9th, and 10th stimuli; however, the 9th event is clearly a result of 2 quantal responses (see enlarged *inset*). These 2 trials were a result of 40-Hz stimulation of the nerve terminal. Note that the occurrences of events are more prevalent toward the end of the pulse train. Overall average fEPSP responses from 1,000 stimulation trials also depict that more events occur toward the end the train for both the 40- and 60-Hz stimulus trains (*B*). The ensemble average also displays that 60-Hz trains (*D*) enhance vesicular release more so than 40-Hz (*C*) stimulus trains, because the magnitudes of the responses are greater throughout the train. Because more events occur throughout the pulse trains for a small patch of the nerve terminal, the composite of the entire terminal presents a graded enhancement for the intracellular measured EPSPs throughout each single stimulus train.

enhanced calcium signal, by use of calcium-sensitive indicators, within the axon of this preparation, but no substantial changes in calcium were observed in the terminals (19). Perhaps at the time that these earlier experiments were conducted, temporal and spatial resolutions were not optimal to measure rapid spikes of calcium directly at the inner face of synapses close to docked vesicles within the terminal. In *Aplysia brasiliana*, 5-HT enhances calcium entry into the soma of motoneurons through P-type calcium channels (64). It would be of interest to know whether selective toxins that block calcium channels should be used at the crayfish NMJ with a low, but not zero, extracellular calcium to ensure that evoked release is blocked before exposure of terminals to 5-HT.

In crayfish motor nerve terminals, 5-HT shortens the latency of release for the population of vesicles (54). The release of more vesicles increases the size of the postsynaptic potentials. Quantal analysis revealed a strong presynaptic component for the actions of 5-HT by an increase in the number of vesicles releasing transmitter. No effect on the frequency or size of spontaneous release was detected at 100 nM exposure to 5-HT; however, at higher concentrations (1 μM), spontaneous release will be enhanced at both crayfish and lobster NMJs. Thus 5-HT does influence synaptic transmission presynaptically, leading to release of more vesicles at a faster rate than before 5-HT exposure. 5-HT may even act independently of calcium regulation and instead may affect the process of vesicle priming and docking in anticipation of calcium entry. Because 5-HT involves an IP_3 cascade (19, 23), various protein kinases could also be activated. The emerging notion for 5-HT's action is that induction of IP_3 leads to the phosphorylation of vesicle docking proteins such as Munc-18-1 or synaptophysin or even *N*-ethylmaleimide sensitive factor (37). Phosphorylation of Munc-18-1 and synaptophysin has been shown in vitro to inhibit the ability of these proteins to interact with the *N*-ethylmaleimide sensitive factor attachment protein receptor SNARE that they are proposed to regulate (syntaxin for Munc-18- and synaptobrevin for synaptophysin) (52, 53). 5-HT could also lead to phosphorylation of synapsins, resulting in release of vesicle pools from the cytoskeleton, which would increase the pool of free synaptic vesicles available for secretion. Such a mechanism is supported by biochemical analysis in *Aplysia* neurons (1). However, the details remain to be examined in the crayfish motor nerve terminals. The crayfish preparation is feasible for investigation because the machinery of vesicle docking appears to be similar to those of insects and mammals (4–6, 10, 37). It would be of interest to know how long 5-HT promotes the effect of vesicular release for given window of time of exposure and subsequently followed by washing of 5-HT away from the preparation in the absence of electrical stimulation. Further experiments of this type would allow a better understanding of how long the residual effects by second messengers persisted. Possible vesicles are promoted to dock by 5-HT would remain at a high level of readiness for release long after 5-HT is cleared from the preparation. A full pharmacological quantitative comparison of 5-HT effects and various period of exposure is needed to substantiate the mechanisms in detail.

When the EPSP amplitudes plateau, there is likely a steady rate of recycling of vesicles and repackaging of transmitter that could contribute to the relatively constant number of vesicle fusion events taking place for each pulse toward the end of the

Table 1. *Influence of 5-HT on quantal release parameters: mean quantal content*

Events	1st Pulse Obs	2nd Pulse Obs	3rd Pulse Obs	4th Pulse Obs	5th Pulse Obs	6th Pulse Obs	7th Pulse Obs	8th Pulse Obs	9th Pulse Obs	10th Pulse Obs
					Saline 40 Hz					
0	980	946	905	833	777	679	608	568	531	449
1	17	51	89	162	212	298	359	390	461	479
2	2	3	3	2	9	19	29	38	47	67
3	1		2	2	2	3	3	3	4	5
4			1	1		1				
5										
6							1	1		
m	0.024	0.057	0.10	0.176	0.236	0.349	0.432	0.481	0.567	0.628
					Saline 60 Hz					
0	978	955	904	833	785	667	578	499	431	405
1	22	43	93	161	205	319	397	465	527	536
2		1	2	6	9	14	24	32	38	56
3		1	1		1		1	4	4	2
4										1
m	0.022	0.048	0.1	0.173	0.226	0.347	0.448	0.541	0.615	0.658
					5-HT 40 Hz					
0	886	808	706	632	557	499	458	389	344	317
1	114	191	290	361	435	491	526	595	637	660
2		1	4	7	7	10	16	15	19	22
3					1					1
4								1		
m	0.114	0.193	0.298	0.375	0.452	0.511	0.558	0.629	0.675	0.707
					5-HT 60 Hz					
0	749	557	364	261	115	90	37	32	8	2
1	249	433	622	704	822	843	883	849	873	843
2	2	10	14	32	60	65	73	103	108	143
3				3	3	2	6	6	11	11
4							1			1
m	0.251	0.453	0.650	0.775	0.951	0.979	1.051	1.073	1.122	1.166

Direct counts of the quantal events recorded before and during exposure to serotonin (5-HT; 100 nM) for each stimulus within the 40- and 60-Hz trains. The first column states the number of discrete events observed (0, failures; 1, single events; 2 double events; etc.) that occurred for the 1,000 trials. The remaining columns state the observed number of occurrences during the stimulus events within the 10-pulse train for 1,000 trials. The direct counts are shown before and during exposure to 5-HT at 40 and 60 Hz. The mean quantal content (*m*) for each pulse within the train is shown for each condition. Obs, observed.

20-stimuli pulse train. Alternately, vesicles within a reserve pool may be recruited during this period within the stimulus train, and the ones that just fused may recycle through a rapid path which would then provide a means to rapidly replenish the readily releasable vesicle pool. Electrical activity has been shown to alter the number of vesicles docked at synapses based in tetrodotoxin studies in fish electric organs (42) as well as during LTP induction in the hippocampus of rats (2). Regardless, the addition of 5-HT rapidly recruits more vesicles into use and modulates vesicle kinetics of docking and release. This suggests that a reserve pool of vesicles exists during the plateau phase of both the 40- and 60-Hz pulse trains. Because the 5-HT enhances the synaptic response more at 40 Hz than at 60 Hz, a smaller amount of reserve pool vesicles may be available for recruitment because so many are being utilized to maintain the output needed during the 60-Hz stimulation. It is just as likely that the rate-limiting step could be the number of available docking sites, because at 60 Hz more sites could possibly be in use. Likely a combination of the two possibilities is at play. It may even be possible that the pool of vesicles recruited with electrical depolarization is different than those recruited with 5-HT.

When a vesicle recycles after release, it is postulated to follow two different routes for recycling: a rapid loop and a slower one that reprocess the vesicles within the endoplasmic reticulum (39, 40, 50, 56). Two slightly different pools of reserve vesicles might be produced by these alternate recycling mechanisms. In the crayfish motor nerve terminals, one route might be differentially influenced by 5-HT, via secondary messenger activation, and the other may be more tightly regulated by electrical activity patterns of the terminal. Such a differential regulation is feasible because, as shown in this study, incubation of the NMJ with 5-HT in the absence of sustained electrical activity results in more vesicles being released with a single stimulus or within a train of stimuli.

Possibly the use of vesicle loading dyes, such as FM1-43, could resolve this issue. However, there is still some concern with the use of FM1-43 because the fluorescence may overestimate the time of vesicle fusion and recycling because the styryl dye might not be fully released from the synaptic vesicle and cleft on vesicle fusion compared with a nonmembrane soluble dye (39). However, more recent studies suggest that the dye might be distributed in the endosomal compartment or released by various means to account for longer retention times of the dye (3, 34, 51). These recent findings of vesicular kinetics and recycling support the notion of differential regulation, and thus the paths may be targeted independently by cellular processes, such as those by 5-HT-triggered IP_3-in-

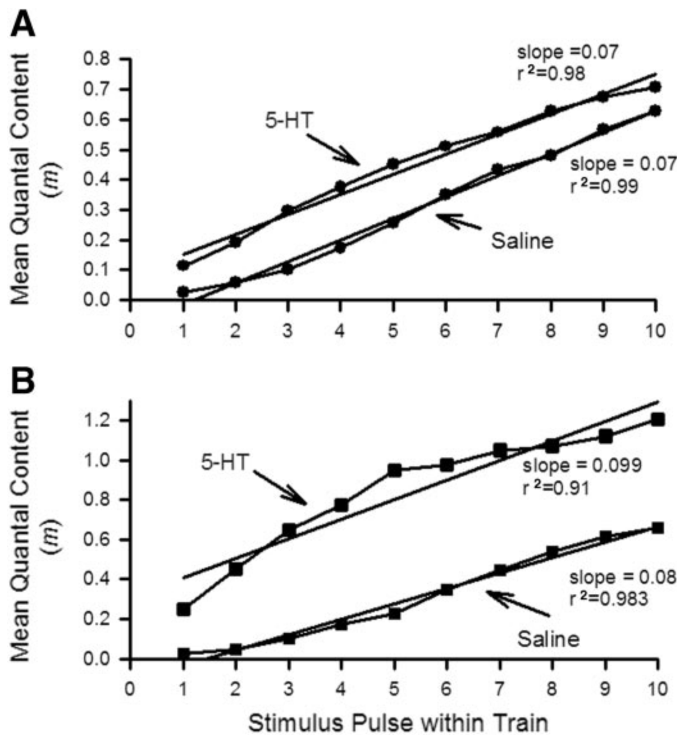

Fig. 6. Mean quantal content (*m*), as determined by direct counts, increases linearly throughout the 40-Hz (*A*) and 60-Hz (*B*) stimulus trains. Application of 5-HT (100 nM) enhances the *m* throughout the stimulus train in a fairly uniform manner. A linear least squares fit of the data is superimposed on the scatter plots, and the slopes are reported. The *m* values were obtained from a 1,000 trials and counted for each of the 10 stimuli within the pulse train.

duced phosphorylation and direct electrical depolarization for calcium-induced cellular responses (63). In depressed NMJs of crayfish, release is promoted by 5-HT, as determined by FM1-43 and electrophysiological studies, suggesting that different pools and paths are present (63). Both such processes would be feasible in the crustacean NMJ system in our presented study.

If more calcium enters when increasing the stimulation frequency (i.e., 40 to 60 Hz), via voltage-sensitive calcium channels, the influx can exceed the efflux until a new equilibrium is set, as we have shown. Very-high-output terminals, such as those of crustacean phasic motor neurons, generally show little facilitation during stimulation trains even though there is still a substantial electrical chemical driving gradient (8, 9). Exposure to 5-HT (100 nM) can enhance vesicular release in the range of a 100% in phasic high-output motor nerve terminals of the leg extensor in crayfish, demonstrating again that vesicular fusion can be further enhanced even in very-high-output terminals through neuromodulation even though frequency facilitation may not produce as large an effect (9). This phenomenon is pertinent to various types of synapses within the varicosities of tonic motor nerve terminals because some synapses are likely high output compared with others because of the number and spacing of AZs within given synapses (12, 14). Figure 7*A* illustrates a high-output synapse (Sy1) with two AZs and a low-output synapse (Sy2) with only one AZ. Increasing the stimulation frequency would likely alter the probability of release differentially among such synapses of varying synaptic complexity. In the example shown (Fig. 7, *A* and *B*), the synapse with two AZs (Sy1) close together will have a greater probability of release than the simple synapse (Sy2) when the stimulation frequency is increased, whereas the action of 5-HT would likely affect both equally in promoting vesicle priming (Fig. 7, *C* and *D*).

Differences in the responses obtained from EPSPs and fEPSPs may also reflect some subtle differences depending on the location where one obtains the fEPSPs. The results obtained from the fEPSPs indicate that 5-HT had the greatest effect for the 60-Hz trials in enhancing the mean quantal content. In comparison, the responses obtained from the EPSPs demonstrate the 5-HT has the most pronounced effect for the

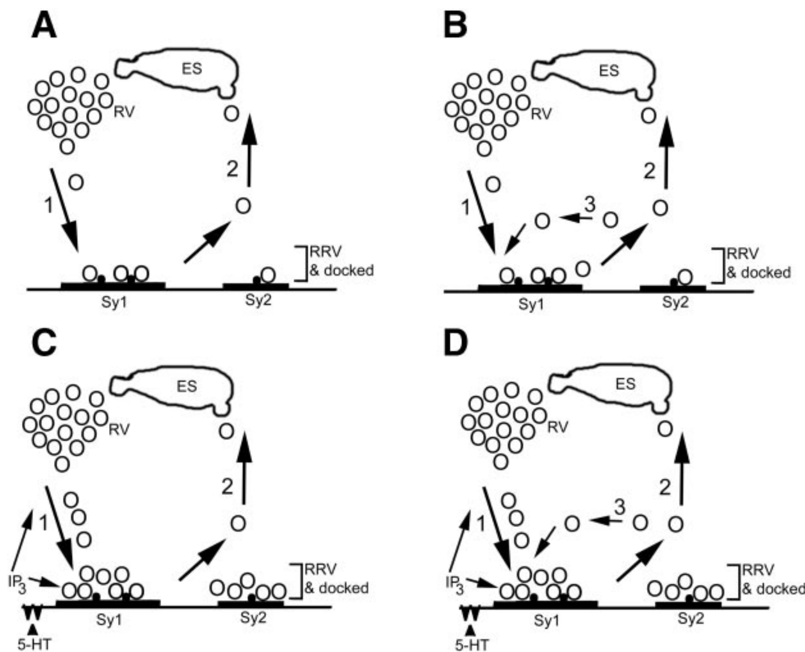

Fig. 7. Schematic representation of the vesicle pathways within the presynaptic motor nerve terminal. *A*: in the absence of electrical stimulation (i.e., Inactive) of the nerve terminal, few vesicles will spontaneously be released at a slow rate; thus a slow recycling path (*path 1* to *2*) will be utilized. *B*: on electrical stimulation (i.e., Active) the high-output synapse (Sy1), the one with 2 active zones as shown with dark hemispheres sitting on the inner face of the synapse, will be recruited before the low-output synapse (Sy2) with only 1 active zone. Vesicles may be recruited from the reserve vesicle pool (RV, *path 1*) to the readily releasable vesicle pool (RRV) for docking and fusion with the presynaptic membrane as well during electrical stimulation. The vesicles may then recycle through a slow process and intermediate endosomal (ES) stage (*path 2*), as well as a different path (*path 3*) that is relatively rapid in recharging the vesicles with transmitter before the vesicle ends up back at the RRV pool. *C*: when the nerve terminal is not electrically stimulated but is exposed to 5-HT (i.e., 5-HT and Inactive), at a low concentration (100 nM), there is a priming of the synapses by promoting *path 1*. The activation of the second messenger inositol 1,4,5-trisphosphate (IP_3) can recruit vesicles from RV as well as enhance priming and docking of vesicles at the synapses within the RRV by phosphorylating synaptically relevant proteins. In this case, both the high-output (Sy1) and low-output (Sy2) synapses will be influenced. *D*: when electrical activity and exposure to 5-HT are combined (i.e., 5-HT and Active) a marked enhancement of transmission will occur that will activate *path 1* and *path 3* for rapid recycling.

40-Hz stimulus trains. Because the EPSPs represent an ensemble average of the terminals innervating the entire muscle, they may best represent the effective motor output to regulate behavior of the animal. The focal recordings only sample a very small fraction of the terminal (i.e., one varicosity out of hundreds on a muscle fiber), but they allow a level of resolution of discrete quantal events. Previously, it was shown that there are substantial differences along the length of a given terminal in synaptic structure and responses to evoked stimuli (10, 12, 13). Thus there may well be differences in the responsiveness to 5-HT or the effects of assessing frequency of stimulation in conjunction with the effects of 5-HT along the length of the nerve terminals. There is recent evidence that microdomains in second messenger signaling cascades are present in neurons, so it is feasible they might even exist along the length of a nerve terminal (21). To resolve this issue, an extensive study would need to be undertaken to give the degree of variation among preparations and muscle fibers within a preparation (11, 43).

In summary, we postulate that the plateau in the EPSP amplitudes during the stimulus train is likely a response to steady state of influx, buffering, and efflux of calcium ions. The efflux is probably via the Na^+/Ca^{2+} exchanger. The larger the calcium influx, the greater the driving forces for the Na^+/Ca^{2+} exchanger, although maybe not in a linear fashion. In addition, the steady recycling process of vesicles being used for maintaining release during the long trains of stimulation may be augmented by recruiting vesicles from the reserve vesicle pool (RV, Fig. 7*B*). 5-HT enhances the number of vesicles entering into the rapid recycling pool in the absence (Fig. 7*C*) as well as in the presence of electrical stimulation (Fig. 7*D*). However, when more vesicles enter the rapid recycling pool, induced by higher frequency stimulus trains, fewer vesicles are available for recruitment by 5-HT or possible a docking inference might occur. This scenario is schematically represented in Fig. 7 to illustrate these points. Generally, in the absence of electrical stimulation and absence of 5-HT a few spontaneous releases occur resulting in vesicles to recycle via a slow process (*path 1* to *2*, Fig. 7*A*). With electrical stimulation such as with pulse trains we used, a vesicle that was just recaptured from the presynaptic membrane may also recycle through an endosome intermediate to enter and reside in an RV (*path 1* to *2*, Fig. 7*B*). This rather outdated model for the nerve terminal is not likely the process at work here for two reasons: first, the kinetics measured in various studies for dye-filled vesicle recycling does not fit with the duration of time it would require (although low stimulation frequencies show slower recycling rates); second, such endosomal structures are observed in only a few types of presynaptic nerve terminals (3, 17, 18), and they have yet to be clearly delineated in the motor nerve terminals of crayfish. Thus it is likely that *path 3* (Fig. 7*B*) is utilized during rapid sustained transmission. Possibly some homeostasis is obtained with repetitive high-frequency trains of stimuli because *path 3* can speed up and slow down, depending on the rate of fusion at the presynaptic membrane. Thus going from 40-Hz trains to 60-Hz trains results in increased residual calcium, a higher probability of vesicle fusion, and enhanced recycling. In addition, some of the vesicles in the RV pool will be recruited, reducing the RV pool potential for 5-HT to act on. We postulate that the raised plateau in EPSPs on stimulation at 60 Hz occurs because of a new equilibrium in the Na^+/Ca^{2+} exchanger that develops as a response to handling the higher calcium influx. In the case of the 60-Hz stimulation trains, the RV is reduced; thus 5-HT has lower probability of using the RV. In the absence of electrical activity, the action of 5-HT would still favor the direction of the RRV from RV (*path 1*, Fig. 7*C*). Thus even low-output synapses would be primed (Sy2) as well as high-output synapses (Sy1). When synapses are primed by the effects of 5-HT, the electrical stimulated *path 3* (Fig. 7*D*) would be recruited into action to maintain the high recycling. Thus the recycling paths induced by electrical activity and 5-HT may complement each other by acting on distinct steps in the process. For example, 5-HT acting through IP_3 by phosphorylating key proteins to promote both vesicles to be released from synapsins as well as enhanced vesicle docking would be additive to the paths stimulated by electrical activity. The neuromodulation of synaptic function described in this report may well apply to synapses in central nervous systems as well as at other NMJs.

ACKNOWLEDGMENTS

We are very thankful to Dr. Josef Dudel (Physiologisches Institut der Ludwig-Maximilians-Universität München) for suggestions and continual interaction during this study. Appreciation is given to Andy Johnstone for critical editing and suggestions on the manuscript.

GRANTS

Funding was provided by National Science Foundation Grant IBN-0131459 (to R. L. Cooper), a G. Ribble Fellowship for undergraduate studies in the School of Biological Sciences at the University of Kentucky (to G. M. Sparks), and a Undergraduate Research Scholarship awarded by the Arnold and Mabel Beckman Foundation (to G. M. Sparks).

REFERENCES

1. **Angers A, Fioravante D, Chin J, Cleary LJ, Bean AJ, and Byrne JH.** Serotonin stimulates phosphorylation of *Aplysia* synapsin and alters its subcellular distribution in sensory neurons. *J Neurosci* 22: 5412–5422, 2002.
2. **Applegate MD, Kerr DS, and Landfield PW.** Redistribution of synaptic vesicles during long-term potentiation in the hippocampus. *Brain Res* 401: 401–406, 1987.
3. **Aravanis AM, Pyle JL, and Tsien RW.** Single synaptic vesicles fusing transiently and successively without loss of identity. *Nature* 423: 643–647, 2003.
4. **Atwood HL and Cooper RL.** Functional and structural parallels in crustaceans and *Drosophila* neuromuscular systems. *Am Zool* 35: 556–565, 1995.
5. **Atwood HL and Cooper RL.** Assessing ultrastructure of crustacean and insect neuromuscular junctions. *J Neurosci Methods* 69: 51–58, 1996.
6. **Atwood HL and Cooper RL.** Synaptic diversity and differentiation: crustacean neuromuscular junctions. *Invert Neurosci* 1: 291–307, 1996.
7. **Atwood HL and Wojtowicz JM.** Short-term plasticity and physiological differentiation of crustacean motor synapses. *Int Rev Neurobiol* 28: 275–362, 1986.
8. **Bradacs H, Cooper RL, Msghina M, and Atwood HL.** Differential physiology and morphology of phasic and tonic motor axons in a crayfish limb extensor muscle. *J Exp Biol* 200: 677–691, 1997.
9. **Cooper RL, Donmezer A, and Shearer J.** Intrinsic differences in sensitivity to 5-HT between high- and low-output terminals innervating the same target. *Neurosci Res* 45: 163–172, 2003.
10. **Cooper RL, Hampson D, and Atwood HL.** Synaptotagmin-like expression in the motor nerve terminals of crayfish. *Brain Res* 703: 214–216, 1995.
11. **Cooper RL, Harrington CC, Marin L, and Atwood HL.** Quantal release at visualized terminals of a crayfish motor axon: intraterminal and regional differences. *J Comp Neurol* 375: 583–600, 1996.

12. **Cooper RL, Marin L, and Atwood HL.** Synaptic differentiation of a single motor neuron: conjoint definition of transmitter release, presynaptic calcium signals, and ultrastructure. *J Neurosci* 15: 4209–4222, 1995.
13. **Cooper RL, Stewart BA, Wojtowicz JM, Wang S, and Atwood HL.** Quantal measurement and analysis methods compared for crayfish and *Drosophila* neuromuscular junctions and rat hippocampus. *J Neurosci Methods* 61: 67–78, 1995.
14. **Cooper RL, Winslow J, Govind CK, and Atwood HL.** Synaptic structural complexity as a factor enhancing probability of calcium-mediated transmitter release. *J Neurophysiol* 75: 2451–2466, 1996.
15. **Crider ME and Cooper RL.** The importance of the stimulation paradigm in determining facilitation and effects of neuromodulation. *Brain Res* 842: 324–331, 1999.
16. **Crider ME and Cooper RL.** Differentially facilitation of high- and low-output nerve terminals from a single motor neuron. *J Appl Physiol* 88: 987–996, 2000.
17. **De Camilli P and Takei K.** Molecular mechanisms in synaptic vesicle endocytosis and recycling. *Neuron* 16: 481–486, 1996.
18. **De Camilli P, Haucke V, Takei K, Mugnaini E.** The structure of synapses. In: *Synapses*, edited by Cowan M, Südhof T, and Stevens CF. Baltimore, MD: The Johns Hopkins Univ. Press, 2001, p. 89–133.
19. **Delaney K, Tank DW, and Zucker RS.** Presynaptic calcium and serotonin-mediated enhancement of transmitter release at crayfish neuromuscular junction. *J Neurosci* 11: 2631–2643, 1991.
20. **Del Castillo J and Katz B.** Quantal components of the end-plate potential. *J Physiol* 124: 560–573, 1954.
21. **Delmas P, Crest M, and Brown DA.** Functional organization of PLC signaling microdomains in neurons. *Trends Neurosci* 27: 41–47, 2004.
22. **Dixon D and Atwood HL.** Crayfish motor nerve terminal's response to serotonin examined by intracellular microelectrode. *J Neurobiol* 16: 409–424, 1985.
23. **Dixon D and Atwood HL.** Conjoint action of phosphoinositol and adenylate cyclase systems in serotonin-induced facilitation at the crayfish neuromuscular junction. *J Neurophysiol* 62: 1251–1259, 1989.
24. **Dudel J.** Facilitatory effects of 5-hydroxy-tryptamine on the crayfish neuromuscular junction. *Naunyn-Schmiedebergs Arch Pharmacol* 249: 515–528, 1965.
25. **Dudel J.** Potential changes in the crayfish motor nerve terminal during repetitive stimulation. *Pflügers Arch* 282: 323–337, 1965.
26. **Dudel J.** The effect of reduced calcium on quantal unit current and release at the crayfish neuromuscular junction. *Pflügers Arch* 391: 35–40, 1981.
27. **Dudel J.** Modulation of quantal synaptic release by serotonin and forskolin in crayfish motor nerve terminals. In: *NATO Advanced Research Workshop on Modulation of Synaptic Transmission and Plasticity in Nervous Systems (1987: Castelvecchio Pascoli, Italy)*, edited by Hertting G and Spatz HC. Berlin: Springer-Verlag, 1988, vol. H19, p. 259–270.
28. **Dudel J.** Calcium and depolarization dependence of twin-pulse facilitation of synaptic release at nerve terminal of crayfish and frog muscle. *Pflügers Arch* 415: 304–309, 1989.
29. **Dudel J.** Calcium dependence of quantal release triggered by graded depolarization pulses to nerve terminals on crayfish and frog muscle. *Pflügers Arch* 415: 289–298, 1989.
30. **Dudel J.** Shifts in the voltage dependence of synaptic release due to changes in the extracellular calcium concentration at nerve terminals on muscle of crayfish and frogs. *Pflügers Arch* 415: 299–303, 1989.
31. **Dudel J.** Twin pulse facilitation in dependence on pulse duration and calcium concentration at motor nerve terminals of crayfish and frog. *Pflügers Arch* 415: 310–315, 1989.
32. **Dudel J and Kuffler SW.** The quantal nature of transmission and spontaneous miniature potentials at the crayfish neuromuscular junction. *J Physiol* 55: 514–529, 1961.
33. **Dudel J, Parnas I, and Parnas H.** Neurotransmitter release and its facilitation in crayfish muscle. VI. Release determined by both, intracellular calcium concentration and depolarization of the nerve terminal. *Pflügers Arch* 399: 1–10, 1983.
34. **Gandhi SP and Stevens CF.** Three modes of synaptic vesicular recycling revealed by single-vesicle imaging. *Nature* 423: 607–613, 2003.
35. **Glusman S and Kravitz EA.** The action of serotonin on excitatory nerve terminals in lobster nerve-muscle preparations. *J Physiol* 325: 223–241, 1982.
36. **Haas HL and Jefferys JG.** Low-calcium field burst discharges of CA1 pyramidal neurones in rat hippocampal slices. *J Physiol* 354: 185–201, 1984.
37. **He P, Southard RC, Whiteheart SW, and Cooper RL.** Role of α-SNAP in promoting efficient neurotransmission at the crayfish neuromuscular junction. *J Neurophysiol* 82: 3406–3416, 1999.
38. **Katz B and Miledi R.** The role of calcium in neuromuscular facilitation. *J Physiol* 195: 481–492, 1968.
39. **Klingauf J, Kavalali ET, and Tsien RW.** Kinetics and regulation of fast endocytosis at hippocampal synapses. *Nature* 394: 581–585, 1998.
40. **Kuromi H and Kidokoro Y.** Selective replenishment of two vesicle pools depends on the source of Ca^{2+} at the Drosophila synapse. *Neuron* 35: 333–343, 2002.
41. **Magrassi L, Purves D, and Lichtman JW.** Fluorescent probes that stain living nerve terminals. *J Neurosci* 7: 1207–1214, 1987.
42. **Maler L and Mathieson WB.** The effect of nerve activity on the distribution of synaptic vesicles. *Cell Mol Neurobiol* 5: 373–387, 1985.
43. **Mykles DL, Medler SA, Koenders A, and Cooper RL.** Myofibrillar protein isoform expression is correlated with synaptic efficacy in slow fibres of the claw and leg opener muscles of crayfish and lobster. *J Exp Biol* 205: 513–522, 2002.
44. **Ohnuma K, Kazawa T, Ogawa S, Suzuki N, Miwa A, and Kijima H.** Cooperative Ca^{2+} removal from presynaptic terminals of the spiny lobster neuromuscular junction. *Biophys J* 76: 1819–1834, 1999.
45. **Parnas H, Dudel J, and Parnas I.** Neurotransmitter release and its facilitation in crayfish. I. Saturation kinetics of release, and of entry and removal of calcium. *Pflügers Arch* 393: 1–14, 1982.
46. **Parnas H, Dudel J, and Parnas I.** Neurotransmitter release and its facilitation in crayfish. IV. The effect of Mg^{2+} ions on the duration of facilitation. *Pflügers Arch* 395: 1–5, 1982.
47. **Parnas I, Parnas H, and Dudel J.** Neurotransmitter release and its facilitation in crayfish muscle. II. Duration of facilitation and removal processes of calcium from the terminal. *Pflügers Arch* 393: 323–236, 1982.
48. **Parnas I, Parnas H, and Dudel J.** Neurotransmitter release and its facilitation in crayfish muscle. V. Basis for synapse differentiation of the fast and slow type in one axon. *Pflügers Arch* 395: 261–270, 1982.
49. **Rheuben M.** Quantitative comparison of the structural features of slow and fast neuromuscular junctions in *Manduca*. *J Neurosci* 5: 1704–1716, 1985.
50. **Richards DA, Guatimosim C, and Betz WJ.** Two endocytic recycling routes selectively fill two vesicle pools in frog motor nerve terminals. *Neuron* 27: 551–559, 2000.
51. **Rizzoli SO and Betz WJ.** Neurobiology: all change at the synapse. *Nature* 423: 591–592, 2003.
52. **Rubenstein JL, Greengard P, and Czernik AJ.** Calcium-dependent serine phosphorylation of synaptophysin. *Synapse* 13: 161–172, 1993.
53. **Shuang R, Zhang L, Fletcher A, Groblewski GE, Pevsner J, and Stuenkel EL.** Regulation of Munc-18/syntaxin 1A interaction by cyclin-dependent kinase 5 in nerve endings. *J Biol Chem* 273: 4957–4966, 1998.
54. **Southard RC, Haggard J, Crider ME, Whiteheart SW, and Cooper RL.** Influence of serotonin on the kinetics of vesicular release. *Brain Res* 871: 16–28, 2000.
55. **Sparks GM, Brailoiu E, Brailoiu GC, Dun NJ, Tabor J, and Cooper RL.** Effects of m-CPP in altering neuronal function: blocking depolarization in invertebrate motor and sensory neurons but exciting rat dorsal root neurons. *Brain Res* 969: 14–26, 2003.
56. **Stevens CF and Williams JH.** "Kiss and run" exocytosis at hippocampal synapses. *Proc Natl Acad Sci USA* 97: 12828–12833, 2000.
57. **Tabor J and Cooper RL.** Physiologically identified 5-HT_2-like receptors at the crayfish neuromuscular junction. *Brain Res* 932: 91–98, 2002.
58. **Undem BJ, Oh EJ, Lancaster E, and Weinreich D.** Effect of extracellular calcium on excitability of guinea pig airway vagal afferent nerves. *J Neurophysiol* 89: 1196–1204, 2003.
59. **Verma V.** The presynaptic active zones in three different types of fibres in frog muscle. *Proc R Soc Lond B Biol Sci* 221: 369–373, 1984.
60. **Viele K, Stromberg A, and Cooper RL.** Estimating the number of release sites within the nerve terminal by statistical analysis of synaptic charge. *Synapse* 47: 15–25, 2003.
61. **Walrond JP, Govind CK, and Heustis S.** Two structural adaptations for regulating transmitter release at lobster neuromuscular synapses. *J Neurosci* 13: 4831–4845, 1993.
62. **Walrond JP and Reese TS.** Structure of axon terminals and active zones at synapses on lizard twitch and tonic muscle fibers. *J Neurosci* 5: 1118–1131, 1985.

63. **Wang C and Zucker RS.** Regulation of synaptic vesicle recycling by calcium and serotonin. *Neuron* 21: 155–167, 1998.
64. **Yu B, Gamkrelidze GN, Laurienti PJ, and Blakenship JE.** Serotonin directly increases a calcium current in swim motoneurons of *Aplysia brasiliana*. *Am Zool* 41: 1009–1025, 2001.
65. **Zefirov AL, Mukhamedzyanov RD, Minlebaev MG, Cheranov SY, Abdrakhmanov MM, and Grigor'ev PN.** Transmitter secretion in the frog neuromuscular synapse after prolonged exposure to calcium-free solutions. *Neurosci Behav Physiol* 33: 613–622, 2003.
66. **Zucker RS.** Characteristics of crayfish neuromuscular facilitation and their calcium dependence. *J Physiol* 241: 91–110, 1974.
67. **Zucker RS and Lara-Estrella LO.** Post-tetanic decay of evoked and spontaneous transmitter release and a residual-calcium model of synaptic facilitation at crayfish neuromuscular junctions. *J Gen Physiol* 81: 355–372, 1983.