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Influence of Neuromodulators and Vesicle Docking Related Proteins on Quantal Release

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Abstract

We assessed the mechanisms and related kinetics of vesicular release during the enhancement and depression of synaptic efficacy by the exogenous application of neuromodulators as well as altering the levels of protein related to vesicle fusion within nerve terminals. In order for a vesicle to be released, it must first dock at the presynaptic membrane; then, in the presence of Ca^{2+}, a fusion pore for evoked transmitter release opens. To determine if the presence of synaptically relevant proteins, such as α-SNAP, can alter the number of evoked quantal units and their rate of occurrence, α-SNAP was injected into the nerve terminals and the kinetics of release were measured. The enhancement of α-SNAP within the nerve terminal increases the number of rapidly occurring quantal events. The neuromodulator serotonin (5-HT), known to stimulate an IP_3 cascade, also causes an enhancement of evoked quantal events. The presence of 5-HT or α-SNAP at the terminal results in a faster rate of release for multiple events without a detectable change in the initial rate of primary events. Interestingly, the molt-related hormone, ecdysone (20-HE), causes a decrease in the rate of multiple events and results in an increase in latency jitter of vesicular release, thus indicating that 20-HE may act as a neuromodulator that depresses release. Investigating proteins and modulators of synaptic function will hopefully allow one to understand some of the mechanisms underlying synaptic differentiation and selective actions of neuromodulators, individually and in combination.

Introduction

The nerve cell is chemically integrated with other cells at morphologically identified locations called synapses. Neuromodulators endogenously released within an animal may enhance or suppress synaptic efficacy, in turn affecting the animal's behavior. Neuromodulators are composed of hormones, peptides, or biogenic amines. They affect cellular processes by stimulating various signaling

cascades mediated by different classes of ligand receptors. Such cellular changes are believed to play roles in learning and memory, as well as in behavior.

The crayfish neuromuscular junction (NMJ) easily lends itself to experimentation in synaptic plasticity. The activity of individual varicosities on identified single cells can be analyzed, the varicosities and cells marked, and those same synaptic sites later identified for structural investigation by electron microscopy. The opener muscle of the crayfish legs provides direct correlation of structure and function at individual synapses of identified single cells. These neuromuscular preparations not only make it possible to assess effects of neuromodulators on well-characterized behaviors, but also allow direct correlation between identified synapses and certain behavioral components. They permit the identification of specific cellular mechanisms responsible for the various manifestations of synaptic differentiation and function.

With the use of recently developed proteins and fluorescent dyes, we have begun to investigate mechanistic questions of neurotransmitter release. Intracellular injection of vesicular docking proteins into the large axons of crustacean motor neurons in the absence or presence of neuromodulators allows one to investigate if the intracellular signaling systems involved work independently or synergistically to effect synaptic efficacy and structure. The opener muscle in crayfish has provided a great deal of insight into the basic mechanisms of synaptic transmission because it allows neurotransmitter release to be directly related to synaptic physiology and structure (Atwood et al. 1994; Atwood and Cooper 1995, 1996a,b; Cooper et al. 1995a,b, 1996a,b). In this preparation, the relatively low output of each varicosity along the nerve terminals allows the use of quantal analysis and statistical evaluation of individual vesicular release events.

Molecules thought to have an action in neurotransmission include synaptic membrane SNARE proteins (SNAP receptor) comprising membrane-bound proteins of the synaptic vesicles (called v-SNAREs, e.g., VAMPs/synaptobrevins) and those on the presynaptic membrane (called t-SNAREs, e.g., syntaxins and SNAP23/25's; Littleton et al. 1998;Weber et al. 1998). The protein-protein interactions of the SNARE proteins are regulated by cytosolic proteins called SNAPs (soluble NSF attachment proteins) and NSF (N-ethylmaleimide sensitive factor). NSF is a soluble protein within the cytoplasm and aids in priming docked vesicles for calcium-triggered fusion (Kawasaki et al. 1998). α-SNAPs are also cytoplasmic and are thought to bind to the SNARE complex to then act as adaptors to promote NSF binding (Weidman et al. 1989). Injection studies using squid axons have shown that SNAPs do play a role in neurotransmission by increasing the output of a terminal (DeBello et al. 1995). The working model from various reports indicate that NSF and SNAPs are participating in SNARE priming, prior to fusion, and in SNARE recycling after membrane fusion (Littleton et al. 1998). 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 into nerve terminals at higher than normal levels. A dominant negative α-SNAP(L294A) mutant protein was also used as a competitive blocker of endogenous α-SNAP.

The distribution of nerve terminals containing biogenic amines in invertebrate and vertebrate peripheral nervous systems suggests that amines are not directed at

discrete postsynaptic targets but rather that they are disseminated over a wider area, interacting with receptors on several different target cells. Depending on the physiological state of the animal, the modulators may be released acutely or chronically. Such neuromodulators have been implicated in the production of relatively slow, long-lasting modifications of the efficacy of transmission at synapses where the transmitters are chemically quite different (Kupfermann 1979).

Little is known about the direct mechanisms of 5-HT action on crayfish neurons, with the exception of a few studies showing that 5-HT works through inositol triphosphate (IP_3) and cyclic AMP (cAMP) (Dixon and Atwood 1989a,b; Delaney et al. 1991). Prior studies addressed the effects on neurotransmission in general by recording muscle potentials arising from thousands of synapses (Grundfest and Reuben 1961; Dudel 1965; Kravitz et al. 1976, 1980; Wheal and Kerut 1976; Florey and Rathmayer 1978; Fisher and Florey 1983). With techniques now available, direct measurement of synaptic parameters in presynaptic motor nerve terminals that influence release can be implemented (Cooper et al. 1995b,c). Serotonin's ability to maintain elevated release for up to 1 h at the crayfish neuromuscular junction was reported to be due to IP_3 (Dixon and Atwood 1989b) and with calcium-sensitive indicators it was determined that the IP_3 was not increasing intercellular calcium levels (Delaney et al. 1991). Instead, the IP_3 cascade may be working in other ways to enhance transmission, such as by phosphorylation of molecules involved in the priming and the probability of vesicular release.

Recently studies investigating the nongenomic actions of molt-related compounds among crustaceans and insects suggests that they regulate premolt, molt and postmolt behaviors. Recent evidence has shown that the active form of ecdysone, 20-hydroxyecdysone (20-HE), has a rapid nongenomic action at neuromuscular preparations in the crustacean neuromuscular junctions and in *Drosophila* (Cooper and Ruffner 1998; Cromarty and Kass-Simon 1998; Ruffner et al. 1999). 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 changed rate of spontaneous quantal release and increased latency jitter of evoked release suggested a presynaptic mechanism.

Our objectives in the field of neuromodulation are to determine if neuromodulators affect the kinetics of transmission. In addition, we are interested in whether synaptically relevant proteins and neuromodulators may work in concert to regulate synaptic efficacy. The current question we are tackling is whether the neuromodulators studied here (5-HT and 20-HE) act before or after the formation of the readily releasable pool.

Methods

The Crayfish Neuromuscular Junction Preparation

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 (Dudel and Kuffler 1961) and recorded in a standard manner (Cooper et al. 1995b). 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 20-HE, 5-HT, and all chemicals listed were obtained from Sigma Chemical.

Protein Microinjection

The proteins were stored in dialysis buffer at −80 °C prior to injection. Immediately prior to injection the proteins were mixed with a Texas red dextran solution (70 kDa, Molecular Probes, Eugene, Oregon) to a final concentration of 0.5 mg ml-protein and 0.05% of Texas red dextran. This solution was then loaded into the microelectrode by capillary action from the back of the electrode until the tip of the electrode was sufficiently filled for the wire from the electrode holder to touch the solution interface. The microelectrode was then placed into the excitatory axon of the opener muscle close to the axon bifurcation. Within 30 min of the start of pressure injections, the 70 kDa Texas red dextran entered nerve terminals and loaded the varicosities as described recently (He et al. 1999).

Evoked Postsynaptic Potential Measurements:

Intracellular muscle recordings were made with a 3 M KCl-containing microelectrode placed in a centrally located fiber in the opener muscle. Axons were stimulated by a train of ten pulses given at the indicated frequencies. The stimulation frequency was kept constant for each preparation. The range in frequencies used varied from 40 to 60 Hz with a train interval of 10 s. The frequency chosen was determined at the time of the experiment to insure an EPSP response by the fourth pulse within the train, so that quantitative measures could be made. Measurements and analysis were performed by standard procedures (Cooper et al. 1995b, 1996a).

To quantitatively compare the change caused by the injected agents in the various preparations, the measurements were normalized to a percent change, which was calculated using the difference among the average of the first 100 EPSP events during the baseline recording prior to injection and the average of the 100 EPSP events around the maximum response during the injection procedure, and then dividing the result by the baseline value as shown in the following equation: (|Baseline-maximum response|)/Baseline] x 100% = % Change.

The tenth EPSP peaks within a train were used to calculate a facilitation index (Fe) with respect to the earlier EPSP amplitudes by standard procedures (Crider and Cooper 1999, 2000).

Evoked Postsynaptic Current Measurements

Focal macropatch recording was used to measure synaptic currents. The living nerve terminals were visualized with the vital fluorescent dye (4-Di-2-ASP; Molecular Probes, Eugene, Oregon). The preparation was exposed to the dye

solution for 2 to 5 min, followed by washing in crayfish saline. The synaptic currents were obtained using the loose patch technique by lightly placing a 10- to 20-µm diameter, fire-polished, glass electrode directly over a single, spatially isolated varicosity along the vital dye-visualized nerve terminal. The synaptic transmission was quantified by standard procedures (Cooper et al. 1995b, 1996a). 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) (Cooper et al. 1995c, 1996a).

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 second events). A representative plot of the frequency of occurrence at various latencies is provided and a normalized graph for the occurrence of events is shown for the two curves, as plotted in cumulative frequency for the latencies measured.

Preparation of α-SNAP

The α-SNAPL294A mutant and recombinant α-SNAP were obtained and purified as described earlier (He et al. 1999). Mutation of leucine 294 to alanine [α-SNAP(L294A)] was shown to decrease the ability to stimulate NSF ATPase activity but had no effect on the ability of this mutant to bind NSF (Barnard et al. 1997).

Results

5-HT

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 (Dudel 1965; Fisher and Florey 1983; Dixon and Atwood 1989a,b). All the studies to date show an enhancement in the amplitude of the excitatory postsynaptic potentials (EPSPs) upon application of 5-HT. The increase is seen for each of the EPSPs within the 40 Hz train (Fig. 1). We set out to determine the presynaptic contribution by direct quantal counts and latency of release for this enhancement. These measures allow one to examine the mechanisms of action on quantal release.

Single evoked quantal events (i.e., synaptic currents) were recorded with the use of a focal macropatch electrode placed over visualized motor nerve terminal varicosities. Both the excitatory and inhibitory terminals can be visualized with 4-Di-2-Asp, but since the excitatory axon is stimulated selectively, only excitatory varicosities will reveal evoked synaptic currents. After staining with the vital dye, the terminals look like those shown in Fig. 2A.

5-HT

Before

5 mV

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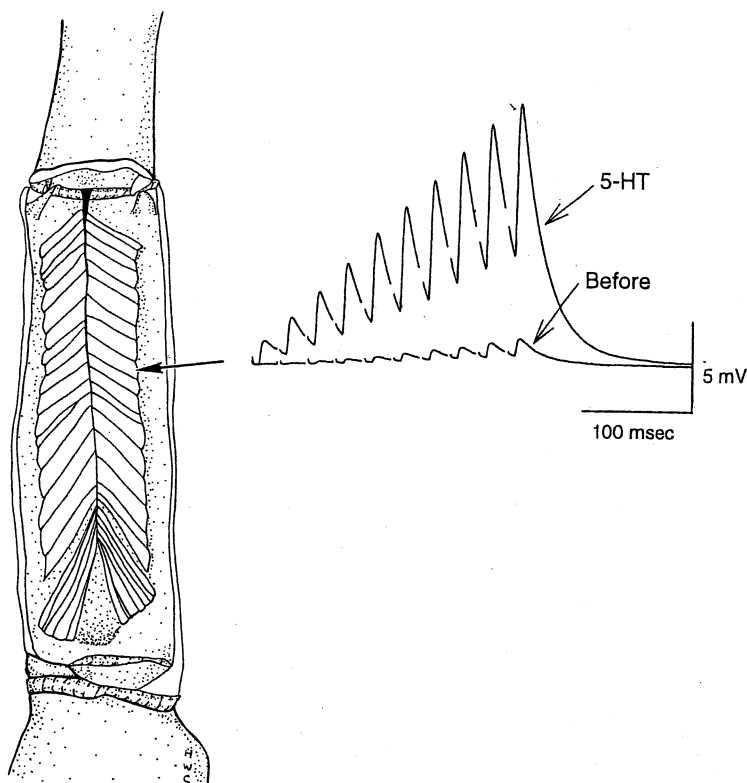
Fig. 1. Excitatory postsynaptic potentials (EPSPs) before and after the exposure of 100 nM 5-HT. A schematic of a ventral view of the opener muscle in a first walking leg to indicate the central region of the muscle that was used in these studies. The preparation was exposed to the bath containing 5-HT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

Individual macropatch current recording from a terminal is represented in Fig. 2B. From such a current recording, a stimulus artifact, the extracellular response of the action potential from the terminal (i.e., spike), and evoked quantal events can be observed. In addition, the latency of synaptic events can be measured from the starting point of the spike to the starting point of an evoked event in the EPSC records. Analysis of single sweeps may reveal a variety of responses with some containing a failure of an evoked response (one of the traces in Fig. 2 C_1) or an evoked response with multiple releases (the single trace in Fig. 2C_2). Fig. 2C_1 (saline) and C_2 (100 nM 5-HT) represent the general findings: there are more evoked, excitatory postsynaptic currents (EPSC) in the presence of 5-HT. The increase in the number of events is even more apparent in the numbers obtained from direct counts of events. The gradual change in the occurrences of EPSCs are demonstrated in the mean quantal content (m_{co}) values shown in Fig. 2D. The direct method of counting quantal events to determine m_{co} (m determined by direct counts) alleviates difficulties associated with changes in the seal resistance, which alters the determination of the mean quantal content determined from either peak amplitude or charge of the responses (Cooper et al. 1995c).
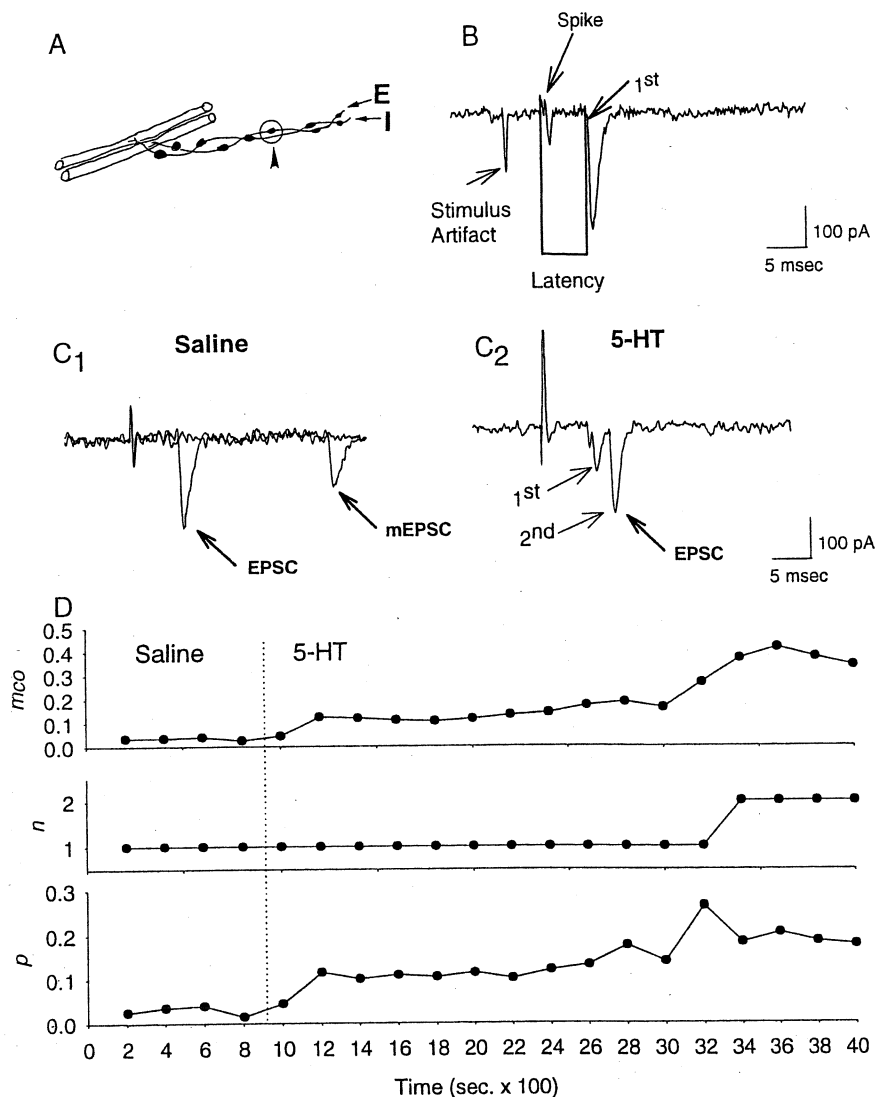
Fig. 2 A-D. Influence of 5-HT on evoked synaptic currents from discrete single varicosities. **A** The recorded location (*arrowhead*) shown in a trace of the varicose nerve terminal; the *circle* represents the rim of a focal macropatch electrode. **B** Synaptic latency of vesicular release is measured from the beginning of the spike (extracelluarly recorded action potential of the terminal) to the initial deflection of the synaptic current. In the recording 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. **C** Representative single sweeps which show the evoked excitatory postsynaptic currents (*EPSC*) and a miniature excitatory postsynaptic current (*mEPSC*). The recordings were made prior (C_1) and after (C_2) exposure to 5-HT (100 nM). The individual evoked quantal events are shown in C_2 for a response that happened to release two quantal units with different latency. **D** Changes in the quantal parameters as a result of the addition of 5-HT. Mean quantal content (*mco*) determined by direct counts revealed a increase after addition of 5-HT. The quantal parameters n and p were determined by the maximum likelihood estimation (MLE, Cooper et al. 1995c)

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, define the effects that neuromodulators have on these parameters (Cooper et al. 1995c, Southard et al. 2000). Gradual or abrupt changes can also be picked up by analyzing groups of 200 events as well as an entire set of 1000 events (Fig. 2D). For the most part, the individual bins of 200 events mimic the larger grouped data set. It is apparent that the m_{co} increases in the presence of 5-HT due to increases in both n and p.
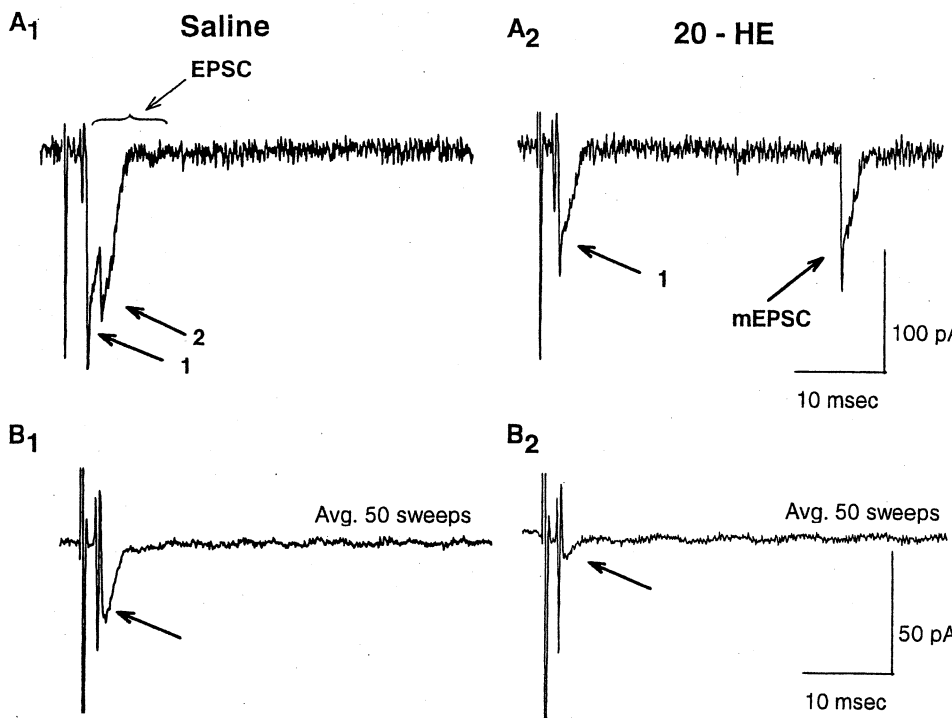
Fig. 3 A, B. The effects of 20-HE on synaptic currents as measured with a focal macropatch electrode over a discrete varicosity on a terminal of the opener muscle. Single evoked responses are shown prior (A_1) and after (A_2) exposure to 20-HE (10 μM). Note the spontaneous release that results in a miniature excitatory postsynaptic current (mEPSC). Average of 50 trials is saline (B_1) and after 15 min of exposure to 20-HE (B_2)

20-HE

In examining the role of the molt-related hormone 20-HE in inducing rapid changes in behavior and EPSP responses in lobsters (Cromarty and Kass-Simon 1998) as well as in crayfish (Cooper and Ruffner 1998), we decided to investigate the mechanistic actions on the presynaptic release machinery by examining whether changes occurred in the quantal release parameters and in latency of evoked vesicular release. Fig. 3 shows the general effect when viewing single sweeps Fig. (A_1 and A_2): fewer events are released over time. An average of 50 traces before the application of 20-HE and after 15 min are shown in Fig. 3 B_1 and B_2, respectively. The reduced average EPSC clearly demonstrates the increase in failure to evoke release. Quantal analysis showed a substantial decrease in the mean quantal content (m_{co}) over time with the presence of 20-HE, along with a reduction in the probability (p) of release and the number of release sites (n) (Table 1). When 5-HT containing bathing medium was added, the mean quantal content revived in all cases along with substantial increases in p. In some cases (four of five), n also increased.

Table 1 Effects of 20-HE and 5-HT on quantal parameters

	Saline to 20-HE			20-HE to 5-HT		
	% Δm_{co}	% Δp	n	% Δm_{co}	% Δp	n
site 1	↓ 43	↓ 13	4→2	↑ 254	↑ 73	2→3
site 2	↓ 28	↓ 48	2→3	↑ 114	↑ 221	3→2
site 3	↓ 48	↓ 48	3→2	↑ 288	↑ 114	2→3

The quantal parameters were determined in each case from 1000 evoked trials. The values used during the 20-HE (10 μM) addition were obtained after the terminal had been exposed for 20 min. The values before and right after 5-HT (100 nM) exposure were used to determine the changes. See Methods for details on quantal estimates. (After Cooper and Ruffner 1998)

Loading of the Terminal with α-SNAP

During co-injection of α-SNAP and a 70 kD Texas red dextran, the axon and terminals were viewed periodically while at the same time the axon was stimulated by a train of ten pulses at 40 Hz with an interval of 10 s. The EPSPs were recorded in the central muscle fibers closest to loaded terminals. When the terminal was loaded with α-SNAP, the EPSP amplitudes started to become enhanced in amplitude (Fig. 4A). The α-SNAPL294A mutant protein had the opposite effect, causing a decrease in the EPSP amplitudes (He et al. 1999). The carrier injections resulted in a slight increase in the amplitude of the EPSPs. This indicates that the injection process may have a positive effect on EPSP amplitudes, but less than that obtained with additional α-SNAP.

A

B
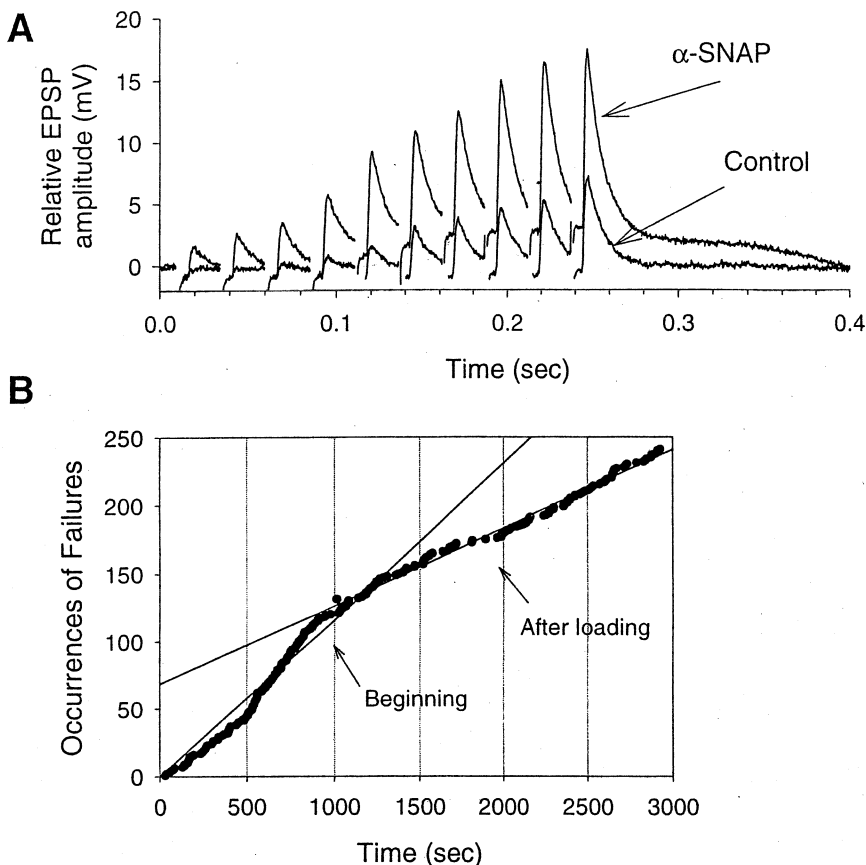

Fig. 4 A, B. The influence of α-SNAP on synaptic transmission. **A** EPSPs from a train at 40 Hz stimulation for 250 msec before the start of injection (*bottom*) and after the terminals were well loaded with α-SNAP (*top*). **B** The rate at which the failures occurred in synaptic current recordings obtained with the focal macropatch electrode before injection and after injection of α-SNAP revealed that fewer failures occurred with the addition of α-SNAP in the terminals

The fact that α-SNAP has a positive effect on EPSP amplitude and the dominant negative α-SNAPL294A mutant had an inhibitory effect suggests that the mammalian proteins are functional in the crayfish NMJ. This justifies the use of these proteins to study the role of α-SNAP in transmitter release at the NMJ. α-SNAP increases the EPSP amplitudes of crayfish NMJ, consistent with the effect on squid axons described by DeBello et al. (1995). Taking advantage of the crayfish system, we turned to quantal analysis to determine the potential mechanisms by which α-SNAP causes this increase. The number of evoked events and failures before and during α-SNAP injection were counted directly as already illustrated for 5-HT and 20-HE neuromodulation. α-SNAP injection had effects on the occurrences of evoked events and the number of failures. The numbers of failures decreased after the dye began to reach nerve terminals and remained at low levels throughout the injection period (Fig. 4B). The rate at which the failures decreased showed some interesting results. The rate tapers down when the dye
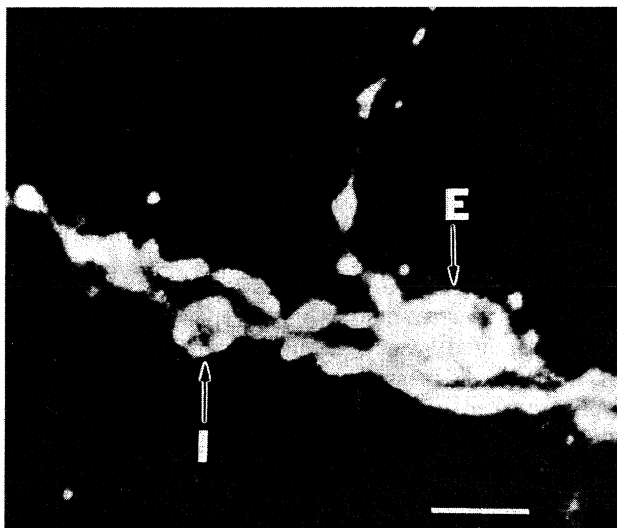
Fig. 5. Immunohistochemistry of motor nerve terminals of the opener muscle. Both inhibitory (*I*) and excitatory (*E*) terminals show enhanced accumulation of α-SNAP within the varicosities (*arrows*) in which pools of synaptic vesicles and synapses are located. Bar: 5 μm
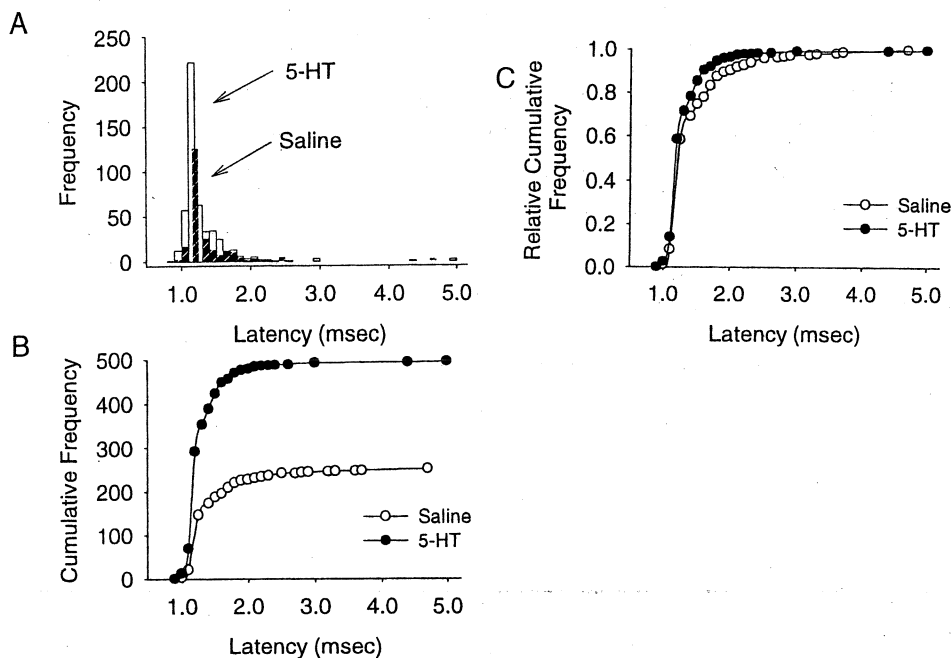

Fig. 6 A-C. Synaptic latency of vesicular release measured before and after application to 5-HT. For the same number of nerve stimulli, the total number of evoked events increases after addition of 5-HT, as shown in a histogram distribution of latencies (**A**) and for cumulative frequency (**B**). The relative cumulative frequency also indicates that there is a higher percent of quantal units released with a shorter latency in the presence of 5-HT (**C**). (After Southard et al. 2000)

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first reaches the terminals and then remains constant for the duration of the experiment. Typically, n did not increase significantly but p increased dramatically, suggesting that α-SNAP increases the probability of release at a site, but does not increase the number of release sites. α-SNAP is normally present within the terminals as examined by both whole mounts (Fig. 5) and Western blots of ventral nerve cord tissue (data not shown, but presented in He et al. 1999).

Latency Measures

To determine whether particular steps in the vesicular release process are affected by the presence of neuromodulators or alterations in the amounts of cytoplasmic docking complex proteins, we measured latency of quantal events, indicative of vesicular release (Fig. 2B). Latency was measured from the starting point of the spike to the starting point of an evoked event in the EPSC records; this was used as an estimate of the time that it took for an evoked event to occur. In earlier works (Katz and Miledi 1965), the time at the peak of action potential to the beginning of the synaptic event was defined as latency, but since neuromodulators could alter the width of the action potential, we chose to measure at the onset. Most of the time between depolarization of the nerve terminal and occurrence of a postsynaptic event is consumed in the process of vesicle fusion, since transmitter diffusion time in the synaptic cleft is very short.

The fastest events had latencies of 1 to 2 ms. To view the change in latency of release that occurred due to an alteration in the release machinery, we plotted histograms of the distribution and cumulative frequency distributions for the various treatments. If greater or fewer numbers of vesicles are released, the total cumulative value will be altered. For comparative purposes, an equal number of stimulus trials was used. The total cumulative plots permit trends in the absolute numbers of quantal events to be seen. However, it is difficult to compare the relative change in latencies when there is a change in the number of events. Therefore, relative cumulative frequency plots were also calculated to compare shifts in the distribution in latencies of the first evoked events. The exchange of the bathing medium with one containing 100 nM 5-HT resulted in a substantial increase in the number of quanta measured (Fig. 6A) but also in a substantial increase in the number of quanta with a minimal delay (Fig. 6B) (Southard et al. 2000). This produces a leftward shift in the curve (Fig. 6C). In general, the opposite trends were observed when standard saline was exchanged for one containing 10 μM 20-HE. Fewer quanta were released and there was a slight rightward shift in the latency of the distribution around 2 ms, although the shift is not significant as examined with Kolmogorov-Smirnov statistic at 95% confidence (Zar 1999). Some preparations revealed more extreme changes than these, but these figures are representative of the majority of experiments for 5-HT and 20-HE. Since 20-HE effects were not reversible upon washout with saline over a 10-min period, and the decrease in the numbers of evoked events continued, it appeared that the nerve may have been affected in such a manner that vesicle release could not be revived; but upon exposure to 5-HT, the nerve terminal quickly regained its ability to enhance transmitter release, since the number of quantal units increased (Fig. 7A) and latencies became shorter (Fig. 7B).

A

B
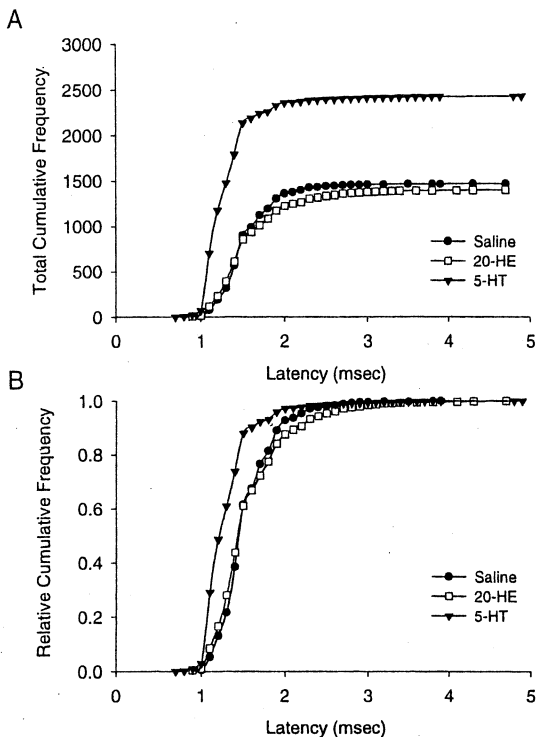
Fig. 7 A, B. To examine the influence of 5-HT after depression due to 20-HE, 20-HE was first exposed for 20 min followed by 5-HT. The total number of events decreased (**A**) with no real change in latency (**B**) for 20-HE whereas the exchange of the bath to one containing 5-HT still increases the total number of events (**A**) and produces leftward very small shifts in the latency (**B**)

Superimposed histograms indicate that more events had shorter latencies after α-SNAP was loaded into the terminals, but also more events occurred (Fig. 8A). The relative cumulative distributions also indicate that more events had short latencies afterα -SNAP was loaded into the terminals (Fig. 8B). Knowing that injected α-SNAP must have reached a saturated level in the terminals for interaction in forming the fusion complex, it was unlikely that a further increase in the number of vesicles released could occur with 5-HT. Much to our surprise, 5-HT (100 nM) resulted in a further increase in vesicular release without large shifts in latencies (Fig. 8B).

In summary, 20-HE alters presynaptic release of vesicles in a rapid nonconventional steroid action (i.e., nongenomic). The response is not reversible by washout, but continues over time. Application of 5-HT can quickly reverse the reduced synaptic efficacy induced by 20-HE. These neuromodulatory actions of 20-HE and 5-HT are also reflected in EPSPs measured by intracellular recordings. The injections of α-SNAP resulted in an increase in the number of evoked events. Furthermore, even after loading a large amount of α-SNAP into the nerve terminal, the addition of 5-HT produced an even greater recruitment of vesicles for rapid evoked release.
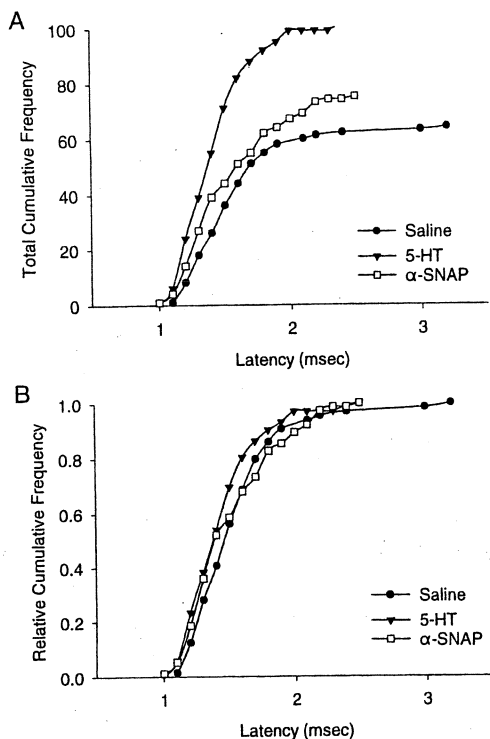
Fig. 8 A, B. The effect of α-SNAP on the release of transmitter. α-SNAP results mainly in an increase in the number of evoked events and small changes on latency (**A**). Application of 5-HT in addition to higher levels of α-SNAP in the terminals can further increase the number of events but only slightly affects synaptic latency (**B**)

Discussion

Mechanistic Actions on Vesicle Priming and Docking

The current view of vesicle priming and docking is based on the SNARE hypothesis (Weber et al. 1998). From this hypothesis, we pose the following schematic representation of the actions of α-SNAP, 5-HT, and 20-HE on the dynamics of vesicle release during evoked synaptic transmission (Fig. 9). Slight modifications from the original views are based on the fact that the distribution of t- and v-SNAREs are not discretely arranged on the vesicles and presynaptic membrane since complexes of v- and t-SNARE have been demonstrated in vesicles. In addition, endosome-endosome fusion studies suggest that SNAPs and NSF are involved in activating the SNAREs prior to SNARE-SNARE binding and membrane fusion (Ungermann et al. 1998). The results in our study suggest that α-SNAP leads to an increase in the fusion-competent pool of synaptic vesicles as deduced from latency measures. This so-called readily releasable pool is in a poised state ready for the calcium influx to trigger membrane fusion.
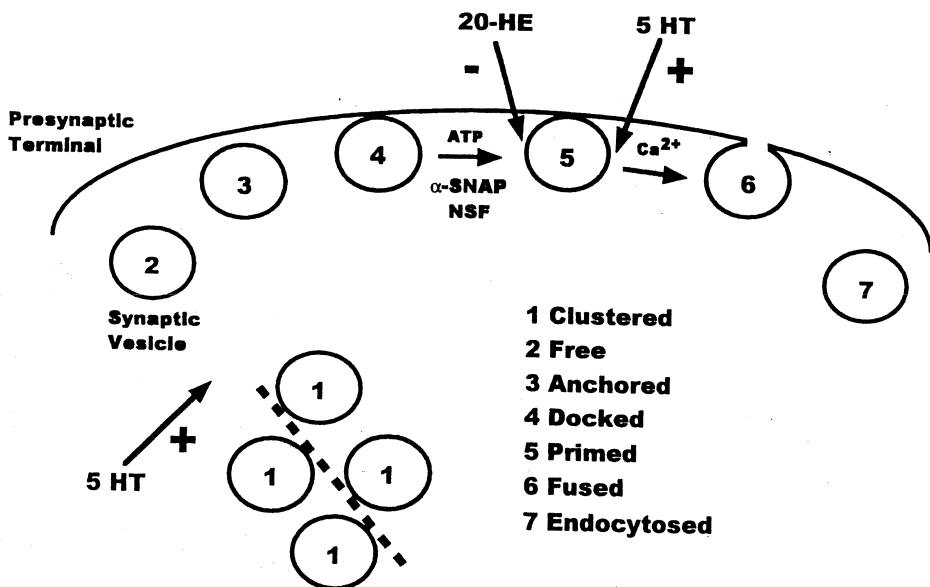
Fig. 9. Schematic representation of the potential sites of action for 5-HT, 20-HE and α-SNAP based on the quantal release results obtained at the motor nerve terminal. 5-HT could phosphorylate synapsins, resulting in release of vesicles from the cytoskeleton, which would increase the pool of free vesicles. Also, 5-HT could affect the Ca^{2+}-dependent steps of neurotransmission via phosphorylation. 20-HE appears to work by opposing vesicle docking and release, but there is not enough structural or biochemical information to state which step(s) are affected. It appears that α-SNAP leads to an increase in the fusion-competent pool of synaptic vesicles

There are two possible mechanisms which could account for the increase in poised vesicles. α-SNAP and NSF could activate the SNARE molecules so that they can bind to each other in the opposing bilayers. Following disruption of the homo-7S complexes present in the vesicle and target membranes, SNARE complexes could form between opposing membranes to form a new 7S complex (hetero-7S-complex). This hetero-7S-complex could facilitate fusion of the two membranes or be held in check by a calcium-sensitive block, as proposed for synaptotagmin (Südhof 1995).

The results of our study with the use of 5-HT also suggest that the readily releasable pool of vesicles is increased even more than when α-SNAP is injected into the axon and reaches the nerve terminal. 5-HT could also affect other processes, since it is known to work through an IP_3 cascade (Dixon and Atwood

1989a,b) which would lead to the activation of various protein kinases. 5-HT could induce the phosphorylation of molecules such as Munc-18-1 or synaptophysin. 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) (Rubenstein et al. 1993; Shuang et al. 1998). 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 SVs available for secretion. While these are possible mechanisms, were they true, the effect of 5-HT would be expected to cause an increase in docking and/or priming analogous to the effects of α-SNAP. From the data presented, it would seem more likely that 5-HT could affect the Ca^{2+}-dependent step of neurotransmission either by inducing the phosphorylation of synaptotagmin (Davletov et al. 1993) or perhaps the phosphorylation of the Ca^{2+} channel itself (Gray et al. 1998).

Physiological Actions of 20-HE

At this point in time we can only suggest that 20-HE may work in some way to oppose vesicle docking and release since no second-messenger systems are known to be activated or depressed by the presence of 20-HE. Surprisingly few studies investigating the nongenomic actions of molt-related steroid compounds among crustaceans and insects are present (Cooper and Ruffner 1998; Cromarty and Kass-Simon 1998; Ruffner et al. 1999). These model systems in neurobiology have played in the past, and continue today to play important roles in helping to answer questions involved with the regulation of chemical synaptic transmission. Further investigations of steroid action in crustaceans will be fruitful in providing answers to the mechanistic actions of steroids known to be present in these animals and possible steroid action in mammals. Behavioral changes occur in insects and crustaceans in preparation for and immediately after molting, but these mechanisms are not fully understood. There is an indication that the molt related hormone may induce the behaviors associated with this period (Cromarty 1998). Our recent studies on neuromuscular preparations in which the axon had been severed from its cell body have shown that ecdysone elicits a rapid response at the nerve terminal. The rate at which this response occurred suggests a direct action without transcriptional regulation within the presynaptic terminal or possibly in the muscle fibers. In an isolated crayfish nerve-muscle preparation exposed to 20-HE, there was a pronounced reduction in the size of the EPSPs recorded in the muscle. This study also showed that quick changes in the quantal release of synaptic transmission are seen in response to 20-HE at crayfish neuromuscular junctions (Cooper and Ruffner 1998) and neuromuscular junctions of *Drosophila melanogaster* (Ruffner et al., 1999). EJP amplitudes are seen to be depressed in the presence of 20-HE, due to a reduction in EJCs, thus demonstrating that 20-HE behaves as a neuromodulator. From quantal analysis, we observed that the site of nongenomic action of 20-HE is presynaptic. The results help to explain the behavior of the animals at the time of molt when the molt hormones are at their peak, in that the animals exhibit a reduction in overall movements at that time.

Interaction of 20-HE and 5-HT

Since most biologically active steroids in mammals have been shown to have non-genomic actions (Tuohimaa et al. 1996), it is of interest to understand the mechanism of action and consequences in altering synaptic transmission among neurons in the CNS and at the neuromuscular junction. The *Drosophila* and crayfish neuromuscular junctions continue to serve as ideal preparations for examining glutaminergic chemical transmission at the level of kinetics in quantal events and direct synaptic structural correlation. The interactions of 20-HE, which depresses presynaptic transmission, and serotonin (5-HT), which enhances presynaptic release, is an interesting observation. Emphasis of the mechanisms of action for these neuromodulators and the interactions of signaling pathways would reveal novel avenues of nongenomic and neuromodulatory actions within nerve terminals. Since both 20-HE and 5-HT are normal substances released into the crustacean hemolymph at various times in the molt cycle or daily cycle, they may account for hormone-driven behaviors.

Interaction of 5-HT and -SNAP

The alteration in the levels of synaptic vesicle-related docking proteins, such as α-SNAP, allows one to assess its physiological function and examine if it may be a limiting component in regulating synaptic efficacy. In combining neuromodulator actions in terminals overloaded with α-SNAP, mechanistic questions of the actions of these compounds are able to be addressed.

The 5-HT alone and the α-SNAP alone or in combination with 5-HT increased in the quantal parameter p because most likely more vesicles are in the primed state ready for evoked release. The presence of 5-HT also increased the quantal parameter n, which can be interpreted as more sites for release. Ultrastructural analysis of synapses in this type of terminal have revealed an array of vesicles around each active zone at the synapse (Govind et al. 1994). This would indicate that several vesicles may be primed for release around each active zone, but, in addition, the structural observations also suggest that a 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 primed state at a single active zone. The cytoplasmic proteins such as α-SNAP then may provide the fine tuning in synaptic efficacy at the established active zones. Facilitation of presynaptic release may be related to alterations in docking and priming of vesicles, since short-term facilitation observed in the crayfish opener cannot be attributed solely to calcium loading, due to the short life time of residual calcium after evoked release (Parnas et al. 1982; Atwood and Wojtowicz 1986; see also Zucker and Lara-Estrella 1983). In the presence of 5-HT, which is known to work through IP_3 (Dixon and Atwood 1989a,b), it is possible that a cellular cascade could lead to phosphorylation of molecules that promote vesicle docking/priming for release (Rubenstein et al. 1993; Shuang et al. 1998). While these are possible mechanisms, detailed experiments are needed to address the underlying causes of enhanced synaptic release during short-term and long-term

synaptic facilitation and among terminals that show differences in synaptic efficacy (King et al. 1996; Bradacs et al. 1997; LaFramboise et al. 2000).

The physiological data indicate convincingly that 5-HT and α-SNAP work to enhance docking/priming, although we were are not able to assess if 5-HT and α-SNAP may possibly be altering vesicle recycling. This remains to be tested by possibly loading recycling vesicles with fluorescent indicators viewed with light microscopy (i.e., FM1-43; Msghina et al. 1998; Wang and Zucker 1998; Quigley et al. 1999) or with HRP loading and subsequent electron microscopy (Thompson and Atwood 1984; Listerman et al. 1999). Likewise, the action of 20-HE is apparently involved in altering vesicle release, so structural examination or monitoring vesicle dynamics would be beneficial.

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