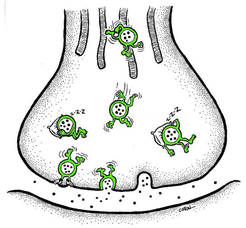


Capture, rather than delivery, limits accumulation of synaptic neuropeptide vesicles.

Arvonn Tully, Dinara Shakiryanova, Edwin S. Levitan
Department of Pharmacology,University of Pittsburgh, Pittsburgh, PA

Abstract:

Release of a GFP-tagged neuropeptide in transgenic *Drosophila melanogaster* occurs on the timescale of minutes, and is sutained by undocked mobilized vesicles in presynaptic terminals (Shakiryanova et al., Nature Neurosci. In press). Although these features are widely conserved, individual bouton types contain widely varying levels of peptidergic vesicles. To gain insight into this diversity, we examined peptidergic vesicle motion in living neuromuscular junctions.

In synaptic boutons, peptidergic vesicle motion is unpolarized and does not appear to use the motors or microtubule tracks that support axonal transport. Delivery of new peptidergic vesicles by axonal transport was measured by following recovery of peptide fluorescence after photobleaching entire synaptic boutons. This also made it possible to visualize and track individual peptidergic vesicles as they entered boutons from the axon. These experiments indicate that most peptidergic vesicles pass straight though en passant boutons without stopping, and that capture of newly transported vesicles occurs on a time scale of hours. Fast transport through boutons can also be seen without photobleaching. Thus, vesicle capture and retention, rather than delivery, limit neuropeptide accumulation in resting boutons.

Introduction

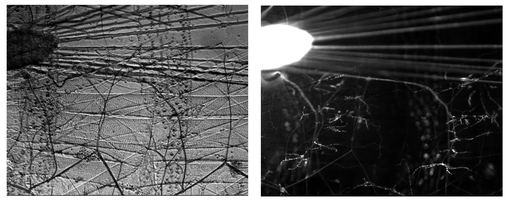
Neuropeptides are stored in dense core vesicles (DCVs) and released from nerve terminals during bursts of activity, to trigger changes in behaviors. This release depends on previous neuropeptide synthesis and packaging in the soma, and axonal transport to nerve terminals. Therefore, accumulation synaptic neuropeptide stores requires significant time and effort.

We have previously shown that intrabouton DCV mobility is calcium dependent, and that this motion sustains release over several minutes. Neuropeptide secretion is also limited by the number of vesicles at the release site. However, the mechanism for targeting neuropetides to a terminal to be released is not known. Using GFP-tagged neuropeptide (anf-GFP) in *Drosophilia* larval neuromuscular junctions, we studied *in vivo* how peptidergic vesicles accumulate in synaptic boutons.

Methods

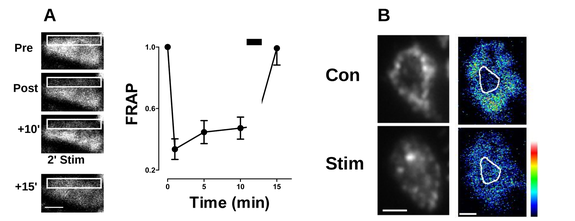
Type Ib and III motor neuron boutons from muscles 6,7, 12, or 13 of segments A4 in 3rd instar larva of transgenic *Drosophila melanogaster* expressing Emerald GFP-tagged proAtrial Natriuretic Factor (ANF, also call proAtrial Natriuretic Peptide)were observed on an upright microscope with a water immersion objective and a cooled ccd digital camera(Innovision Software), or by confocal microscopy(Zeiss LSM Pascal). Dissections where performed in standard HL3 or DM drosophila media. Long term measurements were performed in HL3 with a 3% Bovine Calf Serum. 0 Ca salines were prepared by omitting Ca, and adding 0.5 mM EGTA, and correcting pH to 7.2.

Figure 1. Transgeneic Drosphila express anf-GFP contstruct targeted to nervous system



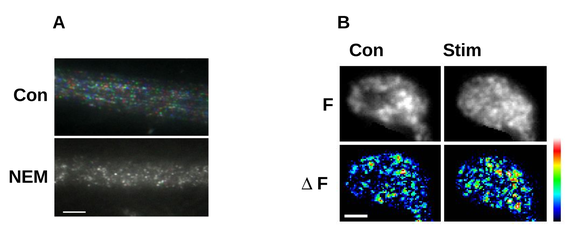
Left, Wide field, whitelight view of a filleted drosophilia 3rd instar larva, showing ventral ganglia connected to segmented muscles via nerve fibers. Right, Same prep in fluorescent light, shows how the elav promoter targets the anf-GFP construct to the neuromuscular junctions in in the body wall muscles.

Figure 2. Synaptic DCV movement is unpolarized after liberation.



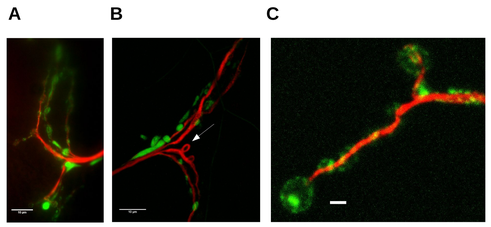
A. Depolarization (Stim, indicated by bar in right graph) increases FRAP following tangential lengthwise photobleaching (N=5). B, C. Redistribution of neuropeptide vesicles in type Ib synaptic boutons evoked by 2 minutes of stimulation (Stim). B. Wide field images before and after bursting electrical stimulation. C. Confocal images before and after depolarization of a large posterior segment bouton. Equatorial optical sections of confocal stacks are shown. Note that peptidergic vesicles moved into the central region of the bouton indicated by the white outline. Size bars equal 2 microns.

Figure 3. Synaptic DCV mobilization persists without axonal transport motor activity.



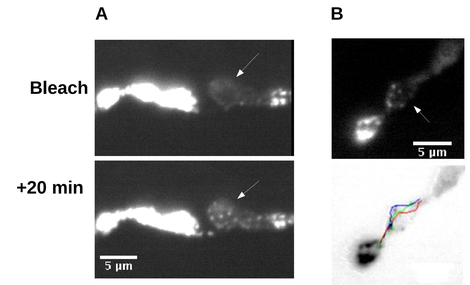
A. N-ethylmaleimide(NEM) inhibits axonal transport of peptidergic vesicles. Sequential images of a motor nerve, acquired every 3 seconds, were color-coded red, green and blue, and then superimposed. Note that vesicle images are white in NEM-treated preparation, indicating that the vesicles were immobile in axons. Size bar equals 5 microns. B. Panels show F and ΔF data from a representative NEM-treated type Ib bouton. In ΔF panels, color indicates intrabouton DCV motion, which is intact with NEM treatment. Size bars equal 2 microns.

Figure 4. The distribution of microtubules is suited for inter-, but not intra-, bouton DCV transport.



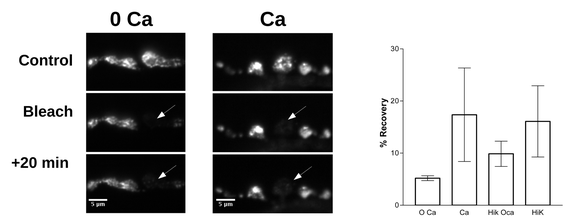
The 22C10 Futch antibody and anti-mouse TRITC conjugate staining of transgenic elav-anf::GFP drosophila; green indicates neuropeptide, and red indicates microtubules in boutons(A, B, C). A. Wide field images of muscle 12 NMJ. B.Confocal reconstruction of muscle 12 NMJ, showing loops (arrow) of microtubules in some type Ib boutons. C.Confocal reconstruction of muscle 6 type 1b boutons demonstrates that microtubules minimally colocalize with DCVs, suggesting that microtubules only support interbouton, but not intrabouton vesicle transport(Bar = 2 μm).

Figure 5. Photobleaching reveals inefficient capture of new DCVs by single boutons.



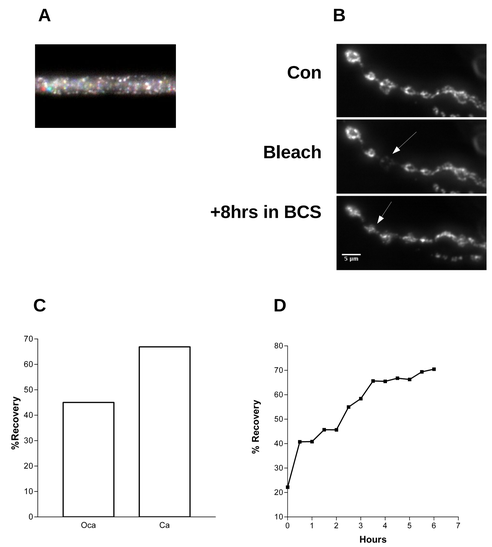
A. Wide field image of GFP-neuropeptide fluorescence in larval neuromuscular junction showing limited accumulation new DCVs within 20 minutes in a photobleached bouton (arrow). B.Top,Image of a photo bleached bouton (arrow). Bottom, Trajectories of 3 DCVs overlaid negative image of boutons. Colored lines showing paths of DCVs that passed through the photobleached bouton within 0.5 minute.

Figure 6. Calcium independence of vesicle capture after 20 minutes



After 20 minutes, average FRAP is statistically and visually indistinguishable, in the presence and absence of bath calcium. Normalized fluorescence recovery, 20 minutes after photobleaching. N=4 for each condition.

Figure 7. DCV capture by synaptic boutons occurs on the timescale of hours.



A. DCV axonal transport in motor nerve shown by three consecutive color-coded (red, blue, green) images which have been superimposed. White indicates no motion, color indicates motion. Axon shows slowed motion, but the prep is still alive after 8 hours in HL3 media supplemented 3% bovine calf serum(BCS). B. Top panel, control boutons from muscle 6/7. Middle panel, shows the nerve branch immediately after photobleaching. Bottom panel shows that new vesicles have been captured by the bouton after 8 hours in the BCS media. C. Fluorescence recovery (normalized to neighboring bouton) in 8 hours. For each condition, n=2. D. Time course of fluorescence recovery over 6 hours, measured every ½ hour from a single preparation.

Conclusions

Intrabouton motion of DCVs does not utilize axonal transport motors that support delivery of new peptidergic vesicles.

Accumulation of DCVs by resting boutons is not limited by synthesis or delivery. Indeed, the majority of DCVs pass through en passant boutons without contributing to the steady state level of DCVs. Thus, DCV capture and retention limit neuropeptide storage in nerve terminals.

Our studies suggest that DCV content in resting synaptic boutons is at a steady state with residence and replacement half lives of hours. This contrasts with delivery that occurs on the time scale of minutes.

Future experiments will test whether synaptic capture of DCVs is activity dependent.