Synaptic neuropeptide release induced by octopamine without Ca^{2+} entry into the nerve terminal

Dinara Shakiryanova[a], Geoffrey M. Zettel[a], Tingting Gu[b], Randall S. Hewes[b], and Edwin S. Levitan[a,1]

[a]Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, PA 15261; and [b]Department of Zoology, University of Oklahoma, Norman, OK 73019

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Synaptic release of neurotransmitters is evoked by activity-dependent Ca^{2+} entry into the nerve terminal. However, here it is shown that robust synaptic neuropeptide release from *Drosophila* motoneurons is evoked in the absence of extracellular Ca^{2+} by octopamine, the arthropod homolog to norepinephrine. Genetic and pharmacology experiments demonstrate that this surprising peptidergic transmission requires cAMP-dependent protein kinase, with only a minor contribution of exchange protein activated by cAMP (epac). Octopamine-evoked neuropeptide release also requires endoplasmic reticulum Ca^{2+} mobilization by the ryanodine receptor and the inositol trisphosphate receptor. Hence, rather than relying exclusively on activity-dependent Ca^{2+} entry into the nerve terminal, a behaviorally important neuromodulator uses synergistic cAMP-dependent protein kinase and endoplasmic reticulum Ca^{2+} signaling to induce synaptic neuropeptide release.

GCaMP | GFP | monoamine | neuromuscular junction | neurotransmission

Neuromodulators induce presynaptic signaling to regulate fast neurotransmission triggered by activity-induced Ca^{2+} entry into the nerve terminal. Neuromodulators also influence the very low rate of spontaneous quantal release of classical transmitters. However, because spontaneous release is functionally relevant only in specialized cases (1), neuromodulators are not believed to typically induce physiologically significant release in the absence of extracellular Ca^{2+}. Studies of endocrine cells suggest that release of peptides packaged in large dense-core vesicles (LDCVs) also requires Ca^{2+} entry because of the rarity of spontaneous LDCV fusion and the inefficient permeation of peptides through the LDCV-fusion pore (2, 3). However, because it is difficult to measure peptide release at intact synapses, a much more limited dataset supports the conclusion that Ca^{2+} influx into the nerve terminal is absolutely required for peptidergic transmission. Thus, it remains unclear how neuromodulators control synaptic neuropeptide release.

With this background in mind, we set out to study regulation of neuropeptide release at the *Drosophila* neuromuscular junction (NMJ) by octopamine-induced cAMP signaling. Octopamine, the homolog of norepinephrine in *Drosophila*, controls many behaviors by activating central G protein-coupled receptors that induce adenylyl cyclase activation and intracellular Ca^{2+} release (4, 5). Octopamine is also in NMJ boutons at muscles 12 and 13 and can have complex effects at adjacent muscle 6 and 7 NMJs: octopamine inhibits phasic transmission and facilitates tonic release induced by K^+, with the latter effect requiring cAMP-dependent protein kinase (PKA) (6–8). *Drosophila* motoneurons also contain neuropeptides, which are released in response to nerve stimulation, depolarization, and developmental cues (9–13). However, the impact of octopamine and cAMP on synaptic neuropeptide release is unknown, even though such regulation could be relevant to the effects of octopamine on central peptidergic neurons involved in controlling metabolism, development, and circadian behavior (14, 15).

Experiments in *Drosophila* have established that the fluorescence from presynaptically expressed GFP-tagged atrial natriuretic factor (AnfGFP) reports native neuropeptide content and release (12, 16–19). Here, this optical assay was applied to *Drosophila* larvae to study regulation of neuropeptide release at the muscle 6/7 NMJ. Although the expectation was that Ca^{2+} entry would be required, octopamine was found to induce robust synaptic neuropeptide release in the absence of extracellular Ca^{2+}. Genetics and pharmacology were then combined to identify the presynaptic signaling responsible for this noncanonical neurosecretion.

Results

Synaptic neuropeptide release at the muscle 6/7 NMJ was measured as the percentage-change in AnfGFP fluorescence ($\Delta F\%$), with a positive number indicating a drop in content indicative of release. As expected, release induced by 70-Hz nerve stimulation for 15 s was abolished by replacing extracellular Ca^{2+} with the Ca^{2+} chelator EGTA (Fig. 1*A*). However, at the same terminal in the absence of extracellular Ca^{2+}, the adenylyl cyclase activator forskolin (FSK) evoked neuropeptide release (Fig. 1 *B* and *C*, closed circles). Indeed, this release was comparable to that seen with minutes of intense electrical activity in the presence of extracellular Ca^{2+} (13). The inactive analog dideoxyforskolin did not mimic this response (Fig. 1*C*, open circles), but the membrane permeant phosphodiesterase-resistant cAMP analog 8-parachlorophenylthio-cAMP (cpt-cAMP) also induced neuropeptide release in the Ca^{2+}-free solution (Fig. 1*D*). Hence, cAMP produced by adenylyl cyclase evoked robust synaptic neuropeptide release without Ca^{2+} entry into the nerve terminal.

To explore the roles of cAMP effectors, analogs that specifically stimulate PKA or epac (exchange protein activated by cAMP) were applied in the absence of extracellular Ca^{2+}. Both the PKA activator N^6-Benzoyl-cAMP (N6) and the epac activator 8-(4-methoxyphenylthio)-2′-O-methyl-cAMP (Me) evoked synaptic neuropeptide release (Fig. 2*A*). Although the epac activator produced a larger response in WT NMJs, this could reflect its greater concentration (200 vs. 100 μM) and membrane permeability. Therefore, we set out to determine whether epac and PKA are necessary for neuropeptide release induced by the adenylyl cyclase activator. There is no known specific chemical epac inhibitor, but there is a fly line with a P element insertion near the C-terminal cyclic nucleotide binding domain (allele $Epac^{KG00434}$). The epac activator failed to induce neuropeptide release in this line (Fig. 2*B*, compare WT+Me to mut+Me), showing that the mutation disrupts epac function. Nevertheless, FSK still evoked robust release in the epac mutant (Fig. 2*B*, mut+FSK). In contrast, H89 (which inhibits PKA, but not epac) markedly reduced FSK-evoked release (Fig. 2*B*, compare WT+FSK to WT+FSK+H89). Hence, epac had at most a minor contribution to cAMP-evoked synaptic neuropeptide release without Ca^{2+} entry. Furthermore, these results

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The authors declare no conflict of interest.

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[1]To whom correspondence should be addressed. E-mail: elevitan@pitt.edu.

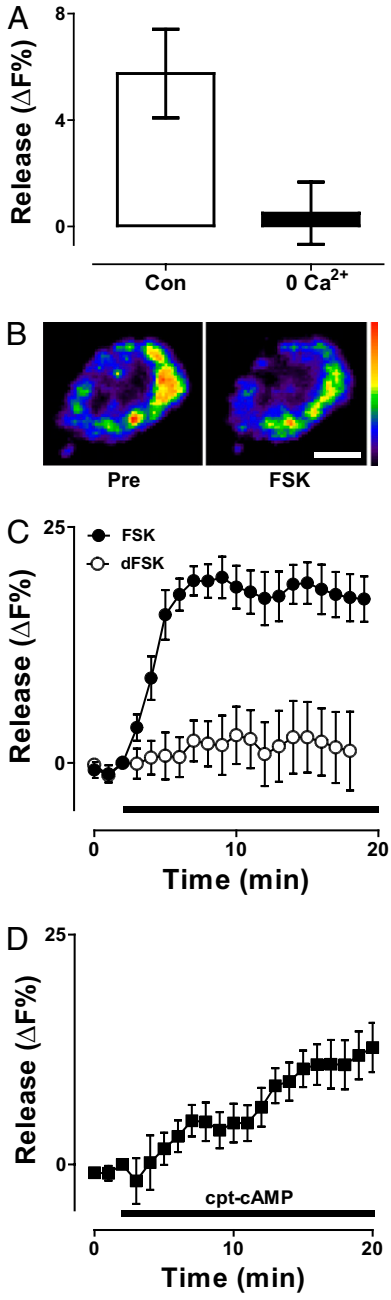

Fig. 1. Synaptic neuropeptide release is induced by cAMP in the absence of extracellular Ca^{2+}. (*A*) Removing extracellular Ca^{2+} abolishes neuropeptide release from type Ib boutons induced by 70-Hz stimulation for 15 s. Note that the response in 0 Ca^{2+} (n = 4) differed from control (Con) (n = 4, $P < 0.05$). Release was measured as the drop in AnfGFP fluorescence (ΔF%). (*B*) Pseudocolor images showing neuropeptide content in 0 Ca^{2+} before (Pre) and after application of 50 μM FSK (FSK) for 10 min. (Scale bar, 2 μm.) The decrease in presynaptic neuropeptide signal indicative of release is shown as shift from warm colors to cool colors. (*C*) Time course of neuropeptide release in the absence of extracellular Ca^{2+} induced by FSK (n = 8) and dideoxy-forskolin (dFSK, n = 7) (application indicated by bar). (*D*) Time course of neuropeptide release in the absence of extracellular Ca^{2+} induced by 1 mM cpt-cAMP (n = 10).

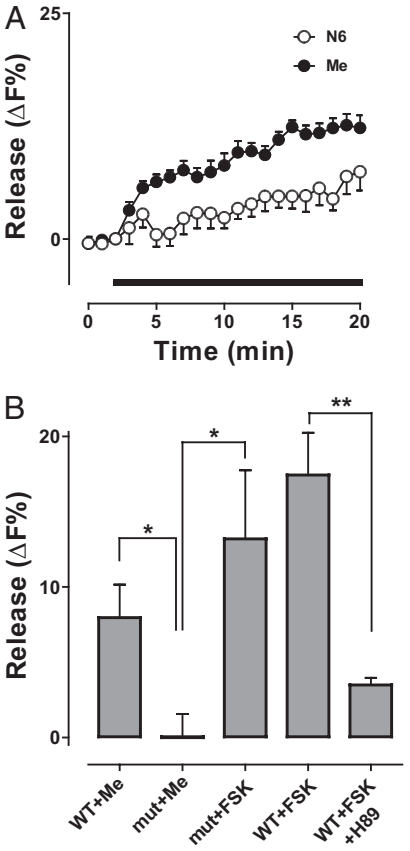

Fig. 2. Roles of PKA and epac in cAMP-evoked synaptic neuropeptide release. (*A*) Time course of release evoked by specific activators of PKA (100 μM N6, n = 7) and epac (200 μM Me, n = 6). (*B*) PKA-mediated neuropeptide release in the absence of epac function. Release was quantified after application of drugs for 10 min. Release induced by the epac activator (WT+Me, n = 5) was abolished in epac mutant flies (mut+Me, n = 5), but FSK-evoked release in WT animals (WT+FSK, n = 8) was still apparent in the epac mutant (mut+FSK, n = 3). In contrast, FSK-evoked neuropeptide release was inhibited by 5 μM H89 (WT+FSK+H89, n = 7). *$P < 0.05$, **$P < 0.01$.

are consistent with the conclusion that cAMP-induced release was mediated mainly by PKA.

Induction of synaptic neuropeptide release without Ca^{2+} entry by pharmacological PKA activators led us to study octopamine, a native neuromodulator that acts presynaptically via PKA at the *Drosophila* NMJ (8). Octopamine also evoked neuropeptide release from motoneuron boutons in Ca^{2+}-free medium (Fig. 3 *A* and *B*, filled circles). Furthermore, release elicited by octopamine was inhibited with H89 (Fig. 3 *B* and *C*) ($P < 0.001$). However, there are two limitations associated with bath applied H89: it could have acted on both sides of the synapse or had nonspecific effects in addition to inhibiting PKA. Therefore, a selective dominant-negative regulatory PKA subunit (20) was expressed only in neurons to specifically inhibit presynaptic PKA. With two chromosomal insertions of the dominant-negative gene (BDK22 and BDK35), octopamine-induced release was inhibited (Fig. 3*C*). Hence, presynaptic PKA is required for Ca^{2+} entry-independent neuropeptide release by the native neuromodulator.

In addition to activating PKA, octopamine can evoke Ca^{2+} release from internal stores (4). Releasing presynaptic endoplasmic reticulum (ER) Ca^{2+} with caffeine (21) for 3 min elicited release of 24 ± 4% of synaptic neuropeptide content, suggesting a possible role for ER Ca^{2+} in presynaptic octopamine action. Therefore, to genetically probe whether ER Ca^{2+} stores participated in the octopamine effect, neuropeptide release was studied in the $CaP60A^{Kum170}$ (Kum170) mutant, in which the sarco-ER Ca^{2+} ATPase (SERCA) is persistently inhibited by brief exposure to 40 °C (22). Although octopamine evoked release under permissive conditions, heating Kum170 animals for 8 min to deplete ER Ca^{2+} stores abolished release measured

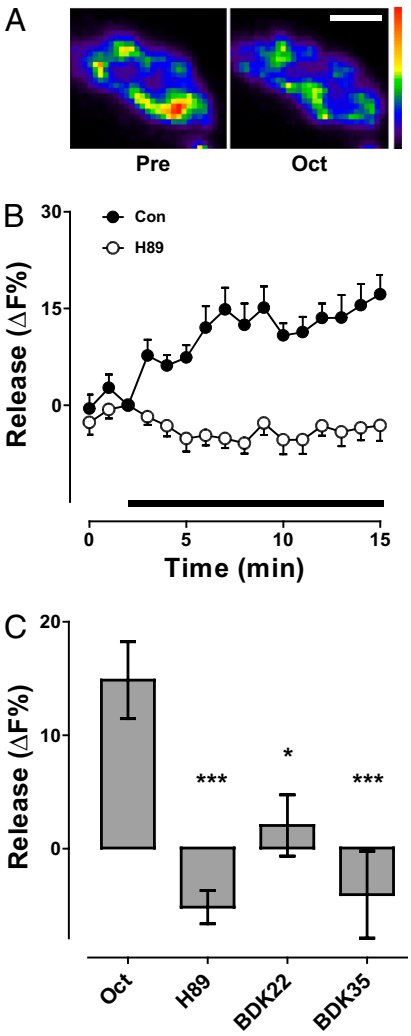

Fig. 3. Octopamine acts via PKA to evoke synaptic neuropeptide release. (*A*) Pseudocolor image showing neuropeptide content before (Pre) and after application of 100 μM octopamine for 10 min (Oct). (Scale bar, 2 μm.) (*B*) Time course of neuropeptide release induced by octopamine (indicated by bar) in the absence (Con, ●, $n = 9$) and presence of H89 (○, $n = 8$). (*C*) Release induced by octopamine in 5 min (Con, $n = 9$) was inhibited by H89 ($n = 8$) or expression of a dominant-negative PKA subunit [BDK22 ($n = 5$) and BDK35 ($n = 6$)]. $^{*}P < 0.05$, $^{***}P < 0.001$.

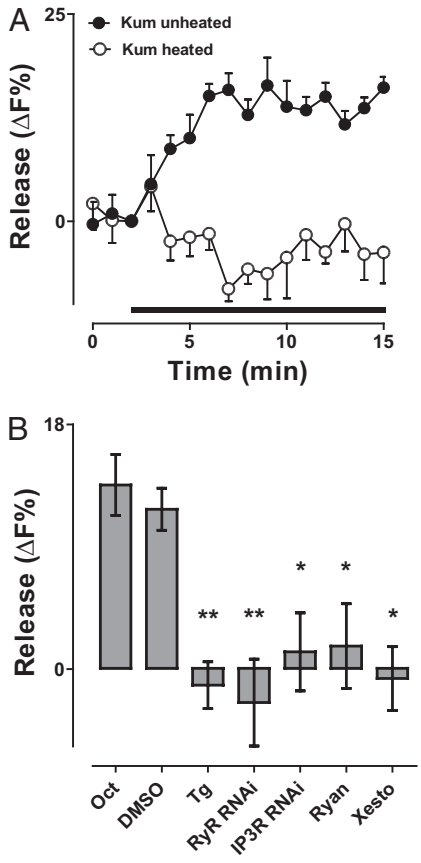

Fig. 4. ER Ca^{2+} is required for octopamine-induced neuropeptide release. (*A*) Time course of octopamine-induced neuropeptide release in unheated or briefly heated Kum170 flies. Octopamine application indicated by bar. (*B*) ER Ca^{2+} channels are required for octopamine-induced release. Neuropeptide release evoked by octopamine for 10 min (Con, $n = 9$) was evident in the presence of DMSO ($n = 3$), but was abolished by 20 μM Tg ($n = 3$), RyR RNAi ($n = 6$), IP3R RNAi ($n = 5$), 100 μM Ryan ($n = 5$), and 0.1 μM Xesto ($n = 4$). DMSO served as the control for Tg and Xesto, and Con was the control for statistical analysis of Ryan and the RNAis. $^{*}P < 0.05$, $^{**}P < 0.01$.

subsequently at room temperature (Fig. 4*A*). A similar effect was produced by acute application of the SERCA inhibitor thapsigargin (Tg) (Fig. 4*B*), excluding developmental or pleiotropic effects of the mutation. Hence, ER Ca^{2+} was required for octopamine-induced synaptic neuropeptide release.

Therefore, we tested whether octopamine-induced release depended on ER Ca^{2+} channels [i.e., the ryanodine receptor (RyR) and the inositol trisphosphate receptor (IP3R)]. Because null RyR and IP3R mutants are lethal and dominant-negative subunits are not available, RNA interference was used initially. Specifically, neuronal expression of RyR RNAi or IP3R RNAi each inhibited the octopamine response (Fig. 4*B*). To exclude off-target or developmental effects, ER Ca^{2+} channels were acutely blocked by specific inhibitors known to be effective in *Drosophila* (23). Inhibiting RyRs with ryanodine (Ryan) or IP3Rs with xestospongin C (Xesto) blocked octopamine-induced neuropeptide release in the absence of extracellular Ca^{2+} (Fig. 4*B*). Hence, independent pharmacology and genetic experiments showed that octopamine required presynaptic RyRs and IP3Rs.

Finally, to test whether octopamine elevates presynaptic Ca^{2+}, the GCaMP3 Ca^{2+} indicator (24) was expressed in neurons and imaged in the nerve terminal in the absence of extracellular Ca^{2+}. Under these conditions, octopamine induced a transient increase in cytoplasmic Ca^{2+} (Fig. 5*A*). Taken together, the above results show that octopamine activated presynaptic ER Ca^{2+} channels to induce synaptic neuropeptide release.

Given that Ca^{2+} can induce neuropeptide release directly, why is PKA also required for octopamine-induced neuropeptide release? One potential explanation could be that PKA promotes activation of RyRs and IP3Rs, as occurs in muscle (25, 26). If this happened in the nerve terminal, then inhibiting PKA would reduce octopamine-induced Ca^{2+} mobilization. However, in contrast to neuropeptide release (Fig. 3*B*), the octopamine Ca^{2+} response was unaffected by H89 (Fig. 5*A*). Hence, ER Ca^{2+} mobilization was independent of PKA. The basis of the PKA requirement became evident, however, with quantitative consideration of the Ca^{2+} data. First, the octopamine Ca^{2+} response, although detectable, was minuscule compared with electrical activity in the presence of extracellular Ca^{2+} (Fig. 5*B*). Second, the octopamine-induced increase in Ca^{2+} was brief compared with evoked neuropeptide release (compare Figs. 3*B* and 4*A* to 5*A*). Apparently, the extent and duration of octopamine-induced Ca^{2+} mobilization are not sufficient, suggesting that synergistic

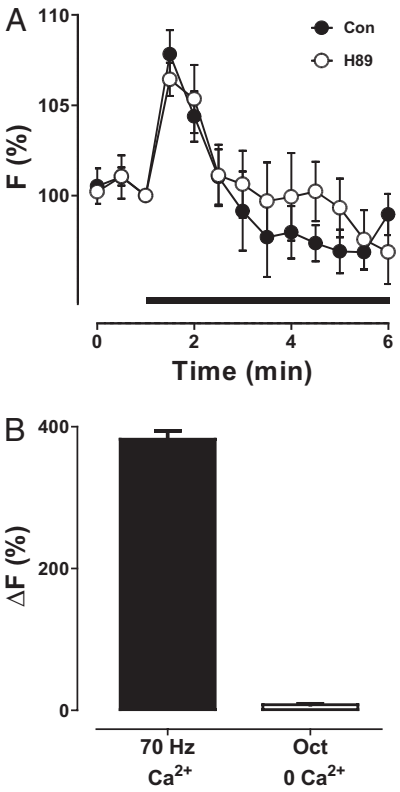

Fig. 5. Octopamine induced mobilization of intracellular Ca^{2+}. (*A*) Time course of GCaMP3 fluorescence (F) without extracellular Ca^{2+} induced by octopamine (indicated by bar) in the presence ($n = 4$) and absence ($n = 4$) of the PKA inhibitor H89. (*B*) Comparison of the peak changes in Ca^{2+} (ΔF) induced by octopamine in the absence of extracellular Ca^{2+} ($n = 4$) or by 15 s of 70-Hz stimulation in the presence of extracellular Ca^{2+} ($n = 8$).

signaling with PKA is required to evoke robust neuropeptide release in the absence of Ca^{2+} entry into the nerve terminal.

Discussion

Neuromodulators regulate synaptic release induced by Ca^{2+} entry into the nerve terminal. However, here a monoamine was found to evoke neuropeptide release without Ca^{2+} entry into the terminal. Given that neuropeptides and other LDCV cargoes, such as neurotrophins, activate postsynaptic signaling, neuropeptide release induced by signaling instead of activity may serve to alter postsynaptic function. It is also possible that the effect of cAMP on synaptic development (27) may be caused in part by neuromodulator-induced synaptic release of neuropeptides and other bioactive proteins packaged in LDCVs. Furthermore, the induction of synaptic neuropeptide release in the absence of activity-dependent Ca^{2+} entry at central synapses could be important in regulation of neuropeptide-dependent insect behavior by octopamine (5, 14, 15). Indeed, given the importance of monoamines and neuropeptides in the control of mood and behavior in mammals, a similar mechanism could operate in the brain.

Synaptic neuropeptide release induction by a neuromodulator is reminiscent of presynaptic facilitation of spontaneous quantal release of classical transmitters, which also occurs without Ca^{2+} entry. However, quantal "minis", which are produced by rare exocytosis of individual small synaptic vesicles, are typically insufficient for conventional neurotransmission (1). Nevertheless, it is possible that the same mechanism is responsible for neuromodulator-induced synaptic neuropeptide release and spontaneous minis, but with LDCVs being more sensitive to presynaptic signaling than small synaptic vesicles. According to this reasoning, there may be significant release of neuropeptide cotransmitters at mammalian synapses with known presynaptic facilitation of spontaneous minis. Such unconventional peptidergic transmission, which could now be revealed with GFP imaging, may have been undetected because neuropeptides do not typically evoke obvious postsynaptic electrophysiological responses.

It is surprising that a small, transient ER Ca^{2+} response is required for sustained octopamine-induced neuropeptide release. Of course, bulk cytoplasmic Ca^{2+} measurements may not accurately reflect local Ca^{2+} levels at sites of exocytosis. However, an alternative explanation is that Ca^{2+} from the ER does not directly evoke exocytosis, but rather promotes PKA action. For example, such a synergistic effect could occur on a PKA substrate that is phosphorylated more efficiently after binding Ca^{2+}. In this case, the nonphysiological direct adenylyl cyclase activator FSK could on its own induce substrate phosphorylation, but the native neuromodulator, which acts via receptors and G proteins to more weakly activate adenylyl cyclase, could require synergistic activation of ER Ca^{2+} channels and PKA to produce a significant effect.

Genetic and pharmacological experiments established that PKA plays a much larger role in the octopamine effect than epac, even though either cAMP effector can induce Ca^{2+} entry-independent synaptic neuropeptide release. Because the cAMP affinities of epac and PKA are similar (28), the prominence of PKA may reflect that phosphorylation can be long lasting compared with more transient interactions of epac with its targets. Alternatively, PKA may be positioned to interact more efficiently with adenylyl cyclase, neuropeptide-containing LDCVs, or sites of neuropeptide release. Accordingly, it will be interesting to determine whether epac is significant in other presynaptic cAMP effects, including regulating conventional Ca^{2+}-dependent neurotransmission and neuropeptide release induced by activity.

Materials and Methods

Unless otherwise indicated, *Drosophila melanogaster* third instar larvae were filleted and imaged in Ca^{2+}-free HL3 in which 1.5 mM Ca^{2+} was substituted with 0.5 mM EGTA, a Ca^{2+} chelator (70 mM NaCl, 5 mM KCl, 0.5 mM Na_3EGTA, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM trehalose, 115 mM sucrose, 5 mM sodium Hepes, pH 7.2). AnfGFP fluorescence was detected with wide-field epifluorescence microscopy in muscle 6 and 7 type Ib boutons of *elav-GAL4 UAS-AnfGFP* flies using an Olympus BX microscope equipped with a 60× 1.1 NA water-immersion objective, fluorescein filters, and a Hamamatsu digital-cooled CCD camera, as described previously (13, 19). The same optics were used to measure Ca^{2+} in flies generated by crossing Ok6-GAL4 with *UAS-GCaMP3* (24), which were kindly provided by Loren Looger (Janelia Farm, Ashburn, VA). Epac function was disrupted by generating *elav-GAL4 UAS-AnfGFP* flies that were homozygous for a P-element insertion in the gene (allele $Epac^{KG00434}$; Bloomington stock 13663). Single crosses were performed with *elav-GAL4 UAS-AnfGFP* flies and RNAi lines targeting the RyR (VDRC 109631) and the IP3R (Bloomington stock 25937). Similarly, presynaptic expression of the dominant-negative PKA subunit was accomplished with single crosses of BDK22 and BDK35 flies (20), which were kindly provided by Daniel Kalderon (Columbia University, New York, NY), to flies expressing AnfGFP driven by *elav-Gal4* or Geneswitch 3550–2 with RU486 feeding (28, 29). Nerve stimulation was performed in Ca^{2+}-free HL3 or Ca^{2+}-containing HL3, as described previously (13, 19). Inhibitors of signaling, such as H89, Tg, Xesto, and Ryan, were applied for 15 to 25 min before application of octopamine or FSK. SERCA was also inactivated by heating Kum170 larvae for 8 min at 40 °C. Signaling chemicals were purchased from Biolog Life Sciences, Axxora, and Sigma.

Release was quantified as the percent-change in AnfGFP fluorescence, with a positive number indicating a decrease in AnfGFP content indicative of release [i.e., 100*(Fo − F)/Fo, where Fo was the initial fluorescence and F was the fluorescence after stimulation]. Pseudocolor scales are presented in arbitrary units, with warm colors indicating greater content. Thus, release is shown as a shift to cooler colors. Statistical significance was determined with Student's *t* test for two experimental groups and ANOVA and the Bonferroni posttest for more experimental groups.

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