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Living Synaptic Vesicle Marker: Synaptotagmin-GFP

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Synapses are the site of chemical communication between neurons and between neurons and muscles. The synaptic vesicle (SV) is a prominent presynaptic organelle which contains chemical neurotransmitters and fuses with the plasma membrane to mediate neurotransmission. There are about 50 or so synaptic proteins which are either integral vesicle membrane proteins (e.g., synaptotagmin, syt; and synaptobrevin, syb) or vesicle-associated proteins (e.g., cysteine string protein, CSP; Fernandez-Chacon and Sudhof, 1999). We have transformed *Drosophila* with a novel syt-eGFP (enhanced GFP) fusion protein, the fluorescence pattern of which colocalizes with native SV proteins at synapses, suggesting that the syt-eGFP fusion protein is correctly localized as an integral SV protein and therefore a good SV marker in living synapses. We demonstrate that the syt-eGFP line can be used to study SV dynamics in vivo by fluorescence recovery after photobleach (FRAP).

The syt-eGFP fusion was constructed as shown in Figure 1. The eGFP carries double substitution of Phe 64 to Leu and Ser 65 to Thr and fluoresces 35-fold more intensely than wild-type GFP when excited at 488 nm, based on spectral analysis of equal amounts of soluble protein (Cormack *et al.*, 1996). Four syt-eGFP transgenic lines were generated; one with insertion on the X chromosome, two on the second chromosome, and one on the third chromosome. All of these lines produced clear fluorescence when crossed to a pan-neuronal GAL4 driver (elav-GAL4, see Fig. 2) or a subset neuronal GAL4 driver 4G-GAL4 (data not shown). 4G-GAL4 is identified from an enhancer trap screen for neuronal-specific genes; it starts expression at late embryogenesis panneuronally, but in a subset of motor neurons in the third instar larvae, and enriched in mushroom body in adult brain. The eGFP-positive animals, from embryos to adults, can be readily recognizable under a fluorescence dissecting scope. Stocks with expression of syt-eGFP in all neurons (recombinant chromosome carrying both *elav-GAL4* and *syt-eGFP* on the X chromosome) or subset of neurons (recombinant chromosome carrying both *4G-GAL4* and *syt-eGFP* on the second chromosome) were established.

Multiple lines of evidence indicate that syt-eGFP is present in SVs, with expression similar to the native syt (Fig. 2). First, syt-eGFP is highly enriched in the neuropil region of the ventral nerve cord (VNC) of the embryo

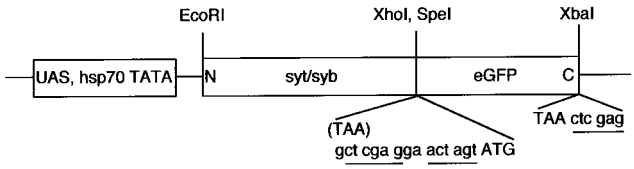

FIG. 1. Map of syt-eGFP and syb-eGFP fusion constructs. Syt (accession number M55048) or syb (neuronal synaptobrevin, accession number S66686) coding region was fused to the N-terminal of eGFP (enhanced GFP, catalog number 6084-1 from ClonTech, Palo Alto, CA; sequence accession number U55763) with EcoRI and XhoI. Syt cDNA (encoding a protein of 475 amino acids) was PCR-amplified and sequence-confirmed with a pair of primers syt. 1: gggaa ttcat taggg gcaac aacac agc (EcoRI) and syt. 3: ccctc gagc c ttcat gttct tcagg atctc (XhoI). n-Syb (encoding 180 amino acids) was PCR-amplified and sequence-confirmed with a pair of primers syb1: acagc cgaat tcgct gaggc (EcoRI) and primer syb2: tcctc gagcc acgcc gccgt gatcg ccag (XhoI). The eGFP fusion cassettes were then introduced into *Drosophila* transformation vector *p{UAST}* (see Flybase at http://flybase.bio.indiana.edu/) under the control of UAS and hsp70 TATA sequence. Restriction sites are labeled in the upper part; sequences at junctions are spelled out in the lower part with restriction sites underlined. The stop codon TAA (parenthesized) of syt or syb is replaced with gct. N and C indicate the N-terminal and the C-terminal ends of the eGFP fusions, respectively. For syt-eGFP, the N-terminus is in the vesicle lumen and C-terminus in the cytoplasm; for syb-eGFP, N-terminus is in the cytoplasm and C-terminus is in the vesicle lumen.

(data not shown) and larva (Fig. 2A, left), as well as in the axonal lobes of the larval mushroom body (Fig. 2A, right). These neuropil regions are densely packed with neuronal synapses. Second, at neuromuscular junction (NMJ) synapses, where we have higher resolution of single synaptic boutons, the syt-eGFP pattern perfectly matches the staining pattern seen with antibodies against SV-associated proteins (Fig. 2B). This suggests that syt-eGFP is tightly linked to SVs. Third, to further

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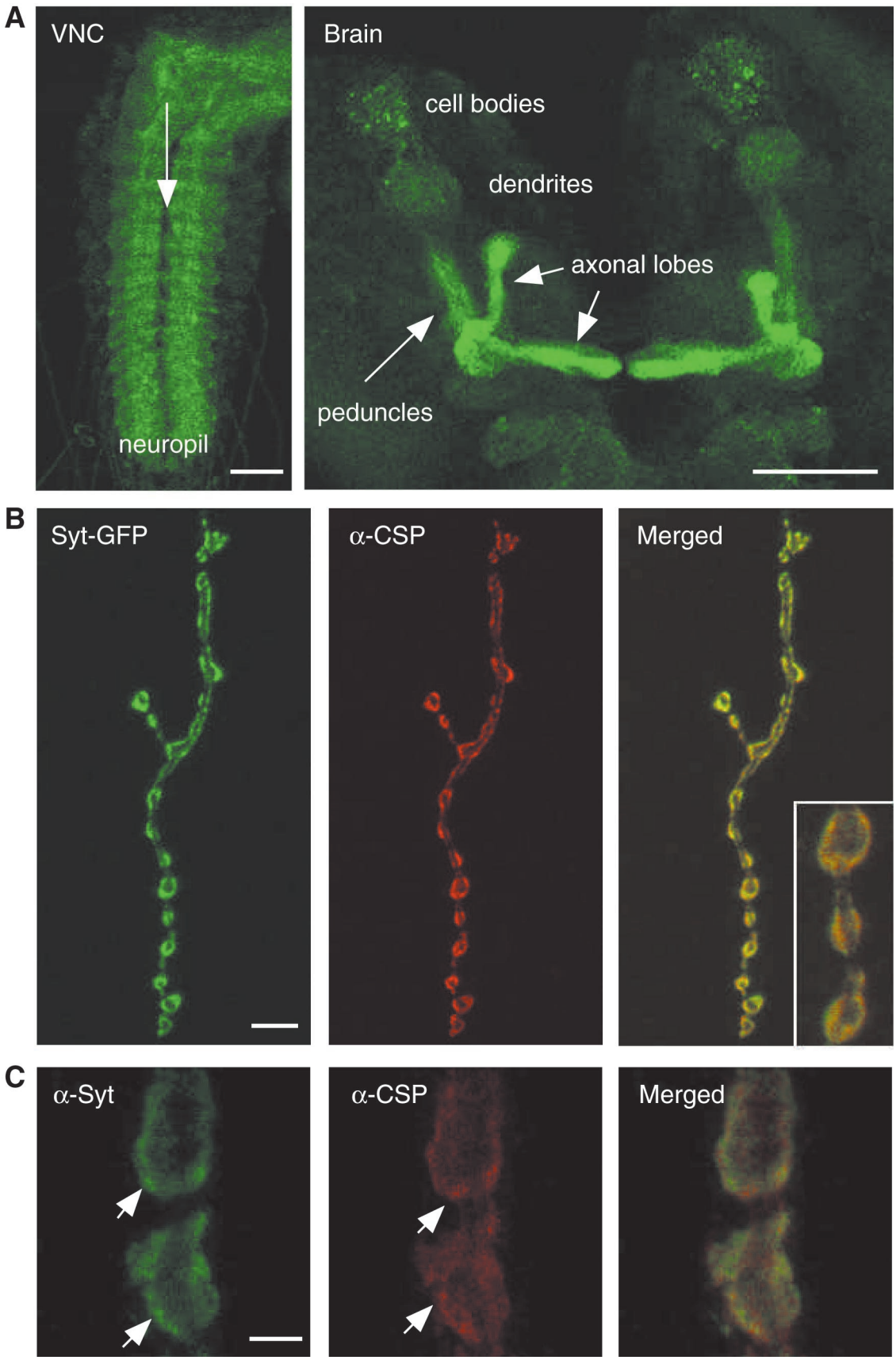
A
VNC
neuropil
Brain
cell bodies
dendrites
axonal lobes
peduncles
B
Syt-GFP
α-CSP
Merged
C
α-Syt
α-CSP
Merged

FIG. 2

demonstrate that syt-eGFP is specifically present in SVs, we took advantage of a dynamin mutant *shi*^{ts} to drive fusion in the absence of vesicle recycling (Estes *et al.*, 1996). *shi*^{ts} mutants block SV endocytosis at restrictive temperature (35°C), causing SV proteins to trap in the plasma membrane. We made a stock that carries *shi*^{ts}, *elav-GAL4,* and *syt-eGFP.* As shown in Figure 2C, syt-eGFP is redistributed to the periphery of synaptic boutons at the restrictive temperature, consistent with it being restricted to SVs. Based on the enrichment of syt-eGFP in synaptic regions of CNS, identical localization with endogenous SV proteins at NMJ synapses, and the peripheral redistribution of syt-eGFP at *shi*^{ts} NMJ synapses at restrictive temperature, we conclude that syt-eGFP is restricted to SVs, similar to the native syt.

While synaptic activity can be directly measured using electrophysiology and synaptic vesicle recycling can be studied using uptake of fluorescent marker dyes (Ryan *et al.*, 1996), both of these techniques require that vesicles take part in a full recycle of exocytosis and endocytosis. Therefore, mutations that severely alter the processing of SVs or their mobilization will contain SV subpopulations that are inaccessible to these conventional techniques. Genetically engineered syt-eGFP-labeled SV markers, however, can be used to visualize SVs in mutants that have severe effects on SV release, clustering, transport, or recycling. Therefore, these markers complement activity-dependent dye loading and electrophysiology, techniques that are both better suited for study of mutations which have relatively mild effects on synaptic activity. Moreover, FRAP analyses of syt-eGFP-labeled SVs is best used to measure the dynamic intermixing of fluorescently labeled SVs with photobleached compartments within synaptic boutons (Fig. 3), which cannot be achieved by classical electrophysiological assays or dye loading.

In addition to the syt-eGFP lines, we have also made syb-eGFP transgenic lines (see Fig. 1). In total, eight independent transgenic lines with insertions on all major chromosomes (the X, the second, and the third chromosomes) were obtained. All eight lines, when crossed to either elav-GAL4 or 4G-GAL4, produce eGFP-positive animals. While syb-GFP(S65T) transgenic flies have become available recently (Estes *et al.*, 2000), we expect that the syb-eGFP line described here will be better suited for some experiments, e.g., biogenesis of SVs, as eGFP matures faster to the fluorescent form and folds more efficiently than GFP(S65T) (Cormack *et al.*, 1996). In parallel experiments, we observed that the fluorescence of syt-eGFP is consistently brighter than that of syb-eGFP. One plausible explanation is that the fluorescence of eGFP is dimmer at lower pH (Tsien, 1998), the lumen of vesicles is acidic (pH 5.6; Miesenbock *et al.*, 1998), and therefore it quenches the fluorescence of eGFP, as the eGFP end of syb-GFP is inside the lumen (see Fig. 1 Legend). The eGFP end of syt-eGFP, however, is located at the cytoplasmic side of the synaptic membrane (see Fig. 1 Legend). Therefore, the fluorescence of syt-eGFP will not be affected by the reduced pH in the vesicle lumen.

We observed that animals with expression of syt-eGFP driven by 4G-GAL4 are fully viable, whereas animals carrying 4G-GAL4, syb-eGFP are lethal at the pupal stages. The reason for the syb-eGFP lethality is currently unknown. One possibility is that syb is a component of the core complex of membrane fusion (Fernandez-Chacon and Sudhof, 1999) and the overexpression of syb in the form of syb-eGFP in neurons might have a dominant-negative effect on the core complex. We expect that the novel SV marker of syt-eGFP with increased intensity and less deleterious effects will prove to be an important tool for addressing a number of essential neurobiological questions: biogenesis of SVs, axonal transport and dynamics of SVs, synaptogenesis, and synaptic development and function in flies. The syt-eGFP line is especially suited for imaging of live samples, as illustrated in Figure

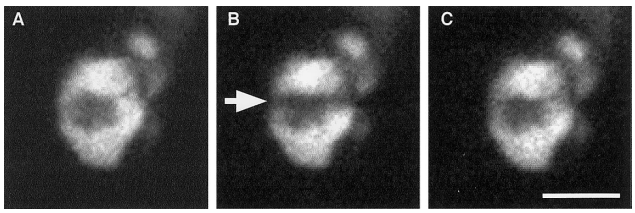

FIG. 3. Imaging of in vivo SV dynamics with fluorescence recovery after photobleach (FRAP). Individual NMJ synaptic boutons of third instar larvae of *elav-GAL4, syt-eGFP* were photobleached with 100% 488 nm, 514 nm laser power for 100 milliseconds. Photobleaching and serial image collecting were done on a Zeiss LSM510 laser-scanning confocal microscope (Oberkochen, Germany). **A:** An NMJ synaptic bouton before photobleaching. **B,C:** The same bouton 0 and 1 s after photobleaching. The focally bleached area across the bouton middle is indicated by an arrow in **B** and is refilled with fluorescent SVs in about 1 s after bleaching (**C**). The FRAP assay on the syt-eGFP line can be employed to dissect in vivo SV dynamics with a pharmacological and/or genetic approach. Scale bar = 4 μm.

FIG. 2. syt-eGFP is associated with SVs and labels the neuropils in the central nervous system as well as NMJ synaptic terminals in a pattern identical to antibody staining against synaptic vesicle protein. **A:** Syt-eGFP driven by elav-GAL4 reveals neuropil in the VNC of a third instar larva (left). Arrow indicates ventral midline. Scale = 20 μm. In the third instar larval brain (right), syt-eGFP reveals the entire mushroom body (MB). Different parts of the MB are annotated accordingly (Liu *et al.*, 2000). The two axonal lobes are highly enriched with the syt-eGFP marker. Scale = 50 μm. **B:** Syt-eGFP colocalizes with α-CSP staining in the NMJ synapses. Syt-eGFP is green; α-CSP in red. Scale = 5 μm. **C:** Syt-eGFP and endogenous syt localizes to the peripheral of NMJ synapses when exocytosis was stimulated with high [K^+] of 90 mM and endocytosis is blocked under restrictive temperature (35°C, 5 min) in the dynamin mutant background of *shi*^{1}. For a detailed procedure of high [K^+] stimulation of exocytosis and high temperature blockage of endocytosis of NMJ terminals, see Estes *et al.* (1996). The punctate staining nature (shown by arrow) of α-syt (in green) or α-CSP (in red) is evident and indicated by arrows. Scale = 3 μm. The genotype for A-C is *shi*^{ts}, *elav-GAL4, syt-eGFP* on the X chromosome. Antibody staining for larval preparations (larval CNS in **A** and larval NMJ synapses in **B–C**) and image collection were performed as previously described (Zhang *et al.*, 2001).

3. This reagent will be applicable to mammals with minor modification.

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