Neuroscience Research 75 (2013) 275–282

Contents lists available at SciVerse ScienceDirect

Neuroscience Research

journal homepage: www.elsevier.com/locate/neures

Physiological separation of vesicle pools in low- and high-output nerve terminals

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ARTICLE INFO

Article history:
Received 19 November 2012
Received in revised form 18 January 2013
Accepted 18 January 2013
Available online 8 February 2013

Keywords:
Synapse
Reserve pool
Readily releasable pool
Bafilomycin A1
Serotonin

ABSTRACT

Physiological differences in low- (tonic like) and high-output (phasic like) synapses match many of the expected anatomical features of these terminals. However, investigation in the recruitment of synaptic vesicles from a reserve pool (RP) to a readily releasable pool (RRP) of synaptic vesicles within these types of nerve terminals has not been fully addressed. This study highlights physiological differences and differential modulation of the vesicles in a RP for maintaining synaptic output during evoked depression of the RRP. With the use of bafilomycin A1, a vacuolar ATPase blocker, recycling vesicles are blocked in refilling with transmitter. The tonic terminal is fatigue resistant due to a large RRP, whereas the phasic depresses rapidly upon continuous stimulation. These differences in rates of depression appear to be in the size and degree of utilization of the RRP of vesicles. The working model is that upon depression of the tonic terminal, serotonin (5-HT) has a large RP to act on in order to recruit vesicles to the RRP; whereas, the phasic terminal, 5-HT can recruit RP vesicles to the RRP prior to synaptic depression but not after depression. The vesicle pools are physiologically differentiated between phasic and tonic output terminals.

1. Introduction

Chemical synaptic communication at neuromuscular junctions (NMJs) generally occurs by synaptic vesicles, packaged with a transmitter, fusing with the presynaptic plasma membrane to release the transmitter into a synaptic cleft for the postsynaptic receptors to receive and respond accordingly. How vesicles fuse with the presynaptic membrane is an active area of investigation on a comparative scale as it is assumed there are a variety of ways this process may occur from kiss-and-run to full exocytosis of vesicles (Rosenmund and Stevens, 1996; Aravanis et al., 2003; Rizzoli et al., 2003; Sudhof, 2004; Rizzoli and Betz, 2005; Fredj and Burrone, 2009). The recycling and repacking of the vesicles are also of substantial interest particularly given that there appear to be different pools of vesicles for various functions within presynaptic nerve terminals and unique processes as well as serving similar roles among various animal species (Atwood and Cooper, 1996a,b; Sudhof, 2004; Rizzoli and Betz, 2005; Denker et al., 2011a,b).

Recently it was suggested that synaptic vesicles may not just serve as a means of packaging transmitter but also providing essential proteins as a buffer source for use when needed (Denker et al., 2011a,b). In addition, a large influx of Ca^{2+} can depress synaptic transmission (Katz and Miledi, 1969; Heuser et al., 1971; Ohta and Kuba, 1980). Also, acidification within the nerve terminals depresses vesicles endocytosis (Lindgren et al., 1997). These processes may serve as potential negative feedback mechanisms. Such observations raise questions about the functional needs of reserve pool (RP) and readily releasable pool (RRP) of vesicles and their roles. To determine the functional differences, in terms of vesicle recycling and recruitment in the RP and the RRP between phasic (high-output) and tonic (low-output) motor nerve terminals, the packaging of neurotransmitter was pharmacologically blocked in recycling vesicles and the action of the well-established modulator 5-HT that enhances synaptic efficacy at crustacean NMJ was investigated in this study.

Crustaceans have played a major contribution for investigating structure and function relationships in synaptic transmission that have aided in understanding synapses in general for all animals (Atwood, 1976, 1982a,b; Jahromi and Atwood, 1974; Atwood and Cooper, 1995, 1996a,b; Cooper et al., 1995a,b, 1996a,b; Walrond et al., 1993; Johnstone et al., 2008, 2011; Denker et al., 2011a). An advantage of many NMJs in the crayfish is that they are graded in transmission as many crustacean muscles do not produce action potentials (Atwood, 1967, 1976). This allows one to follow a rise or decrease in synaptic efficacy over time as well as influences in modulation of the synaptic function with quantal analysis (Dudel and Kuffler, 1961; Cooper et al., 1995b, 2003; Djokaj et al., 2001).

Selective axonal stimulation first studied in crayfish leg extensor yielded two different types of muscle contraction: one a fast twitch-like, the other one with a slower response but depression

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http://dx.doi.org/10.1016/j.neures.2013.01.007

resistant (Lucas, 1907, 1917; Blaschko et al., 1931; Wiersma, 1933; Van Harreveld and Wiersma, 1936). Later the same contractile pattern and physiology was also identified in crayfish abdomen extensor and flexor musculature (Kennedy and Takeda, 1965a,b; Parnas and Atwood, 1966). Physiological and histological studies suggested that this difference is not only due to the types of motor neurons (phasic/high-output and tonic/low-output), but also the structure of postsynaptic targets (fast and slow muscles) (Baierlein et al., 2011).

The morphological and physiological differences of tonic and phasic nerve terminals have been studied in the crayfish model (Atwood, 1963, 2008) and particularly well in the leg extensor for comparisons (King et al., 1996; Bradacs et al., 1997; Msghina et al., 1998). In this leg preparation both types of nerve terminals innervate the same postsynaptic muscle fiber and give rise to stark differences in postsynaptic responses. An advantage of this preparation is that the target is the same fiber so comparisons in neuronal communication can be probed. In this preparation the small varicosities have a high mean quantal content and the synapses depress relatively quickly within the phasic terminals. However, the larger varicosities of the tonic nerve terminal have a low mean quantal content and show marked facilitation with resistance to depression (Wu and Cooper, 2010). Given that the nerve terminals in this preparation do innervate the same fiber there might be feedback from the fiber being stimulated by one neuron to the other non-stimulated neuron or an alteration in receptor sensitivity. So, for our current study we chose to use distinctly separate muscles in the crayfish abdomen that fit phasic and tonic profiles to avoid interaction of the muscle activity.

The abdominal extensor musculature has been well described in *Procambarus clarkii* and other closely related species (Pilgrim and Wiersma, 1963; Parnas and Atwood, 1966; Sohn et al., 2000). All three deep extensor muscles (medial – DEM, lateral 1 – DEL1, lateral 2 – DEL2) are composed of phasic type of muscle fibers (fast-contracting fibers) with short sarcomeres less than 5 μm (Parnas and Atwood, 1966). DEM is a twisted helix muscle. Most of the DEL1 and DEL2 muscle fibers are straight. Kennedy and Takeda (1965a,b) had identified the superficial extensors medial – SEM and lateral – SEL and their innervation profiles. Both the superficial muscles contain tonic muscle fibers (slow-contracting fibers) with longer sarcomeres ranging 9–11 μm (Parnas and Atwood, 1966) (see Fig. 1 for details).

A recent study of the tonic NMJs on the crayfish opener muscle in the walking leg did demonstrate that blocking the vesicular glutamate transporter (VGlut) with bafilomycin A1 depressed synaptic transmission faster than without its presence and that the rate of synaptic depression is stimulation dependant (Wu and Cooper, 2012a). Also, a working dose of 4 μM bafilomycin A1 was demonstrated to work well without functional damage to crayfish NMJs. The opener NMJ has a substantial RP which can be recruited by 5-HT application after the induction of synaptic depression, suggesting a functional separation in vesicles of the RRP and RP. In this study, the high-output terminals on the DEL1 muscle fibers were compared to the low-output terminals in SEL muscle fibers within the same segment of abdominal musculature. Based on previous reports of the morphological and physiological characteristics of the tonic and phasic terminals, a slower depression of the tonic terminals with or without bafilomycin A1 treatment was predicted. Also, we expected that treatments with bafilomycin A1 would depress both the terminals faster than without the drug; however, we also predicted phasic terminals would show a much greater rate of depression than tonic terminals in the presence of bafilomycin A1. Given the recent results of tonic terminals on the leg opener NMJs responding to 5-HT after the induction of depression, we expected to be able to recruit vesicles from RP in the tonic terminal to a greater extent than the phasic terminals in the abdominal preparations. Working models in the functional difference of the vesicle pools in low- and high-output terminals are presented.

2. Materials and methods

General. All the experiments were carried out in the midsize crayfish (*P. clarkii*) measuring 6–10 cm in body length. They were individually housed in plastic containers with oxygenized water. The temperature of the animal room was controlled at 20–21 °C. The animals were fed with dry fish food and water changed on weekly basis.

Dissection. The dissection is described in Sohn et al. (2000). All the connective tissues and residual flexor muscles were removed to better visualize DEL1, DEL2, DEM and SEL. Only DEL1 and SEL in segment A2, A3, A4 were used (Fig. 1). Dissected preparations were maintained in crayfish saline, a modified Van Harreveld's solution

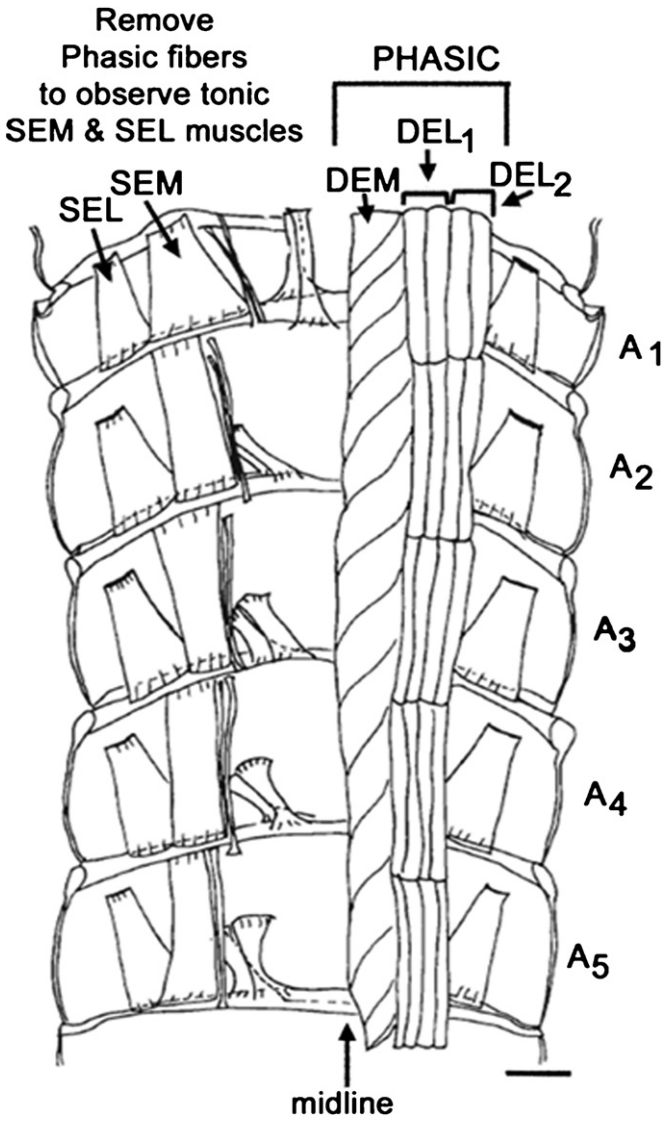

Fig. 1. Schematic presentation of crayfish abdomen extensor musculature. Each side of each segment contains deep extensor medial muscle (DEM), deep extensor lateral muscle 1 (DEL1), deep extensor lateral muscle 2 (DEL2), superficial extensor lateral muscle (SEL), superficial extensor medial muscle (SEM). On the left side of the figure, dorsal SEL and SEM are viewed by removing DEM, DEL1, and DEL2. DEM, DEL1 and DEL2 are phasic muscles whereas SEM and SEL are tonic in nature. A1–A5 refers to abdomen segments. Scale bar = 2.35 mm.
The figure is modified from Sohn et al. (2000).

(in mM: 205 NaCl; 5.3 KCl; 13.5 $CaCl_2 \cdot 2H_2O$; 2.45 $MgCl_2 \cdot 6H_2O$; 5 HEPES adjusted to pH7.4).

Pharmacology. All chemicals were obtained from Sigma–Aldrich Chemical (St. Louis, MO). Bafilomycin A1 (B1793) solution was made by dissolving 10 μg powder in 20 μl DMSO (99.9%), then adding crayfish saline to the desired concentrations based on experimental conditions. The solution was stored at −20 °C and used within 3 months. 4 μM bafilomycin A1, 2.5 h incubation was used based on the dose–response relation found in leg opener preparation of crayfish (Wu and Cooper, 2012a). Afterwards, they were stimulated by recruiting the motor neurons with the use of a stimulating suction electrode. During the 2.5 h incubation time, the bafilomycin A1 solution was circulated in the dish every 30 min. 1 μM 5-HT was made in crayfish saline from frozen stock of 1 mM 5-HT. It was added to the preparation to exchange with bafilomycin A1 solution after synaptic depression occurred.

Physiology. Only segments A2, A3 and A4 were used. The nerve bundles were stimulated in one segment above for phasic muscle (DEL1) recording or in the same segment for tonic muscle (SEL) recording by a suction electrode connected to a Grass stimulator. The effects of bafilomycin A1 on the rate of synaptic depression were compared between the phasic and tonic nerve terminals using continuous stimulation at 5 Hz. In the 5-HT sensitivity assay prior to depression, the nerve was stimulated at 0.5 Hz while SEL or DEL1 in the same segment was recorded immediately for 1 min or less in saline then during the application of 5-HT. EPSPs were recorded following standard procedures (Cooper et al., 1995a; Crider and Cooper, 2000; Baierlein et al., 2011).

Analysis. The amplitude of the EPSP every 5 min or 1 min in tonic preparations and every 1 s in phasic preparations was measured. Scatter plots of the EPSP amplitudes were graphed. The time to reach a 50% reduction in the peak EPSP amplitude was used as an index to calculate the half depression time. The averages of time to 50% decline were compared between phasic and tonic nerve terminals. For the 5-HT effect before synaptic depression, the percentage EPSP amplitude change before and after adding 5-HT was calculated.

3. Results

3.1. Tonic nerve terminals

When the segmental nerve that innervates SEL muscle fibers was stimulated at 5 Hz in crayfish saline, the average time to 50% depression is 384.4 min ($n = 5$, ±13.7 min SEM, Fig. 2A). Five out of five preparations showed the restoration of synaptic transmission after depression with 5-HT (1 μM) application ($p < 0.05$ non-parametric rank sum test). Preparations responded in varying degrees to the 5-HT application; however, the amplitude of EPSPs was markedly increased in comparison with the depressed state (see Fig. 2A example).

3.2. Tonic nerve terminals in 4 μM bafilomycin A1

The average time to 50% depression is 58.9 min ($n = 5$, ±11.8 min SEM, Fig. 2B) using continuous stimulation at 5 Hz in the presence of 4 μM bafilomycin A1. As for the preparations bathed in crayfish saline that depressed, all the preparations exposed to 4 μM bafilomycin A1 showed an enhanced effect to 5-HT as indicated by an increase in the EPSP amplitude ($p < 0.05$ non-parametric rank sum test).

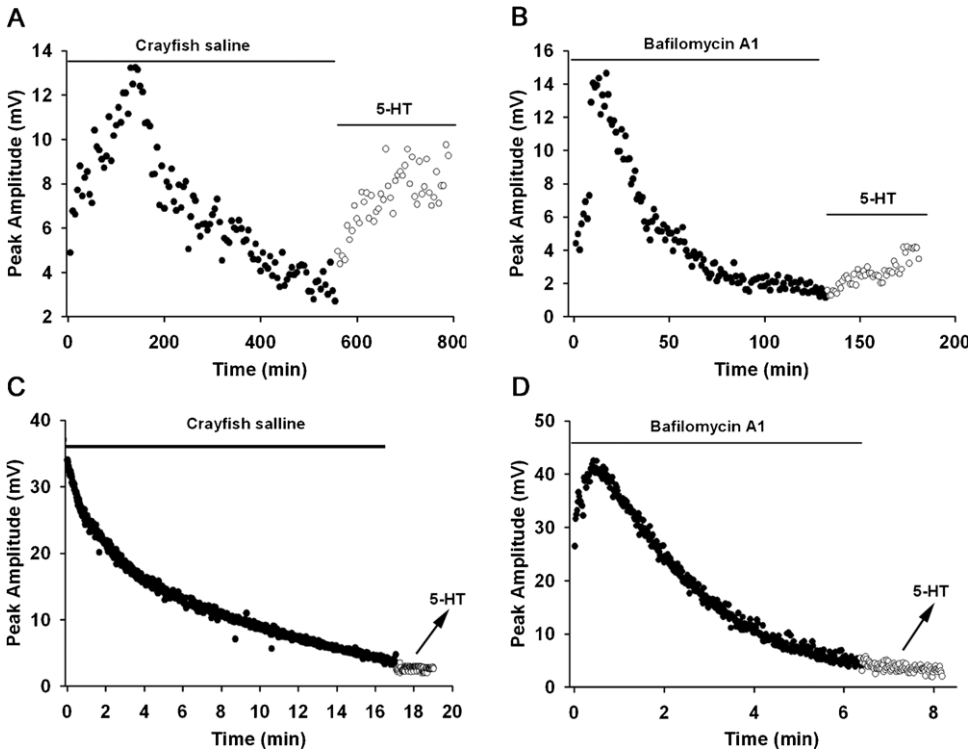

Fig. 2. The synaptic transmission of abdominal tonic and phasic motor nerve terminals in crayfish saline and a 4 μM bafilomycin A1 containing saline. (A) Representative scatter plot of EPSP amplitude (mV) over time serve as a saline control for tonic terminals. After substantial depression was observed, 5-HT containing saline was exchanged for the saline bath. The 5-HT exposure resulted in the amplitudes of the EPSPs increasing. (B) The synaptic activity of abdomen tonic nerve terminals in 4 μM bafilomycin A1. The EPSPs depress sooner than controls and after substantial depression exposure to 5-HT also produced an increase in the EPSP amplitude. (C) The synaptic activity of abdomen phasic nerve terminals in crayfish saline depress rapidly as indicated by the decline in EPSP amplitude. After substantial depression was observed exposure to 5-HT did not produce an increase in the EPSP amplitude. (D) The EPSP amplitudes produced by the abdomen phasic nerve terminals decreased faster than saline controls when incubated with 4 μM bafilomycin A1. As with the saline control preparations, the EPSPs did not restore with subsequent exposure to 5-HT after depression occurred.

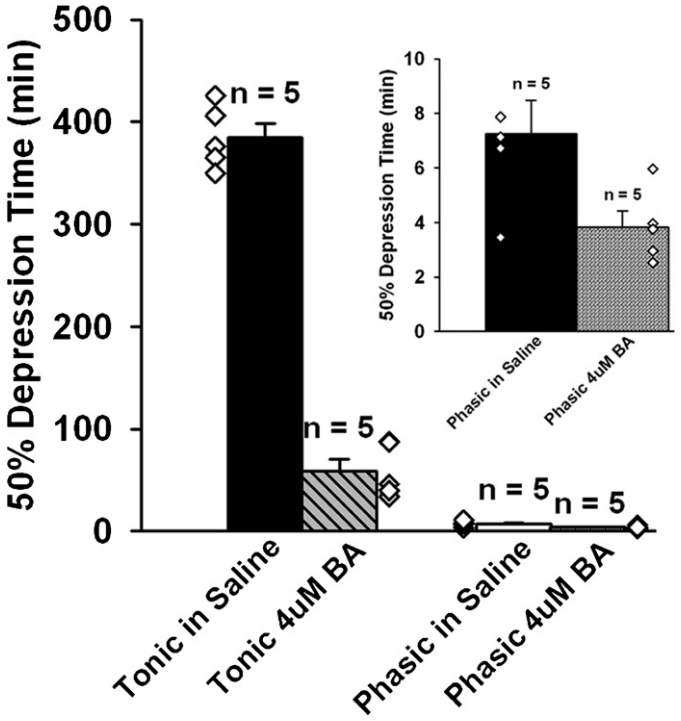

Fig. 3. Bar graphs depicting the average time to 50% depression time in the EPSP amplitude for 5 preparations. The average depression time is 384.4 min (±13.7 min SEM, $n = 5$) for tonic NMJs exposed to saline only. When the tonic NMJs are incubated in bafilomycin A1 the depression time is 58.9 min (±11.8 min SEM, $n = 5$). The phasic terminals treated as a saline control depressed on average 7.2 min (±1.2 min SEM, $n = 5$). When the phasic terminals were treated with bafilomycin A1 they depressed on average 3.8 min (±0.6 min SEM, $n = 5$). The diamonds are the values for each individual preparation. The enlarged inset in the bar chart for the phasic terminals illustrates the small amount of time. The main graph is presented with the same time scale for the phasic and tonic NMJs for ease of comparison.

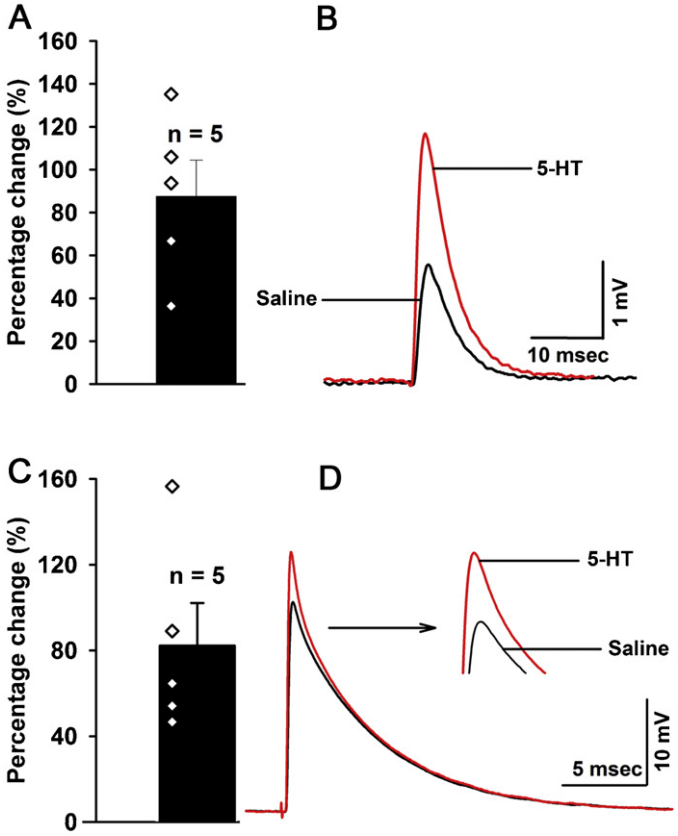

Fig. 4. The tonic low-output and phasic high-output NMJs response to 5-HT prior to evoked depression. Five out of five preparations show an enhancement in the EPSP amplitude to application of 5-HT for both terminal types (A-tonic, C-phasic). The increase in peak amplitude of the EPSP, due to 5-HT exposure (red traces), is obvious prior to synaptic depression in the representative traces shown (B-tonic, D-phasic).

3.3. Phasic nerve terminals in crayfish saline

The segmental nerve was stimulated continuously at 5 Hz while the very anterior part of DEL1 muscle in the next posterior segment was recorded. By recording in the next segment helps to insure recording the activity from a single phasic motor neuron and not a group of phasic neurons that innervate the same fibers within a segment (see Cooper et al., 1998). The average time to 50% depression time is 7.2 min ($n = 5$, ±1.2 min SEM, Fig. 2C). After depression, not one of the five preparations showed the rejuvenation of synaptic transmission with exposure to 5-HT, as illustrated in the representative scatter plot (Fig. 2C).

3.4. Phasic nerve terminals in 4 μM bafilomycin A1

In the presence of 4 μM bafilomycin A1, the phasic nerve terminals depressed much faster. The average time to 50% depression time is 3.8 min ($n = 5$, ±0.6 min SEM, Fig. 2D). Like in saline alone for these phasic terminals, exposure to 5-HT did not enhance the synaptic transmission in any of the 5 preparations.

3.5. Overall results

In both tonic and phasic preparations, 4 μM bafilomycin A1 can depress the terminals markedly faster (Fig. 3). After synaptic depression, exposure to 5-HT enhanced synaptic transmission in the tonic NMJs. However, the phasic NMJs which were either exposed or not to bafilomycin A1 did not show an increase in EPSP amplitude after 5-HT application.

Both tonic and phasic NMJs respond to 5-HT exposure prior to depression (Fig. 4) which indicates that both motor nerve terminals have receptors for 5-HT and have the potential to increase synaptic efficacy due to 5-HT exposure. The focus point for this study is that the phasic terminals do not show any enhancement after depression but they have the ability to respond prior to depression. This is a similar finding in 5-HT effects for other phasic and tonic NMJs for crayfish (Cooper et al., 2003; Johnstone et al., 2008). In five out of five preparations both the tonic and phasic NMJs produced an enhanced response to 5-HT application prior to the induction of depression. These five preparations were only used for the 5-HT sensitivity assay prior to depression. These preparations had minimal stimulation before application of 5-HT with a 0.5 Hz rate of stimulation for a minute or less in saline and then exposed to a saline containing 5-HT. An average percent change in the EPSP amplitude in response to application of 5-HT for these tonic NMJs is 87.55% (±16.88 SEM, $n = 5$) and phasic NMJs show an increase of 82.36% (±19.91 SEM, $n = 5$).

4. Discussion

In this study we have demonstrated that bafilomycin A1 produces synaptic depression sooner than without exposure to the drug at the NMJs. Synaptic depression is rapid for the phasic terminals as compared to the tonic terminals; however, after depression of the tonic terminals application of 5-HT rejuvenates the EPSP responses as the amplitude increases. After the high-output phasic terminals fully depress there are apparently no RP vesicles to recruit to the RRP, because no further enhancement by 5-HT was

observed. Prior to depression of the phasic terminals, 5-HT does enhance the synaptic responses. Thus, the evidence is suggestive that the high-output terminals utilize what might be considered a RP during evoked stimulation. In this case, the RP might be considered as a 5-HT sensitive pool as long as synaptic depression has not already occurred. The high-output terminals make use of all available vesicles for maintaining evoked release during the late stages of depression. However, the tonic terminals remain sensitive to enhancing transmission by 5-HT even after substantial depression. A model to explain this phenomenon is that the RP are present in tonic terminal and still available for modulation after depression; however, the RP is small in the phasic terminal and the RP is being used during evoked stimulation so there are few, if any, available for modulation after synaptic depression occurs (Wu and Cooper, 2012b)

Bafilomycin A1 belongs to a family of antibiotics. The "bafilomycins" and "concanamycins" were identified as specific vacuolar ATPase inhibitors (Bowman et al., 1988) and have proved to be useful pharmacological tools to investigate the function of compartmental acidification, since the hydrogen ion pump is blocked by bafilomycin A1, which prevents repackaging of the synaptic vesicle with transmitter after exocytosis–endocytosis

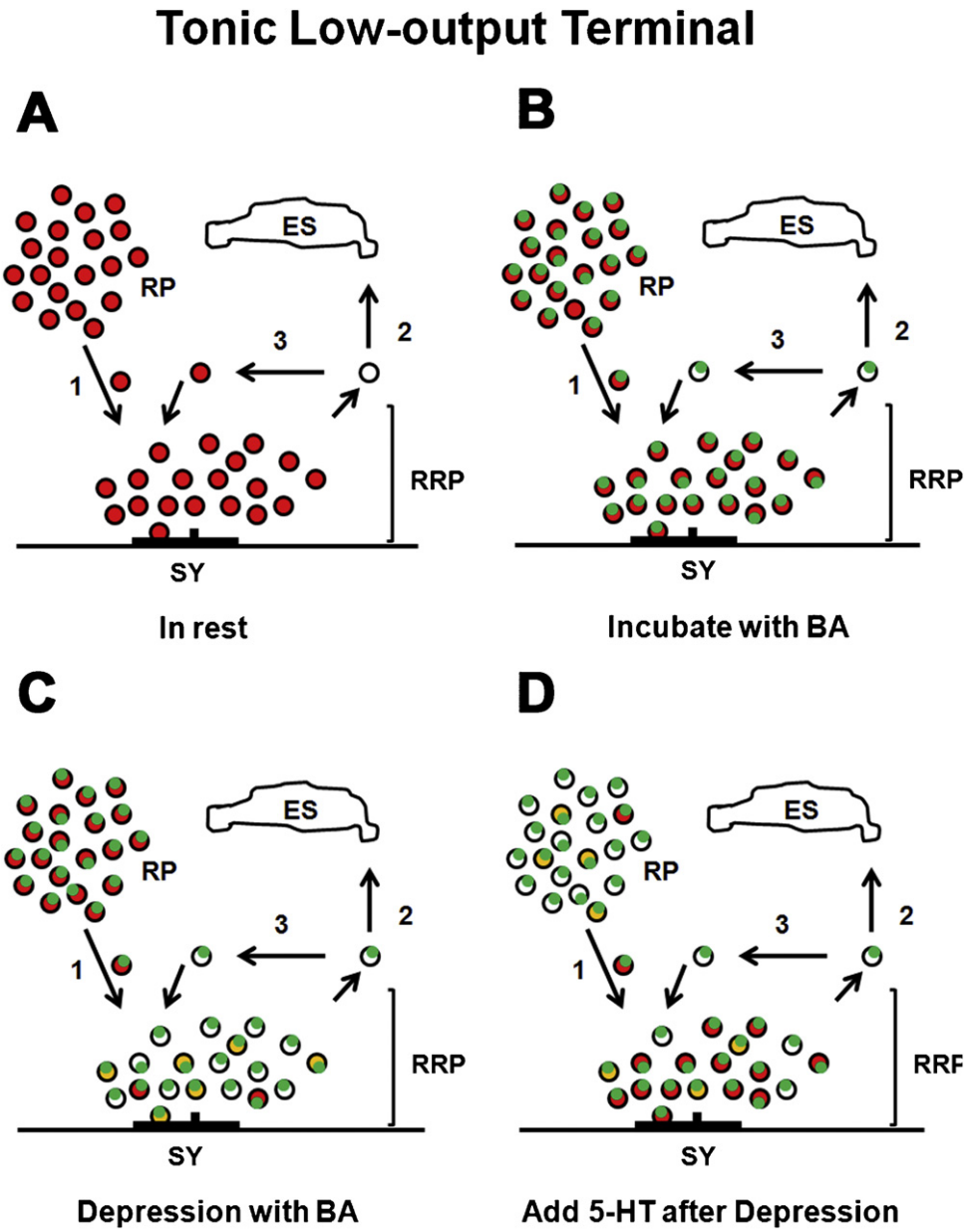

Fig. 5. Model of vesicle recycling between RP and RRP and the effect of bafilomycin A1 and 5-HT on transmitter release with the associated vesicle dynamics in low-output NMJs. (A) Synaptic vesicles are separated into RRP and RP over a synapse and with electrical stimulation vesicles in RP can slowly join in to the RRP (1), and then recycle back to RP either through or bypass endosome (2 or 3). (B) In an active synapse, in addition to the slow recycling loop, vesicles in RRP recycle within the RRP which is a quick recycling loop (3). Recycling vesicles are refilled with glutamate (red vesicles are filled with glutamate) but incubating with bafilomycin A1 (green dots represent bafilomycin A1) the recycling vesicles cannot refill (clear vesicles). (C) With repetitive stimulation, the vesicles in the RRP are depleted of glutamate and with bafilomycin A1 the packaging is depressed at a considerably faster rate. (D) However, application of 5-HT recruits vesicles from the RP to the RRP. In the presences of bafilomycin A1 the RRP and vesicles recycling to the RP will become depleted of transmitter. The effect of 5-HT on increasing EPSP amplitude may also be accounted for by an increase in docking of RRP. These low-output synapses have a larger RP to recruit from than the high-output synapses. RP, reserve pool; RRP, readily releasable pool; SY, synapse; BA, bafilomycin A1; ES, endosome.

cycle (Cavelier and Attwell, 2007; Kidokoro et al., 2004). The recovery of the tonic nerve terminals by 5-HT after they are treated with bafilomycin A1 and subsequent depression is present implies that mitochondria ATP production is not impaired by bafilomycin A1 within the time frame of these experiments. This is implied since vesicle docking and recycling is an ATP dependent process (Tolar and Pallanck, 1998). Longer exposure time may lead to effects not investigated in this study. We have shown previously that 8 μM bafilomycin A1 appears to have some unwanted effects in a crayfish NMJ as the nerve would fail to conduct electrical signals; although, motor neurons function in *Drosophila* larvae exposed to 16 μM (Wu and Cooper, 2012a). Generally, bafilomycin A1 appears to work as suggested for crayfish NMJs (Wu and Cooper, 2012a,b) since the results are fitting in a dose dependent manner and for incubation times as well as parallel actions on synaptic responses observed for *Drosophila* NMJs (Kuromi and Kidokoro, 2000; Denker et al., 2011a) and mammalian brain slices (Cavelier and Attwell, 2007). So this compound can likely be utilized in other crustacean models for similar experimental manipulations.

The action of 5-HT at NMJs in a variety of invertebrates reveals a range of responses from excitation to inhibition depending on the species (Wu and Cooper, 2012b). However, at crayfish NMJs 5-HT has always been demonstrated to enhance synaptic transmission (Florey and Florey, 1954; Dudel, 1965; Fischer and Florey, 1983), for the inhibitory (GABA) as well as excitatory (glutamate) motor neurons (Johnstone et al., 2008; Vyshedskiy et al., 1998; Wang and Zucker, 1998). The tonic like walking leg opener muscle in crayfish responds to 5-HT with an increase in the EPSP amplitudes of

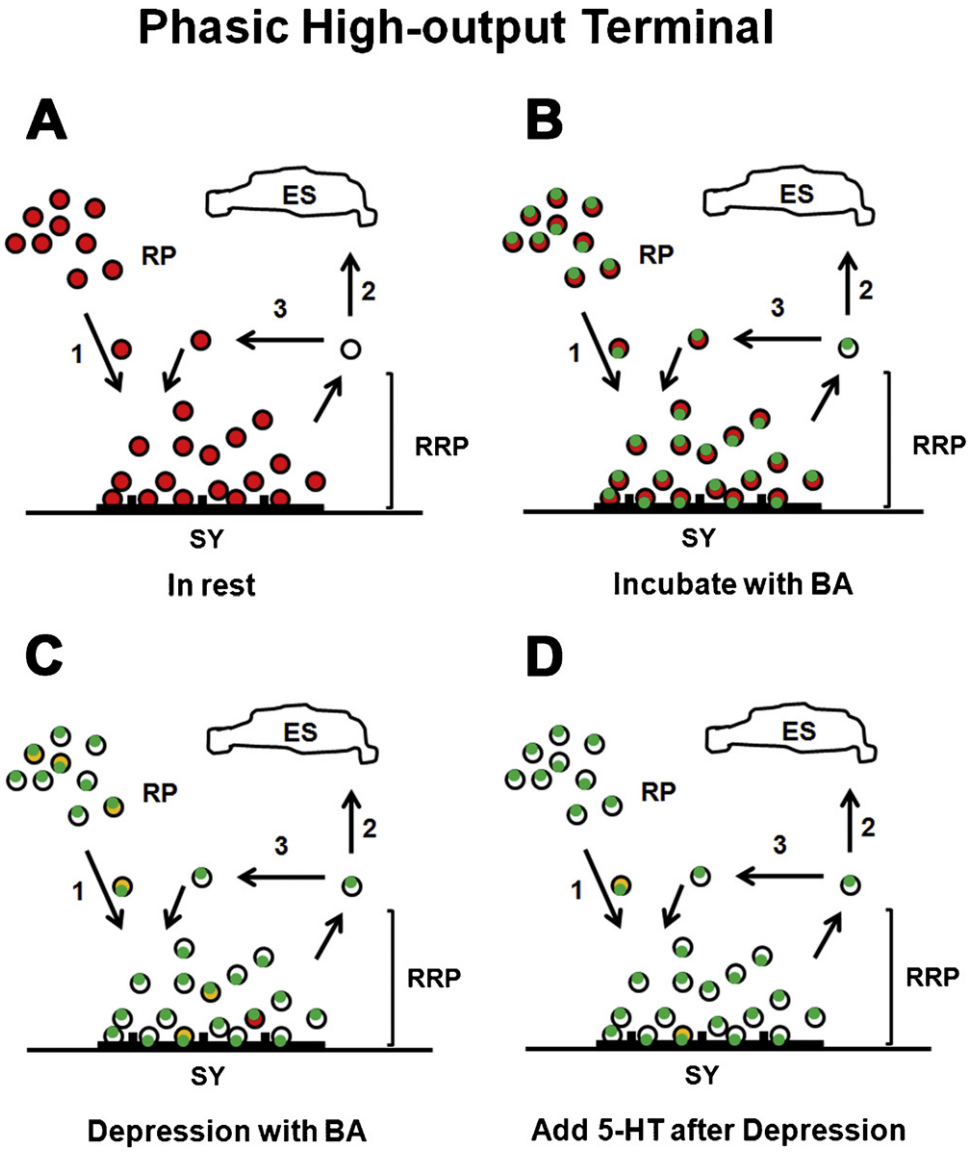

Fig. 6. Model of vesicle recycling between RP and RRP and the effect of bafilomycin A1 and 5-HT on transmitter release with the associated vesicle dynamics in high-output NMJs. (A) Synaptic vesicles are separated into RRP and RP over a synapse and with electrical stimulation vesicles in RP can slowly join in to the RRP (1), and then recycle back to RP either through or bypass endosome (2 or 3). High-output terminals differ in the number of active releasing sites. (B) In an active synapse, in addition to the slow recycling loop, vesicles in RRP recycle quickly within the RRP by a quick recycling loop (3). Recycling vesicles are refilled with glutamate (red vesicles are filled with glutamate) but incubating with bafilomycin A1 (green dots represent bafilomycin A1) the recycling vesicles cannot refill (clear vesicles). Since the high-output synapses have more vesicles in the RRP a larger postsynaptic effect is observed for the initial stimulations of the terminal. The effect of 5-HT in increasing the EPSP amplitude on non-depressed may also be accounted for by an increase in docking of RRP. (C) With repetitive stimulation the vesicles in the RRP are depleted of glutamate and with bafilomycin A1 the packaging is depressed at a considerably faster rate. Since more vesicles are recycling, the RRP pool is affected at a greater rate than for low-output synapses. (D) However, application of 5-HT recruits vesicles from the RP to the RRP. After synaptic depression occurs the vesicles in RP as well as the RRP are depleted of glutamate. Hence, 5-HT has little effect in recruiting any packaged vesicles from the RP. RP, reserve pool; RRP, readily releasable pool; SY, synapse; ES, endosome.

the excitatory nerve. This effect is mostly due to an increase in the mean quantal content related to more synaptic vesicles docking and fusing (Cooper et al., 2001; Djokaj et al., 2001; Sparks and Cooper, 2004; Logsdon et al., 2006). Likewise, in the walking leg of crayfish, the extensor muscle is innervated by phasic and tonic terminals and both motor nerve terminals increase in synaptic efficacy with 5-HT application (Cooper et al., 2003). However the tonic terminal shows a higher sensitivity to 5-HT as compared to the phasic even when the basal synaptic output is decreased by lowered extracellular Ca^{2+} (Cooper et al., 2003). Even though the phasic terminals do not reach their maximal output in vesicle fusion with lower evoked release, with a lowered bathing Ca^{2+}, the EPSP responses do not appear to reach a maximal level in synaptic output with 100 nM 5-HT. In addition, the percent increase in synaptic output was similar for normal bathing saline (13.5 mM Ca^{2+}) as for the saline containing a reduced Ca^{2+} (6.75 mM), suggesting that a similar degree of second messenger systems is utilized in the two conditions.

Recently it was demonstrated that the 5-HT mediated responses at crayfish NMJs are in part mediated by PLC. Blocking PLC activation by pre-treatment of U73122 (50 μM) solution drastically reduced the enhancement of the EPSP amplitude observed with 5-HT application (Wu and Cooper, 2012b). Investigation of PLC inhibition and 5-HT treatment for phasic terminals has not been approached yet; however, we expect a dampened response with such inhibition. The phasic terminals in the leg extensor do show an enhancement in the EPSP amplitude prior to synaptic depression by application of 5-HT (Johnstone et al., 2008), just as for the abdominal extensor muscle used in the study herein. Likewise, little enhancement occurs by 5-HT application after synaptic depression is induced in either of these phasic motor terminal preparations. A descriptive model helps to visualize what appears to be the differences in recruitment of vesicles from a dynamic RP, or for argument sake, vesicles that are sensitive to being recruited by modulation of downstream effects induced by 5-HT for tonic and phasic like motor nerve terminals. Figs. 5 and 6 highlight a physiological model of RP and RRP vesicle utilization with evoked stimulation based on the observations to date in tonic low-output and phasic high-output NMJs of the crayfish model. Due to the large enhancement in the EPSP amplitude induced by 5-HT prior or after synaptic depression occurs, the prediction is that there is a larger RP of vesicles for the tonic terminals. This is also supported by the fact that bafilomycin A1 inhibits the recycling pool from repackaging with neurotransmitter and a RP can still be recruited by 5-HT. However, for the phasic terminals if synaptic depression is induced by evoked stimulation with or without bafilomycin A1 treatments there is little if any RP to be modulated by downstream actions of exposure to 5-HT. Considering, that high-output terminals in multiple types of crayfish preparations are slightly enhanced, as compared to tonic terminals, in synaptic efficacy by 5-HT prior to evoked synaptic depression may indicate that a small RP of vesicles exists in phasic terminals but are rapidly utilized during evoked activity of the terminal. Given that treatment with bafilomycin A1 produces rapid depression in high-output terminals is indicative that a large percentage of vesicles are utilized for the initial stimulation of the nerve terminal.

As to why such differences in sensitivity to 5-HT occurs for evoking an enhancement of the EPSP amplitudes between the tonic and phasic terminals further investigation into 5-HT receptor density, subtypes, and second messenger recruitment as well as potential intracellular Ca^{2+} release from the endoplasmic reticulum needs to be addressed. Different 5-HT receptor subtypes use varying cellular cascades is possible (Hensler, 2002) and it is known that there are differences in the amount of the calcium binding protein frequenin (Jeromin et al., 1999) in tonic and phasic terminals of crayfish as well as differences in the amount of Ca^{2+} influx with evoked stimulation (Msghina et al., 1999). The varying degree of Ca^{2+} entry with stimulation may have a role in the recruitment of RP into the RRP for the phasic terminals since the Ca^{2+} influx is greater (Msghina et al., 1999). For potential roles of second messenger systems induced by 5-HT or Ca^{2+} in crayfish motor nerve terminals see earlier reports (He et al., 1999; Dropic et al., 2005; Tabor and Cooper, 2002; Wu and Cooper, 2012a,b). The effect of 5-HT increasing EPSP amplitude on non-depressed and even depressed tonic terminals may also be accounted for by an increase in docking of RRP. In this case, the probability of vesicular fusion could account in part for a larger EPSP (Southard et al., 2000; Strawn et al., 2000). This can potentially occur by various mechanisms as outlined in a review on 5-HT effects on invertebrate NMJs (Wu and Cooper, 2012a).

As for potential mechanisms in evoked stimulation inducing recruitment of vesicles, in the absence of exogenous neuromodulation, see reports by Akbergenova and Bykhovskaia, 2009, Aravanis et al. (2003), Denker et al. (2011a,b), Desai-Shah and Cooper (2009, 2010), Fiumara et al. (2004), Kuromi and Kidokoro (2000), Sudhof (2004) and Yamashita (2012). The usage of the RRP and RP of these tonic and phasic NMJs in the crayfish abdomen are fitting to their physiological profiles for the musculature involved in rapid tail flips or slow movements in postural control (Cooper et al., 1998; Mykles et al., 2002).

Acknowledgments

We thank Mr. Zana R. Majeed for comments on the manuscript. This project was supported in part by the Department of Biology, University of Kentucky and personal funds by RLC.

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