

Synaptic neuropeptide release by dynamin-dependent partial release from circulating vesicles

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ABSTRACT Neurons release neuropeptides, enzymes, and neurotrophins by exocytosis of dense-core vesicles (DCVs). Peptide release from individual DCVs has been imaged in vitro with endocrine cells and at the neuron soma, growth cones, neurites, axons, and dendrites but not at nerve terminals, where peptidergic neurotransmission occurs. Single presynaptic DCVs have, however, been tracked in native terminals with simultaneous photobleaching and imaging (SPAIM) to show that DCVs undergo anterograde and retrograde capture as they circulate through en passant boutons. Here dynamin (encoded by the *shibire* gene) is shown to enhance activity-evoked peptide release at the *Drosophila* neuromuscular junction. SPAIM demonstrates that activity depletes only a portion of a single presynaptic DCV's content. Activity initiates exocytosis within seconds, but subsequent release occurs slowly. Synaptic neuropeptide release is further sustained by DCVs undergoing multiple rounds of exocytosis. Synaptic neuropeptide release is surprisingly similar regardless of anterograde or retrograde DCV transport into boutons, bouton location, and time of arrival in the terminal. Thus vesicle circulation and bidirectional capture supply synapses with functionally competent DCVs. These results show that activity-evoked synaptic neuropeptide release is independent of a DCV's past traffic and occurs by slow, dynamin-dependent partial emptying of DCVs, suggestive of kiss-and-run exocytosis.

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INTRODUCTION

Neuropeptides, neurotrophins, and enzymes are packaged in dense-core vesicles (DCVs) for synaptic release by Ca²⁺-induced exocytosis. In contrast to rapid neurotransmission by small synaptic vesicles (SSVs) at active zones, peptidergic transmission by DCVs typically requires bouts of activity, occurs away from active zones, and is slow, suggesting that presynaptic SSVs and DCVs differ in their release properties. Based on green fluorescent protein (GFP) imaging in cultured cells, peptide release from single DCVs has

been detected at the neuron soma, growth cones, neurites, axons, and dendrites (Han *et al.* 1999; Ng *et al.*, 2003; Lochner *et al.*, 2006; de Wit *et al.*, 2009; Matsuda *et al.*, 2009; Xia *et al.*, 2009). These in vitro studies have shown variable properties for release from single DCVs, ranging from complete release, indicative of full-collapse fusion, to partial release, suggestive of kiss-and-run exocytosis, which is accepted for DCVs but controversial for SSVs (Wu *et al.*, 2014). However, activity-induced release from single neuronal DCVs has not been imaged in vivo from nerve terminals. Thus the kinetics and exocytic mechanism underlying peptidergic neurotransmission remain unknown.

Dynamin, in addition to its role in recycling SSVs by pinching off endocytic vesicles after exocytosis at active zones, participates in endocrine DCV exocytosis. For example, dynamin promotes kiss-and-run exocytic peptide release by pancreatic β -cells (Min *et al.*, 2007). Furthermore, many studies suggest that dynamin facilitates dilation of the fusion pore to promote kiss-and-run release from adrenal chromaffin cells (Fulop *et al.*, 2008; Anantharam *et al.*, 2011; Gonzalez-Jamett *et al.*, 2013; Trouillon and Ewing, 2013). However, dynamin has also been proposed to limit release of catecholamines (Graham *et al.*, 2002) and tissue plasminogen activator (Tsuboi *et al.*, 2004), the latter of which is slow because of its interaction with the fusion

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Abbreviations used: ANF, atrial natriuretic factor; DCV, dense-core vesicle; Dilp2, *Drosophila* insulin-like peptide 2; NMJ, neuromuscular junction; *shi*, *shibire*; SPAIM, simultaneous photobleaching and imaging.

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pore from inside the DCV (Weiss *et al.*, 2014). Given these diverse effects in endocrine cells, it is interesting that dynamin's role in synaptic neuropeptide release is unknown.

The lack of mechanistic understanding of presynaptic DCV exocytosis during peptidergic neurotransmission led us to image the intact *Drosophila* larval neuromuscular junction (NMJ). Type Ib motoneuron terminals in this preparation possess glutamatergic SSVs and DCVs containing neuropeptides and a bone morphogenic protein (Anderson *et al.*, 1988; James *et al.*, 2014). Furthermore, a temperature-sensitive dynamin mutant (*shibire^{ts1}* [*shi^{ts1}*]) is effective at this synapse (e.g., Ramaswami *et al.*, 1994). Finally, simultaneous photobleaching and imaging (SPAIM) can track individual presynaptic DCVs for minutes in the intact *Drosophila* NMJ (Wong *et al.*, 2012). Although SPAIM was used to understand the supply of DCVs to en passant boutons by vesicle circulation and capture, we reasoned that this method could be used to detect release from individual presynaptic DCVs. Here experiments with mutant dynamin animals and SPAIM are performed to study neuropeptide release at the intact NMJ. Activity is found to induce dynamin-dependent partial emptying of DCVs. Kinetic and traffic studies are integrated to address how activity induces slow sustained synaptic peptide release.

RESULTS

Dynamin enhances synaptic peptide release

To test whether dynamin affects activity-dependent neuropeptide release at the *Drosophila* NMJ, we expressed GFP-tagged prepro-*Drosophila* insulin-like peptide 2 (Dilp2) in wild-type (WT) animals and the temperature-sensitive dynamin mutant *shi^{ts1}*. Release at the larval neuromuscular junction was evoked by tetanic nerve stimulation (70 Hz, 1 min) at 22°C (i.e., a permissive temperature) or after heating to 30°C to acutely inactivate *shi^{ts1}*. Imaging showed that the temperature change did not affect synaptic neuropeptide release in WT animals (Figure 1, A and B). However, temperature-dependent dynamin inhibition halved activity-evoked peptide release (Figure 1, C and D). Temperature-dependent dynamin inhibition does not affect presynaptic Ca²⁺ responses to activity (Macleod *et al.*, 2004). Therefore, at a native synapse, dynamin enhances activity-evoked peptide release.

Fractional release from synaptic DCVs

Dynamin dependence stimulated us to consider the hypothesis that neuropeptide release occurs by kiss-and-run exocytosis. Therefore we set out to determine whether release incompletely empties single presynaptic DCVs. However, because total internal reflection microscopy and oil immersion objectives are not amenable to imaging the intact NMJ in filleted larva, simultaneous photobleaching and imaging (SPAIM) on a confocal microscope (Wong *et al.*, 2012) was used to image nerve terminal DCVs. Of importance, a single punctum in these experiments likely corresponds to a single DCV. First, previous analysis suggested that a single fluorescent punctum represents a single DCV detected by electron microscopy (Shakiryanova *et al.*, 2006). Second, in SPAIM experiments, DCVs are tracked as they enter a large photobleached area, are transported rapidly into a bouton, and eventually become immobilized (i.e., docked) before being stimulated to undergo exocytosis. Throughout this extended tracking, puncta never split or merged, suggesting that they comprise one DCV. Indeed, to our knowledge, there is no evidence from electron microscopy or subresolution light microscopy (Scalettar *et al.*, 2014) that demonstrates that neuronal DCVs travel or localize in clusters. Therefore SPAIM was used to analyze release from single DCVs.

In unstimulated preparations, Dilp2-GFP DCV fluorescence did not change (Figure 2Ai). Furthermore, although nerve stimulation (70 Hz, 60 s) did not affect all DCVs (Figure 2Aii), most DCVs released in response to activity (Figure 2Aiii). Indeed, for DCVs located near the bouton edge in the plane of focus, 20 of 22 DCVs responded. Therefore synaptic neuropeptide release is initiated reliably by activity, especially for membrane proximal DCVs. However, quantification of release in 33 DCVs showed that depletion was always incomplete (i.e., <50%; Figure 2B).

Variable kinetics of synaptic peptide release by individual DCVs

Further insights came from examining the kinetics of release from individual Dilp2-GFP DCVs. In unstimulated preparations, time courses were noisy but showed no significant change (Figure 3Ai). Time courses of some DCVs looked similar to unstimulated controls (Figure 3Aii) implying that they did not respond. However, most DCVs displayed release responses soon after initiation of activity (Figure 3, iii–v, dotted line shows start of tetani). However, responses had varying kinetics: in some cases, there were stepwise changes in content, implying rapid release (Figure 3Aiii), whereas others were slower stepwise (Figure 3Aiv) or gradual (Figure 3v) events. The latter events were irreversible, monotonic, and not correlated with movement, precluding an artifact due to increased DCV diffusion as occurs with mobilization (Shakiryanova *et al.*, 2005). Furthermore, because *Drosophila* SSVs and DCVs are not very acidic (Sturman *et al.*, 2006) and the emerald GFP variant used here is not sensitive to pH, these responses cannot be attributed to proton flux through the fusion pore. Rather, slow release is likely limited by permeation through the fusion pore or dispersion in the synaptic cleft. Of greatest importance, these data show that the kinetics of activity-evoked partial peptide release from single DCVs at a native synapse is surprisingly variable.

Postfusion steps limit the rate of synaptic peptide release

To further analyze release kinetics, ensemble averages from single Dilp2-GFP DCV records were calculated. Of interest, the extent of release deduced from single DCVs (Figure 3B) was comparable (~20%) to that obtained from macroscopic whole-bouton measurements (Figure 3C). Similarly, release kinetics was similar for ensemble-averaged single-DCV data (Figure 3B) and data from whole boutons (Figure 3C). Thus single DCVs revealed by SPAIM, which arrived within minutes, are representative of the typical behavior of the hundreds of DCVs present in type Ib boutons that have a half-life of many hours (Shakiryanova *et al.*, 2006). This finding is surprising, given the less efficient release of older DCVs in endocrine cells (e.g., Duncan *et al.*, 2003).

DCVs in cultured hippocampal neurons have a long latency for the onset of individual release events ($\tau = 16$ s; Xia *et al.*, 2009). If the onset of exocytosis was rate limiting for activity-dependent peptide release at the NMJ, then latencies in single Dilp2-GFP DCV records should have a mean similar to the time constant derived from an exponential fit of ensemble averaged records (21.5 s in Figure 3B). However, the latency of onset of unitary synaptic Dilp2-GFP events at the NMJ was much shorter (2.4 ± 0.5 s, $N = 25$). The dramatic difference in values derived from the latency in single DCV records and the time constant from ensemble averages and the individual DCV time courses (Figure 3A) shows that initiation of exocytosis is rapid compared with subsequent slow release.

Multiple rounds of release from single presynaptic DCVs

Resident DCVs in the *Drosophila* NMJ turn over slowly ($t_{1/2} \sim 6$ h; Shakiryanova *et al.*, 2006). Therefore maintaining peptidergic

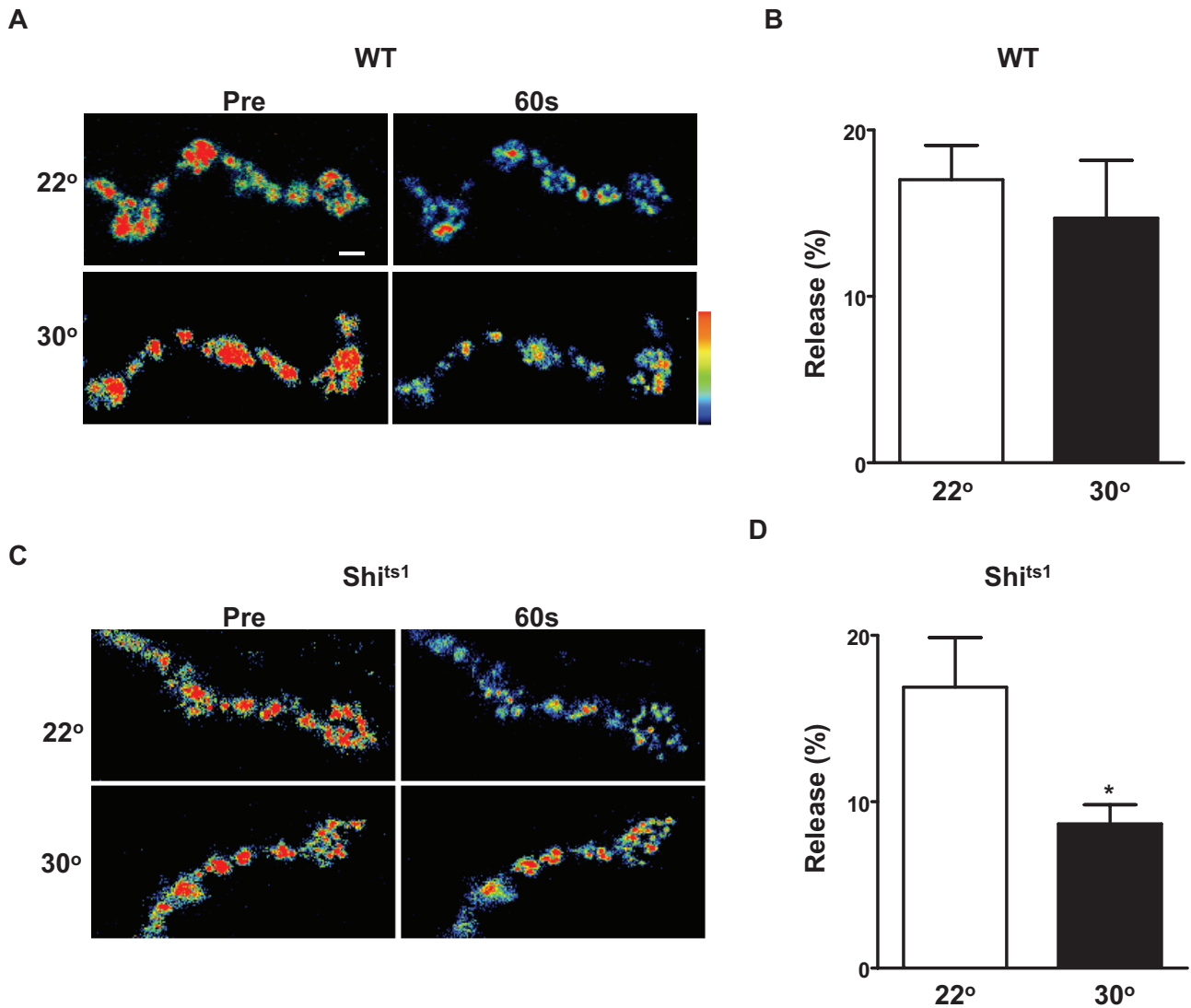


FIGURE 1: Dynamin enhances activity-dependent synaptic neuropeptide release. (A) Pseudocolor images of Dilp2-GFP in WT motoneuron synaptic boutons before and after 60 s of activity at 22°C (top) or 30°C (bottom). Bar, 2 μ m. (B) Quantification of activity-induced release by WT boutons at 22°C ($N = 7$) and 30°C ($N = 5$). Note absence of temperature effect. (C) Pseudocolor images of Dilp2-GFP in *shi^{ts1}* motoneuron synaptic boutons before and after 60 s of activity at 22°C (top) or 30°C (bottom). Bar, 2 μ m. (D) Quantification of the effect of temperature on synaptic peptide release at *shi^{ts1}* NMJs ($N = 10$ for 22°C; $N = 11$ for 30°C). * $p < 0.05$.

transmission would be problematic if each DCV could undergo exocytosis only once. In fact, no such limitation was detected at the intact NMJ. First, the effect of stimulating motor nerves twice with 10-s tetani was examined. Specifically, an ensemble average was generated from Dilp2-GFP DCVs that produced a stepwise response to the first bout of stimulation. Examination of the resultant time course showed that, when stimulated again 1 min later, a second response was elicited (Figure 4A). Indeed, two separate responses could be detected from a single DCV (Figure 4B). Second, single Dilp2-GFP DCVs subjected to prolonged tetanic stimulation (e.g., for 60 s) sometimes (5 of 33 DCVs) produced two responses (Figure 4C, i and ii). Such double responses are unlikely to have been produced by two independent, coincidentally colocalized DCVs because, as described earlier, a single punctum likely corresponds to a single DCV. Furthermore, the latency to first exocytosis is short (see earlier discussion) and never exceeded 9 s, but longer

latencies were sometimes evident for the second event evoked by a prolonged tetanus (Figure 4C). This would not be expected for two independent DCVs but is consistent with repeated kiss-and-run release from a single DCV with loss of VAMP (synaptobrevin) and synaptotagmin (Tsuboi et al., 2004). In addition, the size of responses varied dramatically between DCVs but not among two responses in a single experiment (i.e., large events in Figure 4Ci, small events in Figure 4Cii), which is not expected for independent DCVs. Finally, there was no correlation between fluorescence intensity and the presence of two release events even though a pair of DCVs should have double the fluorescence of a single DCV. Therefore the simplest interpretation of the experimental data is that single DCVs can undergo two rounds of activity-evoked partial release.

The capacity for multiple responses (Figure 4) and the range of single responses (Figure 3A) suggest that the three peaks in the histogram (Figure 2B) can be interpreted as representing

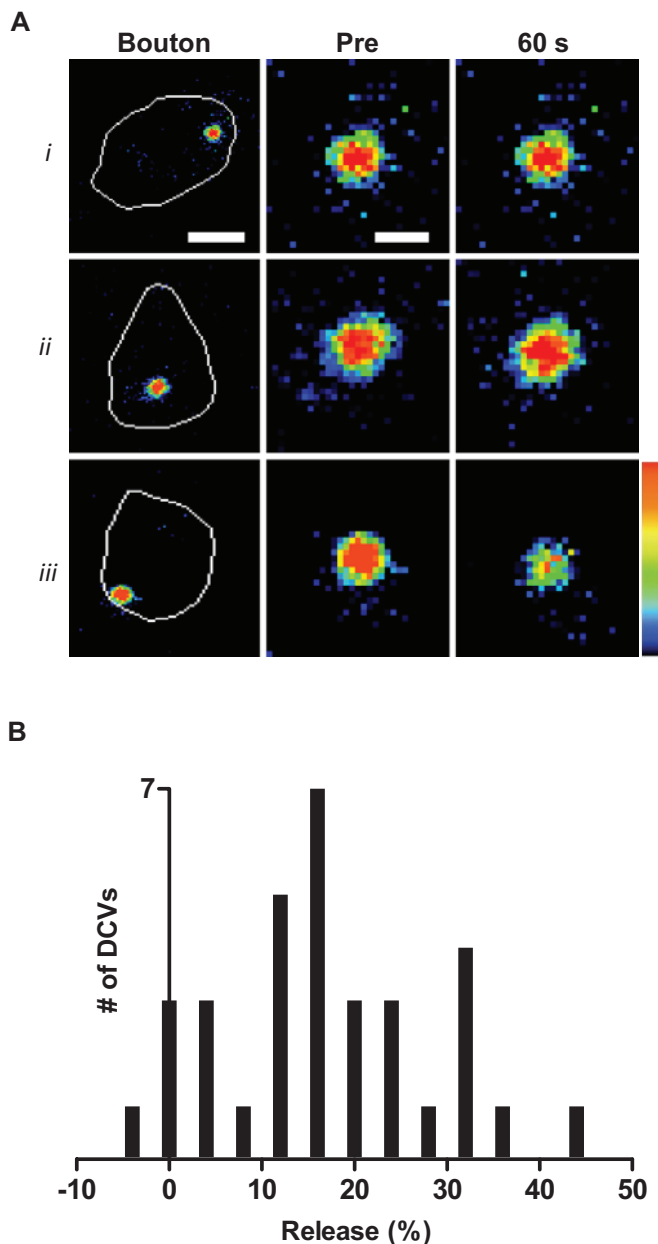


FIGURE 2: Activity evokes partial emptying of DCVs in the nerve terminal. (A) Pseudocolor images of single Dilp2-GFP DCVs in type Ib boutons, which are outlined in white in left images, before (Pre) and after 60 s. (i) DCV in an unstimulated terminal; (ii) unresponsive DCV localized in a bouton that was stimulated for 60 s; (iii) responsive DCV in a bouton that was stimulated for 60 s. Note the drop in signal for iii only. Bars, 1.75 μm (bouton images on the left), 0.5 μm (magnified DCV images). (B) Histogram quantifying the range of responses to stimulation in 33 DCVs. No DCV released more than half of its neuropeptide content.

unresponsive DCVs, DCVs that responded to activity once with $\sim 16\%$ release, and DCVs that responded twice with $\sim 32\%$ release (i.e., 2×16). In terms of synaptic function, multiple bouts of partial release by individual DCVs are significant because they further sustain activity-evoked release and provide a means for DCVs to function throughout their long lives in the nerve terminal. Finally, multiple release events are notable because they exclude a model in

which DCVs completely empty by full-collapse fusion but peptide is retained at the membrane due to failure to disperse (i.e., binding); in such a model, a second response could not be initiated because peptide is already outside of the bouton after the first round of exocytosis. Therefore, although there may be some effect of slow or incomplete dispersion after release, multiple unitary responses demonstrate that release is partial and can occur repeatedly from a single presynaptic DCV to sustain peptidergic transmission.

Newly arrived DCVs are competent for activity-evoked synaptic release

On entering synaptic boutons, some DCVs are captured, whereas others visit the bouton transiently (i.e., they enter the bouton and then leave within 2 min; Wong *et al.*, 2012). However, release from each of these subpopulations has not been measured. Therefore, to distinguish whether captured DCVs are optimized for release, atrial natriuretic factor (ANF)-GFP DCV records were sorted based on whether DCVs at the onset of stimulation had been in a bouton for at least 2 min (i.e., the threshold for capture) or < 1 min. To simplify kinetic analysis and improve signal-to-noise ratio for these dimmer vesicles, we calculated ensemble time courses using data combined in 5-s periods (i.e., time binned) for the top 70% of responses (i.e., for ANF-GFP DCVs, $> 18\%$ release identified responders). Furthermore, to take into account that not all DCVs fell into this class, we also quantified the fraction of responders (i.e., release probability). This quantification showed that release probability, kinetics, and extent were similar in visiting and captured DCVs (Figure 5, A and B). Therefore DCVs are competent for synaptic release soon after their arrival in synaptic boutons.

Peptide release is consistent throughout the terminal

Synaptic release of glutamate packaged in SSVs is greatest at the most distal bouton, which is characterized by higher Ca^{2+} responses to single action potentials and tetani (Guerrero *et al.*, 2005; Lnenicka *et al.*, 2006). To determine whether location in the terminal affects synaptic neuropeptide release similarly, we sorted single anterograde ANF-GFP DCV records according to the bouton location at which capture occurred; the most distal bouton was assigned number 1, and proximal boutons were numbered in order (numbers 2–4). This analysis revealed no obvious difference in release probability among the most distal and proximal boutons (Figure 6A). Similarly, the time course and average release by responders were indistinguishable between proximal and most distal boutons (Figure 6B). Therefore, in contrast to glutamatergic transmission by SSVs at active zones, there is no detectable difference in activity-induced synaptic neuropeptide release based on location in the terminal.

DCVs supplied by anterograde and retrograde transport are competent for release

DCVs circulate bidirectionally through the motoneuron terminal (Shakiryanova *et al.*, 2006; Wong *et al.*, 2012). However, the release properties of anterograde and retrograde DCVs are unknown. Therefore SPAIM experiments were set up to examine whether the direction of entry into the bouton affects release. Ensemble averages from single ANF-GFP DCVs showed that release probability (Figure 7A) and time course and extent of release (Figure 7B) were unaffected by whether the DCVs entered the bouton by anterograde or retrograde transport. This functional equivalence implies that bidirectional capture of circulating DCVs effectively supplies en passant boutons with functionally competent DCVs for activity-induced synaptic neuropeptide release.

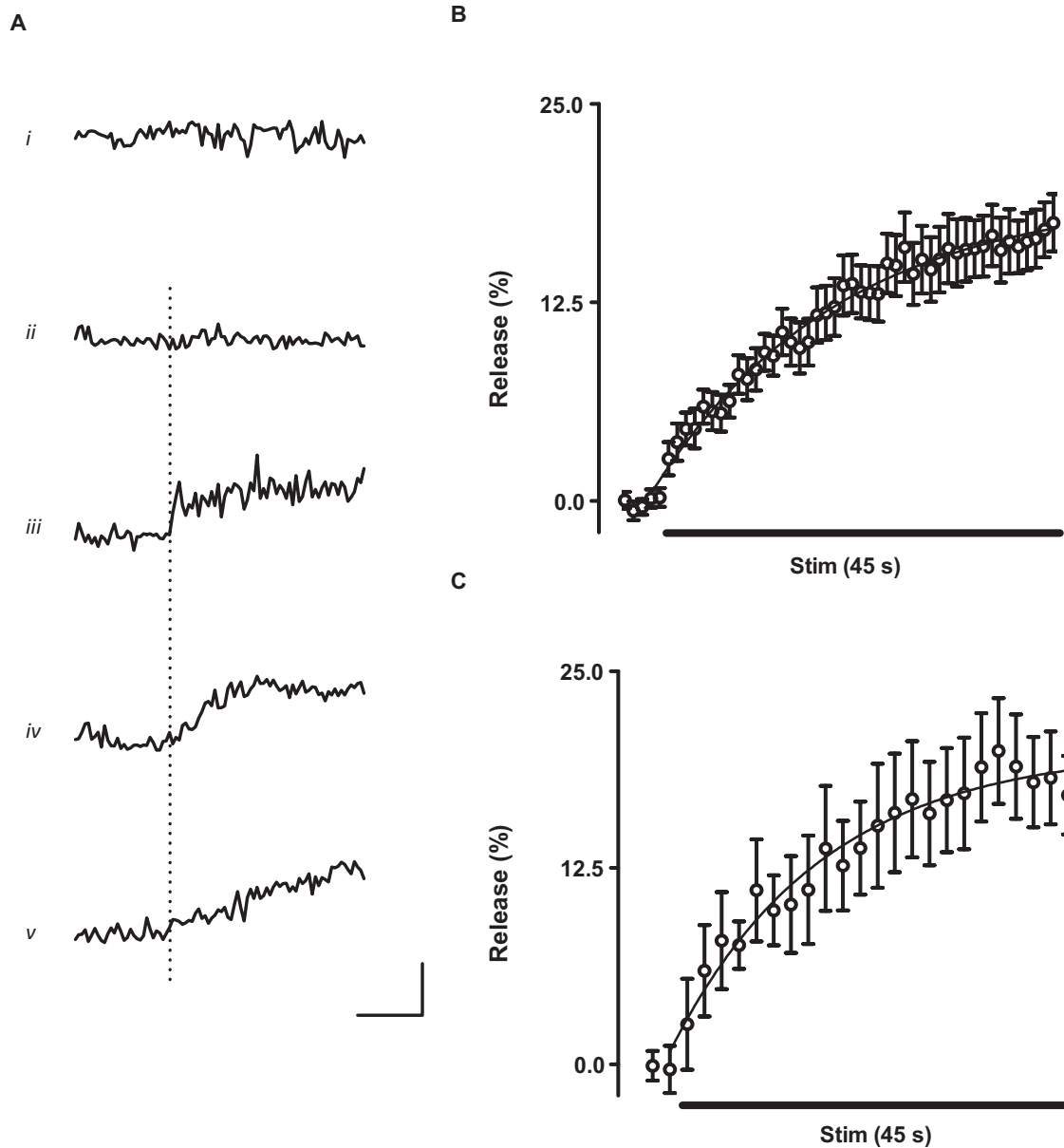


FIGURE 3: Kinetics of neuropeptide release from single DCVs. (A) Time courses from single Dilp2-GFP DCVs. Horizontal bar, 20 s; vertical bar, 25%. Dotted line indicates start of 70-Hz stimulation. Release is plotted as a positive (upward) change. (i) Data from an unstimulated synapse; (ii–v) Data from stimulated synapses. (B) Ensemble-average time course from 33 DCVs. Curve is a single-exponential fit of data ($\tau = 21.5$ s). (C) Average response from seven boutons. Curve is a single-exponential fit of data ($\tau = 17.6$ s). Horizontal bars in B and C indicate 45 s of 70-Hz stimulation.

DISCUSSION

Previous studies with cultured cells revealed great variation in single-DCV-mediated peptide release between experimental preparations. Even with the same peptide (ANF-GFP), extent and latency to initiation of release varied greatly according to cell type and neuronal compartment (Han *et al.*, 1999; Barg *et al.*, 2002; Duncan *et al.*, 2003; Ng *et al.*, 2003; Xia *et al.*, 2009). However, past *in vitro* studies did not examine nerve terminals, the location of peptidergic synaptic transmission. Furthermore, results in culture may have been affected by the absence of the normal surroundings found *in vivo* that could influence release and subsequent dispersal of neuropeptides in the synaptic cleft. Finally, many prior studies used unphysiological sustained stimuli, such as bath-applied K^+ , nicotine, and

long-lasting voltage clamp steps. Therefore, here, peptide release from single DCVs was induced by activity at the intact *Drosophila* NMJ. Imaging showed that synaptic neuropeptide release from single DCVs is dependent on dynamin and always incomplete, which are traits associated with kiss-and-run exocytosis (Wu *et al.*, 2014). The limited depletion of DCVs is reminiscent of depolarization-evoked release from the hippocampal neuron soma (Xia *et al.*, 2009). However, the latency to initiation of release was closer to that found with robust release from endocrine DCVs (Barg *et al.*, 2002) than from cultured hippocampal neurons (Xia *et al.*, 2009). Indeed, initiation of exocytosis is not rate limiting, as electrical activity reliably initiates synaptic peptide release and can induce by multiple rounds of partial emptying of DCVs. Thus synaptic release can be

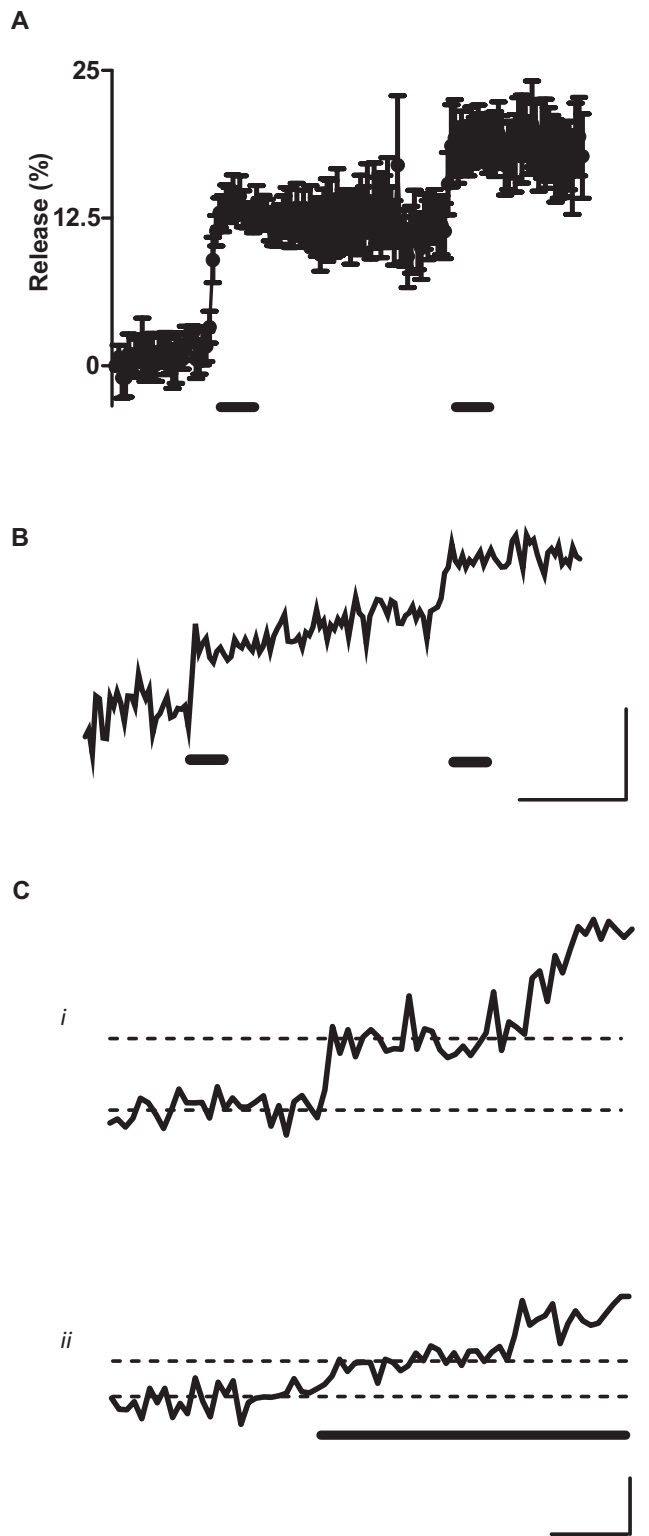


FIGURE 4: A single DCV can release twice. (A) Ensemble average from Dilp2-GFP DCVs stimulated twice for 10 s at 70 Hz (indicated by thick horizontal bars). Data are from six DCVs that displayed stepwise responses to the first tetanus. (B) Single-DCV time course with two stepwise responses to the double stimulation protocol in A. (C) Time courses of single Dilp2-GFP DCVs responding twice during a single prolonged tetanus (indicated by thick horizontal bar). Both responses were relatively large in i and small in ii. Horizontal dashed lines distinguish the baseline and signal after the first release event. Thin horizontal bars, 10 s; thin vertical bars, 10% release.

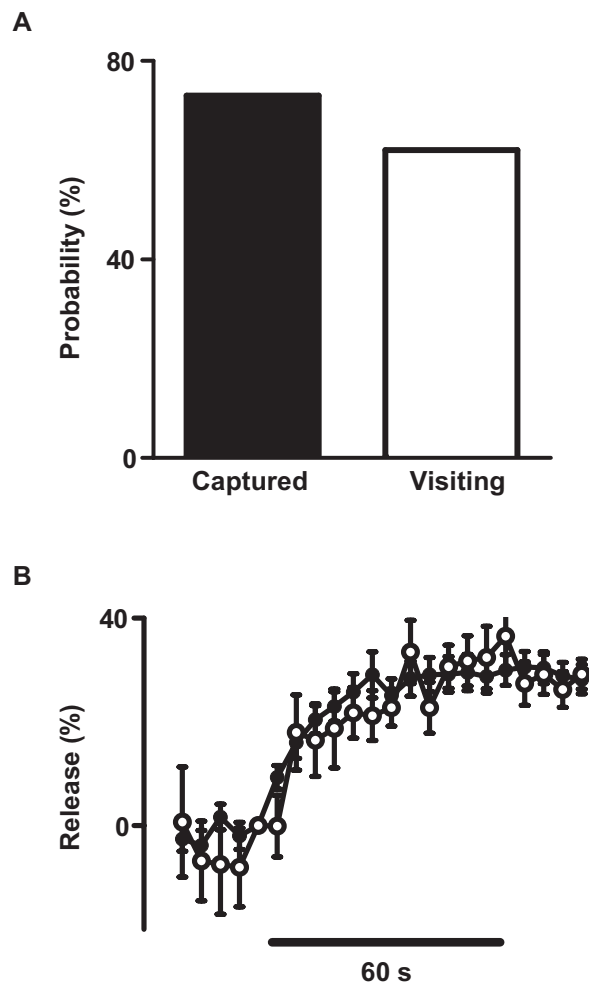


FIGURE 5: Comparable release from visiting and captured DCVs. At the onset of tetanic stimulation, visiting DCVs had been present in the bouton for <1 min, whereas captured DCVs had been present >2 min. (A) Release probability for 26 captured and 16 visiting ANF-GFP DCVs. (B) Time courses of release from 19 captured (filled circles) and 8 visiting (open circles) ANF-GFP DCVs.

sustained without completely emptying DCVs, which bypasses the need to replace every used DCV by axonal transport from the distant soma. In this way, the apparent exclusive use of kiss-and-run exocytosis (and the corollary absence of full-collapse fusion) is well suited for peptidergic transmission.

Of interest, variable release kinetics from single DCVs had not been reported previously based on expression of GFP-tagged neuropeptides (e.g., neuropeptide Y and ANF). However, slow neuropeptide release can be produced by other peptides packaged in the same DCV (de Wit *et al.*, 2009). Such an effect could reflect action from the DCV lumen to regulate fusion pore dilation (Weiss *et al.*, 2014) or prevention of peptide dispersion from the matrix after release (de Wit *et al.*, 2009). Alternatively, variability in release could occur with a single cargo if fusion pore dilation and peptide dispersion are affected by the highly structured synaptic cleft, which features many synapse-spanning proteins. Regardless of its cause, the relatively slow release from presynaptic DCVs is, along with partial emptying and repeated exocytosis, well suited for peptidergic neurotransmission and release of neurotrophic factors and enzymes that influence synaptic development, maintenance, and remodeling.

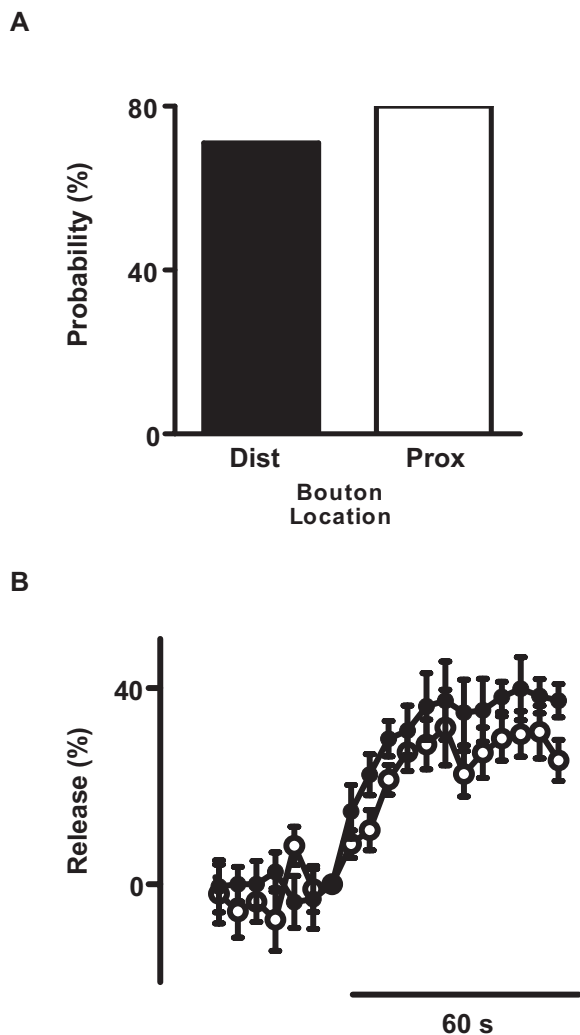


FIGURE 6: Single-DCV release is similar in proximal and the most distal boutons. (A) Single ANF-GFP DCV release probability in the most distal bouton (Dist, $N = 7$) and proximal boutons 2–4 (Prox, $N = 10$). (B) Ensemble-average time course from five distally (closed circles) and eight proximally (open circles) captured ANF-GFP DCVs. Data were time binned in 5-s intervals. All DCVs here arrived by anterograde transport.

Imaging single presynaptic DCVs was also used to determine the dependence of release on location in the nerve terminal. In contrast to SSV-mediated transmission in the same preparation (Guerrero *et al.*, 2005), peptide release was not polarized (i.e., greater at the most distal bouton). This was surprising because the location dependence of glutamate release was attributed to differences in presynaptic Ca^{2+} (Guerrero *et al.*, 2005; Lnenicka *et al.*, 2006), which also triggers DCV exocytosis. However, a toxin that blocks Ca_v2 channels and SSV exocytosis does not affect neuropeptide release at the larval NMJ (James *et al.*, 2014), suggesting that another Ca^{2+} channel mediates DCV exocytosis. Hence, total Ca^{2+} may not reflect the activity of channels that selectively mediate synaptic neuropeptide release, which occurs away from active zones (Zupanc, 2006). Of interest, lack of polarized release fits with the fact that released peptides can diffuse over large distances to produce neuromodulation.

This study explored whether single DCV release is affected by routing to or residence time in synaptic boutons. Traditionally, DCVs

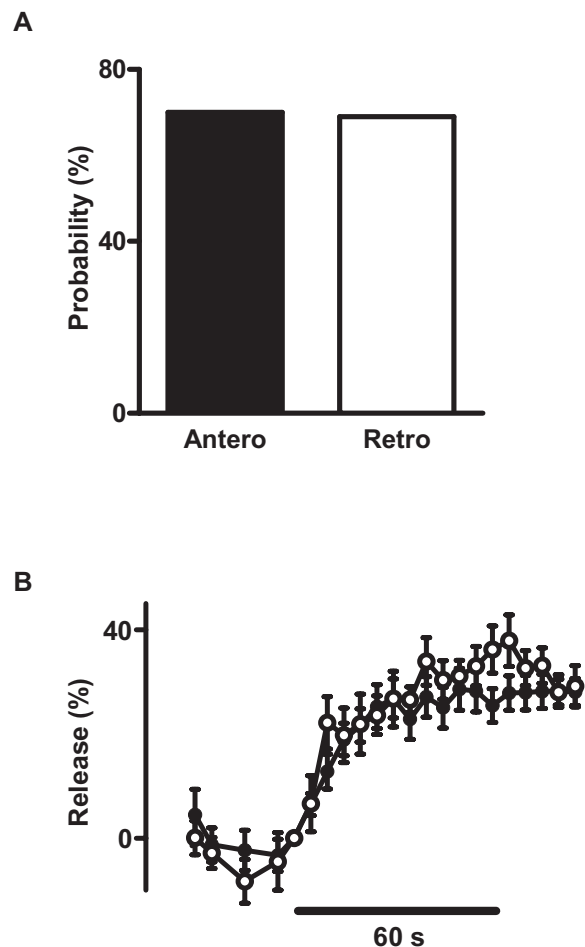


FIGURE 7: DCVs delivered by anterograde and retrograde transport are functionally equivalent. (A) Single ANF-GFP release probability for DCVs that entered boutons by anterograde (Antero, $N = 23$) or retrograde (retrograde, $N = 16$) transport. (B) Ensemble-average time course from 16 anterograde (filled circles) and 11 retrograde (open circles) ANF-GFP DCVs. Data were time binned in 5-s intervals.

were believed to be delivered to the nerve terminal by anterograde axonal transport, with the time for transitioning between axonal transport and capability for release being unknown. However, SPAIM experiments recently revealed that DCVs are distributed among en passant boutons by being captured as they circulate back and forth between the proximal axon and the most distal bouton in the terminal (Wong *et al.*, 2012). In the type Ib boutons studied here, capture is inefficient, so that DCVs often visit boutons only transiently. Indeed, it is the combination of routing by vesicle circulation and inefficient capture that ensures that DCVs are distributed evenly to en passant boutons (Wong *et al.*, 2012; Kuznetsov and Kuznetsov, 2013; Bulgari *et al.*, 2014). The effectiveness of this mechanism in supporting peptidergic transmission depends, however, on the maintenance of DCV functionality while circulating over long distances. However, the effect of vesicle circulation on DCV competence for release was unknown. Here this issue was addressed by showing that anterograde and retrograde DCVs have indistinguishable release properties. Thus vesicle circulation and bidirectional capture effectively provide functionally competent DCVs for activity-dependent synaptic peptide release.

Single-DCV experiments also suggest that newly arrived DCVs are as functionally competent as resident DCVs. First, the time

course and extent of release deduced from ensemble averages deduced from single DCVs that had arrived within minutes after photobleaching were comparable to release measured by imaging whole boutons, which are dominated by DCVs that are hours old (Shakiryanova *et al.*, 2006). Second, sorting of single-DCV records showed that release is similar for newly arrived DCVs (<1 min) and DCVs that were in boutons for >2 min, the threshold for capture (Wong *et al.*, 2012). Therefore the transition from axonal transport to capability for exocytosis occurs rapidly upon entering the bouton. This finding supports the proposal that activity-dependent capture supplies DCVs to immediately support release (Shakiryanova *et al.*, 2006; Frischknecht *et al.*, 2008). In addition to maintaining release of constitutively expressed neuropeptides, the secretory competence of newly arrived DCVs also reduces the delay between synthesis of newly induced neuropeptides and their release. Such efficiency is desirable because many neuropeptide genes are subject to dynamic transcriptional control. Therefore the competence of recently arrived DCVs may enable peptidergic transmission to rapidly reflect plasticity in neuropeptide gene expression.

Despite this utility, the functional equivalence of recently arrived and resident DCVs was not expected, as newly synthesized DCVs dock and release more efficiently in endocrine cells (Duncan *et al.*, 2003). This difference could reflect the increase in cytoplasmic DCV mobility induced by activity in nerve terminals (Shakiryanova *et al.*, 2005, 2007), which may allow resident and newly arrived DCVs to dock to the membrane equivalently. In other words, when activity is intense, nerve terminal DCVs are not held in reserve by immobilization in the cytoplasm (Burke *et al.*, 1997). In addition, it should be noted that because of vesicle circulation (Wong *et al.*, 2012), newly arrived DCVs may have not been newly synthesized. Apparently, to allow axonal transport to terminals, which can take days in humans, the effect of time on neuronal DCVs is effectively suspended to prevent the age discrimination found in endocrine cells.

The *shits1* dynamin mutant completely halts endocytosis and fast transmission by SSVs at active zones but was shown here to attenuate but not abolish synaptic neuropeptide release. This is of practical interest because selective expression of *shits1* is used to identify neurons that affect behavior in *Drosophila* (Kitamoto, 2001). Our results show, however, that this mutant cannot distinguish whether a neuron influences behavior by virtue of its SSVs or DCVs. They also suggest that dynamin is not absolutely required for synaptic peptide release but instead plays a regulatory role for DCVs. In agreement with this proposal, preliminary single-DCV experiments failed to reveal a qualitative change in peptide release kinetics with the reduced release produced by inhibiting dynamin. Therefore more investigation will be required to fully discern the function of dynamin on synaptic DCVs (e.g., to test for effects on fusion pore dilation and docking). Nevertheless, the studies here establish *shits1* as a tool for studying peptidergic transmission and demonstrate that dynamin facilitates synaptic DCV-mediated peptide release, which is mediated exclusively by kiss-and-run exocytosis.

MATERIALS AND METHODS

Stimulation of peptide release

Prior *in vitro* studies detected robust release of ANF-GFP from single DCVs in chromaffin cells, undifferentiated PC12 cells, and differentiated PC12 cell growth cones and more limited ANF-GFP release from cultured hippocampal neurons (Han *et al.*, 1999; Barg *et al.*, 2002; Duncan *et al.*, 2003; Ng *et al.*, 2003; Xia *et al.*, 2009). However, the stimuli used in these studies are not amenable to imaging transgenically expressed ANF-GFP at the native *Drosophila* NMJ: bath-applied K⁺ directly evokes muscle contraction that inter-

feres with presynaptic imaging, nicotine is not effective at this glutamatergic synapse, and type Ib synaptic boutons, which are surrounded by the subsynaptic reticulum of the postsynaptic muscle, have not been voltage clamped. Therefore, as described previously (Levitan *et al.*, 2007), activity-evoked release at *Drosophila* third-instar larval type Ib boutons on muscle 6/7 was induced by 70-Hz electrical nerve stimulation in the presence of extracellular glutamate to desensitize postsynaptic receptors and inhibit muscle contraction.

Imaging single DCVs

Fluorescent puncta likely representing single DCVs (Shakiryanova *et al.*, 2006) entering type Ib boutons were imaged at 1 Hz by SPAIM on an upright Olympus (Center Valley, PA) FluoView 1000 microscope with a dipping water immersion objective (60x, numerical aperture 1.1) as described previously (Wong *et al.*, 2012). Because DCVs had to stay in view for >1 min in these experiments, studied DCVs tended to display little mobility. The axial resolution of these optics (i.e., the vertical thickness of an optical section) is comparable to the size of a bouton. Therefore it was not possible to ascertain whether a DCV was close to the bouton plasma membrane above or below the plane of focus. However, resolution in the plane of focus is much greater, making it possible to detect when DCVs were relatively close to the bouton edge that was evident before bleaching. Limitations in spatial resolution, however, do not affect measuring changes of fluorescence intensity, which are instead limited by the signal-to-noise ratio. Single DCVs in *elav-GAL4 UAS-ANF-GFP* (*elav>ANF-GFP*) larva (Rao *et al.*, 2001) are dim. Therefore 1-Hz data from those animals were averaged in 5-s periods to improve signal-to-noise ratio. Furthermore, time courses included only DCVs that stayed within a bouton throughout the whole period shown.

To produce brighter DCVs with greater signal-to-noise ratio, a new chromosome 2 recombinant line was generated featuring a stronger motoneuron driver (*OK6-GAL4*) and a construct based on inserting GFP into *Drosophila* proinsulin-like peptide 2 (Wong *et al.*, 2012), which is released natively by neurons. In the latter line (i.e., *OK6>UAS-Dilp2-GFP/CyO*), fluorescence from single DCVs in type Ib boutons is approximately double that seen in *elav>ANF-GFP* animals.

Because the emerald GFP variant used in both constructs is insensitive to pH, release was measured as the percentage loss in fluorescence ($100 \Delta F/F_0$, where $\Delta F = F_0 - F_t$). Accordingly, the loss of GFP fluorescence is quantified as an increase in release (i.e., a positive number). Peptide release was comparable in the two lines.

Dynamin mutant experiments

Drosophila expresses only one gene for dynamin, *shibire* (*shi*). Because the temperature-sensitive *shits1* mutation is recessive and located on the X chromosome, the X chromosome recombinant *elav>ANF-GFP* line was not convenient for examining the effect of dynamin on peptide release. Therefore single crosses were performed with *OK6>UAS-Dilp2-GFP/CyO* males and virgin *shits1* females at 18°C. GFP-expressing male larva (i.e., *shits1/y; Ok6-GAL4 UAS-Dilp2-GFP/+*) were used for confocal imaging of boutons, which was performed at 22°C or after heating to 30°C. Dynamin inhibition at the elevated temperature in these flies was verified by the block of depolarization-evoked FM4-64 (Molecular Probes, Eugene, OR) uptake into SSVs (Ramaswami *et al.*, 1994).

Statistics

Data are presented as mean \pm SEM. Pairwise statistics were calculated with the Student's *t* test.

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