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THE REGULATION AND PACKAGING OF SYNAPTIC VESICLES AS RELATED TO RECRUITMENT WITHIN GLUTAMATERGIC SYNAPSES

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Abstract—The reserve pool (RP) and readily releasable pool (RRP) of synaptic vesicles within presynaptic nerve terminals, at crayfish and larval *Drosophila* neuromuscular junctions (NMJs), were examined for physiological differentiation into distinctly separate functional groups. These NMJs are glutamatergic and produce graded excitatory postsynaptic potentials (EPSPs). The packaging of glutamate was perturbed by blocking the vesicular glutamate transporter (VGlut) with bafilomycin A1. Various frequencies of motor nerve stimulation, exposure time, and concentration of bafilomycin A1 were examined. The low-output tonic opener NMJs in crayfish exposed to 4 μM bafilomycin A1 and 20-Hz continuous stimulation decreased the EPSP amplitude to 50% in ~30 min with controls lasting 3 h. After activity and bafilomycin A1-induced synaptic depression, the EPSPs were rapidly revitalized by serotonin (5-HT, 1 μM) in the crayfish preparations. The 5-HT action can be blocked with a PLC inhibitor. We postulate 5-HT recruits unused vesicles from the RP. The perception is the RRP is selectively activated during rapid electrical stimulation (20 Hz) sparing the RP. When stimulation frequency is high (40 Hz) the RP is recruited to the RRP and dampens subsequent recruitment with 5-HT. The higher output synapses of the larval *Drosophila* NMJ when stimulated at 1 Hz or 5 Hz and exposed to 4 μM of bafilomycin A1 showed a depression rate of 50% within ~10 min with controls lasting ~40 min. After low frequency depression and/or exposure to bafilomycin A1 a burst of higher frequency (10 Hz) can recruit vesicles from the RP to the RRP.

Key words: synapse, reserve pool, readily releasable pool, bafilomycin A1, serotonin.

INTRODUCTION

In the early 1960s presynaptic vesicles were placed into different groups based on their spatial distribution and physiological properties (Birks and MacIntosh, 1961; Elmquist and Quastel, 1965). Various nomenclatures have been used; therefore, no absolute definitions have been established with anatomical distinctions (Rosenmund and Stevens, 1996; Palfrey and Artalejo, 1998; Li and Schwarz, 1999; Kuromi and Kidokoro, 2000; Rizzoli et al., 2003; Rizzoli and Betz, 2005; Fdez and Hilfiker, 2006). Vesicles are distributed inside a nerve terminal without a clear anatomical line to separate readily releasable pool (RRP) from reserve pool (RP). Nevertheless, the physiological and anatomical separation of a RP and RRP is widely utilized for working models to explain and study synaptic transmission (Ruiz et al., 2011). RRP is defined as a group of vesicles close to the synaptically active zones which will fuse and release neurotransmitter responding to Ca^{2+} influx, while the RP is a group of vesicles slightly farther away from the synaptic face. Kidokoro et al. (2004) defined an immediately releasing pool (IRP) at *Drosophila* neuromuscular junctions (NMJs) as distinctly different from a RRP based on vesicles docked for initial fusion and as compared to ones that can undergo rapid exocytosis and endocytosis. Generally, the IRP is grouped within the RRP (Aravanis et al., 2003; Sudhof, 2004) and for our purposes, we grouped these two pools together as we examined longer term exocytosis and endocytosis processes. The vesicles in the RP pool are recruited under particular circumstances such as high frequency stimulation or in the presence of neuromodulators like 5-HT for crayfish NMJs (Wang and Zucker, 1998; Quigley et al., 1999; Logsdon et al., 2006). With transmission electron microscopy (TEM), Johnstone et al. (2008, 2011) showed the structure of a crayfish nerve terminal with single vesicle resolution and an approach to estimate a range, with incorporating stereological errors, in measuring the location of vesicles in regard to the presynaptic membrane. Rarely are errors in location or distance dealt with from measurements utilizing TEM; however, such considerations are valuable (Atwood and Cooper, 1996; Feuerverger et al., 2000; Kim et al., 2000). Since a number of disease states afflicting humans are related to presynaptic function, it is of interest to understand the fundamental properties that are most likely common to all animals as well as those that are different (Waites and Garner, 2011).

Several experiments have used various ways to physiologically separate vesicle pools. Rosenmund and Stevens (1996) and Bykhovskaia et al. (2001) used high osmolarity shock to deplete RRP. Such osmotic pressure changes on the nerve terminal were shown in

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Abbreviations: 5-HT, serotonin; CaM-K, CaM-kinase; CICR, Ca^{2+}-induced Ca^{2+}-release; EPSPs, excitatory postsynaptic potentials; GluT, glutamate transporter; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IP3, inositol 1,4,5-trisphosphate; IRP, immediately releasing pool; mEPPs, miniature end-plate potentials; NMJs, neuromuscular junctions; RP, reserve pool; RRP, readily releasable pool; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; TBOA, DL-threo-β-benzyloxyaspartic acid; TEM, transmission electron microscopy.

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the 1960s to cause transmitter release (Hubbard et al., 1968). Millar et al. (2002) employed a rapid vesicle depletion technique (200-Hz train stimulation) with a Cs^+ substitution, to deplete docked vesicles in crayfish tonic and phasic extensor NMJs. Schneggenburger et al. (1999) also demonstrated, in synapses at the Calyx of Held, that only a small fraction of the RRP is used by a single presynaptic action potential under physiological conditions but with a large percentage of vesicles that can be induced to fuse to the membrane if presynaptic calcium rises quickly. Akbergenova and Bykhovskaia (2009) identified RRP in *Drosophila* NMJ from TEM micrographs. In their study, the recycled vesicles, loaded with FM1-43, after intense stimulation were primarily localized within a group close to the synapses. Also, using a mutation in *Drosophila* to block recycling of vesicles revealed two distinct pools of vesicles (Kuromi and Kidokoro, 1998). In previous research in our laboratory, Logsdon et al. (2006) depleted the vesicles in RRP in crayfish opener NMJ by moderately stimulating a motor nerve axon in the presence of TBOA (DL-threo-β-benzyloxyaspartic acid), an inhibitor of a glutamate transporter (GluT) located on the plasma membrane. After synaptic depression occurred, it was demonstrated that the depressed state could be reversed by adding 5-HT. The interpretation is that 5-HT had the ability to promote the recruitment of vesicles from RP into RRP (Logsdon et al., 2006). Dudel (1965) first showed that synaptic transmission is enhanced at the crayfish NMJ by 5-HT and since then there have been a number of studies addressing the potential mechanisms of 5-HT's action (Enyeart, 1981; Glusman and Kravitz, 1982; Dixon and Atwood, 1985, 1989). The compiled findings indicated that two vesicle pools can be distinguished physiologically with one pool as the RRP and the other RP that can be recruited by 5-HT into the RRP.

It is postulated that new vesicles form the RP from an endosome and are packaged with neurotransmitter. It is implied that the RP can be spared and may not intermix with the RRP upon low-stimulation frequency (Kuromi and Kidokoro, 1998). Based on this view, Logsdon et al. (2006) proposed for the crayfish opener excitatory motor neuron that with repetitive trains of stimulation, vesicles can be quickly recycled within RRP and be depleted prior to recruiting from the RP. Thus, synaptic depression occurs due to the depletion of filled vesicles in RRP, while most vesicles in RP remain fully packaged with neurotransmitter. When evoked excitatory postsynaptic potentials (EPSPs), that showed depression, are rescued with 5-HT, not only will the evoked EPSPs grow in amplitude but also the spontaneous quantal events become intermixed in size. Normal-sized quantal events induced by 5-HT and reduced ones from the depressed state appear together as compared to just the smaller sized quanta responses during depression. A working model to explain this phenomenon was put forth, such that fully packaged vesicles from the RP are recruited, by actions of 5-HT, to the RRP which contains partially filled vesicles that are rapidly recycling during the high-stimulation frequency.

There are two main sources of glutamate that can be utilized to fill recycling vesicles in the nerve terminals. Glutamate can be directly taken back up from the synaptic cleft through the plasma membrane transporter (GluT). This is the glutamate that was released during synaptic transmission. At the crayfish and *Drosophila* NMJs, as compared to the vertebrate CNS, glutamate does not cycle through a glutamine path via a glia cell. A second source of glutamate is the glutamate already in the cytoplasm, within the nerve terminals, that may not have been immediately taken up across the membrane during synaptic activity. The NMJ on the opener muscle in crayfish is used here to compare the previous findings related to glutamate uptake and to build on studies that addressed actions of 5-HT in enhancing synaptic transmission (Dudel, 1965; Dixon and Atwood, 1989; Crider and Cooper, 2000; Sparks et al., 2004). The NMJs on this tonic muscle are relatively low in synaptic efficacy as compared to higher output phasic motor nerve terminals in the crayfish (Cooper et al., 1995a, 2003) or even those of the larval *Drosophila* NMJs (Cooper et al., 1995b) presented herein. Previous results indicate a significant role for glutamate uptake from synaptic cleft occurs in the process of refilling recycled vesicles. TBOA-treated NMJs preparations of the crayfish opener muscle showed a depression 2 h sooner, measured by EPSP amplitudes, as compared to controls. TBOA treatment did not block cytoplasmic glutamate from packaging in the vesicles nor could one address the issue if the vesicles were recycling within the RRP and RP. The purpose of this current study is to address this point by the use of bafilomycin A1.

As shown in past studies, 5-HT effect on enhancing synaptic transmission is partially induced by inositol 1,4,5-trisphosphate (IP3)-signaling pathway (Dixon and Atwood, 1989). In the study herein, we confirmed that blocking PLC, which in turn could lead to a decrease in IP3 formation, substantially blocked the 5-HT-induced response. Given that the 5-HT receptors at this nerve terminal appear to share pharmacological similarities to the mammalian 5-HT_2 subfamily (Tabor and Cooper, 2002), the mechanism of PLC activating IP3 formation for the downstream effect in promoting synaptic transmission is practical (Dropic et al., 2005). The novel aspect in this study compared to previous studies is the RP is being recruited following depression of the RRP and the mechanism of recruitment from a depressed synaptic state is being addressed.

The larval *Drosophila* NMJs also utilize glutamate as a neurotransmitter (Lee et al., 2009); however, synaptic transmission is not enhanced by exposure to 5-HT (Dasari and Cooper, 2004). We used the larval *Drosophila* NMJ as a comparison for the effects of bafilomycin A1 on the crayfish preparation. Kidokoro et al. (2004) used 20 μM at the *Drosophila* NMJ which is high compared to studies with vertebrate brain slices. Such high concentration appears to be toxic for the crayfish NMJs. Thus, we used lower concentrations throughout our studies. Since we examined actions of bafilomycin A1 with crayfish, a model species for comparative studies, various concentrations and stimulation paradigms were utilized.

EXPERIMENTAL PROCEDURES

General

The experiments on crayfish were carried out in the first or second walking legs of midsize animals (*Procambarus clarkii*), measuring 6–10 cm in body length. They were individually housed in plastic containers with aerated water and fed dry fish food. The temperature of the animal room was controlled at 20–21 °C. The water was changed on a weekly basis. Crayfish were induced to autotomize the first or second walking leg by forceful pinching at the ischiopodite segment. The details on the dissection of the opener muscle preparations are in video format (Cooper and Cooper, 2009). Dissected preparations were maintained in crayfish saline, a modified Van Harreveld's solution (in mM: 205 NaCl; 5.3 KCl; 13.5 $CaCl_2$ $2H_2O$; 2.45 $MgCl_2$ $6H_2O$; 5 Hepes adjusted to pH 7.4). The larval *Drosophila* preparations were performed as previously described (Li et al., 2001) for early 3rd instars. The standard HL3 saline was used for physiological measures (Stewart et al., 1994; Ball et al., 2003). All experiments conformed to a named local as well as International Guidelines on the ethical use of animals and that all efforts were made to minimise the number of animals used and their suffering.

Pharmacology

All chemicals were obtained from Sigma Chemical (St. Louis, MO). Bafilomycin A1 (B1793) solution was made by dissolving 10 μg powder in 20 μl DMSO (99.9%), then adding crayfish or *Drosophila* saline to obtain the desired concentrations. The solution was stored at −20 °C no longer than 3 months. Various concentrations ranging from 4 nM to 16 μM were used. The crayfish preparations without incubation were compared to the ones with 2.5-h incubation. In the group without incubation, electrical stimulation was applied immediately after switching from saline to bafilomycin A1 containing saline. The groups with 2.5-h incubation in bafilomycin A1 were electrically stimulated after the 2.5 h. During the 2.5-h incubation time, the bafilomycin A1 solution was mixed in the recording dish every 30 min. 5-HT (1 μM) was made in crayfish saline from frozen stock of 1 mM. The bafilomycin A1 saline was used to make up the 5-HT containing saline. 5-HT was only examined in the crayfish preparations as 5-HT does not enhance synaptic activity at the *Drosophila* NMJs. U73122 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione and U73343 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidinedione were obtained from Tocris Bioscience (Minneapolis, MN). Five millimolar U73122 and U73343 stock solutions were made by dissolving the chemicals in DMSO (99.9% pure). The desired concentrations were obtained by adding crayfish saline. The solutions were freshly made for all the trials. The preparations were incubated for 1 h while the train stimulation was applied. A 500 nM 5-HT solution made from 1 mM stock was added to the preparations after 1-h incubation with either U73122 or U73343.

Physiology

The excitatory axon innervating the opener muscle in the crayfish was selectively stimulated in the merus segment by placing a branch of the leg nerve into a suction electrode connected to a Grass stimulator (Dudel and Kuffler, 1961). Continuous stimulation at 20 or 40 Hz was applied to the excitatory nerve to compare the effects of bafilomycin A1. A train-stimulation of 20 EPSPs at 40 Hz every 10 s was applied to examine the effect by the PLC inhibitor and the inactive PLC inhibitor analog. Intracellular EPSP recordings were performed by standard procedures (Cooper et al., 1995a; Crider and Cooper, 2000). To be consistent, the distal muscle fibers were always used in this study, because anatomical, physiological properties of opener muscle differ in proximal, medial, and distal regions (Cooper et al., 1995a; Mykles et al., 2002). The synaptic responses at the larval *Drosophila* NMJs were recorded by standard procedures (Lee et al., 2009). All the experiments were performed at room temperature (20–21 °C). Continuous stimulation at 1 and 5 Hz was applied to the excitatory nerve to examine the bafilomycin A1 effect. Miniature end-plate potentials (mEPPs) were recorded for 5 min before applying 5-Hz stimulation either in saline or 8 μM bafilomycin A1 and for 10 min after 50% depression occurred. To separate RRP from RP, a similar stimulation paradigm as presented by Kuromi and Kidokoro (1998, 2000) was used. Preparations were stimulated at 1 or 5 Hz for 30 min followed by 10-Hz stimulation for 10 s, then returned to 1 Hz for at least 20 s. Both EPSPs and mEPPs were measured by intracellular recordings. Electrical signals were recorded online to a computer via a PowerLab/4s interface.

Analysis

On examining the effect of bafilomycin A1, the amplitude of the last EPSP every 1 or 10 s in various experimental paradigms was measured (software Chart Version 5; ADInstruments) for the crayfish responses. When the largest facilitated EPSP responses were present, 60 events were averaged and normalized to 100%. Because of the variation in the time to peak facilitation among preparations, the time it took from the beginning of the stimulation to the time to reach 50% of the maximum amplitude was used as an index for depression time. The mean time of 50% decline was compared among different experimental conditions. In the crayfish preparations, after the EPSP amplitudes were less than 50% and nearly undetectable from back ground noise, the saline was exchanged to 1 μM 5-HT. The mean time of 50% decline at 20-Hz continuous stimulation with 2.5-h incubation in 8 μM, 4 μM, 400 nM, 4 nM, bafilomycin A1 solutions as well as a control saline were used to provide a dose–response relationship. For the experiments of PLC inhibitor the 20th EPSP in a pulse train for totally 20 consecutive trains before and during exposure to 5-HT were compared. A percentage change was measured. In *Drosophila* preparations, the analysis of the responses for depression experiments was similar with the exception that only 1 and 5 Hz stimulation paradigms were used. The percentage change was measured by comparing the average amplitude of 100 mEPPs before applying stimulation and after 50% depression or comparing the average amplitude of 20 EPSPs before and after 10-Hz stimulation. The coefficient of variation (C_V) was determined by the ratio of the standard deviation to the mean. Statistical significance was assessed by paired, unpaired *t*-test or Mann–Whitney rank sum test. One, two and three asterisks indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. All the data are presented as mean ± SEM.

RESULTS

Depression at crayfish NMJs for 20- and 40-Hz stimulation

The depression time to 50% depression was taken as an index for measuring the effect of bafilomycin A1 on synaptic transmission as compared to controls. A 20-Hz continuous stimulation was used in the opener muscle preparation as performed in previous experimentation using this preparation (Logsdon et al., 2006). A 40-Hz continuous stimulation was also used for comparative purposes to determine if depression time would be decreased as compared to 20-Hz continuous stimulation in the absence and presence of bafilomycin A1.

The viability of crayfish neuromuscular preparations in minimal saline is well known. Even with nerve stimulation, excised preparations are robust in nature. When exposed to crayfish saline with 20-Hz continuous stimulation, the average depression time to 50% of the peak EPSP amplitude is 192 min ($n = 5$, ±33 min SEM, Fig. 1A1 and C). After depression was induced, exposure to 5-HT revitalized synaptic transmission in all cases with the 20-Hz stimulation paradigm (Table 1). This rejuvenation demonstrates the nerve terminals are not dead or badly damaged by the prolonged time of stimulation.

Preparations continuously stimulated at 40 Hz in crayfish saline had an average 50% depression time of 42 min ($n = 7$, ±8 min SEM, Fig. 1A2 and C). After depression, only three preparations showed recovery of synaptic transmission with the presence of 5-HT (Table 1). This is probably because RP vesicles were already recruited to the RRP at a high frequency of stimulation. These results indicated that 40-Hz stimulation depresses synaptic transmission quicker than 20 Hz ($p = 0.003$, Mann–Whitney rank sum test), which is consistent with the expectation because vesicular recycling is facilitated at a higher stimulation frequency.

Effect of stimulation rate and exposure time to bafilomycin A1

In examining the effects of bafilomycin A1 to produce a 50% depression in EPSP amplitude, two different exposure paradigms were used. Six preparations at 20-Hz continuous stimulation in 4 μM bafilomycin A1, without a preincubation, took 62 min on average to depress to 50% ($n = 6$, ±20 min SEM, Fig. 1B1 and C). Four out of six preparations were rejuvenated with 5-HT after depression (Table 1).

In contrast to 20-Hz continuous stimulation, preparations were continuously stimulated at 40 Hz, in the presence of 4 μM bafilomycin A1 without pre-incubation. In this set of experiments it took an average of 30 min ($n = 6$, ±8 min SEM, Fig. 1B2 and C) to depress the EPSP amplitudes to 50% but in only one out of six preparations did the EPSPs reappear with the 5-HT exposure (Table 1). Therefore, 20-Hz continuous stimulation was used for all the following experiments in crayfish preparations since they still had a response to 5-HT after depression.

Since there are no previous reports on the action of bafilomycin A1 at the crayfish NMJ, we examined various concentrations and exposure times. The use of 4 μM BA with 2.5-h incubation was used for brain slices of rodents (Cavelier and Attwell, 2007). We examined if an incubation time of 2.5 h was necessary or if acute application was sufficient to have an effect on the recycling RRP. In this series of studies, 4 μM, 20-Hz continuous stimulation with 2.5-h prior incubation was compared to 4 μM, 20-Hz continuous stimulation without prior incubation but allowing the preparation to remain in saline for 2.5 h as for the sham control preparations.

With 20-Hz continuous stimulation and 2.5-h incubation in 4 μM bafilomycin A1, it took on average 31 min to depress the EPSP amplitude to 50% ($n = 6$, ±4 min SEM, Fig. 1C); an enhancement of the EPSP amplitude induced by 5-HT after depression was observed in five out of six preparations (Table 1). By contrast, a 20-Hz continuous stimulation without incubation in 4 μM bafilomycin A1 took on average 62 min to depress to 50% ($n = 6$, ±20 min SEM, Fig. 1C). As expected, there was a large degree of variation among these preparations. After depression, only four out of six preparations showed EPSPs regaining their amplitude with exposure to 5-HT (Table 1). These results showed that the synaptic transmission in the group without 2.5 h of incubation took longer to depress (almost double the time) than the group with 2.5 h of incubation. In addition, the results of this group without incubation were highly variable in the time to depression as compared to the group with incubation. The results of 2.5 h of pre-incubation appeared to be more reliable than without incubation for the effect of bafilomycin A1. This paradigm was used as standard procedure for obtaining a dose–response relationship to synaptic depression time.

Comparing the effect of bafilomycin A1 treatment to time of synaptic depression indicated that depression induced at 20 Hz is quicker ($p = 0.004$, Mann–Whitney rank sum test) with exposure to bafilomycin A1 (Fig. 1C and Table 1). The higher stimulation rate of 40 Hz also induced depression more rapidly as compared to 20 Hz when not being exposed to bafilomycin A1. Also, with 40 Hz and exposure to bafilomycin A1, the depression rate was rapid (Fig. 1C). One interpretation is that bafilomycin A1 can efficiently block vesicles refilling under repetitively high frequency stimulation. Also, we discovered that 2.5 h incubation resulted in less variability in the depression rate most likely because of an even distribution of the drug inside of the nerve terminal prior to rapid stimulation.

Dose–response relationship

Since these are novel experiments of bafilomycin A1 exposure in the crayfish model preparation, constructing a dose–response relationship helped in determining a practical dosage range for a specific cellular function. For example, it is conceivable that bafilomycin A1 might block ATP production by acting on mitochondrial ATPase H^+ pump, and, therefore, has a spurious action on synaptic transmission. However, an enhanced action of 5-HT on synaptic transmission after the induction of synaptic depression suggests that inhibition of ATP production was not a potential side effect for a given concentration. Given that bafilomycin A1 was used at *Drosophila* NMJs to address a similar topic (Kidokoro et al., 2004), we expected that this compound would also be suitable for other arthropod NMJs (i.e., crayfish).

Four micromolar bafilomycin A1. Four micromolar bafilomycin A1 was used with the standard stimulation paradigm described above in section 'Effect of stimulation rate and exposure time to bafilomycin A1'. A total of six preparations were examined. On average, it took about 31 min to depress to 50% from the peak amplitude ($n = 6$, ±4 min SEM, Fig. 2B1); after

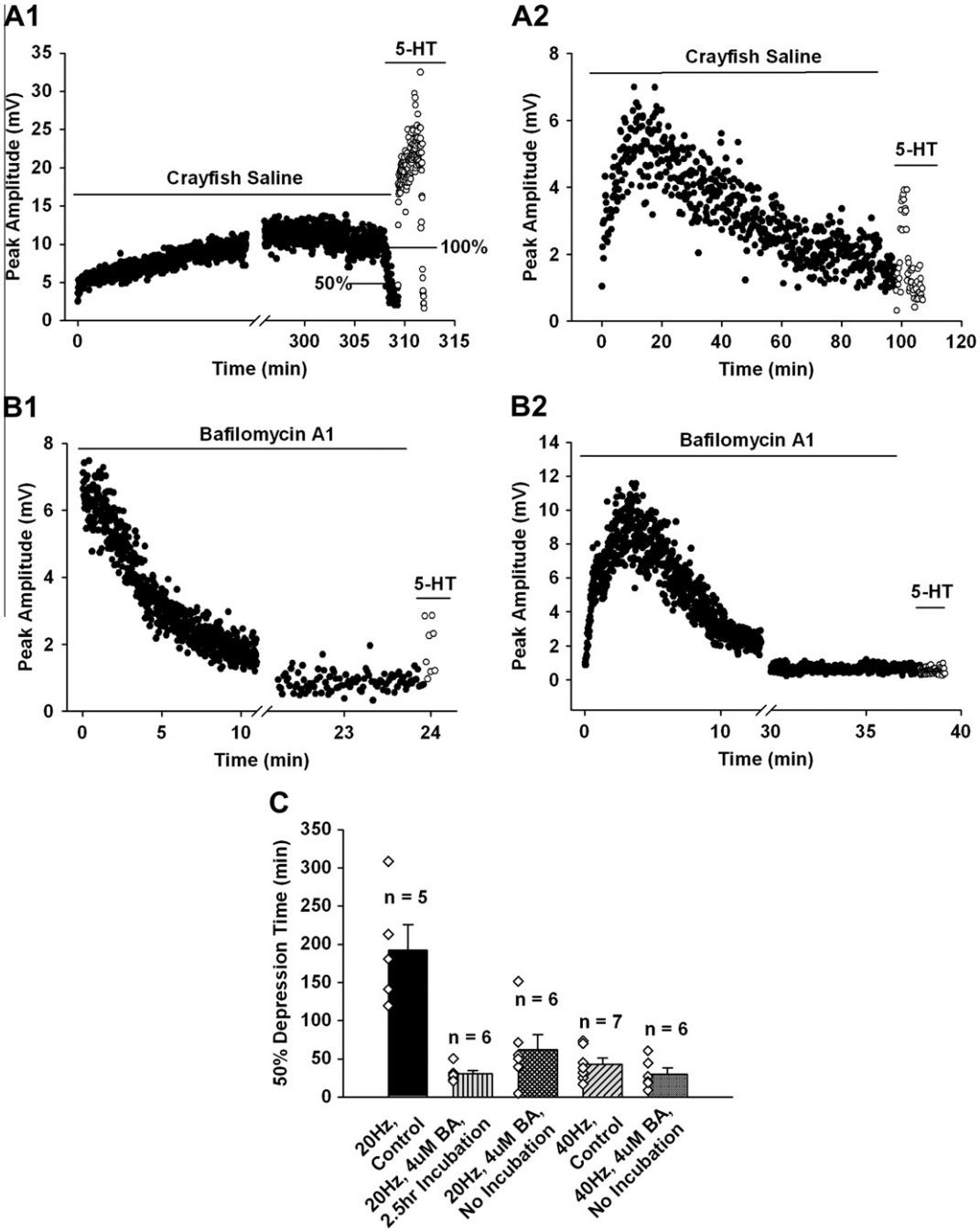

Fig. 1. Effect of stimulation rate and exposure time to bafilomycin A1 at 20-Hz and 40-Hz continuous stimulation. (A) Representative scatter plot of the EPSP peak amplitudes (mV) at 20-Hz (A1) and 40-Hz (A2) continuous stimulation in crayfish saline. Filled circles are EPSPs in crayfish saline. After substantial depression in the EPSP amplitude a 5-HT containing saline replaced the saline bath. EPSPs reappeared as indicated in open circles. (B) Representative scatter plot of the EPSP peak amplitudes (mV) at 20-Hz (B1) and 40-Hz (B2) continuous stimulation in 4 μM bafilomycin A1 without pre-incubation. After substantial depression in the EPSP amplitude a 5-HT containing saline replaced the saline bath. EPSPs reappeared as indicated in open circles. (C) Composite results comparing 50% depression times. Bar graph representing the various experimental conditions (*n* represents the sample size, BA is bafilomycin A1).

substantial depression was induced, 5-HT still enhanced the amplitude in EPSPs in five out of six preparations.

Eight micromolar bafilomycin A1 is toxic to crayfish NMJs. To test whether higher concentrations can work more effectively, the concentration of bafilomycin A1 was doubled from 4 to 8 μM. Two preparations failed to show synaptic responses during the incubation. In order to test the time to damaging the health of the NMJ and whether the lack of responsiveness was caused by the failure of neurotransmission or other side effects of this drug, three preparations were stimulated for 1 s every 10 min while being exposed to 8 μM bafilomycin A1 (Fig. 2A1 and A2). The EPSPs in all three preparations failed within 1.5 h, however, the EPSPs recorded right before failures were normal. We

Table 1. The values in 50% depression time and the number of preparations that showed an increase in the amplitudes of EPSPs with 5-HT exposure after depression. BA, bafilomycin A1

Stimulation	Number of preparations	Time depress to 50% (min)	w/5-HT
0 μm BA (control); 20-Hz continuous	5	192.46	5/5
4 μm BA; 2.5 h incubation 20-Hz continuous	6	30.51	5/6
4 μm BA; w/o incubation 20-Hz continuous	6	62.08	4/6
0 μm BA (control); 40-Hz continuous	7	42.87	3/7
4 μm BA; w/o incubation 40-Hz continuous	6	30.07	1/6

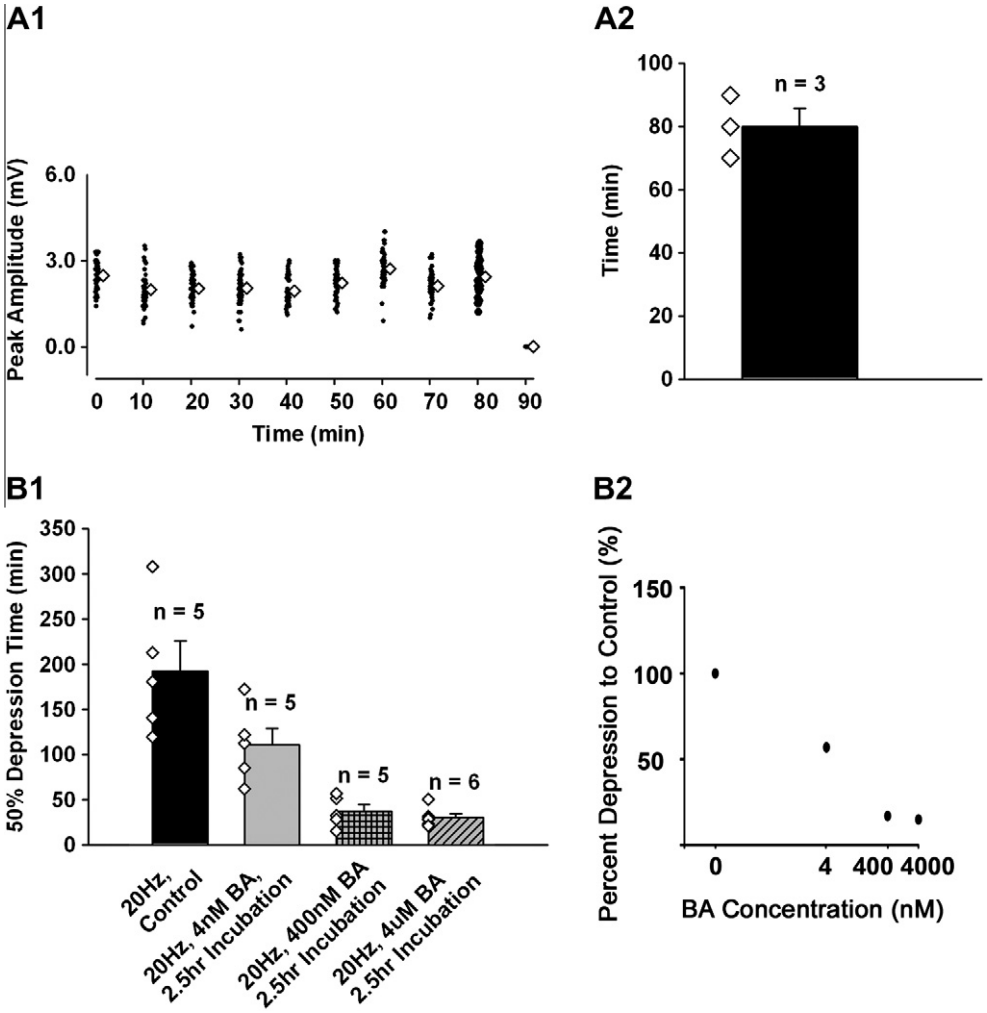

Fig. 2. Dose–response relationship. (A) Representative scatter plot (A1) of the EPSP peak amplitudes (mV) during the incubation with 8 μM bafilomycin A1 in which the preparations were stimulated for 1 min measured every 10 min. Filled circles are individual EPSPs and the open diamonds are the average amplitudes. Bar graph (A2) is the time when preparations stopped producing EPSPs. The average time for all three preparations is 80 min (±6 min SEM). The open diamonds represent the three individual preparations. (B) Bar graph (B1) of the 50% depression time in relation to the concentration of bafilomycin A1 exposure. All the experiments were performed with 20-Hz continuous stimulation preceded by 2.5-h pre-incubation in 0 nM, 4 nM, 400 nM, and 4 μM bafilomycin A1 solution (*n* represents sample size, BA is bafilomycin A1). Dose–response relationship (B2) measures the rate to 50% depression with 0 nM, 4 nM, 400 nM, and 4 μM bafilomycin A1 exposure. Eight micromolar bafilomycin A1 is toxic and is not depicted in the graph.

interpret these results as a toxic effect rather than a failure of presynaptic neurotransmission to block the packaging of vesicles.

Four nanomolar and 400 nM bafilomycin A1. Two lower concentrations of bafilomycin A1 (4 nM and 400 nM) were also examined for the effect on vesicular repackaging inhibition. With 4 nM bafilomycin A1, the average depression time to 50% is 111 min ($n = 5$, ±19 min SEM, Fig. 2B1). All the preparations showed synaptic recovery with 5-HT after depression. The average 50% depression time is 30 min ($n = 5$, ±9 min

SEM, Fig. 2B1) for 400 nM. All the preparations showed synaptic recovery with 5-HT after depression.

Overall results of dose–response effect. In normal crayfish saline, the average depression time to 50% peak amplitude is 192 min ($n = 5$, ±33 min SEM, Fig. 2B1). After depression, 5-HT always produced an enhanced response. The 2.5 h of pre-incubation without stimulation for the following concentrations 8 μM, 4 μM, 400 nM, 4 nM of bafilomycin A1 and sham control followed by continuous 20-Hz stimulation was used to graph a dose–response curve of the time required to depress the EPSPs to 50%. As mentioned above, 8 μM of bafilomycin A1 exposure is lethal so data with 8 μM bafilomycin A1 are not included in the dose–response curve. The incubations at 4 μM took about 30 min while control preparations lasted on average 3 h. The 400 nM bafilomycin A1 had an effect similar to 4 μM. The depression time from 4 nM bafilomycin A1 occurred between the times noted for control and 400 nM treatments. Within the dose range examined, the suggested inhibition of vesicular refilling by bafilomycin A1 was correlated with the concentration of drug exposure (Fig. 2B2). It appears that the RP vesicles were protected as the addition of 5-HT always resulted in an enhanced response after synaptic depression was induced.

PLC inhibitor attenuates 5-HT effect on synaptic transmission

The PLC non-selective inhibitor U73122 was used to test the possibility of 5-HT mediating an enhanced recruitment of RP vesicles via a PLC intermediate step. The inactive analog of U73122, U73343, served as a negative control. Doses ranging from nanomolar to millimolar have been employed in various studies of mammalian models. No previous reports are present for these compounds being examined at the crustacean NMJ. Ten micromolar was reported to be sufficient in reducing IP3 production in human platelet cells (Bleasdale et al., 1990). We used 5 and 10 μM, but no significant reduction of 5-HT effect was observed (data not shown). Therefore, 50 μM was used for both U73122 and U73343.

The preparations were recorded in crayfish saline for 1 h before being switched to a 500 nM 5-HT solution. The average amplitude of 20th EPSP in a pulse train for 20 consecutive trains was compared before and after adding 5-HT. The average percentage change increased by 266% ($n = 5$, ±42% SEM, Fig. 3A1 and A2). Instead of crayfish saline only, the preparations were stimulated and recorded while being bathed in 50 μM U73122 solution for 1 h before being exposed to 5-HT in crayfish saline. The average amplitude in the 20th EPSPs for 20 consecutive trains was compared before and after adding 5-HT. The average percentage change was only 14% ($n = 6$, ±6% SEM, Fig. 3B1 and B2). As a negative control, the preparations were recorded in U73343 (50 μM) solution for 1 h before the 5-HT exposure. The average amplitude of 20th EPSPs for the pulse train was compared before and after adding 5-HT. The average percentage change was 191% ($n = 5$, ±23% SEM, Fig. 3C1 and C2).

Actions of bafilomycin A1 at the *Drosophila* NMJ

Low frequencies (1 and 5 Hz) continuous stimulations were used due to the high output property of the *Drosophila* NMJs. Pre-incubation with 4 μM bafilomycin A1 did not result in any significant difference from the ones without pre-incubation (data not shown). There was no significant difference in depression rate for 5 Hz ($n = 5$, 32 min, ±6 min SEM) and 1 Hz ($n = 5$, 37 min, ±3 min SEM) when exposed to normal fly saline only (Fig. 4).

As a comparison to the crayfish preparations, 4 μM bafilomycin A1 was first examined at both the 1-Hz and 5-Hz stimulation paradigm. In the presence of 4 μM bafilomycin A1 the 5 Hz resulted in rapid depression ($n = 8$, 5 min, ±1 min SEM); however, due to the variability, there is no significant difference in the 1-Hz stimulation rate ($n = 5$, 3 min, ±1 min SEM) (Fig. 4). However, the difference in rate of depression is profound with exposure to bafilomycin A1 ($p < 0.001$, Student's *t*-test). This rapid rate of synaptic depression substantiates the paralyzed effect within 2 min of whole larval injection of bafilomycin A1 (~1 μM) (Denker et al., 2011a). To test whether higher concentrations work more effectively, the concentration of bafilomycin A1 was doubled from 4 to 8 μM. Surprisingly, the *Drosophila* preparations did not have a rapid cessation at 8 μM as for the crayfish preparation. All the preparations examined maintained their resting membrane potentials. The mean time of depression appeared to be reduced, however not significantly, at 5 Hz ($n = 8$, 9 min, ±0.6 min SEM) compared to 1 Hz ($n = 5$, 11 min, ±2 min SEM). Exposure to 8 μM did not depress terminals any faster than did the 4 μM exposure (Fig. 4). To further confirm the possibility of saturating the rate of depression at 4 μM concentration, a higher dose of 16 μM was examined at 1-Hz stimulation (11 min, ±0.5 min SEM). No significant difference in time to depression was present between 4 and 8 μM exposure.

The spontaneous vesicular pool at the *Drosophila* NMJ

The mEPPs are produced by single (quanta) vesicular fusion events (Fatt and Katz, 1952). Therefore, the size of mEPP can be used as an indirect measure in the amount of neurotransmitter content released during vesicle fusion as well as receptivity to the transmitter. One would expect the glutamate content of recycled vesicles to decrease if the vesicle is not completely empty after evoked depression in the presence of bafilomycin A1, because the refilling process is impeded. However, intracellular recordings of mEPPs in *Drosophila* muscle did not show significant difference between saline and bafilomycin A1 treatments. The percentage change in the amplitude as well as the coefficient of variation (C_V) after depression was similar in saline changed to saline (control group) or saline changed to bafilomycin A1 (experimental group). There was a similar degree in rundown in the amplitude of the

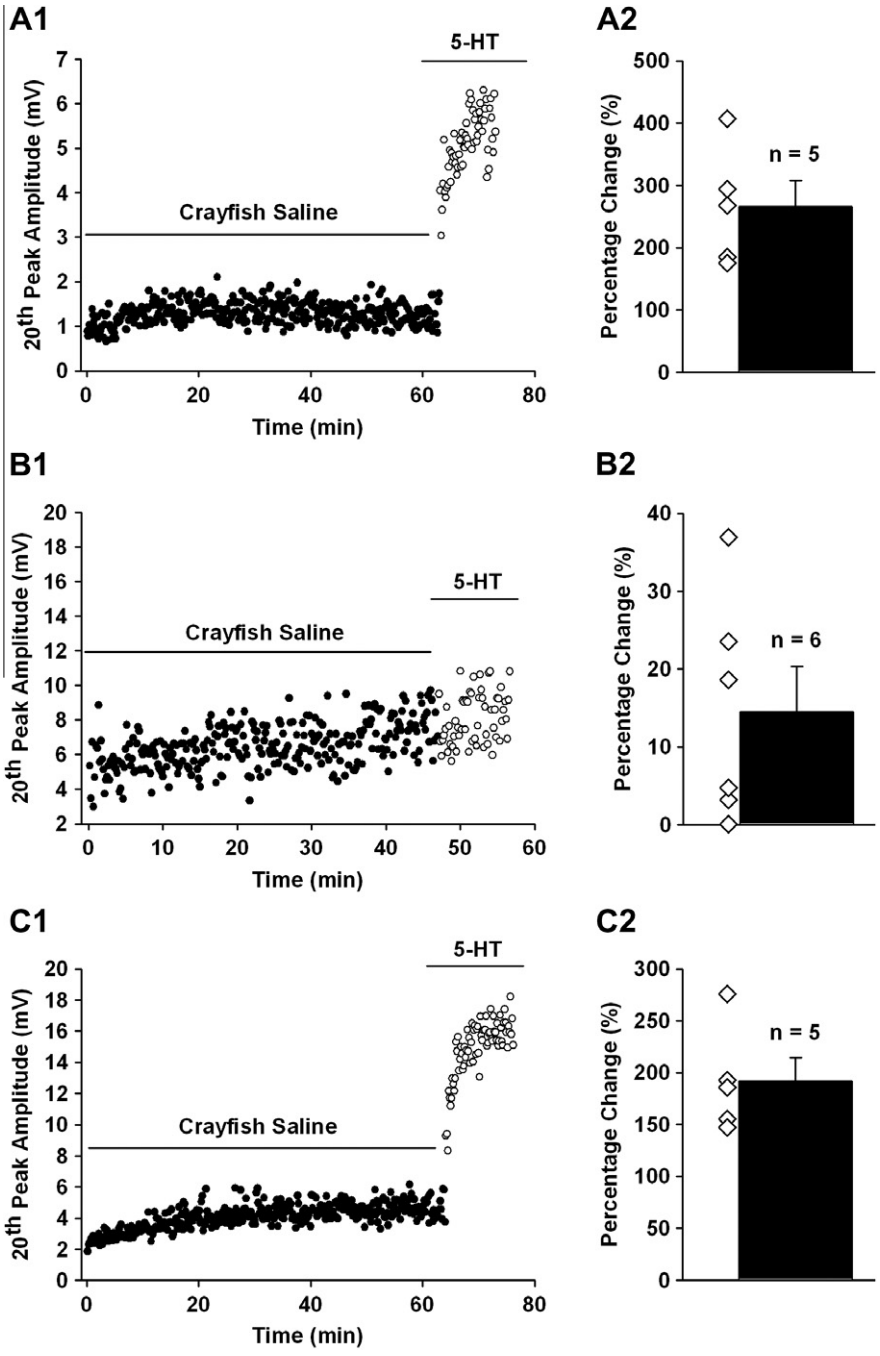

Fig. 3. The effect of U73122 and U73343 on 5-HT induced synaptic transmission. A representative scatter plot for one preparation depicts the amplitude of 20th EPSP in each pulse train through an hour of control (A1), U73122 (B1) and U73343 (C1) treatment followed by 5-HT treatment. 5-HT enhances the amplitude significantly in control and U73343 treated preparations but not U73122 treated group (open circles). In control, U73122 and U73343 treated group, the average percentage change is 266% (n = 5, ±42% SEM) (A2), 14% (n = 6, ±5.8% SEM) (B2) and 191% (n = 5, ±23% SEM) (C2) respectively. The open diamonds is the percentage change for each individual preparation.

spontaneous single quantal events for both conditions (Fig. 5). This is shown as a negative percent change and a positive C_V.

Separation of RP and RRP physiologically at the *Drosophila* NMJ

Following a similar stimulation paradigm as presented by Kuromi and Kidokoro (1998, 2000), which was designed to test if RP vesicles can be recruited into the RRP, a high frequency stimulation (10 Hz) was given for 10 s and then returned to a lower stimulation frequency (1 or 5 Hz) while monitoring the EPSP amplitudes. Unlike crayfish NMJs, 5-HT has no effect on enhancing synaptic transmission at these *Drosophila* NMJs (Dasari and Cooper, 2004) so the high frequency stimulation paradigm was used to recruit from the RP. The two stimulation paradigms used are depicted in Fig. 6A. The

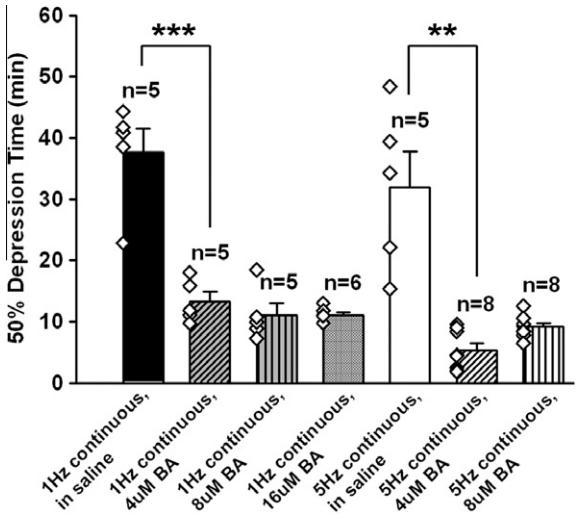

Fig. 4. Actions of bafilomycin A1 at the *Drosophila* NMJ. Bar graph of the 50% depression time in minutes (min) when the preparations were treated with 4 μM, 8 μM bafilomycin A1 for both the 1-Hz and 5-Hz stimulation paradigms (16 μM only at 1 Hz) (*n* represents sample size, BA is bafilomycin A1). The diamonds are the 50% depression time of each individual preparation. Two and three asterisks indicate $p < 0.01$ and $p < 0.001$ respectively.

nerve was stimulated at low frequency (1 or 5 Hz) for 30 min in the presence or absence of 4 μM bafilomycin A1, followed by high frequency stimulation at 10 Hz for 10 s then back to 1 or 5 Hz for at least 20 s. The average amplitude in 20 EPSPs before and after the 10-Hz stimulation was compared.

Consistent with the general findings of Kuromi and Kidokoro (2000), preparations not exposed to bafilomycin A1 showed no difference in the EPSP amplitudes before and after tetanic stimulation (10 Hz) for both paradigms (1 Hz–10 Hz–1 Hz or 5 Hz–10 Hz–5 Hz, Fig. 6B and D). With bafilomycin A1, tetanic stimulation can enhance synaptic transmission from the evoked depressed state (Fig. 6C and D). This suggests that bafilomycin A1 treatment depletes glutamate in RRP vesicles during the 30 min low frequency stimulation and the 10 Hz tetanic stimulation can recruit RP vesicles that are full of glutamate to recover the synaptic activity.

DISCUSSION

The ATPase specific inhibitor bafilomycin A1 has been used in various studies involving packaging of vesicles in mice hippocampal neurons (Cavelier and Attwell, 2007). However, the effect of bafilomycin A1 on vesicular packaging and synaptic transmission in the invertebrate model organism *P. clarkii* has not been previously examined in relation to recruiting a RP with neuromodulation. Bafilomycin A1 exposure at 20 μM on *Drosophila* NMJs was reported earlier and was shown to have substantial effects in reducing evoked synaptic transmission (Kidokoro et al., 2004). In depressed NMJs of the *Drosophila*, in the presence of bafilomycin A1, a burst of 30-Hz stimulation was able to rejuvenate vesicular release for a short period. Thus, a RP was indicated to be independent of the RRP. Likewise, in nerve terminals of the crayfish opener NMJ after the induction of evoked depression in the presence of bafilomycin A1, evoked responses reappeared with modulation by 5-HT. Thus, a physiological separation in the RRP and RP is present for both preparations. Our results also extend the findings that the quantal events by minis appear to be a different pool of vesicles compared to the evoked vesicle pool in the larval *Drosophila* NMJ preparation.

As anticipated, the crayfish preparations stimulated at 40 Hz depressed faster than the ones stimulated at 20 Hz. However, recovery of synaptic activity by 5-HT following depression was more variable in preparations stimulated at 40 Hz compared to the 20 Hz paradigm. Two out of several possibilities could explain the larger variation at the higher frequency stimulation paradigm. First, it is possible that at 40 Hz the RP vesicles were recruited into RRP to support the functional demand; thus, fewer vesicles remained in RP which could be recruited by 5-HT. Secondly, in the presence of bafilomycin A, the accelerated rate of vesicular turnover at 40 Hz reduced the RRP of vesicles that were able to be repackaged with transmitter.

The opener crayfish preparation is lower in synaptic efficacy than the larval M6 *Drosophila* NMJ (Cooper et al., 1995b); however, they both appear to have a means of protecting a reserve pool of vesicles that can be pulled into action when a demand is present if the

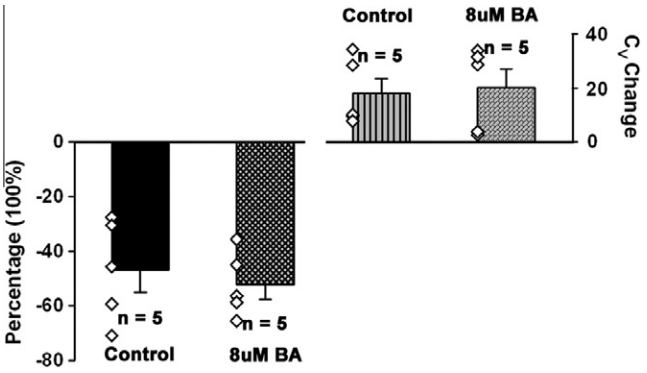

Fig. 5. The spontaneous vesicular pool at the *Drosophila* NMJ. The percentage change of the average amplitude of 100 mEPPs before and after 50% depression and the coefficient of variation (C_V) are shown. Preparations in saline and in 8 μM bafilomycin A1 are compared. The diamonds represent each individual preparation.

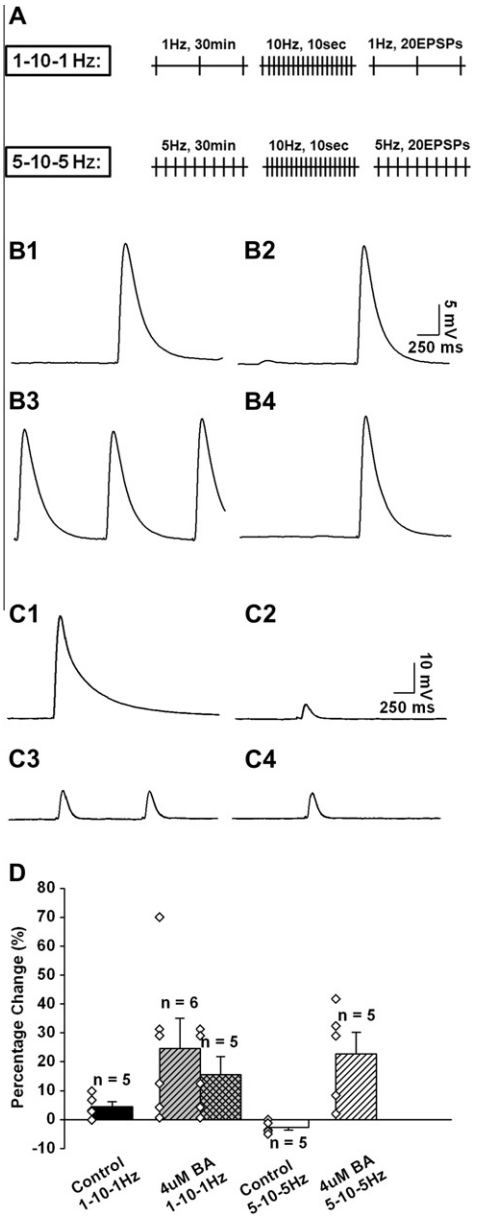

Fig. 6. Separation of RP and RRP physiologically at the *Drosophila* NMJ. (A) The stimulation paradigm used to separate RRP and RP physiologically. 1-Hz and 5-Hz baseline stimulation are compared. The nerve was stimulated at low frequency (1 and 5 Hz) for 30 min in the presence or absence of 4 μM bafilomycin A1, followed by high frequency stimulation at 10 Hz for 10 s then retuned back to 1 or 5 Hz for at least 20 s. (B, C) Representative traces of 1 Hz–10 Hz–1 Hz in control (B) and 1 Hz–10 Hz–1 Hz in preparation treated with 4 μM bafilomycin A1 (C). The trace at 5 Hz–10 Hz–5 Hz group shows the similar pattern. B1, C1 show one EPSP at the beginning of 1-Hz stimulation. After 30 min, the amplitude in control does not change (B2) while bafilomycin A1 treated preparation depresses (C2). With 10 s of 10-Hz tetanic stimulation an enhancement in the EPSP amplitude did not occur in control group (B3) but did so for the bafilomycin A1 treated preparation (C3). The enhancement continues even when stimulation was returned to 1 Hz (C4), whereas the control preparation did not show as much change (B4). (D)The percentage change (average amplitude of 20 EPSPs after 10 Hz compared to the average of 20 EPSPs before 10 Hz) with 1 Hz–10 Hz–1 Hz and 5 Hz–10 Hz–5 Hz stimulation paradigms with and without exposure to 4 μM bafilomycin A1. In both stimulation paradigms, 4 μM bafilomycin A1 causes larger percentage change in EPSP amplitude.

terminals are not highly depressed due to high frequency stimulation. The RP not only is distinguishable from the RRP, but also another discrete pool that maintains spontaneous events is likely present. At least for the *Drosophila* NMJs, when the RRP is functionally depleted of filled vesicles for evoked transmission, minis still occur. The size of the crayfish muscles used in this study did not allow for spontaneous events to be clearly distinguishable for quantitative purposes, with intracellular recordings, to compare with the small *Drosophila* muscles. Possibly with focal macropatch electrode recordings over nerve terminals would be an approach to address this issue at the crayfish NMJs or to use smaller preparations. The larval *Drosophila* muscles used are relatively small which aids in a higher whole membrane resistance allowing the spontaneous event to be readily observed. The larval *Drosophila* NMJ does show rundown in evoked release with the HL3 saline (Stewart et al., 1994). Also, there is some attenuation in the amplitudes of the mEPPs as well if one starts recording within a few minutes of a rapid dissection (total time from start of dissection to recording ~5 min). Kidokoro et al. (2004) noted that in transected segmental nerves, the same NMJ preparation as used in current study, vesicle recycling is reduced as compared to intact segmental nerves. In our study, we were required to drive the axons without intrinsic motor nerve activity induced from an intact CNS. Since the controls and the experimentally treated preparations were handled in a similar manner, direct effect of the experimental manipulation can be compared. It is likely that HL3 is not an optimally suited saline. Other salines, such as HL6, are being examined for NMJ physiology (Macleod et al., 2002). Given that HL6 does contain amino acids, we did not wish to add such cofounding variables to these experimental conditions used in our study with transmitter repackaging. We suggest, this remaining pool of vesicles that were still fusing after the RRP depletion is arriving from the RP vesicles to the synaptic face. In a model for the *Drosophila* NMJ, we suggest a dynamic RP in which vesicles can be recruited depending on the stimulation frequency and even after the RRP is depressed, some RP vesicles could be pulled into action for asynchronous and evoked coordinated fusion.

The presence of mEPPs after depression of evoked transmission does bring up an interesting topic to the source of the mEPPs in relation to evoked vesicular events. In vertebrate CNS slice preparations, it appears the spontaneous occurring events arise from an independent pool of vesicles from the evoked pool (Sara et al., 2005; Fredj and Burrone, 2009; Chung et al., 2010); however, other studies, suggest that the spontaneous events are from the same pool as the evoked (Wilhelm et al., 2010).

The recovery of the depressed crayfish preparations with exposure to 5-HT suggests that mitochondria ATP production is not impaired by bafilomycin A1 because vesicle docking and recycling is an ATP-dependent process (Tolar and Pallanck, 1998). Incubating preparations for 2.5 h might allow bafilomycin A1 to

distribute more evenly inside the terminals, which theoretically would result in more consistent results, compared to acute exposure to bafilomycin A1. This postulation is supported as the variation in depression rates among preparations was greater when preparations were not incubated. Also, with incubation, further fusion events are not compromised, as exposure to 5-HT still enhances release. Likewise, the *Drosophila* NMJs likely have sufficient mitochondrial function after exposure to bafilomycin A1 and low frequency depression since high frequency stimulating bursts still allow vesicular fusion events to occur.

It would be of interest to know if similar cellular mechanisms in recruitment of the vesicles from the RP to the RRP occur for these two preparations. Potentially different cellular mechanism might lead to the same end result, such as phosphorylation of synapsins to increase the pool of free vesicles for docking or possibly actions on calcium influx and release from internal stores (Yang et al., 1999; Dropic et al., 2005). This internal release of Ca^{2+} could activate calmodulin in turn activating CaM-kinase (CaM-K), which can phosphorylate proteins to either activate or deactivate them. In this case, CaM-K could phosphorylate the integral vesicle membrane protein synapsin. In *Aplysia* neurons exposure to 5-HT has been shown to cause phosphorylation of synapsins (Fiumara et al., 2004). This could then result in vesicles being freed from the cytoskeleton to promote docking to the presynaptic membrane. Phosphorylation of mediator proteins involved directly with the docking machinery, such as t-SNARE, v-SNARES, NSFs or MUNC proteins, could also be occurring along with mobilizing vesicles from the RP (for crayfish studies see – Dixon and Atwood, 1989; Tolar and Pallanck, 1998; He et al., 1999; Southard et al., 2000). Possibly, an equivalent form of Bruchpilot-like proteins is distributed in the crayfish and *Drosophila* motor nerve terminals that might form divisions among vesicles in their movements (Kittel et al., 2006) and Ca^{2+} buffering which not only alters fusion but the endocytosis processes as well (Yamashita, 2012). There may indeed be various overlapping cellular processes utilized by 5-HT to pull vesicles from the RP to the RRP as well as independent processes related to electrical activity of the terminal. It was demonstrated that $[Ca^{2+}]_o$ is needed for synaptic release at the frog (Katz and Miledi, 1968) and crustacean NMJs (Fatt and Katz, 1953) and that higher $[Ca^{2+}]_o$ increases synaptic communication. The rise in $[Ca]_i$ from the prolonged Calcium conductance after a series of action potentials is also implied as being one mechanism which rises residual $[Ca]_i$ to account for short-term facilitation at the crayfish NMJs (Fuchs and Getting, 1976; Parnas et al., 1982; Wojtowicz and Atwood, 1984; Atwood and Wojtowicz, 1986; Dudel, 1989a,b; Cooper et al., 1996). However, at very high concentrations of $[Ca^{2+}]_o$ synaptic transmission can be depressed quickly. The $[Ca^{2+}]_i$ can activate calmodulin in turn activating CaM-K as well as potentially activating a Ca^{2+}-induced Ca^{2+}-release (CICR). Release of Ca^{2+} from ER can be regulated by inositol 1,4,5-trisphosphate (IP3) (Berridge, 2005). Activation of ryanodine receptors within presynaptic nerve terminals is known to alter evoked transmitter release (Liu et al., 2005). One might suggest the rate of uptake into the ER by the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and release by CICR will alter vesicle recruitment from the RRP. For review of vesicle pools and various experimental approaches to address their function at the *Drosophila* NMJ see Kidokoro et al. (2004). The role of $[Ca^{2+}]_i$ sequestering and release by the ER and extrusion by pumps and exchangers in crayfish and *Drosophila* NMJs was recently addressed (Desai-Shah and Cooper, 2009, 2010).

In order to illustrate the general findings presented in this study we present a simple model (Fig. 7) that highlights the recruitment of the RP to the RRP by 5-HT and/or Ca^{2+} for the crayfish NMJ and by Ca^{2+} for the *Drosophila* NMJs. The *Drosophila* NMJs are not sensitive to 5-HT; however, there are likely other neuromodulators and second messenger cascades that may enhance transmission. A recent review of 5-HT actions on NMJs of invertebrates indicates various species that do have a response to applied 5-HT (Wu and Cooper, 2012). It would be of interest to know if similar receptor subtypes and secondary messengers are utilized among these species. The presence of intracellular Ca^{2+} in this model occurs through voltage-gated Ca-channels. The presence of minis (i.e., spontaneous quantal events not associated with depolarization of the terminal) may be occurring due to vesicles fusing at the same synaptic sites recruited during electrical depolarization of the terminal or other regions of the synapse (Chung et al., 2010).

Given that inhibition of PLC reduced the effect of 5-HT in recruiting presumable RP vesicles, a likely scenario is that the 5-HT receptors are coupled, through G protein-coupled receptors to PLC. The PLC can activate a number of further cascades such as DAG and IP3. The IP3 might then activate the Ca^{2+} response from the ER and this Ca^{2+} could even then activates CaM-K. A similar situation has been demonstrated for neurons in *Aplysia*, such that exposure of 5-HT resulted in an increase of IP3 levels that correlated with an increase in synaptic activity (Jin et al., 2007). Future studies with compounds such as a kinase inhibitor (staurosporin), a phosphatase inhibitor (okadaic acid) or cytoskeletal disrupting agents would be interesting to determine the impact in recruiting the RP to the RRP after synaptic depression is induced.

In short, this study aided in understanding a dose range of applying bafilomycin A1 on crayfish and *Drosophila* preparations as well as the effects of putatively blocking glutamate repackaging in recycling vesicle pools. The ability to physiologically differentiate the RRP and RP with varied experimental techniques provides additional insight into the underlying mechanisms of how these pools are utilized within nerve terminals. Future investigation into variations of how the pools function in various types of nerve terminals will shed additional light on the molecular regulation in synaptic vesicle dynamics. Currently we are comparing high output phasic type of nerve terminals in crayfish

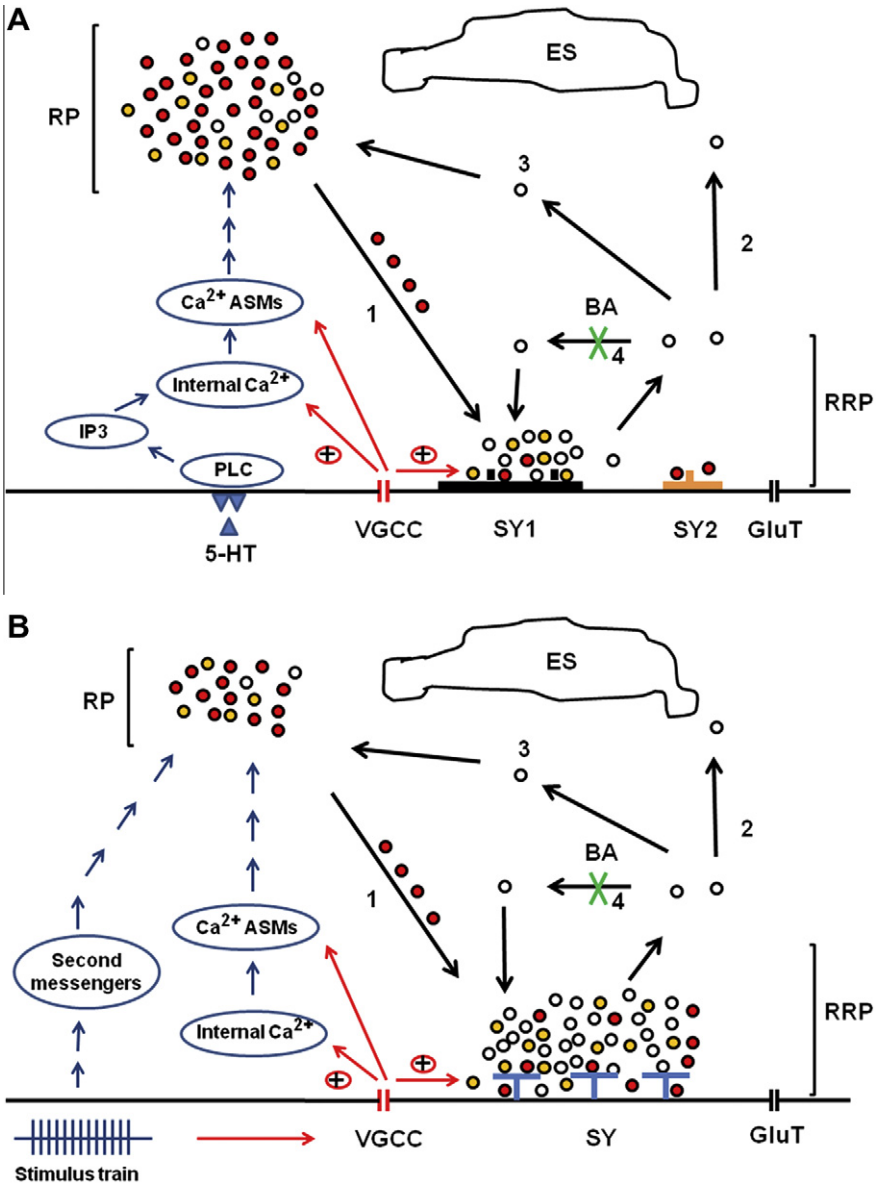

Fig. 7. Mechanistic diagram of vesicle recycling between RP and RRP and the effect of 5-HT or electrical stimulation on recruiting RP vesicles in crayfish and *Drosophila* NMJs. Synaptic vesicles are separated into RRP and RP. Two vesicle recycling pathways have been proposed. In a rest synapse, vesicles in RP can slowly join into the RRP (1), and then recycle back to RP either through or bypass endosome (2 or 3). This is called slow recycling loop. However, in an active synapse, in addition to the slow recycling loop, vesicles in RRP recycle quickly within the RRP (4) which is named quick recycling loop. Recycling vesicles are refilled with glutamate. However, this refilling process can be disrupted by bafilomycin A1 treatment (as shown by an empty vesicle in pathway 4). Synaptic depression occurs sooner with bafilomycin A1 treatment because vesicles in RRP can be depleted of transmitter in time with stimulation while the recycling vesicles can no longer be refilled. (A) In crayfish low output terminals, more RP vesicles comparing to RRP is proposed. After bafilomycin A1 induced synaptic depression, 5-HT is able to recruit RP vesicles to rejuvenate the synaptic activity. One of the possible mechanism is that 5-HT activates IP3 signaling pathway which stimulates Ca^{2+} release from the internal stores such as ER. The elevated $[Ca^{2+}]_i$ then activates other second messengers like CaM kinase. After several steps of cellular activity, RP vesicles are pulled into RRP. In addition, external Ca^{2+} coming through VGCC can increase $[Ca^{2+}]$ in the internal stores, activate Ca^{2+} activated second messengers, also increase the cooperativity of vesicle fusion via synaptotagmin. 5-HT may also activate low probability synapses like SY2. The low probability synapses are likely ones with less complex synaptic structure (Cooper et al., 1996). (B) In *Drosophila* high output terminals, more vesicles are present in RRP. After bafilomycin A1 induced synaptic depression, higher frequency stimulation can pull the RP vesicles into RRP. In this model $[Ca^{2+}]_i$ is elevated by repetitive high frequency stimulation which then increases $[Ca^{2+}]_i$ in the ER (internal stores) and directly activates Ca^{2+} activated second messengers as well as increases the cooperativity of vesicle fusion. Red filled vesicles are one full of transmitter. Orange are partly filled vesicles and blank ones are empty. RP, reserve pool; RRP, readily releasable pool; VGCC, voltage-gated calcium channel; SY, synapse; GluT, glutamate transporter; BA, bafilomycin A1; ES, endosome; Ca^{2+} ASMs, calcium activated second messengers.

preparations and high output *Drosophila* NMJs in populations of vesicles within the RP and RRP in their recycling regulation. Recently, it was demonstrated that only a small percentage of vesicles undergo exocytosis even at high output motor nerve terminals (Denker et al., 2011a,b). It would be of interest to further

investigate why there are fewer reserve vesicles in high output synapses and what the function of a large RRP is when a large percentage of them are apparently not used before synaptic depression occurs.

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