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Regulation of synaptic vesicles pools within motor nerve terminals during short-term facilitation and neuromodulation

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Logsdon, S., A. F. M. Johnstone, K. Viele, and R. L. Cooper. Regulation of synaptic vesicles pools within motor nerve terminals during short-term facilitation and neuromodulation. *J Appl Physiol* 100: 662–671, 2006. First published October 6, 2005; doi:10.1152/japplphysiol.00580.2005.— The reserve pool (RP) and readily releasable pool (RRP) of synaptic vesicles within presynaptic nerve terminals were physiologically differentiated into distinctly separate functional groups. This was accomplished in glutamatergic nerve terminals by blocking the glutamate transporter with DL-threo-β-benzyloxyaspartate (TBOA; 10 μM) during electrical stimulation with either 40 Hz of 10 pulses within a train or 20- or 50-Hz continuous stimulation. The 50-Hz continuous stimulation decreased the excitatory postsynaptic potential amplitude 60 min faster than for the 20-Hz continuous stimulation in the presence of TBOA ($P < 0.05$). There was no significant difference between the train stimulation and 20-Hz continuous stimulation in the run-down time in the presence of TBOA. After TBOA-induced synaptic depression, the excitatory postsynaptic potentials were rapidly (<1 min) revitalized by exposure to serotonin (5-HT, 1 μM) in every preparation tested ($P < 0.05$). At this glutamatergic nerve terminal, 5-HT promotes an increase probability of vesicular docking and fusion. Quantal recordings made directly at nerve terminals revealed smaller quantal sizes with TBOA exposure with a marked increase in quantal size as well as a continual appearance of smaller quanta upon 5-HT treatment after TBOA-induced depression. Thus 5-HT was able to recruit vesicles from the RP that were not rapidly depleted by acute TBOA treatment and electrical stimulation. The results support the notion that the RRP is selectively activated during rapid electrical stimulation sparing the RP; however, the RP can be recruited by the neuromodulator 5-HT. This suggests at least two separate kinetic and distinct regulatory paths for vesicle recycling within the presynaptic nerve terminal.

synapse; calcium; serotonin; glutamate; recycle; potentiation

IN POSTSYNAPTIC TARGETS, the excitatory synaptic potentials (EPSP) are incremental in relation to the numbers of packets of transmitter released below a threshold potential (19). This is generally accepted by a packet of neurotransmitter contained within a vesicle being released from the presynaptic nerve terminal into the synaptic cleft (33, 45). A number of studies have set out to address whether all of the vesicles are equally potent in eliciting quantal postsynaptic responses (52, 56). To resolve some of the issues in vesicular packaging, one needs to determine whether the vesicle pools are equally recruited during electrical activity and in the presence of neuromodulators. Because it is known in a number of preparations that there are populations of vesicles within nerve terminals that behave differently in their fusion and recycling kinetics, we examined the ability of these pools to be recruited during electrical activity of the nerve as well as in the presence of neuromodulators, which have secondary actions on vesicular kinetics. By addressing this in relation to presynaptic function, a better understanding in fluctuations in quantal responses during synaptic depression, facilitation, and synaptic differentiation will hopefully be achieved. In this study, we make use of the known simplicity of the synaptic structure at the crayfish opener neuromuscular junction (NMJ) and its quantal nature of transmitter release to assess discrete synapses within the motor nerve terminals (33, 19, 20). The opener muscle in the crayfish walking leg, as used in this study, is nonspiking, and graded postsynaptic potentials are directly related to the number of presynaptic vesicle fusion events (29–31, 33), as also shown at the frog NMJ when the responses are below the threshold level for an action potential in the muscle (45).

With physiological and pharmacological means, we set out to address whether the uptake of synaptically released glutamate is important in the refilling of the readily releasable pool (RRP) of vesicles that rapidly recycles during maintained stimulation. In addition, we examined whether the reserve pool (RP) of vesicles can be recruited, by the use of serotonin (5-HT), while the RRP remains reduced functionally or is rendered nonfunctional. The goal is also to provide further insight into the regulation of the RP and recycling of the RRP within nerve terminals in a model synaptic preparation.

Because 5-HT at the crayfish NMJ greatly enhances transmitter release (27) presynaptically at NMJs by increasing the probability of vesicular fusion (61) and is thought to do so by recruiting RP of vesicles to the RRP (10, 62), the two populations of vesicles can be differentiated by depleting the function of the RRP by preventing them from being repackaged during rapid recycling. Recruiting the RP with 5-HT while reducing the ability of the rapidly recycling pool from repackaging allows the dissection of the various pools within the terminal. In crayfish motor neurons, 5-HT mediates its rapid effect through the inositol-3-polyphosphate (IP_3) second-messenger system (23, 25). The action of short-term facilitation (STF) in enhancing release was recently addressed from actions of 5-HT at the crayfish NMJ that suggested that different pools of vesicles might reside in the terminal because of alternate recycling mechanisms (62). The recycling of vesicles is generally depicted as following two different routes for recycling: a rapid loop and a slower one that reprocess the vesicles within the endoplasmic reticulum (44, 46, 55, 56, 58, 63).

The rapid recycling process is promoted during repetitive electrical depolarization of the terminal to maintain output. A homeostasis can even exist to recycle vesicles and buffer

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calcium so that a plateau in release is observed; however, a slight alteration in the stimulation frequency or actions of a neuromodulator offset the balance in the various vesicle recycling paths (62). To address whether the RP can be recruited to the RRP during maintained electrical activity of the terminal, the RRP needed to be selectively targeted to be allowed to recycle, but, however, tagged to distinguish release from those of the RP. This can be approached by depleted transmitter within the vesicles of the rapidly recycling pool while not altering the RP within a short window of time.

Rapid functioning synapses clear released transmitter in the synaptic cleft quickly, which is one mechanism to avoid desensitization of postsynaptic receptors to detect subsequent evoked release within several milliseconds (34) and prevent spillover to neighboring synapses (24, 41). The glutamatergic synapses within the vertebrate central nervous system (CNS) clear glutamate by use of glutamate transporters. It is generally accepted that the major recycling path for glutamate is through astrocytes (41). These supportive cells take up the glutamate from the synaptic cleft via glutamate transporters. Within the cell, glutamate is converted to glutamine, which is then released out of the astrocyte. The transporters on the nerve terminal then take up glutamine, which is converted back to glutamate and transported into the vesicles by specific vesicle-associated transporters (9, 65). Thus, for glutamatergic terminals, the glutamate enters back into the terminal by two means: 1) directly via presynaptic transporters and 2) through the use of supportive cells by a glutamate-glutamine cycle.

Vertebrates are known in have multiple types of glutamate transporters. In mice, there appear to be five genes that encode six distinct glutamate transporters (41). The motor nerve terminals at the crayfish NMJ are glutamatergic, and, because glia cells do not enfold with the nerve terminals on the muscle, the variable of supportive cells is eliminated in the process of vesicle repackaging (2, 12). Thus the presynaptic nerve terminal is the likely means to clear glutamate from the synaptic cleft. The reuptake process during normal synaptic transmission is expected to be rapid, because these NMJs can desensitize quickly with exogenously applied glutamate. In the absence of glutamate, the receptors resensitized to be responsive to a subsequent exposure (34). The presynaptic reuptake at the crayfish NMJ is also supported by the fact that the blocker for vertebrate glutamate transporters, DL-threo-β-benzyloxyaspartate (TBOA), can block the reuptake of glutamate at the crayfish NMJ (34). Hence we made use of blocking glutamate reuptake to assess vesicle dynamics within the nerve terminal during rapid release and recycling of the RRP of vesicles.

Through the use of transmission electron microscopy (TEM), one can address whether the recycling pool of vesicles is altered in numbers when the presynaptic transporters are compromised. We examined for differences in the dynamics of the RP and the number of docked vesicles anatomically with TEM to compare with physiological assessments and predictions of vesicle dynamics during blockade of the transporter as well as during recruitment of a RP by 5-HT. Vesicles and vesicle pools are easily identified in nerve terminals in TEM, which provides insight to the state of the nerve terminal before and after pharmacological exposure. In most nerve terminals examined, at a resting state, a small number of vesicles are docked to the active zone accompanied by a close proximity RRP of vesicles and a RP of vesicles located a small distance away (59). After

TBOA induction, the functional responses of the RRP of vesicles are known to become depleted (34). Thus it is of interest to know by TEM whether there is a decrease in docked vesicles or those in the RRP.

This work has previously appeared only in abstract form (47).

METHODS

General. All experiments were performed using the first and second walking legs of 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 dried fish food. Dissected preparations were maintained in crayfish saline, a modified Van Harreveld's solution (in mM: 205 NaCl; 5.3 KCl; 13.5 CaCl$_2$·2H$_2$O; 2.45 MgCl$_2$·6H$_2$O; 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 (33). The STF was induced by providing a train of 10 pulses at 40 Hz, at 3-s intervals, to the excitatory nerve. The selection of stimulation frequencies was chosen to be similar to earlier studies using the same preparation (13, 18). In addition, the distal muscle fibers were always used in this study, as illustrated in Fig. 1, because it is easy to follow these muscle bundles throughout fixation and processing for electron microscopy to obtain physiological and anatomical correlation of similar type synapses (11, 53). Continuous stimulation at 20 and 50 Hz was also applied to the excitatory nerve to compare the effects of TBOA.

Intracellular EPSP recordings were performed by standard procedures (12, 18). The 5-HT (1 μM)-containing saline was made in crayfish saline from frozen stock of 1 mM 5-HT. 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 or to a Dell Latitude D600 computer via a MacLab/4s or a PowerLab/4s interface, respectively.

Field excitatory postsynaptic potentials. In addition, synaptic field potentials were also measured with focal macropatch electrodes to
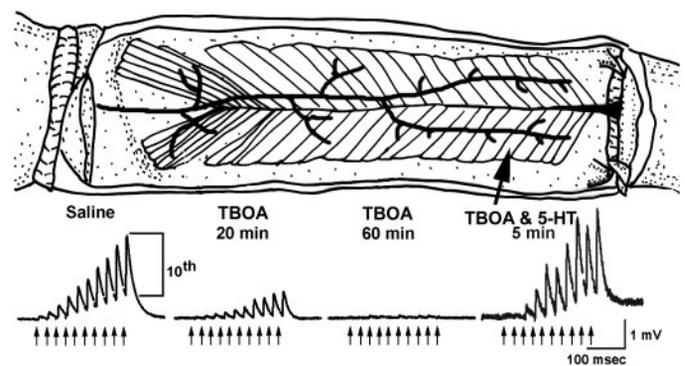

Fig. 1. Effects of DL-threo-β-benzyloxyaspartate (TBOA) on synaptic transmission. A schematic is shown of the opener muscle in the crayfish walking leg with associated excitatory postsynaptic potentials (EPSPs). Recordings were made in distal muscle fibers (arrow). Representative EPSP responses are to a train of 10 stimulation pulses given at 40 Hz before and during exposure to TBOA (10 μM) followed by TBOA and serotonin (5-HT) (*lower* traces). The amplitude of the EPSPs is measured from the trough preceding the EPSP of interest to the peak response, as shown for the 10th pulse during saline exposure. As the preparation is exposed to TBOA, the EPSP amplitudes decrease over time. After the 10th pulse in the train is reduced by at least one-tenth of the initial amplitude, the preparation is exposed to 5-HT (1 μM). 5-HT increases the amplitude of the EPSPs. Arrows in traces of EPSPs represent the 10 stimulation pulses.

assess presynaptic vesicular events. The varicosities on the living terminals were visualized using the vital fluorescent dye 4-Di-2-ASP (Molecular Probes) (12, 48). 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 using 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 EPSPs (fEPSPs) and field miniature EPSPs can be recorded readily (15, 11, 19).

In addition to the direct quantal measures, the area of the evoked and spontaneous events was measured over time in each preparation for comparison within a preparation to determine whether the area of the quantal units was altered. The area of the evoked and spontaneous events was determined by Simpson's mathematical method after the baseline of each trace was controlled for direct current (DC) offset (68). The DC offset was adjusted by setting the average of the baseline to zero. The noise that was added over time in the measures was corrected for by subtraction of the average noise determined within baseline recording. This approach adjusted for the noise independent of the duration measured for each event. The area analysis was managed by converting the traces stored as Scope files to rich text files. The rich text files were then used in conjunction with subroutines written in "R basic." This software is freeware and maintained by CRAN (Comprehensive R Archive Network) and downloadable from http://cran.r-project.org. The computational assessment computed evoked events as any deflection above noise from baseline within the 20-ms window after the action potential spike was recorded as a result of the nerve terminal depolarization. The output files were then imported in Sigma Plot (version 8.0) for graphical purposes and quantifying the measures.

Analysis. The STF for train stimulation was indexed by the ratio in the amplitudes of the 10th and the 5th EPSP within a train (18). The numeral one was subtracted from the ratio to ensure that, if there is no facilitation occurring, the facilitation index will then be zero. The EPSP amplitudes were measured by the difference from the trough preceding the event to the peak amplitude of the event. Using the first EPSP amplitude within a train to calculate facilitation index can lead to erroneously high values when the first EPSP is small, as reported in earlier studies. Thus measures of the 5th to the 10th EPSPs are more reliable for indexing the effect of TBOA. To index a 50% decline in EPSP amplitude, 10 responses were measured from a baseline to the peak at various time intervals. Each 10 pulses were averaged and graphed to ascertain the time point of a 50% decline in amplitude. The times for train stimulation, 20-Hz continuous stimulation, and 50-Hz continuous stimulation were compared. The mean time for a 50% decay was used for statistical comparisons. The induction of depression was stopped when the amplitude of the 10th EPSP in the train reached one-tenth the initial amplitude. Percent change after addition of 5-HT was also calculated and compared between the three stimulation types. An average of 10 consecutive responses was used at varying time intervals to ascertain the effects of TBOA and 5-HT. The average EPSP amplitude directly before 5-HT addition and the largest average EPSP amplitude produced by 5-HT were used to calculate the percent change. The relative change was measured from the depressed EPSP amplitude to the peak response to 5-HT. All exposures to 5-HT occurred after depression of the EPSP amplitude reached one-tenth of their initial value. This was the standard used for all of the preparations. Of course, each preparation took a different amount of time to reach the one-tenth EPSP amplitude. The number of stimuli given to reach a 50% reduction in the EPSP amplitude was also used for indexing purposes. The three groups (40-Hz train stimulation, 20-Hz continuous stimulation, and 50-Hz continuous stimulation) were compared by a one-way ANOVA with follow-up *t*-tests when the assumptions were valid and by a Kruskal-Wallis test when the assumptions were not met. *R* was used for these procedures.

Transmission electron microscopy. All preparations were fixed in a 2.5% glutaraldehyde-, 0.5% formaldehyde-buffered solution (0.1 M sodium cacodylate, 0.022% weight $CaCl_2$, 4% weight sucrose, and adjusted to pH of 7.4) for 1 h with two changes, postfixed with a 2% osmium tetroxide-buffered solution, and embedded in Eponate 812. The samples were serially thin sectioned on a Reichert ultracut microtome and poststained with uranyl acetate and lead citrate. Sections were then viewed on a FEI: Philips Tecnai, Bio Twin 12 model transmission electron microscope at 80 kV.

Measurements for vesicle location and distribution. Each transmission electron micrograph that revealed a synapse was catalogued. In some cases, complete serial sections of synapses were obtained. The presynaptic terminals were classified as excitatory by the shape of the vesicles. Inhibitory terminals reveal oblique shaped vesicles (66) and were not further used for quantification. The appearance of dense bodies associated with synapses within the presynaptic terminal was used to define an active zone (AZ). Within the crayfish NMJ, AZs are places where vesicles cluster in association to fusion with the presynaptic membrane (16). These dense bodies are thought to serve as cytoskeleton attachment points to deliver tethered synaptic vesicles possible from reserve pools.

Synapses at the crayfish NMJ do not have a grid of AZ on the synapses but show synaptic variation such that some synapses may only posses a single AZ, whereas others might have multiple AZs at varying distances from one another (16). We sought to accurately measure docked and RRP by serial sectioning of the synapses around single AZs by ensuring that the counts were not skewed by vesicles associated by near neighboring AZ. Thus three sections to either side of an AZ of interest were viewed, and only sections within one section to either side of the section containing an AZ were used for anatomical measures of vesicle pools. In the crayfish opener preparation, the dense bodies of the excitatory terminals are viewed as hemispheres of ~35–40 nm in diameter, sitting with the cross section of a hemisphere facing the synapse (5). This is documented by the occasional on face view in parallel sectioning of synapses (16). Thus, in 75-nm cross-sectional thickness, a dense body can be contained within a single section. If the dense body is seen within two sections, then sections on either side of these sections were also used for analysis. Care for stereological errors in measuring objects in TEM from two-dimensional images of three-dimensional tissue were implemented as previously described (3, 36, 43).

RESULTS

The crayfish opener muscle in the walking leg is known to have regional variation in the amplitudes of the EPSPs across the muscle fiber, despite being innervated by a single excitatory motoneuron (53). Thus in these experiments the distal muscle fibers were always used as illustrated in Fig. 1. The relatively low-output nature of the toniclike motor nerve of the opener muscle produces a small-amplitude EPSP when a single stimulus is provided. Because the postsynaptic responses are graded and the NMJ in this preparation can have pronounced facilitation (18, 71, 72), a short train of 10 pulses at 40 Hz produces a significant increase in the EPSP amplitudes. The larger amplitude of the 10th response in the train provides an easy measure to examine changes over time in synaptic efficacy by observing an increasing or decreasing amplitude. When the preparation is exposed to saline containing TBOA (10 μM), all of the EPSPs within the train decrease in amplitude over time. With exposure to 5-HT (1 μM), after TBOA treatment, the responses show a pronounced enhancement in all cases and even increase in amplitude greater than during the initial observation in saline (Fig. 1, lower traces). In this experimental paradigm, the preparation is continuously stimulated with the pulse train of 10 stimuli every 3 s during the TBOA exposure.

The time domain for running down the glutamate-loaded vesicle pool varied among preparations. Thus, to compare among preparations, an index of the time for the 10th EPSP within the train to decrease to an average 50% of its initial average value was used. A representative analysis is shown in Fig. 2. All EPSPs were collected for the 50-Hz and 20-Hz continuous-stimulation paradigms; however, analysis of the EPSP amplitude was performed only on sections of the fully observed file, where changes in amplitude occurred. The average time to 50% reduction among all the preparations for each stimulation condition (40-Hz train, 20- and 50-Hz continuous stimulation) are presented in Fig. 3A, whereas the average number of stimuli to a 50% reduction is presented in Fig. 3B. The data in Fig. 3B were far more amenable to ANOVA analysis than the data in Fig. 3A because the data in Fig. 3B could be transformed by a cube root transformation to achieve approximate normality, an assumption of ANOVA (the cube root transformation was confirmed by a Box-Cox transformation analysis as well. Without the transformation, the variances of the three groups are quite unequal). We rejected the hypothesis of equal treatment means ($P = 0.015$) and used follow-up t-tests, which indicate that the 40-Hz train stimulation group is different from the other two groups ($P = 0.008$ for comparing 40 to 20 Hz, and $P = 0.024$ for comparing 40 to 50 Hz). No suitable transformation could be found for the time data in Fig. 3A, which were then analyzed by the less sensitive Kruskal-Wallis nonparametric test. The only significant result achieved ($P = 0.042$) for these data were the pairwise comparison between the 20- and 50-Hz groups. Considering that these are pairwise comparisons, we view this result as indicative, not conclusive, and would hope larger sample sizes would shed more light on this issue.

Because the 50-Hz continuous stimulation rapidly depressed the EPSP amplitudes compared with the 20-Hz stimulation, one would expect that the reserve pool of vesicles would be reduced in the 50-Hz-stimulated preparations compared with the 20-Hz-stimulated preparations. Because 5-HT is thought to mobilize the reserve pool of vesicles to the synapse at the crayfish NMJs (10, 14, 40, 61), we postulated that the 50-Hz-stimulated preparations would not show much, if any alteration, in the synaptic responses when exposed to 5-HT (1 μM) compared with the 20-Hz trains or the preparations stimulated with an intermittent 40-Hz train. The results were surprising in that the 50-Hz depressed synapses showed a pronounced enhancement upon exposure to 5-HT (1 μM), comparable to the
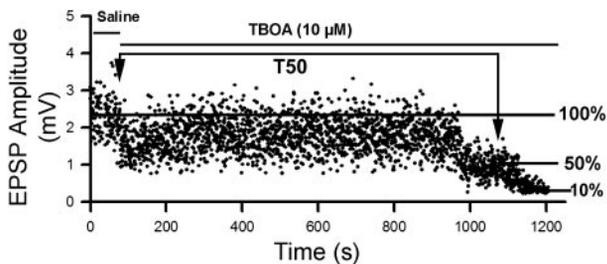

Fig. 2. The presence of TBOA resulted in a reduction of evoked transmission. The 10th EPSP amplitude, during 40-Hz, 10-pulse trains every 3 s, is shown decreasing over time after exposure. The time to 50% reduction (T50) in the 10th EPSP after exposure to TBOA was determined as indicated on the graph for each preparation. The same approach was used for the continuous stimulation of 20- and 50-Hz paradigms.
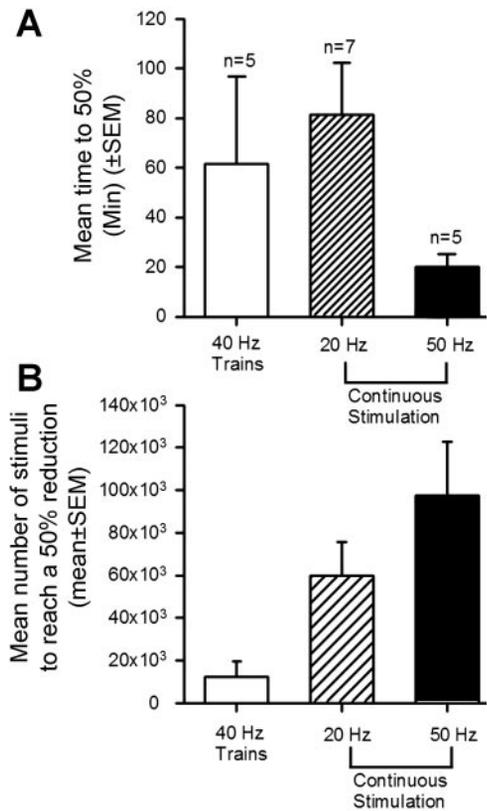

Fig. 3. Time and number of stimuli to run down the EPSP amplitudes by 50% in the presence of TBOA. A: mean run-down time to 50% the initial value for the different stimulation paradigms in the presence of TBOA. B: number of stimuli. Data in B are more amenable to an ANOVA, where we reject the hypothesis of equal means ($P = 0.015$). Follow-up t-tests indicate that the 40-Hz train stimulation group is different from the other 2 groups ($P = 0.008$ for comparing 40 to 20 Hz, and $P = 0.024$ for comparing 40 to 50 Hz). Data in A require less sensitive nonparametric methods, where the only indicative result was the pairwise comparison looking at the difference between the 20- and 50-Hz groups ($P = 0.042$).

depressed terminals stimulated with the intermittent 40-Hz trains and the continuous 20-Hz trains (Fig. 4). The results of 5-HT exposure on electrically depressed nerve terminals is important because this suggests that RP is not depleted. In addition, this implies that the presynaptic sites are not blocked with empty vesicles recycling because of the electrically stimulated depression. There must be ample docking and fusion space on depressed synapses for such a prominent enhancement of vesicle fusion upon 5-HT exposure.

One such mechanistic possibility for synaptic depression is that the glutamate receptors postsynaptically are internalized, as shown for the CNS in vertebrates during the induction of long-term depression (51, 69). In this case, the size or area of the quantal depolarized responses should become smaller over the induction of the depression. This would be the case not only for the evoked quantal events but also for the events that arise because of spontaneous release, assuming that spontaneous events occur from the same synapses that were evoked electrically. To test for such a possibility, the individual quantal events were monitored by a focal macropatch electrode placed directly over visually discrete varicosities on the nerve terminal before and during the induction of depression by TBOA exposure (Fig. 5A). Single stimuli may evoke one or
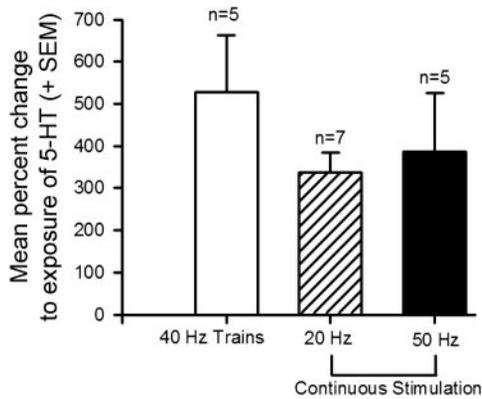
Fig. 4. Enhancement with 5-HT of evoked transmission after TBOA treatment. After the synaptic responses were reduced to at least one-tenth of their initial amplitude, they were exposed to 5-HT. The average percent change to exposure of 5-HT and TBOA from the depressed state during TBOA alone exposure is shown. All depressed preparations were enhanced drastically by 5-HT ($P < 0.05$, Wilcoxon's rank sum test). Thus vesicles in the reserve pool (RP) that are fully loaded with glutamate are able to be recruited into action.

more quantal responses (a singlet, doublet, etc.), or none at all (a failure) (Fig. 5B). Because we were interested in knowing whether the size or area of the quantal responses varied during depression, the area (deflection off baseline) of the quantals was monitored over time (Fig. 5C). The area measures were also monitored during stimuli that did not produce an event but were deemed to be a failure in evoking a response. Because

these NMJs of the opener muscle are very low in synaptic efficacy, this is expected and had been reported previously (11, 16). The normal distribution in the area of the noise for baseline recordings (or failures) centers at zero. This is the prevalent measure during the late phase of exposure to TBOA with only a few evoked events being observed. Upon exposure of the terminal to 5-HT (1 μM), after synaptic depression is maintained, the quantal responses appear rapidly (Fig. 5C). Fewer failures in evoking a response were evident after the treatment with 5-HT. The responses of the macropatch recordings parallel the results obtained with the intracellular recordings, which represent an ensemble average of the discrete regions of the nerve terminals that were monitored with the focal macropatch electrode.

It is apparent that the postsynaptic receptors are not rapidly downregulated or internalized or even for that matter desensitized during the synaptic depression, because quantal responses rapidly arise with exposure 5-HT. Additional evidence supports this notion from the monitoring of spontaneous events before and during depression. The range in the area measures of the minis (i.e., spontaneous events) are not different (Fig. 5D, a and b). Given that the minis might occur from all synapses present and not just the recruited synapses during the stimulation, one would expect a significant portion of the minis to be reduced in size if the postsynaptic receptor array was compromised during depression. Also the minis showed a similar range during the 5-HT exposure, which also suggests

Fig. 5. TBOA is shown to reduce the size of the quantal responses via presynaptic mechanisms. Recordings of individual evoked quantal events and spontaneous events were obtained by use of a focal macropatch electrode placed over visualized varicosities (A). Quantal responses from evoked release and spontaneous release were monitored (B). The area of the evoked field excitatory postsynaptic potentials (fEPSPs) decrease over time with TBOA (depleted vesicles). After synaptic depression occurs, the exposure of 5-HT in the presence of TBOA recruited new populations of vesicles from a RP. When the area of the evoked and spontaneous [i.e., field miniature postsynaptic potentials (fmEPSPs)] are plotted, it was observed that the spontaneous quantal events still occur when evoked transmission is depressed (C). The fmEPSPs during saline (Da), depressed fEPSPs (Db) as well as during 5-HT and TBOA exposure (Dc) indicate that postsynaptic sensitivity is not altered, but the type of vesicle from the rapidly recycling pool or a RP varies. During the 5-HT exposure, the evoked and spontaneous events are mixed in size and area, suggesting the ability to recruit new populations of vesicles from a RP in TBOA-depressed terminals.
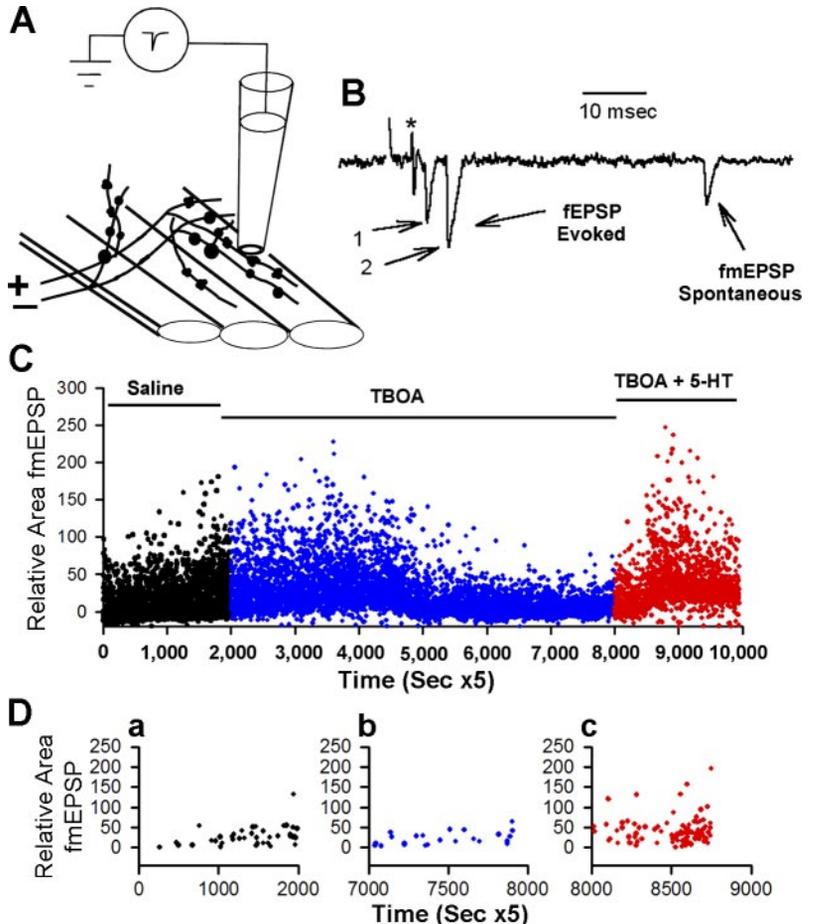
that the same population of synapses are in use (Fig. 5*D*, *c*). The results indicate that the depressed terminals have small quanta, and after 5-HT treatment a mix of very small and normal sized quanta are present. This suggests that some depressed vesicles are indeed mixing with recruited RP vesicles (because of 5-HT), which were fully packaged with glutamate.

To obtain structural evidence for the physiological measures, serial sections of the motor nerve terminals were reconstructed in preparations that were only stimulated in saline and depressed compared with preparations depressed with exposure to TBOA. Three control and three TBOA-treated preparations were examined. The fixation was rapid (<5 s) after stimulation was ceased. The time delay was to remove the ground wire and exchange the saline for a fixation solution in the recording dish. The area surrounding an AZ on crustacean NMJ synapses has a high incidence of docked vesicles and clustering compared with regions of the synapse devoid of AZs (2–4). For this reason, serial sections were obtained on either side of the section containing an AZ, and all three sections were used for the analysis in the number of docked and RRP (within 150 nm of the presynaptic face) vesicles. A electron micrograph and a schematic of the analysis are shown in Fig. 6. To ensure that a neighboring AZ was not present on two sections removed from the section containing the AZ of interest, four to five serial sections were viewed on either side of the AZ in which the measures of docked and RP vesicles were being made. This extra precaution is necessary because vesicles are observed docked ~100 nm distant from an AZ (12, 15). Thus we are confident that the second section removed from the AZ of interest was not close to a subsequent section containing an AZ. The structural analysis for the number of docked and RRP vesicles is presented in Table 1. The lateral extent of the area measured (along the cell membrane) for the vesicle docking was only on synaptic surfaces. There did not appear to be any effect of the TBOA treatment in the number of vesicles in RRP or in the number of docked vesicles. Sometimes an AZ is seen in two serial sections because of being randomly sectioned (11). In these cases, the section order in Table 1 reads 0 and 0 for the sections containing an AZ and −1 or +1 for the section on either side of the one(s) containing an AZ. The splitting of an AZ was present in one preparation, a control, and in two TBOA-treated preparations. A total of six synapses in each condition were examined. Two different synapses in each tissue sample were analyzed in detail as described.
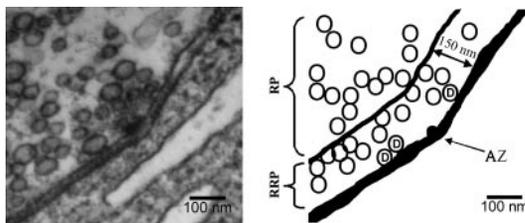

Fig. 6. Docked and readily releasable vesicle pools were determined by transmission electron microscopy. A representative synapse shows vesicles docked and ones neighboring the presynaptic face (*A*). Vesicles and synaptic surface are traced from the photomicrographs for determining the number of docked (D) and readily releasable pool (RRP) of vesicles (*B*). The RRP are vesicles that are not docked but within 150 nm of the presynaptic face. If one-half or more of a bordering vesicle is within the 150 nm line, it is considered to reside in the RRP. Only vesicles touching the synaptic face are considered docked. Only vesicles along the length of synapses were analyzed.

Table 1. *Synaptic structural characteristics measured from nerve terminals that were monitored physiologically*

Preparation	Order	RRP	Docked
Control 1			
SY1	−1	19	5
	0	19	3
	+1	17	6
SY2	−1	20	2
	0	22	4
	+1	20	3
Control 2			
SY1	−1	11	6
	0	13	3
	0	28	5
	+1	14	5
SY2	−1	15	4
	0	18	4
	+1	15	3
Control 3			
SY1	−1	26	4
	0	20	3
	+1	11	3
SY2	−1	19	2
	0	21	4
	+1	17	4
Control Overall Mean		18	3
TBOA 1			
SY1	−1	18	3
	0	12	3
	0	13	5
	+1	13	2
SY2	−1	16	3
	0	20	4
	+1	19	3
TBOA 2			
SY1	−1	12	4
	0	19	2
	+1	30	4
SY2	−1	15	4
	0	22	4
	+1	19	0
TBOA 3			
SY1	−1	23	5
	0	17	3
	+1	11	3
SY2	−1	16	3
	0	20	3
	0	22	3
	+1	11	4
TBOA Overall Mean		17	3

A total of 12 synapses (SY; 2 synapses from each preparation) were fully examined from 3 control and 3 DL-threo-β-benzyloxyaspartate (TBOA)-treated preparations. The sections with defined excitatory synapses that revealed an active zone (AZ) were used as a reference part. These are synapses that contain a dense body in the inner face on the presynaptic side. Two serial sections to either side of a section of interest with an AZ were viewed for analysis. If no other AZ was observed on the same synapse over the 4 or 5 serial sections, then 1 section on either side of the one containing the AZ was used for morphological measures of the vesicle pools. In some sections the dense body of the AZ was observed in 2 sections. The readily releasable (RRP) and reserve vesicle pools are defined in Fig. 6.

DISCUSSION

In this study, we have demonstrated that, within a presynaptic motor nerve terminal of the crayfish NMJ, the RP and the RRP of vesicles are distinguishable, by physiological and pharmacological means, in their recycling paths during electrical activity of the terminals. This was accomplished by the

use of TBOA, a presynaptic reuptake inhibitor of glutamate transporters, and use of the neuromodulator 5-HT to recruit the RP. This synaptic transmission was assessed by intracellular recordings and quantal analysis from recordings of discrete regions on the nerve terminal obtained by focal macropatch electrodes. During exposure to TBOA and continuous stimulation, the evoked single quantal events as well as the spontaneous single events were reduced in size over time. Upon recruiting the RP of vesicles by 5-HT during maintained high-frequency stimulation in the presence of TBOA, the occurrence in normal single events was increased on average along with the smaller size range observed during the TBOA treatment. The wide variation in size and area of the traces for single events after 5-HT induction was compared with the distributions before and during TBOA treatments. Thus some vesicles did not have reduced glutamate content (i.e., RP), whereas others did (i.e., ones rapidly recycling), which suggests that the RP was immune to the treatment of TBOA within the short exposure period but that the rapidly recycling vesicles could not package enough glutamate within their short recycling period.

In a recent study addressing the recruitment of synapses during electrical stimulation alone or in conjunction with 5-HT, it was postulated that, to maintain a plateau in the amplitude of the graded EPSPs in this preparation during continuous stimulation, a steady state of influx, buffering, and efflux of calcium ions is occurring (62). The efflux is probably via the Na^+/Ca^{2+} exchanger. A similar model for the actions of electrical stimulation and for 5-HT on vesicle pools was proposed, as shown in Fig. 7. However, in the study herein, we address the role of the glutamate transporter to further distinguish the rapid recycling vesicle path and the RP path that is enhanced by 5-HT. In the absence of 5-HT

and without electrical stimulation, few spontaneous releases occur, resulting in vesicles recycling via a slow process (*path 1* to *2*, Fig. 7*A*). In the presence of high-frequency electrical stimulation, as we used, a vesicle that was just recaptured from the presynaptic membrane may also recycle through an endosome intermediate to enter and reside in a RP of vesicles (*path 1* to *2*, Fig. 7*B*). As discussed earlier, the endosome-mediated path is somewhat of an outdated model, and supportive evidence in this system is lacking in these motor nerve terminals (62). Mounting evidence supports the notion that *path 3* (Fig. 7*B*) is utilized during repetitive rapid transmission (1, 21, 22). When TBOA is present that is blocking the glutamate transporter, then the readily recycling pools are not fully packaged with glutamate before being released (Fig. 7*C*). An additional path in the rapidly recycling path (*path 3*) induced for electrical activity could be supplemented by vesicles entering from the RP (*path 1*) by the recruitment induced by 5-HT through IP_3 (Fig. 7*D*). This could be mechanistically explained by promoting vesicles to be released from synapsins and enhance docking by phosphorylating key docking-related proteins by IP_3 (14, 40). 5-HT involves an IP_3 cascade in crayfish motor axons (23, 25), and various protein kinases are also known to be activated. The types of kinases that can be activated by 5-HT in mobilizing RP vesicles is a subject worthy of future investigations within this system. For example, in *Aplysia* PKC and PKA have been shown to be utilized during 5-HT-induced facilitation (6, 7, 37, 49). It was also shown in *Aplysia* that 5-HT-induced activation of ApCdc42 is dependent on the phosphatidylinositol 3-kinase kinase and PLC pathways. These then recruit the downstream effectors neuronal Wiskott-Aldrich syndrome protein and p21-Cdc42/Rac-activated kinase. These can regulate the reorganization of the presynaptic actin network (67). Recent studies have also revealed that transmembrane *Aplysia* cell adhesion molecule
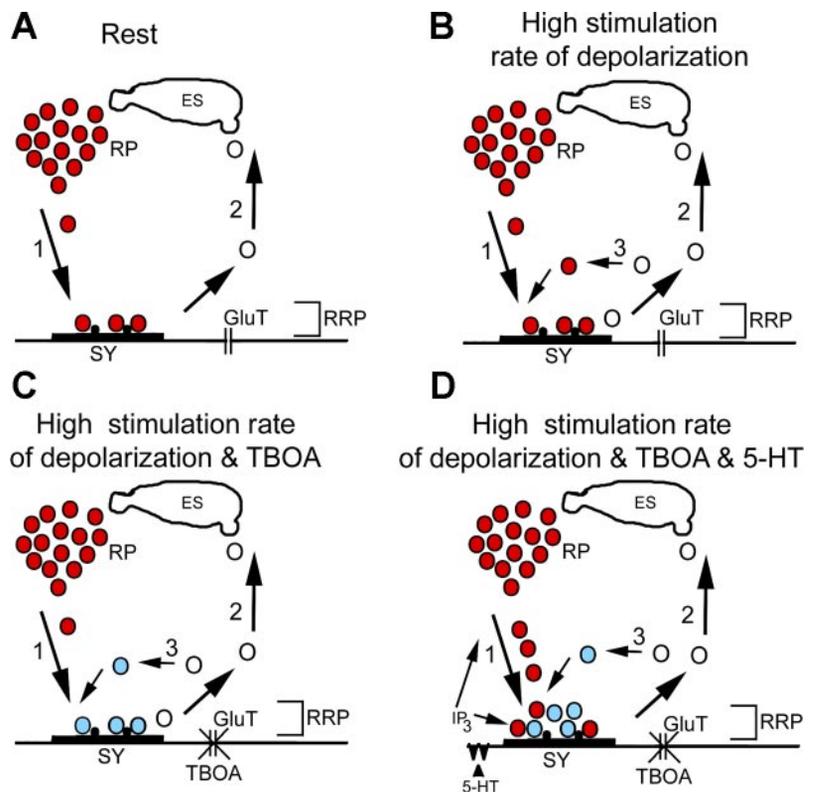

Fig. 7. Schematic representation of the vesicle pathways within the presynaptic motor nerve terminal. In the absence of electrical stimulation of the nerve terminal, few vesicles will spontaneously be released at slow rate; thus a slow recycling path (*path 1* to *2*) will be utilized (*A*). Upon electrical stimulation *path 3* will be recruited into action (*B*). Vesicles may be recruited from the RP (*path 1*) to the RRP 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 *path 3* that is relatively rapid in recharging the vesicles with transmitter before the vesicle recycles back to the RRP. Exposure to TBOA depletes the cytoplasmic glutamate for the rapid recycling vesicles to fill by blocking the glutamate transporter (GluT) (*C*). Blue vesicles represent the partially filled ones. However, after inducing synaptic depression with the presence of TBOA, the depressed synapses (SY) can be recruited by exposure of the terminals to 5-HT. During electrical stimulation the nerve terminal is primed by promoting *path 1*. The additional activation of the second-messenger inositol-3-polyphosphate (IP_3) can recruit vesicles from RP as well as enhance priming and docking of vesicles at the synapses within the RRP by phosphorylating synaptically relevant proteins. When combining electrical activity, incubation with TBOA and exposure to 5-HT *paths 1*, *2*, and *3* are actively producing a mixed response of partly and fully packaged vesicles (*D*).

is associated with structural changes during LTF. Data suggest that the downregulation of transmembrane-*Aplysia* cell adhesion molecule is required to promote both EPSP enhancement in preexisting synapses and varicosity formation in nonsynaptic regions (39). In postnatal rat hippocampal neuronal cultures, it was shown that CaM kinase controls vesicle mobilization at low stimulus frequency, whereas MAPK and calcineurin were active at low and high stimulation frequencies (8). Even though 5-HT has not been linked in these neurons to these kinases, the possibility could exist in other preparations such as the crayfish NMJ.

At 50-Hz stimulation, the vesicle population must continually recycle to maintain the degree of output we observed over time. With a limited number of vesicles present within the terminal, it is very likely that the rapid vesicle recycling path (Fig. 7*B*) is quite active. We have shown with serial electron microscopy of terminals stimulated and exposed to TBOA that vesicles are present in similar quantities in the docking state and slightly less in the RRP compared with control terminals. With the recycling time frame present during the 20-Hz continuous stimulation, vesicles probably have a sufficient time to transport some cytoplasmic glutamate into the vesicles via the glutamate transporters on vesicles for a while longer compared with the 50-Hz stimulation. However, both stimulation paradigms produce depression. Perhaps the 50-Hz stimulation recruits the RP vesicles, and when mobilized they deplete rapidly in the presence of TBOA, whereas during the 20-Hz stimulation a slower but continuous recruitment of the RP occurs, thus providing a longer period before the onset of depression. This is supported by the larger number of stimuli needed to obtain a 50% depression during 50-Hz stimulation. The results suggest that there is not enough cytoplasmic glutamate for the rapid recycling vesicle to fill when TBOA is applied to block the extracellular reuptake (Fig. 7*C*). However, with the application of 5-HT, in the presence of TBOA and rapid stimulation, the vesicles that are fully prepackaged with transmitter are recruited from the RP because their quantal responses are just as large as quantal events before exposure of TBOA (Figs. 6*C* and 7*D*). The evoked as well as spontaneous events observed during 5-HT application are of a mixed population in area of the voltage response, which is expected because partially filled vesicles and those filled before stimulation and exposure to TBOA are being recruited by 5-HT. This model of vesicle recruitment from a RP by actions of 5-HT supports previous findings on vesicular populations and kinetics at the crayfish NMJ (10, 26–28, 61, 62, 64). The paths may be targeted independently by cellular processes, such as those by 5-HT-triggered IP$_3$-induced phosphorylation and direct electrical depolarization for calcium-induced cellular responses (7). In depressed NMJs of crayfish, release has been shown to be promoted by 5-HT through the use of FM1-43 vesicle dye studies and electrophysiological studies, also suggesting that different pools and paths are present (70). The approach with FM1-43 loading of vesicles during long-term facilitation has revealed a nonuniform loading of synapses at the lobster NMJ, suggesting differential synaptic function (42).

There is variation in quantal responses in the absence of TBOA; however, the mean in the area of the single fEPSP is reduced with exposure to TBOA. The decrease in the mean area for single fEPSPs also supports the notion that TBOA is reducing the packaging within the RRP but not altering the RP

within the short time frame of exposure used in this study. As for the general reasons for variation in quantal events in nonpharmacological treated preparations, there are a number of factors that are due to presynaptic as well as postsynaptic properties (35). There are numerous studies that address the mechanisms for quantal variation and nonlinear addition of evoked synaptic potentials (19, 20, 35, 38, 50, 52, 57). The most plausible explanation for the increased variation over time and reduced postsynaptic response during exposure to TBOA reported in this study is that a reduced glutamate reuptake occurs within the presynaptic terminal for use by the rapidly recycling vesicles.

Mechanisms that are proposed to account for quantal fluctuation can be tested in these relatively "simple synapses" of the crayfish NMJ because one can monitor unitary postsynaptic events. It is known that desensitization is not responsible for quantal fluctuation, because very low probability of release is induced, resulting in evoked events interspersed among many failures, and that addition of 5-HT promotes even a greater number of events after TBOA-induced depression occurs. In addition, the peak amplitudes do not show a flattened peak, which would be indicative of receptor saturation. Resensitization is rapid in this preparation (32). These glutamatergic ligand-gated receptor are a quisqualate type with rapid sodium conductance (60). There does not appear to be a wide variation in size among clear core synaptic vesicles in crayfish motor nerve terminals, but one needs to consider stereological problems in assessing vesicle dimensions (3, 36, 43). Also, the size is not informative to the extent of the packaging content. By monitoring single-vesicle-induced fEPSPs from a spatially isolated varicosity by the focal recording technique, one at least knows that the variation is not due to electrotonic spread from distant sites on the muscle, as is the problem with intracellular monitoring of the postsynaptic cell.

One might argue that TBOA is blocking the glutamate receptors postsynaptically and resulting in reduced quantal sizes. Given that the 5-HT exposure occurs in the presence of TBOA at the same concentration as the previous TBOA exposure and the quantal sizes increase on average, it is not likely that TBOA is blocking the glutamate receptors. The potential mechanisms described in this report may well apply to other synapses within the CNS and NMJ of other preparations in which only inference can be made as to the structural and physiological responses because of the lack in the ability to know which nerve terminals are being used or where quantal events are occurring.

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