

# Imaging of Calcium in *Drosophila* Larval Motor Nerve Terminals

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**Karunanithi, Shanker, John Georgiou, Milton P. Charlton, and Harold L. Atwood.** Imaging of calcium in *Drosophila* larval motor nerve terminals. *J. Neurophysiol.* 78: 3465–3467, 1997. Calcium measurements in the presynaptic terminal are essential in the investigation of mechanisms underlying neurotransmitter release. To enhance the genetic analysis of secretory mechanisms, we have developed  $\text{Ca}^{2+}$  imaging techniques for *Drosophila* larval motor nerve terminals. We studied  $\text{Ca}^{2+}$  signals in “big” (type Ib) and “small” (type Is) boutons that innervate ventral longitudinal muscles 6 and 7 in each abdominal segment of Canton-S (CS)-strain 3rd instar larvae. The indicator fluo-3 in conjunction with confocal microscopy was used to detect stimulus-dependent changes in  $[\text{Ca}^{2+}]_i$ . The  $\text{Ca}^{2+}$  signals were reliable and reproducible, and the resting fluorescence remained constant throughout the experiments. The  $\text{Ca}^{2+}$  signals increased with stimulus frequency from 5 to 20 Hz for both bouton types. No significant differences in the  $\text{Ca}^{2+}$  signals were seen between the two bouton types at 5 and 20 Hz, but there was a difference at 10 Hz. The decay of the  $\text{Ca}^{2+}$  signal was more prolonged after 20-Hz stimulation than after 5 and 10 Hz. At the single-synapse level, the secretory efficacy of Is synapses is greater than that of Ib synapses, but our data show that factors other than differences in  $\text{Ca}^{2+}$  entry may govern the strength of synaptic transmission.

## INTRODUCTION

The fruit fly *Drosophila melanogaster* is useful in analysis of the genetics of behavior because studies combining molecular biology, neurophysiology, and behavior can be performed in the same animal. By studying the effects of mutated or nonfunctional genes on physiology and behavior, their role in normal function may be deduced. In studies of synaptic transmission, *Drosophila* mutants have provided valuable information about the role of specific synaptic proteins involved in exocytosis. For instance, the concentration of  $\text{Ca}^{2+}$  ions within the presynaptic terminal following nerve stimulation may control the participation of specific molecules, such as the vesicular  $\text{Ca}^{2+}$  binding protein, synaptotagmin (Bate and Broadie 1995). Imaging techniques have disclosed intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) dynamics in presynaptic terminals including crayfish (Delaney et al. 1989) and frog (Robitaille et al. 1993) neuromuscular junctions and the squid giant synapse (Smith et al. 1993). However, for *Drosophila*, little is known about the  $[\text{Ca}^{2+}]_i$  dynamics in nerve terminals and how they might influence the participation of specific synaptic proteins. We have developed  $\text{Ca}^{2+}$  imaging in *Drosophila* larval motor nerve terminals and studied the dynamics of  $[\text{Ca}^{2+}]_i$  during synaptic activity in two types of nerve terminals.

In 3rd instar larvae, types Ib and Is boutons, which innervate muscles 6 and 7 in each abdominal segment, originate from 2 different motor neurons and differ anatomically: Ib boutons are 3–5  $\mu\text{m}$  diam and possess ~40 synapses on

average, whereas Is boutons are only 1–3  $\mu\text{m}$  diam and possess 7 synapses on average (Atwood et al. 1993). Despite their smaller size and fewer synapses, Is boutons release, on average, about the same number of transmitter quanta per impulse at 1 Hz as do Ib boutons (S. Karunanithi, B. A. Stewart, and H. L. Atwood, unpublished observations), suggesting more effective transmission at the level of individual synapses. By comparing calcium signals in the two terminal types, we addressed the hypothesis that greater effectiveness of transmission for Is synapses results from a larger  $\text{Ca}^{2+}$  signal.

## METHODS

Canton-S *Drosophila melanogaster* wandering 3rd instar larvae reared on cornmeal medium (at 25°C, 60–70% relative humidity) were prepared for recording as described previously (Stewart et al. 1994). Haemolymph-like physiological solution (HL3) was used in experiments. The calcium concentration in the solution was 0.7 mM, unless otherwise specified.

Fluo-3 AM (6  $\mu\text{M}$ ; Molecular Probes, Eugene, OR), was dissolved in HL3 solution containing 0.04% (wt/vol) pluronic acid and *N,N,N',N'*-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN; 20  $\mu\text{M}$  in a final concentration of 0.02% ethanol; Molecular Probes) to a final volume of 1 mL, and the mixture was sonicated. TPEN was used to chelate heavy metals that can affect the fluo-3 responses. The final solution contained 0.8% (vol/vol) dimethyl sulfoxide, which was used to make up the stock concentration of fluo-3 AM (1 mM). The preparation was incubated in this solution in the dark for 40 min at 12°C.

After dye loading, the bath containing the preparation was transferred and secured to the stage of an upright microscope. The experiments were conducted at room temperature (19–22°C), and fluo-3 AM was maintained in the bathing solution. Stimulus pulses were delivered to segmental nerves via a glass suction electrode of 10  $\mu\text{m}$  ID. Stimulus trains of 5 or 10 s duration were delivered at 3- to 4-min intervals.  $\text{Ca}^{2+}$  signals were collected from motor nerve terminals innervating muscle 6 of either segment 3 or 4.

Imaging was performed on an upright microscope (Nikon, Optiphot-2) with a BioRad MRC 600 confocal laser scanner and a  $\times 40$  (0.55 NA) Nikon water immersion objective. The 488-nm excitation line of the argon laser was attenuated to 1% of the maximum power.

Emission was monitored through a low-pass filter with a cutoff at 515 nm. The pinhole of the photomultiplier tube was fully open to allow maximum depth of field and sensitivity. Selected boutons were imaged consecutively (50 times) before, during, and after trains at the different frequencies of stimulation. Images of  $127 \times 170$  pixels were accumulated at 800-ms intervals. The fluorescence response was expressed as the change in fluorescence divided by resting fluorescence ( $F_{\text{rest}}$ ),  $\% \Delta F/F = 100 \times (F_{\text{stim}} - F_{\text{rest}})/F_{\text{rest}}$ .

Comparisons between bouton types were made using a Student's *t*-test, and  $P < 0.05$  was deemed significant. Time constants of the  $\text{Ca}^{2+}$  signal decay were obtained using nonlinear regression (Marquardt-Levenberg algorithm), fitted to the averaged signal

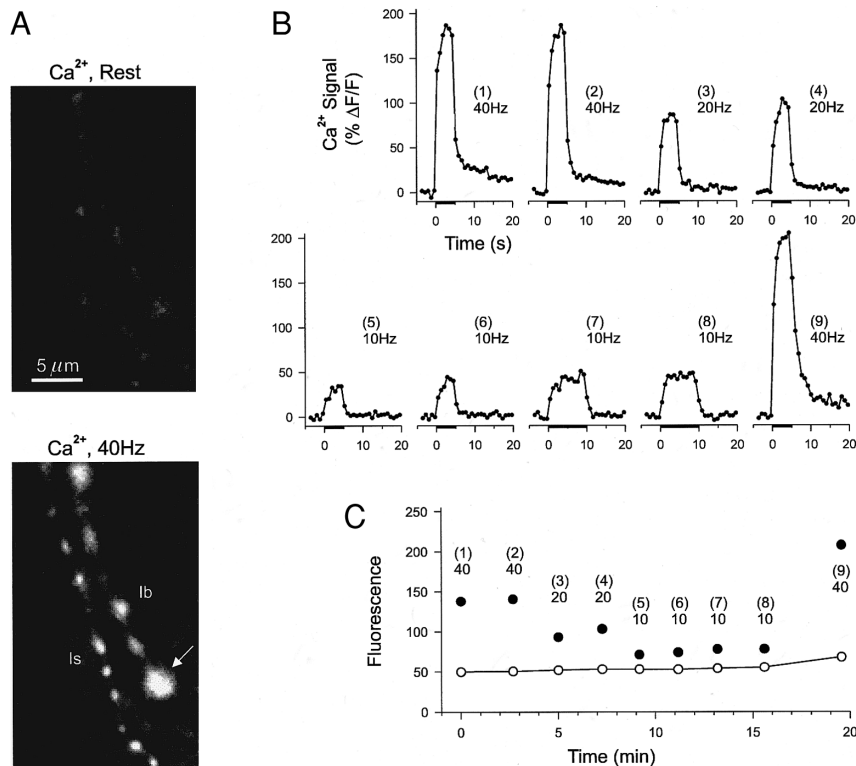


FIG. 1. Stimulation evoked Ca<sup>2+</sup> signals in *Drosophila* boutons. *A*: confocal images from a neuromuscular preparation, showing fluo-3 fluorescence under resting conditions (*top*) and during 40-Hz stimulation (*bottom*). Note the 2 strings of boutons (Type Is on left, Ib on right). *B*: change in Ca<sup>2+</sup> plotted against time for the type Ib bouton indicated by the arrow in *A*, illustrating the effects of stimulation frequency and duration. Trains (sequence number shown in brackets) were delivered at *time 0* (duration indicated by bar) and at the frequency shown. *C*: prestimulus resting fluorescence,  $F_{rest}$  (joined open circles) and the fluorescence value ( $F_{stim}$ ) at the end of each train (filled circles) for each of the 8 stimulus trains (sequence number shown in brackets and frequency below) delivered in *B* and plotted over the time course of the experiment.

following stimulation at each frequency (Fig. 2*B*) using a single or double (sum of 2 single) exponentials (Sigmatat for Windows 1.0). Number of experiments ( $N$ ), number of boutons ( $n$ ), and the standard error of the mean (SE) are included.

## RESULTS

We first determined whether the Ca<sup>2+</sup> signals obtained from individual varicosities were reliable and reproducible. The images in Fig. 1*A* are from a typical experiment and show the fluorescence in both bouton types before (*top*) and during (*bottom*) 40-Hz stimulation. The Ca<sup>2+</sup> signals shown in Fig. 1*B* were obtained by sequential imaging of the Ib bouton (arrow) shown in Fig. 1*A* (*bottom*). Signals obtained at the same stimulus frequencies were reproducible: the time course and magnitude of the Ca<sup>2+</sup> responses were similar in different trains at a given frequency (Fig. 1, *B* and *C*; filled circles). The resting fluorescence,  $F_{rest}$  (Fig. 1*C*, open circles) remained constant throughout the experiment for at least eight trains of stimuli but sometimes increased in longer experiments (Fig. 1*C*, last point). The Ca<sup>2+</sup> signals appeared to be unaffected by photodamage or photobleaching for at least six to eight consecutive trains ( $N = 7$ ).

To confirm that these transients were Ca<sup>2+</sup> dependent, we examined the responses from Ib boutons at two Ca<sup>2+</sup> concentrations. There was a significant increase (paired  $t$ -test) in the average Ca<sup>2+</sup> signal (at the end of stimulation) for two frequencies of stimulation when the Ca<sup>2+</sup> concentration was increased from 0.5 to 0.7 mM (5 Hz:  $24.9 \pm 4.2\%$  to  $62.7 \pm 6.2\%$ , mean  $\pm$  SE,  $P < 0.001$ ; 10 Hz:  $95.4 \pm 3.9\%$  to  $136.8 \pm 5.8\%$ ,  $P < 0.001$ ;  $n = 5$ ). Thus the signals recorded were affected by alterations in Ca<sup>2+</sup> influx. These experiments cannot rule out the possibility that part of the Ca<sup>2+</sup> signal depends on Ca<sup>2+</sup> released from internal stores.

Experiments in which both bouton types were visible in the same field of view (e.g., Figs. 1*A* and 2*A*) were chosen for comparative analysis. Figure 2*B* shows the averaged time course of Ca<sup>2+</sup> signals recorded from Ib and Is boutons at three frequencies of stimulation (5, 10, and 20 Hz) in four experiments. In both types of terminals, the Ca<sup>2+</sup> signal increased significantly (paired  $t$ -test) with stimulus frequency (Fig. 2*B*) from 5 to 10 Hz (Ib:  $P < 0.001$ ; Is:  $P < 0.001$ ), and from 10 to 20 Hz (Ib:  $P = 0.003$ ; Is:  $P < 0.001$ ). There was no significant difference in the average Ca<sup>2+</sup> signal (at the end of stimulation) between the two bouton types at 5 Hz (Ib:  $30.9 \pm 5.6\%$ ; Is:  $20.9 \pm 3.9\%$ ;  $P = 0.15$ ) and 20 Hz (Ib:  $155.5 \pm 26.1\%$ ; Is:  $118.4 \pm 12.9\%$ ;  $P = 0.19$ ), but there was a significant difference at 10 Hz (Ib:  $82.2 \pm 12.8\%$ ; Is:  $48.3 \pm 5.7\%$ ;  $P = 0.02$ ).

In both bouton types, the decay time of the Ca<sup>2+</sup> signal after 20-Hz stimulation (Fig. 2*B*) was prolonged compared with the decays after 5 and 10 Hz. The time constant of decay of the averaged signal after stimulation at 5 and 10 Hz was well fitted by a single exponential (5 Hz:  $\tau_{Ib} = 0.9$  s,  $\tau_{Is} = 0.6$  s; 10 Hz:  $\tau_{Ib} = 1.4$  s,  $\tau_{Is} = 1.1$  s), whereas the recovery after 20-Hz stimulation was well fitted by a double exponential (Ib:  $\tau_1 = 1.7$  s,  $\tau_2 = 27.7$  s; Is:  $\tau_1 = 1.2$  s,  $\tau_2 = 29.5$  s).

## DISCUSSION

Although Ib boutons are larger than Is boutons and have, on average, five times more synapses, the transmission efficacy per synapse is greater for Is boutons. This study indicates that the gross cytoplasmic Ca<sup>2+</sup> signals of the two bouton types do not differ greatly in relative amplitude or rate of onset. However, our data do not preclude the possibil-

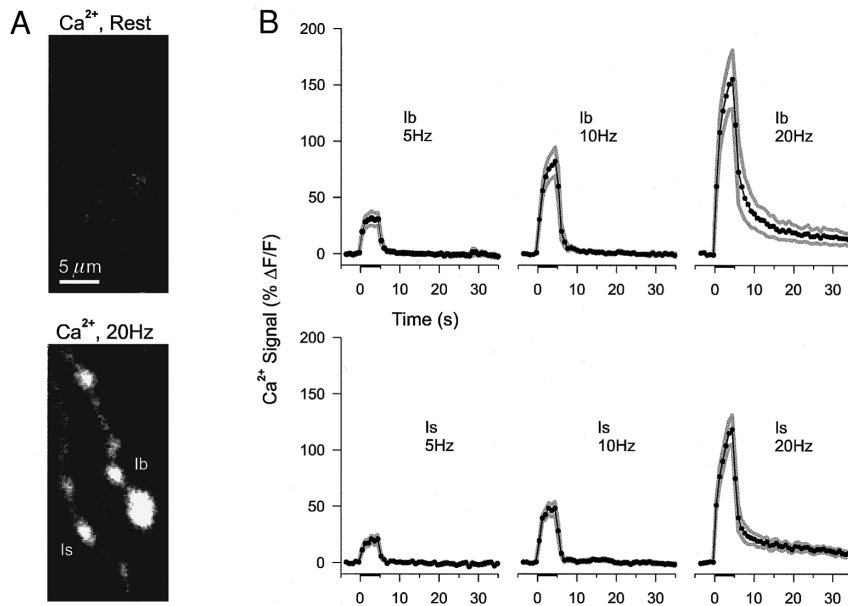


FIG. 2. Comparison of Ca<sup>2+</sup> signals between type Ib and Is boutons. *A*: Ca<sup>2+</sup> fluorescence at rest (*top*) and during a 20-Hz stimulus (*bottom*) for type Ib and Is boutons. *B*: effects of stimulation frequency on Ca<sup>2+</sup> signals averaged for 4 experiments in which both type Ib ( $n = 8$ ) and Is ( $n = 10$ ) boutons were visible and responded in the same field of view, including the experiment imaged in *A*. Trains lasting 5 s were delivered at *time 0* (indicated by bar) at stimulation frequencies of 5, 10, and 20 Hz. The Ca<sup>2+</sup> responses were averaged and plotted by bouton type (black). The top and bottom boundary for SE is plotted in gray.

ity that Ca<sup>2+</sup> signals at Ib and Is active zones differ. Detailed evaluation of the relationship of these signals to synaptic efficacy will require consideration of surface to volume ratio of boutons in which  $[Ca^{2+}]_i$  and transmitter release have been measured and in which ultrastructural analysis of synapse number has been performed. Two nonexclusive explanations for the differences in synaptic efficacy between the two bouton types are as follows. 1) A smaller proportion of Ib synapses may release transmitter even though Ca<sup>2+</sup> entry may occur at all synapses. 2) Intraterminal factors (other than gross cytoplasmic Ca<sup>2+</sup>), including calcium buffering and qualitative or quantitative differences in synaptic proteins, differ in the two bouton types.

For both bouton types, the rate of decay of the Ca<sup>2+</sup> signal following 20-Hz stimulation can be fitted by two time constants, whereas at 5 and 10 Hz, a single exponential describes the decay (Fig. 2*B*). Similar observations in the crayfish neuromuscular junction link the slowly decaying Ca<sup>2+</sup> transients with synaptic augmentation and potentiation (Delaney et al. 1989).

In conclusion, our results show for the first time that Ca<sup>2+</sup> signals can be recorded from *Drosophila* larval motor nerve terminals. The Ca<sup>2+</sup> signals were similar in amplitude and time course for Ib and Is boutons, which suggests that the higher efficacy of Is synapses is governed by other intraterminal factors.

We thank A. Jeromin and A. Shayan for comments on the manuscript.

We also thank T. A. Goldthorpe and Dr. B. S. Jahromi for modifying the software used for analysis.

This work was supported by a National Sciences and Engineering Research Council collaborative program grant to H. L. Atwood, Medical Research Council and Neuroscience Network (Canada) grants to M. P. Charlton, and a Neuroscience Network studentship to J. Georgiou. S. Karunanithi and J. Georgiou contributed equally to this work.

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Received 7 May 1997; accepted in final form 25 July 1997.

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