Brief Communication

Active Zone Localization of Presynaptic Calcium Channels Encoded by the *cacophony* Locus of *Drosophila*

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Presynaptic calcium channels play a central role in chemical synaptic transmission by providing the calcium trigger for evoked neurotransmitter release. These voltage-gated calcium channels are composed of a primary structural subunit, $\alpha1$, as well as auxiliary β and $\alpha2\delta$ subunits. Our previous genetic, molecular, and functional analysis has shown that the *cacophony* (*cac*) gene encodes a primary presynaptic calcium channel $\alpha1$ subunit in *Drosophila*. Here we report that transgenic expression of a *cac*-encoded $\alpha1$ subunit fused with enhanced green fluorescent protein efficiently rescues *cac* lethal mutations and allows *in vivo* analysis of calcium channel localization at active zones. The results reported here further characterize the primary role of *cac*-encoded calcium channels in neurotransmitter release. In addition, these studies provide a unique genetic tool for live imaging of functional presynaptic calcium channels *in vivo* and define a molecular marker for immunolocalization of other presynaptic proteins relative to active zones. These findings are expected to facilitate additional analysis of synaptic development and function in this important model system.

Key words: active zone; cacophony; *Drosophila*; calcium channel; synaptic; GFP

Introduction

Previous studies have described localization of presynaptic calcium channel $\alpha1$ subunits at active zones, where synaptic vesicles dock and fuse with the plasma membrane, and have considered its important functional implications for neurotransmitter release (for review, see Stanley, 1997; Catterall, 1998). The primary vertebrate presynaptic calcium channels are of the N- and P/Q-type for which the $\alpha1$ subunits are encoded by the $\alpha1B$ and $\alpha1A$ genes, respectively. Active zone localization of these channels has been shown at native synapses using labeled neurotoxins and calcium channel antibodies (Robitaille et al., 1990; Westenbroek et al., 1995) and in cultured neurons through the use of an epitope tag (Maximov and Bezprozvanny, 2002). Despite this progress, to date, no genetic tools have been available for live imaging of calcium channel localization *in vivo*. Here we report generation of such a tool and its use in analysis of presynaptic calcium channel distribution and function in *Drosophila*.

The *Drosophila cacophony* (*cac*) locus was originally defined by its role in male courtship behavior (von Schilcher, 1977; Chan et al., 2002) and subsequently found to encode a voltage-gated calcium channel $\alpha1$ subunit related to those of vertebrate presynaptic calcium channels (Smith et al., 1996). More recently, isolation and analysis of a *cac* mutant exhibiting rapid temperature-sensitive paralysis has demonstrated that *cac* encodes a primary presynaptic calcium channel in *Drosophila* (Dellinger et al., 2000; Kawasaki et al., 2000, 2002). To further examine the role of this calcium channel gene product in neurotransmitter release, we used transgenic methods to express enhanced green fluorescent protein (EGFP)-tagged *cac*-encoded $\alpha1$ subunits *in vivo*.

Materials and Methods

Fly stocks. As described previously (Kawasaki et al., 2002), fly stocks included the *cac* lethal alleles [$l(1)L13^{20-3}$ and $l(1)L13^{HC129}$], the X-linked elav-GAL4 enhancer trap line [$P(w+) elav^{C155}$], elav-GAL4 $l(1)L13^{20-3}$, and elav-GAL4 $l(1)L13^{HC129}$. Wild-type flies were *Canton S*. Stocks and crosses for this study were maintained at 25°C.

cac1-EGFP *fusion construct.* Separate PCR products corresponding to the EGFP open reading frame (ORF) and the 3′ end of the *cac* ORF (excluding the stop codon) were generated using Pfu DNA polymerase (Stratagene, La Jolla, CA) with primers designed to include flanking *Eco*RI sites. These products were used to generate a fusion construct in which a two amino acid linker (corresponding to the *Eco*RI site) and the EGFP ORF were appended to the 3′ end of the *cac* ORF (see Fig. 1*A*). The final fusion construct was incorporated into the transformation vector pUAST (Brand and Perrimon, 1993), and transgenic flies were generated as described previously (Kawasaki et al., 2002).

Transformation rescue. Two transgenic lines carrying insertions on the third chromosome, *UAS-cac1-EGFP 786C* and *UAS-cac1-EGFP 247A*, were used to rescue *cac* lethal mutations as described previously (Kawasaki et al., 2002). Briefly, elav-GAL4 $l(1)L13^{HC129}$/FM7i and elav-GAL4 $l(1)L13^{20-3}$/FM7i females were mated to lines homozygous for a *UAS-cac1-EGFP* insertion. Similar control crosses were performed using +/FM7i females. Percentage viability was calculated as follows: (% non-*FM7i* male progeny from the rescue cross/% non-*FM7i* male progeny from the control cross) · 100. Finally, viability of *cac* lethals in the absence of *UAS-cac1 EGFP* was determined similarly in crosses of elav-GAL4 $l(1)L13^{HC129}$/FM7i and elav-GAL4 $l(1)L13^{20-3}$/FM7i females to wild-type (*Canton S*) males.

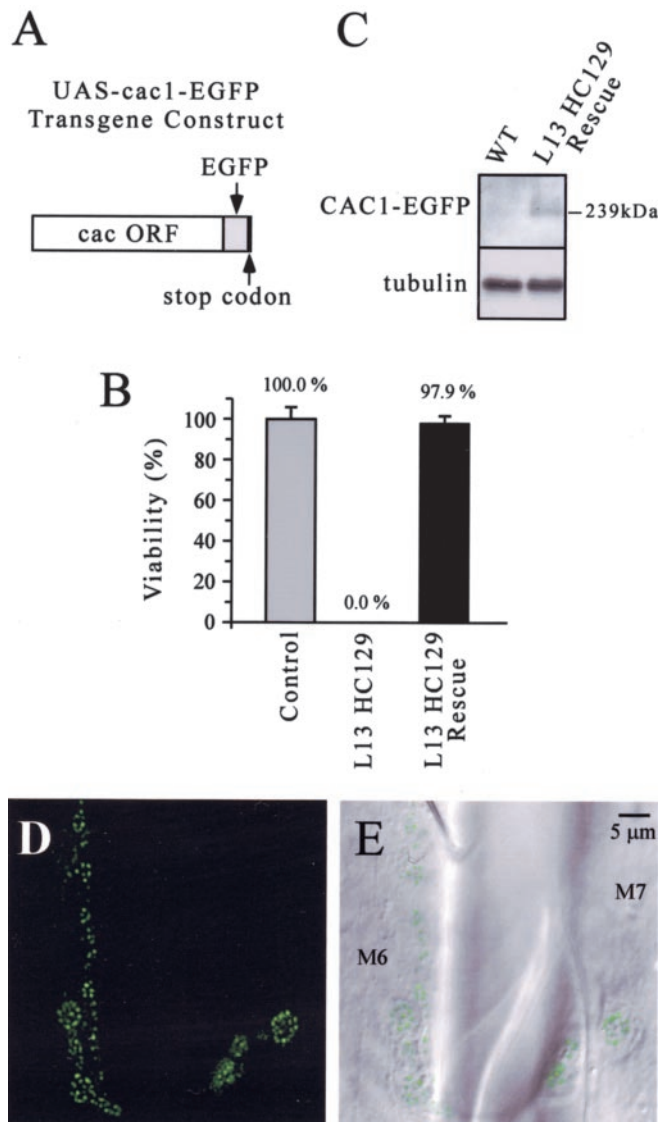*Western blotting.* Conventional Western analysis was performed on

Figure 1. Transformation rescue and live imaging of EGFP-tagged calcium channels. *A*, The *UAS-cac1-EGFP* transgene construct for expression of EGFP-tagged *cac*-encoded α1 subunits. *B*, Rescue of a *cac* lethal mutant. Males carrying a *cac* lethal mutation (L13 HC129) but lacking the transgene exhibited uniform lethality (0% viability); however, *cac* lethal males carrying the transgene (L13 HC129 Rescue) were clearly rescued and exhibited adult viability similar to that of wild-type controls. Adult viability was determined in five independent rescue experiments for each genotype ($n = 5$). Viability of $L13^{HC129}$ rescue males was not significantly different from that of control males ($p > 0.05$). *C*, Western analysis. An anti-GFP antibody detected a single band corresponding to the predicted 239 kDa CAC1-EGFP fusion protein in *cac* lethal flies rescued by transgenic expression of CAC1-EGFP (L13 HC129 Rescue) but not in wild-type (WT) controls. A loading control (tubulin) is also shown. *D, E*, Live epifluorescence and differential interference contrast imaging reveals CAC1-EGFP puncta and their localization within larval neuromuscular presynaptic terminals. Images were obtained from a *cac* lethal mutant (L13 HC129) rescued by neural expression of CAC1-EGFP. M6 and M7 mark identified ventral longitudinal muscles 6 and 7.

samples prepared from fly heads. The equivalent of one fly head was loaded per lane. The primary antibody was an anti-GFP monoclonal (Clontech, Palo Alto, CA) used at a dilution of 1:1000. Detection was accomplished using a horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham Biosciences, Arlington Heights, IL) and enhanced chemiluminescence (Amersham Biosciences ECL Plus Western Blotting Detection System). As an internal loading control, tubulin was detected with an anti-tubulin monoclonal antibody (Sigma, St. Louis, MO) at 1:200,000.

Live imaging. CAC1-EGFP fluorescence was observed at larval neuromuscular synapses. Third-instar larvae were dissected in saline solution (in mM: 128 NaCl, 2 KCl, 4 MgCl₂, 1.8 CaCl₂, 36 sucrose, and 5 HEPES, pH, 7.0), and all nerves projecting from the ventral ganglion were cut to prevent muscle contraction. Epifluorescence images were obtained using a Nikon (Tokyo, Japan) Eclipse E600FN microscope with a Fluor 60× 1.0 numerical aperture water-immersion objective (Nikon) and the following filter set: excitation filter, HQ480/20; dichroic mirror, Q495LP; and emission filter, HQ 525/50 (Chroma, Brattleboro, VT). Images were captured with a CCD camera (ORCA-ER; Hamamatsu Photonics, Hamamatsu, Japan) and acquired and processed using the SimplePCI imaging software package (Compix, Cranberry Township, PA). In this study, all images of larval neuromuscular synapses were obtained from ventral longitudinal muscles 6 and 7 within abdominal segment A2 or A3.

Immunocytochemistry and confocal microscopy. Immunocytochemistry of *Drosophila* neuromuscular synapses was performed by conventional methods with one exception: typically, and in all experiments shown, detection of CAC1-EGFP signals was performed using Tyramide Signal Amplification (Molecular Probes, Eugene, OR) rather than a fluorescently labeled secondary antibody. A typical double-labeling experiment using tyramide enhancement of the CAC1-EGFP signal was performed as described below.

Dissected third-instar larvae and adults were fixed for 30 min in saline solution (see Live imaging) containing 4% paraformaldehyde (the temperature for all incubations was ambient unless specified). After consecutive 10 min washes with (1) saline solution, (2) PBS, and (3) PBT (PBS containing 0.2% Triton X-100), specimens were incubated in blocking buffer (1% bovine serum albumin in PBT) for 1 hr. Blocked samples were incubated for 2 hr with the primary anti-GFP monoclonal antibody (Clontech) at 1:5000 in blocking buffer (for comparison, this antibody was used at 1:500 for conventional detection with a fluorescently labeled secondary antibody). The primary anti-GFP antibody incubation was followed by three washes with PBT (10 min each) and tyramide enhancement essentially according to the instructions of the manufacturer. Two subsequent washes with PBT and one with PBS (10 min each) were followed by a 30 min incubation with PBS containing 5% normal goat serum (NGS blocking solution).

Incubation in the second primary antibody, either a 1:200 dilution of rabbit anti-HRP antibody (Jackson ImmunoResearch, West Grove, PA) or a 1:2000 dilution of rabbit anti-DPAK (*Drosophila* p21-activated kinase) antibody (kindly provided by Nicholas Harden), was performed in NGS blocking solution. After five washes with PBS (12 min each), samples were incubated with a fluorescent secondary antibody, Alexa Fluor 546 goat anti-rabbit antibody (Molecular Probes), diluted 1:100 in NGS blocking solution. Samples were finally washed five times with PBS (12 min each) and mounted in a 1:1 mixture of PBS and glycerol. Preparations were imaged using an Olympus Optical (Tokyo, Japan) FV300 confocal microscope with a PlanApo 60× 1.4 numerical aperture oil objective (Olympus Optical) and a z-step size of 0.2 μm. Images shown in Figure 2 *I–M* were deconvolved and reconstructed using the AutoDeblur and AutoVisualize functions of the Autoquant (Watervliet, NY) software package. The surface projection in Figure 2 *M* was generated using the Voxel Gradient Shading function of the same package.

Electrophysiology. Recordings of EPSCs from dorsal longitudinal flight muscle (DLM) neuromuscular synapses of the adult were obtained and analyzed as described previously (Kawasaki et al., 1998). Recordings were performed at 20°C using 2- to 4-d-old flies. *n* refers to the number of recordings; one recording was obtained from each preparation.

Data analysis. Graphing and analysis of numerical data were performed in Microsoft (Seattle, WA) Excel. All data values in the text and bar graphs are given as mean ± SEM. Statistical analysis was performed using the two-tailed Student's *t* test, and significance was assigned to comparisons for which $p \leq 0.05$.

Results

Transformation rescue and live imaging of EGFP-tagged calcium channels

A transgene construct shown previously to rescue *cac* mutants (Kawasaki et al., 2002) was modified to express a CAC1-EGFP

fusion protein by appending EGFP to the α1 subunit C-terminal cytoplasmic tail (Fig. 1*A*) (see Materials and Methods). As described previously (Kawasaki et al., 2002), the resulting transgene was expressed specifically in the nervous system using the *GAL4-UAS* system and an *elav-GAL4* driver. Rescue of the *cac* embryonic lethal mutations *l(1)L13^{HC129}* and *l(1)L13$^{20-3}$* was examined for two *cac1-EGFP* transgenic lines, and both lines produced efficient rescue of each lethal to adult viability. Rescue of lethal *l(1)L13^{HC129}* by one line, *UAS-cac1-EGFP 786c*, is shown in Figure 1*B*. Consistent with our previous findings, these results indicate that neural expression of a CAC-EGFP α1 subunit is sufficient to provide the essential functions of *cac*-encoded calcium channels. In rescued lethal mutants, the predicted 239 kDa fusion protein was observed by Western analysis using an anti-GFP antibody (Fig. 1*C*). To examine whether expression of the CAC1-EGFP fusion protein allows live imaging of calcium channel localization *in vivo*, rescued lethal mutants were imaged by epifluorescence microscopy. These experiments revealed clear punctuate EGFP fluorescence within live neuromuscular presynaptic terminals of third-instar larvae (Fig. 1*D,E*).

Active zone localization of *cac*-encoded calcium channels

To further characterize the presynaptic localization of *cac*-encoded calcium channels, confocal immunofluorescence imaging was used at larval neuromuscular synapses to examine the distribution of EGFP-tagged α1 subunits. Double labeling using a monoclonal anti-GFP antibody and a neuronal plasma membrane-specific antibody, anti-HRP, was performed in a *cac* lethal mutant rescued by neural expression of CAC1-EGFP. These experiments confirmed the presence of calcium channel puncta within presynaptic boutons of rescued larvae (Fig. 2*A–C*) but not wild-type controls lacking the transgene (Fig. 2*D*). The likely possibility that these CAC1-EGFP puncta correspond to active zones was examined by double labeling with an antibody against DPAK, a well characterized marker for the postsynaptic densities closely apposed to presynaptic active zones (Sone et al., 2000). These experiments revealed extensive colocalization of CAC1-EGFP and DPAK, confirming active zone localization of *cac*-encoded calcium channel α1 subunits (Fig. 2*E–H*). Consistent with previous studies defining the three-dimensional ultrastructure of larval neuromuscular boutons and the surrounding postsynaptic membrane (Atwood et al., 1993; Jia et al., 1993), active zones exhibited an approximately even distribution over the entire surface of a bouton (Fig. 2*I–M*). This is revealed in a series of optical sections through the *z*-axis (Fig. 2*I,K*), as well as the respective maximum projections representing the top and bottom halves of the image stack (Fig. 2*J,L*, respectively) and a surface projection for the same structure (Fig. 2*M*).
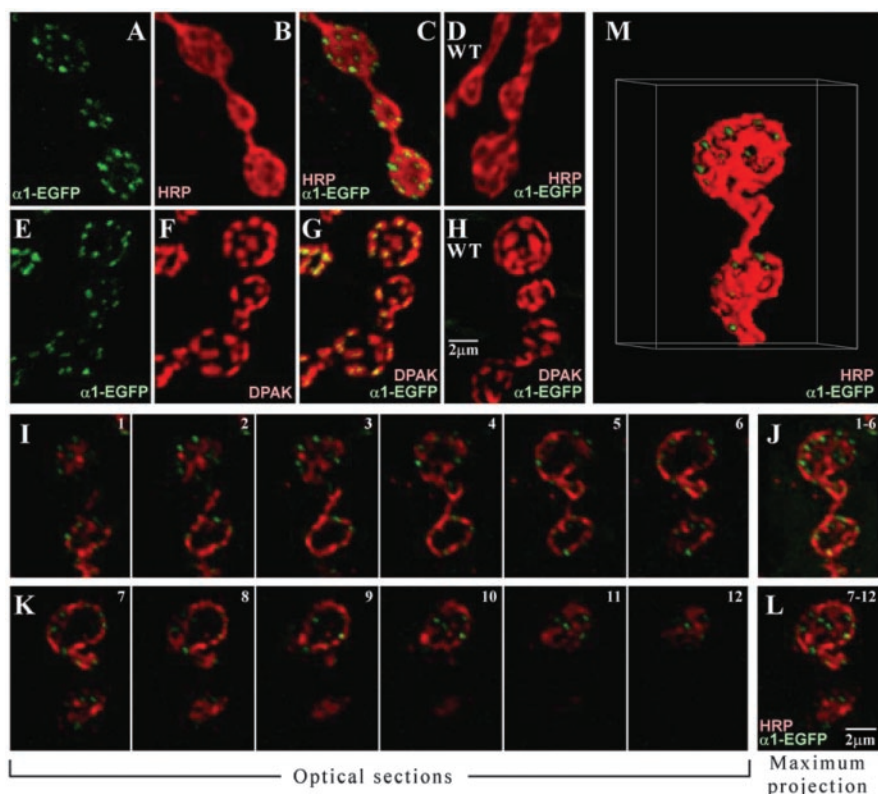

Figure 2. Active zone localization of *cac*-encoded calcium channels. Images were generated by confocal immunofluorescence microscopy at larval neuromuscular synapses of a *cac* lethal mutant (*L13^{HC129}*) rescued by neural expression of CAC1-EGFP. *A–C*, Double labeling with anti-HRP and anti-GFP showing localization of CAC1-EGFP (α1-EGFP) puncta to presynaptic boutons. *D*, A wild-type (WT) control lacking the transgene and thus CAC1-EGFP. *E–G*, Double labeling with anti-DPAK showing active zone localization of CAC1-EGFP puncta. *H*, A wild-type control lacking the transgene. *A–H* are each maximum projections of three consecutive optical z-sections. *I–M*, Analysis of anti-GFP–anti-HRP double labeling showing the three-dimensional distribution of CAC1-EGFP puncta. A series of six optical sections through the *z*-axis representing the top half of the terminal structure (*I*) and a maximum projection of these sections showing the distribution of active zones (*J*). *K, L*, A series of optical sections and the corresponding maximum projection representing the bottom half of the structure. *M*, A surface projection for the entire z-series represented in *I–L*.

cac-encoded calcium channels at adult neuromuscular synapses

Our previous functional analysis of *cac*-encoded presynaptic calcium channels was performed at adult neuromuscular synapses of the DLM (Kawasaki et al., 2000, 2002). To examine calcium channel distribution in the same preparation, imaging of CAC1-EGFP α1 subunits was performed at DLM neuromuscular synapses of a *cac* lethal mutant rescued by neural expression of *cac1-EGFP*. As in the larval preparation, double labeling with anti-GFP and anti-HRP revealed presynaptic CAC1-EGFP puncta (Fig. 3*A–C*), and active zone localization was confirmed by double-labeling with anti-DPAK (Fig. 3*D–F*). At DLM synapses, active zones were typically associated with axonal swellings of 1–2 μm in diameter and were distributed along the axon at intervals of ∼1–2 μm. These results extend our previous findings demonstrating that *cac*-encoded calcium channels function in neurotransmitter release at DLM neuromuscular synapses.

To assess the function of CAC1-EGFP calcium channels in synaptic transmission, analysis of rescued *cac* lethal mutants included voltage-clamp recordings of DLM EPSCs (Fig. 3*G,H*). Evoked synaptic currents from wild type and the rescued *cac* lethal were indistinguishable, exhibiting amplitudes of 2.37 ± 0.18 μA (*n* = 5) and 2.40 ± 0.17 μA (*n* = 5), respectively. The activity dependence of synaptic transmission was examined as
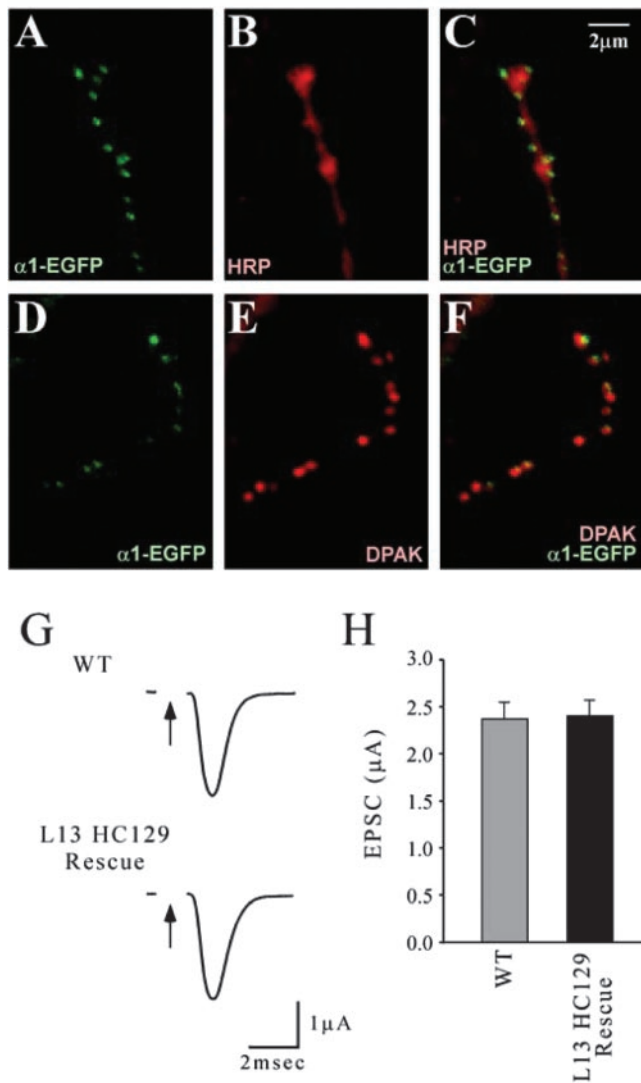
Figure 3. *cac*-encoded calcium channels at adult neuromuscular synapses. Images and recordings were obtained from a *cac* lethal mutant ($L13^{HC129}$) rescued by neural expression of CAC1-EGFP. *A–C*, Double-labeling experiments with anti-GFP (α1-EGFP) and anti-HRP show localization of CAC1-EGFP within presynaptic terminals of DLM neuromuscular synapses. *D–F*, Double labeling with anti-DPAK showing active zone localization of CAC1-EGFP puncta. *G, H*, Voltage-clamp recordings of EPSCs from DLM neuromuscular synapses demonstrate wild-type (WT) synaptic function in the rescued *cac* lethal mutant. Arrows indicate stimulation of the DLM motor axon. Stimulation artifacts have been removed.

Discussion

The results reported here further define the primary role of *cac*-encoded calcium channels in neurotransmitter release and reveal active zone localization of these channels at two different neuromuscular synapses of *Drosophila*. A key aspect of this study is transgenic expression of a EGFP-tagged calcium channel α1 subunit that is targeted to active zones and retains its function in neurotransmitter release. In light of the central importance of presynaptic calcium channels and the utility of *Drosophila* as a model system, the ability to perform live imaging of functional presynaptic calcium channels, as well as immunolocalization of these and other presynaptic proteins relative to active zones, is expected to have general implications for further study of mechanisms operating in the development and function of synapses.

References

Atwood HL, Govind CK, Wu C-F (1993) Differential ultrastructure of synaptic terminals on ventral longitudinal abdominal muscles in *Drosophila* larvae. J Neurobiol 24:1008–1024.

Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401–415.

Catterall WA (1998) Structure and function of neuronal Ca^{2+} channels and their role in neurotransmitter release. Cell Calcium 24:307–323.

Chan B, Villella A, Funes P, Hall JC (2002) Courtship and other behaviors affected by a heat-sensitive, molecularly novel mutation in the *cacophony* calcium-channel gene of *Drosophila*. Genetics 162:135–153.

Dellinger BB, Felling R, Ordway RW (2000) Genetic modifiers of the *Drosophila* NSF mutant, *comatose*, include a temperature-sensitive paralytic allele of the calcium channel α1 subunit gene, *cacophony*. Genetics 155:203–211.

Jia X-X, Gorczyca M, Budnik V (1993) Ultrastructure of neuromuscular junctions in *Drosophila*: comparison of wild type and mutants with increased excitability. J Neurobiol 24:1025–1044.

Kawasaki F, Mattiuz AM, Ordway RW (1998) Synaptic physiology and ultrastructure in *comatose* mutants define an *in vivo* role for NSF in neurotransmitter release. J Neurosci 18:10241–10249.

Kawasaki F, Felling R, Ordway RW (2000) A temperature-sensitive paralytic mutant defines a primary synaptic calcium channel in *Drosophila*. J Neurosci 20:4885–4889.

Kawasaki F, Collins SC, Ordway RW (2002) Synaptic calcium channel function in *Drosophila*: analysis and transformation rescue of temperature-sensitive paralytic and lethal mutations of *cacophony*. J Neurosci 22:5856–5864.

Maximov A, Bezprozvanny I (2002) Synaptic targeting of N-type calcium channels in hippocampal neurons. J Neurosci 22:6939–6952.

Robitaille R, Adler EM, Charlton MP (1990) Strategic location of calcium channels at transmitter release sites of frog neuromuscular synapses. Neuron 5:773–779.

Smith LA, Wang X, Peixoto AA, Neumann EK, Hall LM, Hall JC (1996) A *Drosophila* calcium channel α1 subunit gene maps to a genetic locus associated with behavioral and visual defects. J Neurosci 16:7868–7879.

Sone M, Suzuki E, Hoshino M, Hou D, Kuromi H, Fukata M, Kuroda S, Kaibuchi K, Nabeshima Y-I, Hama C (2000) Synaptic development is controlled in the periactive zones of *Drosophila* synapses. Development 127:4157–4168.

Stanley EF (1997) The calcium channel and the organization of the presynaptic transmitter release face. Trends Neurosci 20:404–409.

von Schilcher F (1977) A mutation which changes courtship song in *Drosophila melanogaster*. Behav Genet 7:251–259.

Westenbroek RE, Sakurai T, Elliott EM, Hell JW, Starr TV, Snutch TP, Catterall WA (1995) Immunochemical identification and subcellular distribution of the alpha 1A subunits of brain calcium channels. J Neurosci 15:6403–6418.

well. In wild type and the rescued *cac* lethal mutant, the respective steady-state EPSC amplitudes after 5 sec of 50 Hz stimulation were $39.1 \pm 0.84\%$ ($n = 4$) and $39.9 \pm 2.38\%$ ($n = 4$) of the initial amplitude. Thus, no indication of altered calcium channel function was observed; however, we cannot rule out subtle effects of the EGFP tag on channel properties. The above results provide direct confirmation of *cac*-encoded calcium channel localization and function at DLM neuromuscular synapses.