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Research report

Influence of serotonin on the kinetics of vesicular release

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Abstract

The mechanisms by which synaptic vesicles are transported and primed to fuse with the presynaptic membrane are important to all chemical synapses. Processes of signal transduction that affect vesicular dynamics, such as the second-messenger cascades induced by neuromodulators, are more readily addressed in assessable synaptic preparations of neuromuscular junctions in the crayfish. We assessed the effects of serotonin (5-HT) through the analysis of the latency jitter and the quantal parameters: *n* and *p* in the opener muscle of the walking leg in crayfish. There is an increase in the size of the postsynaptic currents due to more vesicles being released. Quantal analysis reveals a presynaptic mechanism by an increase in the number of vesicles being released. Latency measures show more events occur with a short latency in the presence of 5-HT. No effect on the frequency or size of spontaneous release was detected. Thus, the influence of 5-HT is presynaptic, leading to a release of more vesicles at a faster rate.

Theme: Excitable membranes and synaptic transmission

Topic: Presynaptic mechanisms

Keywords: Serotonin; Neurotransmission; Crayfish; Neuromuscular junction; Synapse

1. Introduction

Neuromodulators are well recognized as an important class of messengers in all animals investigated. One neuromodulator in particular, serotonin (5-HT), has been determined to be involved in the behavioral expression of dominance and aggression in many animals from crustaceans [36,42–44,59] to mammals [7]. The distribution of nerve terminals containing 5-HT in the peripheral nervous systems of invertebrates and vertebrates suggests that it is not directed at a discrete postsynaptic targets but rather diffuses over a more widespread area. In lobsters it was shown that injection of 5-HT into the circulatory system caused the animal to assume a dominant posture [34,40]. It is known that 5-HT is released into the animal's hemolymph from neuro-secretatory organs [28,44] and that it has an effect on the motor nerve terminals to cause more transmitter release [14–21].

Little is known about the direct mechanisms of action on the neurons, with the exception of a few studies that have shown that 5-HT works through an inositol triphosphate (IP_3) and cyclic AMP (cAMP) second-messenger system [6,18–21]. Presently information is lacking that addresses the influence of the known modulators directly at the *sites* of release among presynaptic neurons to quantify, on a relative basis, the number of release sites and the influence on the *probability* of quantal release at the active synapses. Prior studies addressed the effects on neurotransmission in general by recording muscle potentials arising from thousands of synapses [21,29,30,33,38,39,56].

The role of 5-HT on synapses along a single terminal that show differences in synaptic output can reliably be assessed using a crayfish opener-muscle preparation [8,9,14,15]. As far as we are aware, there has not yet been a study on the examination of neuromodulator effects among synapses of a single motor neuron that display different release characteristics. Using macro-patch recording and quantal analysis to measure synaptic transmission at these sites, we have assessed the effects on transmission by determining the quantal parameters of *m* (mean quantal content), *n* (number of release sites) and *p* (probability of release at a site) in the absence and the presence of 5-HT.

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The beauty of these preparations is that the physiological measures can be directly correlated with synaptic structure directly at the release sites [4]. Incorporating the knowledge of the synaptic structures, such as the number of active zones and locations on a synapse with the location of vesicular populations (docked, readily-releasable, and reserve pools) from which physiological recordings were made, provides significant new mechanistic insights concerning why neuromodulators affect regions differently.

In this study, we demonstrate that 5-HT increases the number of vesicles to be released and that pool of the vesicles are released at relatively faster rates. These factors increase the probability of release occurring and increase the quantal content or in other words, synaptic efficacy is enhanced. Since spontaneous release is not altered in it frequency of release nor in the quantal charge, 5-HT's action is predominantly presynaptic to result in the increased evoked synaptic currents.

Preliminary reports of this work have previously appeared in abstract form [52].

2. Methods

All experiments were performed using the first walking leg of crayfish, *Procambarus clarkii*, measuring 4–6 cm in body length (Atchafalaya Biological Supply Co., Raceland, LA). The opener muscle of the first walking legs was prepared by the standard dissection [26]. The tissue was pinned out in a Slygard dish for viewing with a Nikon, Optiphot-2 upright fluorescent microscope using a 40× (0.55 NA) Nikon water immersion objective. Dissected preparations were maintained in crayfish saline (modified Van Harreveld's solution: 205 mM NaCl; 5.3 mM KCl; 13.5 mM $CaCl_2$; 2.45 mM $MgCl_2$; 0.5 mM Hepes/NaOH, pH 7.4) at 14°C. The entire opener muscle is innervated by a single tonic excitatory motor neuron [8]. To show the innervation pattern and to visualize the nerve terminals for illustration, we used the vital fluorescent dye, 4-[4-(diethylamino) styryl]-*N*-methylpyridinium iodide (4-Di-2-Asp; Molecular Probes, Eugene, OR), to stain the preparation [41]. The living preparation was stained with a 2–5 μM dye solution for 2–5 min, and then washed in crayfish saline before being photographed. The 5-HT, and all chemicals listed were obtained from Sigma Chemical.

2.1. Evoked post-synaptic potentials (EPSPS)

Intracellular muscle recordings were made with a 3 M KCl-containing microelectrode placed in a centrally located fiber in the opener muscle. The responses were amplified with a 1×LU head stage and an Axoclamp 2A amplifier (Axon Instruments). Axons were stimulated by a train of 10 pulses given at the indicated frequencies. The stimulation frequency was kept constant for each preparation. The frequency used was 40 Hz with a train interval of 10 s. All events were measured and calibrated with the MacLab Scope software 3.5.4 version (ADInstruments). Stimulation was obtained by use of a Grass S-88 simulator and a stimulation isolation unit (Grass, SIU) with leads to a standard suction electrode [8].

2.2. Evoked post-synaptic current (EPSCS)

To further our understanding of the enhancement in EPSP amplitudes by 5-HT, we determined the presynaptic contribution of 5-HT's actions by direct quantal counts and latency of release. These measures allow one to examine the mechanisms of action on vesicular release. Focal macropatch recording was used to measure synaptic currents. The synaptic currents were obtained using the loose patch technique by lightly placing a 10 to 15 μm diameter, fire-polished, glass electrodes directly over a single, spatially isolated varicosity along the vital dye-visualized nerve terminal. The living nerve terminals were visualized with the vital fluorescent dye. The preparation was exposed to the dye solution for 2 to 5 min, followed by washing in crayfish saline. The synaptic transmission remained unaltered by this dye as previously shown [8]. The macropatch electrode is specific for current recording within the region of the electrode lumen. The lumen of the patch electrode was filled with the same solution as the bathing medium. The seal resistance was in the range of 100 KΩ to 1 MΩ. Since the seal can easily be lost if muscle twitches under the electrode, stimulation was restricted to 1 Hz. Evoked EPSCs (excitatory postsynaptic currents) and mEPSCs (miniature excitatory postsynaptic currents) were recorded and analyzed to determine the mean quantal content (m), the number of release sites (n), and the probability of release at the sites (p) [9,11]. In each synaptic current recording, a trigger artifact and a nerve spike can be visualized which indicates nerve stimulation. Mean quantal content can be determined by direct counts (*mco*):

$$\text{Direct counts } (mco) = \frac{\sum[0(\text{No. of failures}) + 1(\text{No. of single events}) + 2(\text{No. of double events}) \ldots]}{\text{the total number of sweeps}}$$

Direct counts of the evoked quantal events allowed the distribution of release to be further characterized into the type of distribution in order to estimate n and p. As shown in, Table 1, in some cases, there were no evoked events that followed the nerve terminal spikes. This type of response is called a failure in evoked release, and is counted as a zero. If only one single event occurs after the spike, it is counted as one. When double events occur, it would be referred to as a two, etc.

The direct method of counting quantal events alleviates difficulties associated with changes in the seal resistance as well as in the determination of the mean quantal content as determined by measuring either peak amplitude or charge

Table 1
Effects of 5-HT on quantal parameters *m*, *n* and *p*

		All Sweeps		1–200		201–400		401–600		601–800		801–1000	
		Obs	Pred	Obs	Pred	Obs	Pred	Obs	Pred	Obs	Pred	Obs	Pred
Prep. #1													
Control	0	592	601	113	114	111	114	124	126	130	131	114	116
	1	341	322	69	67	79	70	66	62	55	56	72	67
	2	32	43	9	10	5	11	5	8	6	6	7	10
	3	1	0	0	0	0	0	0	0	1	0	0	0
	m_{co}	0.41		0.435		0.445		0.38		0.35		0.43	
	n	3		2		2		2		4		2	
	p	0.141		0.228		0.228		0.195		0.09		0.223	
5-HT added	0	392	391	58	69	56	67	104	107	81	88	93	98
(0–18 min)	1	545	547	112	88	125	88	94	78	113	87	101	82
	2	23	30	12	28	3	29	2	14	3	22	3	17
	3	2	1	2	0	0	0	0	0	0	0	0	0
	m_{co}	0.602		0.71		0.7		0.48		0.6		0.54	
	c	3		3		2		2		2		2	
	p	0.206		0.257		0.356		0.266		0.302		0.272	
Prep. #2													
Control	0	641	641	119	115	127	128	127	129	132	134	136	137
	1	288	291	47	53	57	56	62	58	62	57	60	55
	2	34	33	17	14	8	9	4	7	3	6	2	6
	3	1	0	0	0	0	0	1	0	0	0	0	0
	m_{co}	0.359		0.405		0.365		0.365		0.34		0.32	
	n	3		2		2		3		2		2	
	p	0.124		0.2		0.19		0.125		0.172		0.162	
5-HT added	0	469	468	108	112	96	101	88	94	88	88	89	94
(0–18 min)	1	452	447	79	70	89	77	98	82	96	96	90	79
	2	35	43	5	11	6	15	7	18	8	8	9	16
	3	3	1	1	0	1	0	0	0	0	0	1	0
	m_{co}	0.531		0.46		0.52		0.56		0.56		0.555	
	n	3		3		3		2		2		3	
	p	0.185		0.159		0.18		0.29		0.292		0.196	
Prep. #3													
Control	0	975		192		196		194		197		196	
	1	23		8		2		6		3		4	
	2	1		0		1		0		0		0	
	m_{co}	0.025		0.04		0.02		0.03		0.015		0.02	
5-HT added	0	966		196		197		198		194		186	
(0–18 min)	1	21		4		3		2		6		6	
	2	1		0		0		0		0		1	
	3	2		0		0		0		0		2	
	m_{co}	0.029		0.02		0.015		0.01		0.03		0.07	
5-HT added	0	916		172		179		189		194		182	
(18–36 min)	1	77		23		19		11		6		18	
	2	2		1		1		0		0		0	
	3	1		1		0		0		0		0	
	m_{co}	0.084		0.14		0.105		0.055		0.03		0.09	
Prep. #4													
Control	0	966	968	194	195	193	193	192	192	196	197	191	191
	1	32	32	5	5	7	7	8	8	3	3	9	9
	2	2	0	1	0	0	0	0	0	1	0	0	0
	m_{co}	0.036		0.035		0.035		0.04		0.025		0.045	
	n	1		1		1		1		1		1	
	p	0.032		0.0251		0.035		0.0399		0.015		0.0449	
5-HT added	0	888	888	176	177	178	180	178	178	179	179	177	177
(0–18 min)	1	109	109	23	23	20	20	22	22	21	21	23	23
	2	3	3	1	0	2	0	0	0	0	0	0	0
	m_{co}	0.115		0.125		0.120		0.110		0.105		0.115	
	n	2		1		1		1		1		1	
	p	0.057		0.116		0.101		0.110		0.105		0.1149	
5-HT added	0	843	852	177	180	174	176	170	174	164	165	168	168
(18–36 min)	1	147	148	20	20	24	24	26	26	35	35	32	32
	2	10	0	3	0	2	0	4	0	1	0	0	0
	m_{co}	0.167		0.130		0.140		0.170		0.185		0.160	
	n	1		1		1		1		1		1	
	p	0.1485		0.1015		0.121		0.1326		0.1759		0.1399	
5-HT added	0	658	667	147	147	130	130	121	121	128	128	132	132
(36–54 min)	1	331	299	53	53	67	67	76	76	70	70	65	65
	2	11	34	0	0	3	3	3	3	2	2	3	3
	m_{co}	0.353		0.265		0.365		0.410		0.370		0.355	
	n	2		1		2		2		2		2	
	p	0.177		0.2645		0.1825		0.2050		0.1850		0.1775	

of the responses. As demonstrated in the middle trace of Fig. 2, there is a problem of underestimating peak amplitudes when latency jitter occurs among events. An alternative approach is to measure the area under the curve for a period of time which encompasses all evoked events, even ones with substantial latency jitter. After obtaining the average area for the mEPSCs the ratio of averaged evoked EPSC and mEPSCs can be used to calculate *m*.

The area under the curve is current-time which is a unit of charge [8]. In previous reports, the three methods (i.e. direct counts, peak and charge measures) were compared and the results showed that charge more closely matched the values calculated by the direct counting method. We have presented only the m_{co} by direct counts and used charge measures in graphical representations to quantify synaptic efficacy, without the use of questionable peak amplitude measures.

The change in the charge measures before and after 5-HT exposure to the nerve terminals are presented along with histograms of the frequency in occurrence in the magnitude of charge for the evoked events. The data sets were tested for a best-fit approximation based on assumptions discussed in earlier reports [9,26]. Binomial distributions are known to represent the quantal nature of release in crayfish neuromuscular junctions [58]. To test for non-uniform binomial distributions, the procedures described earlier were used [58]. The chi-squared statistic (X^2) and a modified Akaike information criterion (AIC) were used to estimate the distribution that best fits the observed distribution of events. Since the exposure to 5-HT produced gradual changes in all the quantal parameters, sample sets of data for every 200 events were used and were found to be sufficient to obtain statistically significant values for quantal predictions instead of using the grouped total of 1000 events. The 1000 sampled grouped data and the 200 event-group sets were compared in Table 1.

2.3. Analysis of latency

Latency was measured as the time period between the starting point of the spike (extracellular recorded action potential) and starting point of evoked events (for the first and the second events).

Representative plots of the frequency of occurrence at various latencies is provided and a normalized graph for the occurrence of events are given. The values were also plotted in cumulative frequency for the latencies measured. Since 5-HT potentially has an effect on the docking of vesicles within the presynaptic terminal, we expected that the number of secondary events would increase and that 5-HT would have an effect on the latency of the second events. Therefore, the number of occurrences and the latency of evoked second events were measured. The latency of second EPSC events will affect the ensemble average of the overall EPSC for the entire muscle. Depending on the rise time and width of all the EPSCs the EPSP will show varying characteristics in its shape. The decay time of EPSPs can have a substantial effect on facilitation when subsequent EPSP events occur before the full decay of the proceeding event [14,15].

3. Results

The central region of the opener muscle in the first pair of walking legs has been used to investigate the effects of serotonin (5-HT) for a number of years [14,30]. All the studies to date show an enhancement in the amplitude of the excitatory postsynaptic potentials (EPSPs) upon application of 5-HT. This is illustrated in the increases seen for each of the EPSPs within the 40 Hz train before and after the bathing medium was rapidly changed to one containing 5-HT (100 nM).

The preparation was allowed to bath for 10 min before repeated measures were taken (Fig. 1). The enhancement in the EPSPs are likely due to a composite of pre- and post-synaptic modifications by 5-HT. Therefore, we set out to determine the presynaptic contribution of 5-HT's actions by direct quantal counts and latency of release.

3.1. EPSCs

Single sweeps may contain a failure or an evoked response with one quantal event or an evoked response with multiple releases (Fig. 2). The speed at which the increase in the number of EPSC's that occur is lost and information on the number of individual events within each trace is not measured with averaging the current traces. Alternatively, with directly counting each quantal event for each trace the gradual changes in the observed quantal occurrences can be compiled (Table 1). These responses indicate an increased number of single and multiple evoked events.

3.2. Quantal parameters

An advantage of directly counting evoked quanta is that one may make estimates regarding the quantal parameters of *n* (number of release sites), and *p* (the average probability of release at the sites) based on the distribution of events, and further, on the effects that neuromodulators have on these parameters. Gradual or abrupt changes can also be picked up by analyzing groups of 200 events as well an entire set of 1000 events (Table 1). The individual bins of 200 events, for the most part mimics the larger grouped data set. It is apparent that the m_{co} increases in the presence of 5-HT due to both an increase in *n* and *p*. Four different preparations were time sakingly analyzed in this manner. Preparations No. 1 and No. 2 were from primary varicosities along a terminal string from two different leg

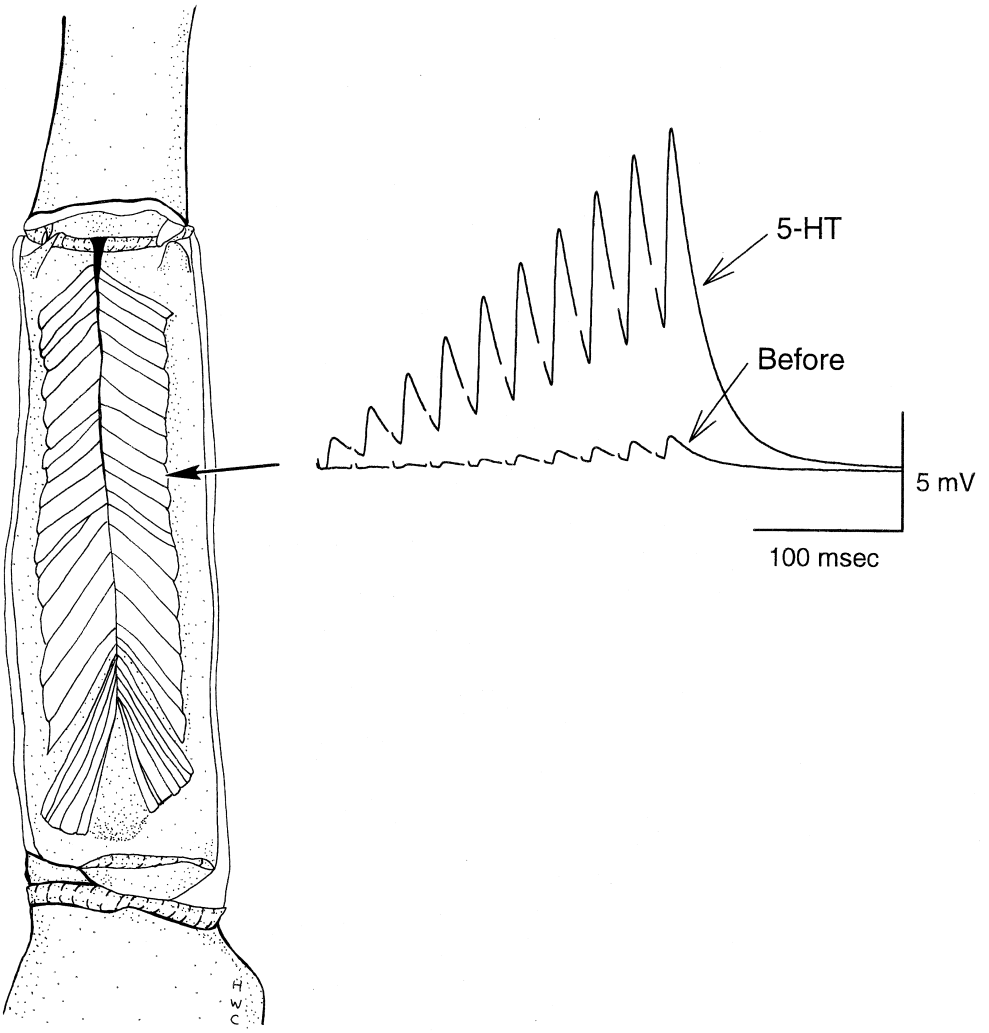

Fig. 1. The excitatory motor nerve to the opener muscle was stimulated with a 40 Hz train that contained ten pulses. Excitatory postsynaptic potentials (EPSPs) before and after the exposure of 100 nM 5-HT. A schematic of a ventral view of the opener muscle in a first walking leg to indicate the central region of the muscle that was used in these studies. The preparation was exposed to the bath containing 5-HT (100 nM) for 10 min before measures were taken. An average of 20 trains were taken for each trace shown.

preparations. Where as preparations No. 3 and No. 4 were from more distal locations along the terminal in other opener neuromuscular preparations.

The values indicated in Table 1 for m_{co} are graphed for demonstrating the temporal effects of 5-HT on mean quantal content (Fig. 3). During 1 Hz stimulation before and after addition of 5-HT shows that the preparations change at varied rates and in some instances there is substantial changes over short periods of time. If only the m_{co} values are shown for each 1000 events the gradual changes will be minimized by averaging. When the synaptic efficacy becomes more pronounced, n did not increase substantially, but the largest change is in the probability of release, p. To assess the rate in activation of this modulator in altering the quantal parameters, every 200 and 1000 evoked events were grouped for quantal analysis.

3.3. Distribution of charge

To add in examining the presynaptic actions of 5-HT on the quantal release in addition to using the golden standard of direct counts [17], the influence on the charge measures for EPSCs and mEPSC were compared before and after application of 5-HT. The influence of 5-HT (100 nM) on evoked EPSC and mEPSC charge shows that only EPSCs were substantially affected, but not the mEPSCs. Only preparations 1–3 contained enough spontaneous events for analysis. A representative distribution shows that 5-HT increases the size of EPSCs which produces a broader EPSC distribution without any substantial effect on means, and medians, of the mEPSCs. The mEPSCs are shown as insets in Fig. 4. The spontaneous events were acquired during the same time experimentation time frame as the

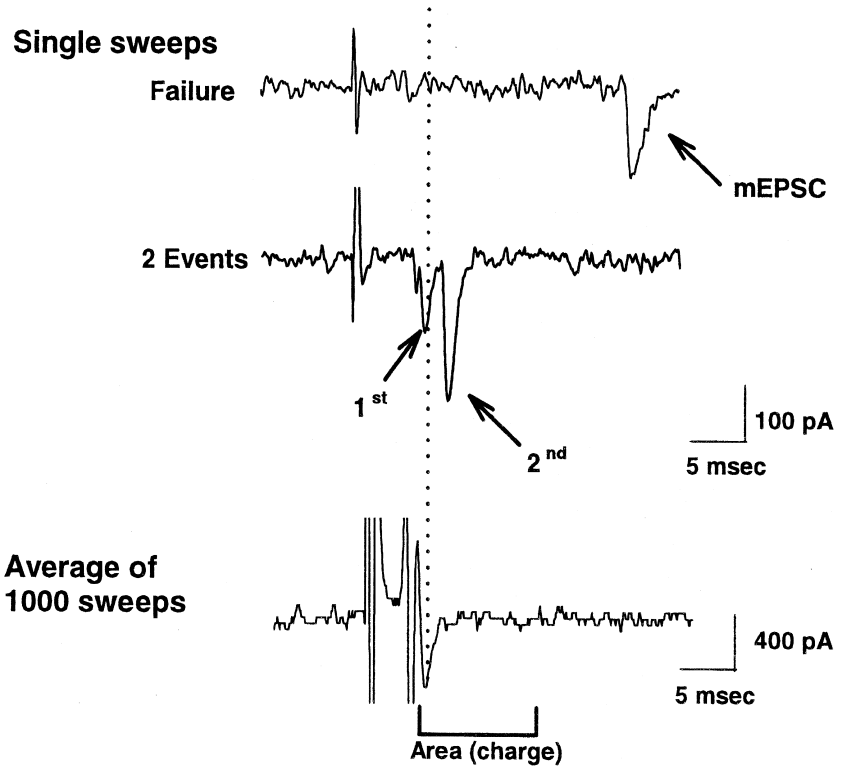

Fig. 2. To determine mean quantal content (m_{co}) one can directly count the number of evoked events: the top single trace shows a failure to evoke a response whereas the next trace shows two evoked events. See Methods for the equation to calculate m_{co}. An alternative approach is to measure the area under the curve for a period of time which encompasses all evoked events, even ones with substantial latency jitter. After obtaining the average area for the mEPSCs the ratio of averaged evoked EPSC and mEPSCs can be used to calculate *m* by current-time (area or charge).

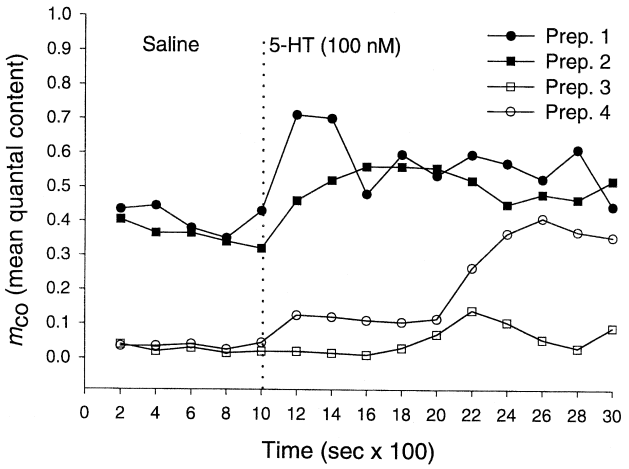

Fig. 3. The temporal effects of 5-HT on mean quantal content by direct counts (m_{co}). Calculating m_{co} for each 200 trials during 1 Hz stimulation before and after addition of 5-HT shows that preparations change at different rates. In some instances there is substantial change over short periods of time which would be minimized if averaging occurred over longer periods of time.

evoked events also shown in the figure for saline and 5-HT exposure. A set of 1000 trials before and immediately after being exposed to 5-HT were used.

We could not observe any consistent change in the distribution of occurrences for spontaneous events as a result of 5-HT application (Table 2). Nor did we observe any real change in the mean charge measures. The coefficient of variation (CV) in the charge measures for the spontaneous events indicates there are differences among experiments in the breadth of the distributions, but in spite of this 5-HT did not result in a consistent trend of altering the mean charge of the mEPSCs (Table 2).

Changes in the evoked charge distributions presented in histogram fashion allows one to examine for relatively large shifts in the distribution, but if there are small changes in charge due to 5-HT, a histogram is difficult for visual assessment (Fig. 4A and B). This is not normally a problem with the application of 5-HT, but in very low-output terminals that are only being stimulated at 1 Hz the occurrence of a few more multiple events and a small enhancement of single events will alter the m_{co} obtained by direct counts with only small changes seen in composite histograms of charge when the majority of events are failures. In order to better represent the increased number

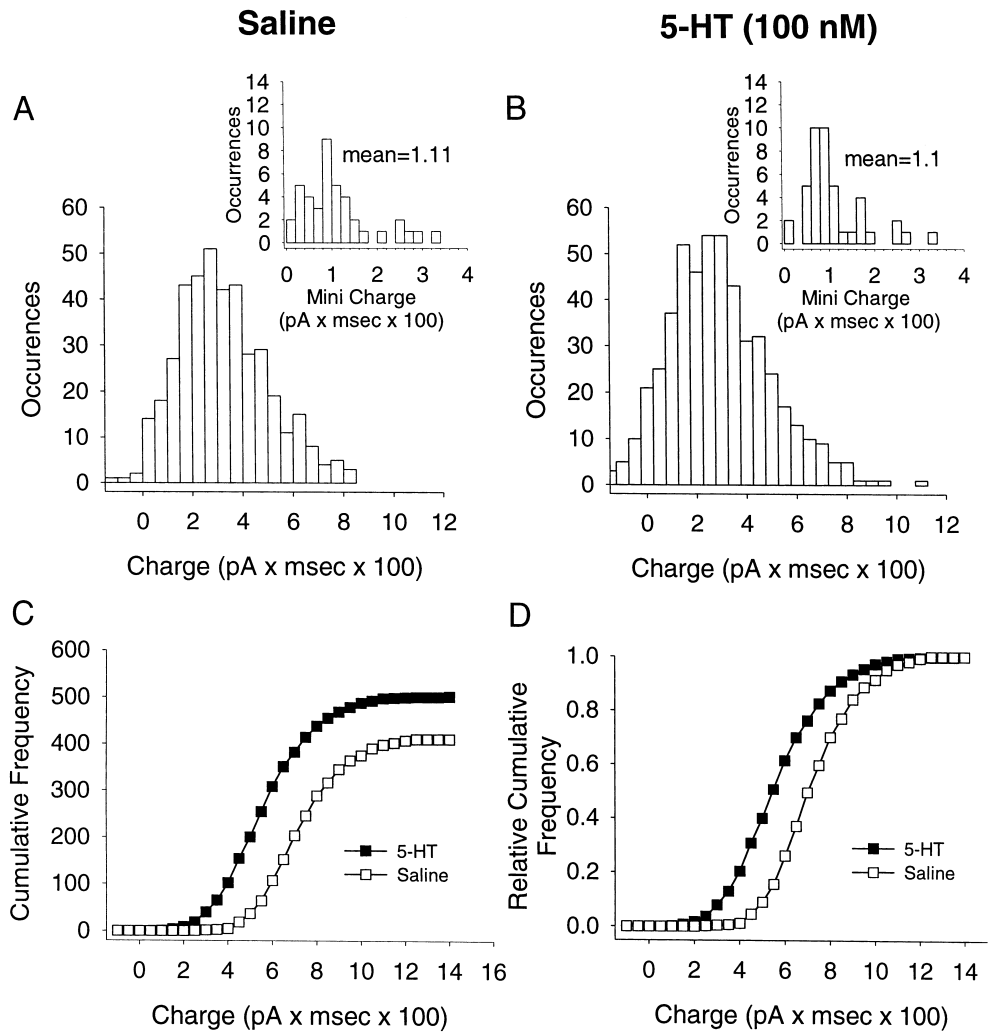

Fig. 4. The influence of 5-HT (100 nM) on evoked EPSC and mEPSC charge shows only an affect on the EPSCs and not the mEPSCs. A set of representative results shows that 5-HT increases the magnitude of EPSCs which produces a shift in the EPSC distribution to the right without any substantial effect on the mEPSCs (insets). These particular results were obtained from a low-output varicosity (preparation No. 2) 1000 trials before and immediately after exposure to 5-HT. The mean values for the EPSC and mEPSC distributions are presented. In order to better represent the increased number of events, cumulative frequency plots for the two distributions can be compared in (C). Since there are more events occurring, due to fewer failures, it is difficult to compare shifts in the occurrence for any particular value of charge due to 5-HT. Therefore, relative cumulative frequency plots were constructed which normalize the total number of events (D). It is apparent that there is a leftward shift in the ranges from 2 to 8 (pA. ms), indicating that 5-HT induced a greater number of events to release with smaller and larger values of charge.

Table 2
Mean charge of spontaneous events recorded before and after exposure to 5-HT (100 nM)

	Mean Charge (*n*) (pA×ms×50)	C.V.	Frequency of mEPSC, Hz
Prep. 1			
Saline	4.437 (41)	68	0.01
5-HT	4.361 (43)	62	0.006
Prep. 2			
Saline	3.056 (18)	49	0.009
5-HT	3.671 (20)	34	0.006
Prep. 3			
Saline	2.364 (76)	69	0.023
5-HT	2.683 (69)	93	0.0345

of evoked charge, cumulative frequency plots for the two distributions from before and after application of 5-HT can be compared as shown in Fig. 4C. This clearly shows the increase in events, due to fewer failures. The small additions offset the curve early and since the curve is adding on the next larger value of charge the difference is only enhanced throughout the ranking of the charge (x-axis).

There is still a difficultly to compare shifts in the value of any particular value of charge due to the increase in events induced by 5-HT since there just more events occurring. Graphical representation of relative cumulative frequency plots which normalize the total number of events to a unitary value of 1 allows for the shifts in charge to be more readily compared independently of the

numbers within the distribution (Fig. 4D). It is apparent that there is a leftward shift in the over all range. Taking the results of Figs. 4C and D, the data indicates that 5-HT induced a greater number of events to be released with smaller and larger values of charge.

3.4. Latency measures

To determine if particular steps in the vesicular release process are effected by the presence of 5-HT, the measure of latency in vesicular release is useful. The latency is the time from when an action potential reaches the nerve terminal, calcium influxes, a primed vesicle fuses to the membrane, transmitter is released to diffuse and bind to the postsynaptic ligand-gated receptors, and sodium current to influx into the muscle cell. Most of the time needed for these event to take place is in the fusion process of vesicle, since diffusion time in the synaptic cleft of transmitter is so short.

Measuring from the starting point of the spike to the starting point of evoked event in the EPSC records was used to calculate time that it took for a evoke event to occur (Fig. 5A). The fastest events took 1 to 2 ms. To view the change in the distribution of occurrences at the various latencies of release due to 5-HT exposure, the latency values are shown as superimposed histograms. The histogram illustrates the dramatic change in the number of occurrences for each range of latency.

To further demonstrate this point, cumulative frequency plots are provided (Fig. 5B) for the same number of trials presented in the histograms before and after 5-HT exposure. Since a greater numbers of vesicles are released the total cumulative value is larger for the 5-HT trials. For comparative purposes the same number of stimulus trials were used (1000) before and after 5-HT exposure. The total cumulative plots can also provide a trend in absolute numbers of release events but it remains difficult to compare the relative change in latencies with more events occurring. So as with the previous distributions for charge measures, relative cumulative frequency plots were also calculated and provided to compare shifts in the distribution in latencies of the first evoked events. The 5-HT resulted in a more substantial increase in the number of vesicles released (Fig. 5B) but also an increase in the

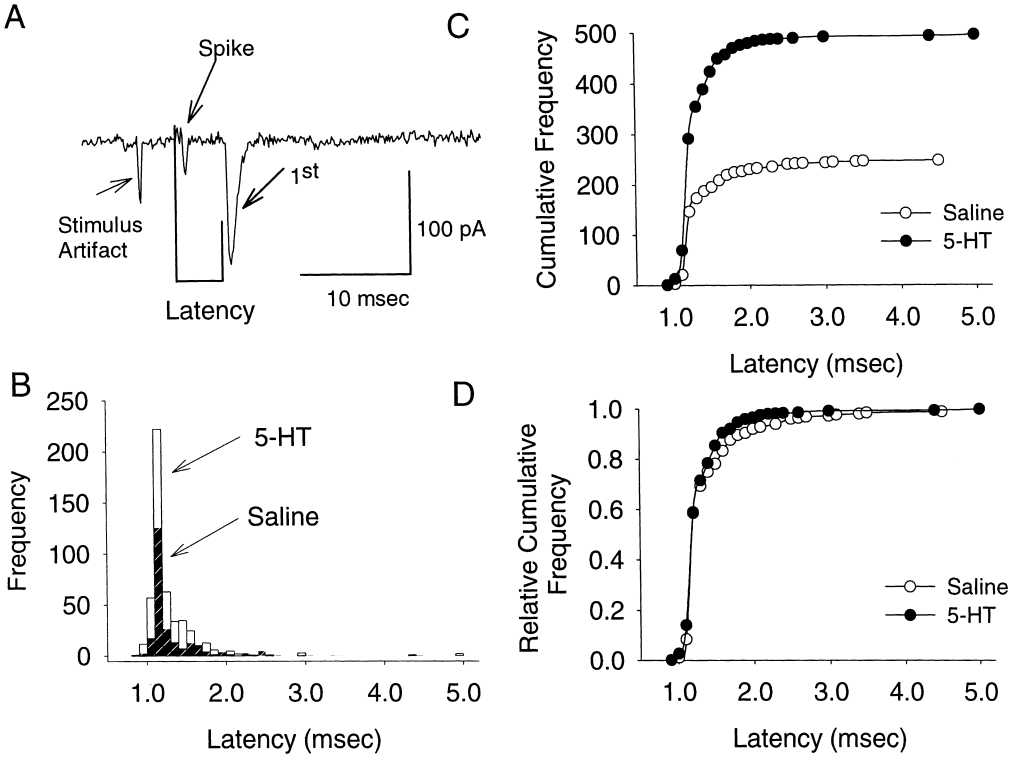

Fig. 5. Synaptic latency of vesicular release measured before and after application to 5-HT for the first evoked events indicates a more vesicles releasing with shorter latencies. Synaptic latency of vesicular release is measured from the beginning of the spike (extracelluarly recorded action potential of the terminal) to the initial deflection of the synaptic current. In (A) a single evoked quantal event occurred with a latency of about 3.5 ms. This is considered a late evoked release only shown for clarity in the measurement procedure. The distribution in the histograms of latency shows a larger number of events occurring within the same time range (B). The latencies for first evoked events within a 1000 trials before and a 1000 trials after 5-HT exposure were used to obtain the distributions. In order to better represent the increased number of first evoked events and latency of release, cumulative frequency plots for the two distributions are compared in (C). Since there are more events occurring, due to fewer failures, it is difficult to compare shifts in the latencies due to 5-HT. Therefore, relative cumulative frequency plots were constructed which normalize the total number of events (D). It is apparent that there is a leftward shift in the ranges from 1.5 to about 3 ms, indicating that 5-HT induced greater number of events to release with shorter latencies within the population.

number of vesicles released with a minimal delay in the range of 1.5–2.2 ms (Fig. 5C). Thus the slight leftward shift in the curve.

For comparison of temporal latency shifts among three preparations, the latency of the first evoked EPSCs are shown as scatter plots over the 1 Hz stimulation time course (Fig. 6). In preparation No. 1 there is a shorter latency for most all events as soon as 5-HT is exposed to the terminals, where as for two other preparations (preparations No. 2 and No. 3) there are more events occurring due to the presence of 5-HT but there are still events occurring with variable latencies (Fig. 6, left panels).

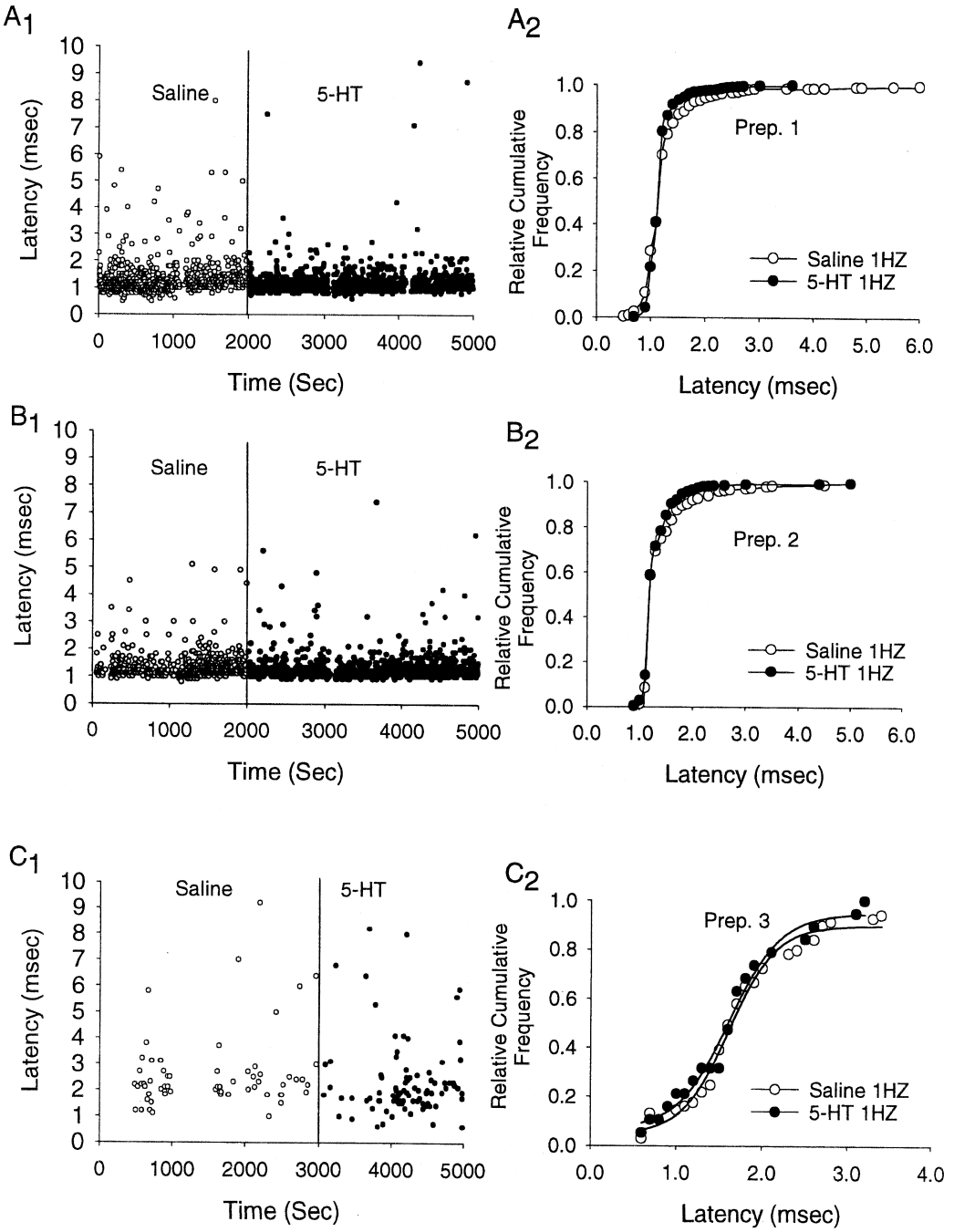

Fig. 6. Comparison of temporal latency shifts among three preparations for the first evoked EPSCs. Scatter plots of the latency for the first events over the stimulation time course of 1 Hz reveals that in some preparations (preparation No. 1) there is a rapid decrease in latency for most all events as soon as 5-HT is exposed to the terminals, where as for two other preparations (preparation No. 2 and No. 3) there are more events occurring due to the presence of 5-HT but there are still events occurring with variable latencies (left panel). Examining the range in latency shifts in which 5-HT has the greatest effect, relative commutative frequency plots demonstrate that from 1.5 to 2.2 the distributions did show a shift toward the left (right panel). The left and right panels are for preparations 1, 2 and 3 from top to bottom.

Examining the range in latency shifts in which 5-HT has the greatest effect, relative commutative frequency plots demonstrates that from 1.5 to 2.2 the distributions did show a shift toward the left for only the first two preparations (Fig. 6, right panels).

To further examine effects of 5-HT on the vesicular kinetics, the second evoked events in traces in which two or more events occurred were also examined and measured for the latency of release before and after 5-HT exposure. Two preparations contained a significant amount of secondary events for this type of analysis (preparations Nos. 1 and 2). In one case, the number of occurrences increased and in the other case there were fewer occurrences during exposure to 5-HT as compare to saline. The latencies did not show any particular shift by the presence of 5-HT (data not shown). Possible at a higher stimulation frequency than 1 Hz or with higher concentrations of 5-HT, latency shifts and the number of occurrences for second events might be altered.

4. Discussion

This study at the crayfish opener neuromuscular junction provides pertinent information because it addresses the role that neuromodulators play physiologically on all of the quantal parameters (*m*, *n* and *p*) which affects the degree in synaptic performance as measured directly at the release sites. From previous detailed studies on the synaptic complexity and physiological studies made from the same sites along various regions of the nerve terminals on the opener muscle, findings can be *directly* correlated to the underlying structural entities which are responsible for efficacy in transmission. We now can add to the knowledge gathered, the effects of 5-HT in altering the parameters that underlie synaptic efficacy. Recent studies report the distinct differences in ultrastructure, synaptic physiology, and calcium responses may explain, in part, why the physiological responses are so varied among regions along a given terminal and among terminals [8,10–12]. How the differences arise, however, is still unresolved [2,3,9–12] and the effects of neuromodulator actions among regions on motor nerve terminals are just now appearing [13–15,35].

In the presence of a neuromodulator such as 5-HT, which is known to work through IP3 cascade in nerve terminals [19,20] and which can lead to phosphorylation of a protein in the nerve–muscle preparations [31], it is possible that molecules associated with vesicle docking/priming are phosphorylated. It is known that several of these proteins can be phosphorylated [49,51]. We choose to examine the possibility in crayfish neural tissue by the following paradigms.

One set of experiments were conducted with the abdominal ventral nerve cords only exposed to saline, to serve as controls. While a second set were exposed to 5-HT (100nM) and a third set to 1 μM. Each set of experiments were run for 15 and 30 min of exposure time to the neuromodulator. All preparations were incubated for 2 h with $^{32}PO_4$ prior to the 5-HT or saline exposure. At this gross level of analysis there was no apparent differences amongst the treatment groups (data not shown). This has also been the case when similar labeling studies were done on synaptosomal preparations (Whiteheart unpublished observation). To examine for specific phosphorylation of neuronal proteins, further immunoprecipitation experiments examining proteins, like SNAP-25, α-SNAP and Munc 18a, are needed and are currently in progress in our laboratories.

Second-messengers also show a role in synaptic transmission based on the degree of electrical activity in the absence of neuromodulators. For example, it is known that in crayfish motor neurons, short-term facilitation depends on Ca^{2+}, and the long-term facilitation is dependant on the induction of cAMP [19]. In the *Aplysia* sensory neurons, long-term, not short-term, facilitation requires protein synthesis [5]. Since an increase in cGMP has been shown in hippocampal pyramidal neurons (glutaminergic) to increase transmitter release independently of changes in the presynaptic action potential [1]. We continue to investigate, by assessing quantal release parameters, to understand better the mechanism of action of neuromodulators. In an earlier report, the active form of ecdysone, 20-hydroxyecdysone (20-HE), that is related with the molting cycle, has a rapid non-genomic action at neuromuscular preparations in the crayfish opener neuromuscular junction and in *Drosophila* [13,50]. The rate at which this response occurred suggested a direct action without transcriptional regulation within the presynaptic terminal or possibly in the muscle fibers. The rate of spontaneous release and in the latency jitter of an evoked release suggested a presynaptic mechanism of action in decreasing synaptic efficacy. These actions of 20-HE are apparently in altering vesicle release so possible structural examination or monitoring vesicle dynamics would be beneficial.

Likewise to determine if particular steps in the vesicular release process are effected by the presence of 5-HT, the measure of latency of vesicular release is useful. The data presented herein is convincing that 5-HT is working to enhance docking/priming, although we were are not able to assess if 5-HT may possibly be altering vesicle recycling. This remains to be tested by possible loading recycling vesicles with flourescent indicators viewed with light microscopy (i.e. FM1-43; [55]) or with HRP loading and subsequent electron microscopy [42,54].

The maintained short latencies for the enhanced number of vesicles released that 5-HT causes would indicate that multiple number of vesicles may be primed for release around each active zone, but in addition the structural observations also suggest that a given limit may be set by conformational constraints to the number of primed/docked vesicles possible around each active zone. The anatomical evidence suggests that regulation of the number

of vesicles to be released at a single synapse may be related to synaptic efficacy and allows for fine tuning in the number of vesicles that are in a prime state at a single active zone [12]. The cytoplasmic proteins such as α-SNAP then may provide the fine tuning in synaptic efficacy at the established active zones such as what has been proposed for synaptotagmin [53].

In a previous study [35], we have tested the functional role of α-SNAP in neurotransmission by investigating the alterations in release kinetics and probability of evoked release when it was introduced at higher than normal levels inside of nerve terminals. A dominant negative α-SNAP(L294A) mutant was also developed as a competitive blocker of endogenous α-SNAP. The results of that study with the use of 5-HT after α-SNAP had been loaded into the terminals also suggest that the 'ready release' pool is increased in number of vesicles even more so then when α-SNAP its self is at a higher concentration in the nerve terminal. 5-HT could also effect other processes since it is known to work through an IP3 cascade [19,20] which could lead to the activation of various protein kinases. 5-HT could induce the phosphorylation of molecules such as Munc-18-1 or synaptophysin or even NSF (Vanaman, Slevin, Whiteheart unpublished).

Phosphorylation of the first two proteins has been shown, in vitro, to inhibit their ability to interact with the SNARE proteins that they are proposed to regulate (syntaxin for Munc18-1 and synaptobrevin for synaptophysin) [49,51]. 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 SV's available for secretion. While these are possible mechanisms (Fig. 7), if they are true, the effect of 5-HT would be expected to cause and increase in docking and/or priming. From the data presented, it would seem more likely that 5-HT could effect the Ca^{2+}-dependent step of neurotransmission either by inducing the phosphorylation of synaptotagmin [16] or perhaps the phosphorylation of the Ca^{2+} channel itself [32]. The potential effects of 5-HT on Ca^{2+} entry and handling needs to be further examined since STF and the latency of evoked events are influenced by Ca^{2+} within the presynaptic nerve terminal [22–27,37,45–48,57,60].

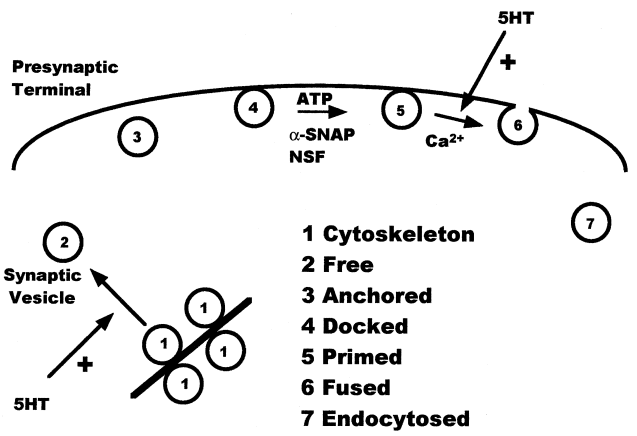

Fig. 7. Schematic representation of the potential sites of action for 5-HT based on the quantal release results obtained at the motor nerve terminal.

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