Presynaptic Mitochondria in Functionally Different Motor Neurons Exhibit Similar Affinities for Ca^{2+} But Exert Little Influence as Ca^{2+} Buffers at Nerve Firing Rates *In Situ*

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Mitochondria accumulate within nerve terminals and support synaptic function, most notably through ATP production. They can also sequester Ca^{2+} during nerve stimulation, but it is unknown whether this limits presynaptic Ca^{2+} levels at physiological nerve firing rates. Similarly, it is unclear whether mitochondrial Ca^{2+} sequestration differs between functionally different nerve terminals. We addressed these questions using a combination of synthetic and genetically encoded Ca^{2+} indicators to examine cytosolic and mitochondrial Ca^{2+} levels in presynaptic terminals of tonic (MN13-Ib) and phasic (MNSNb/d-Is) motor neurons in *Drosophila*, which, as we determined, fire during fictive locomotion at ~ 42 Hz and ~ 8 Hz, respectively. Mitochondrial Ca^{2+} sequestration starts in both terminals at ~ 250 nM, exhibits a similar Ca^{2+} -uptake affinity (~ 410 nM), and does not require Ca^{2+} release from the endoplasmic reticulum. Nonetheless, mitochondrial Ca^{2+} uptake in type Is terminals is more responsive to low-frequency nerve stimulation and this is due to higher cytosolic Ca^{2+} levels. Since type Ib terminals have a higher mitochondrial density than Is terminals, it seemed possible that greater mitochondrial Ca^{2+} sequestration may be responsible for the lower cytosolic Ca^{2+} levels in Ib terminals. However, genetic and pharmacological manipulations of mitochondrial Ca^{2+} uptake did not significantly alter nerve-stimulated elevations in cytosolic Ca^{2+} levels in either terminal type within physiologically relevant rates of stimulation. Our findings indicate that presynaptic mitochondria have a similar affinity for Ca^{2+} in functionally different nerve terminals, but do not limit cytosolic Ca^{2+} levels within the range of motor neuron firing rates *in situ*.

Introduction

Presynaptic mitochondria may support synaptic function by producing ATP, sequestering Ca^{2+} , releasing Ca^{2+} , and generating reactive oxygen species (ROS). Mitochondrial Ca^{2+} sequestration in response to depolarization-induced increases in cytosolic Ca^{2+} levels has been well documented in various systems (Kann and Kovacs, 2007). However, it remains largely unknown whether mitochondrial Ca^{2+} sequestration constitutes an effective and possibly universal mechanism for buffering presynaptic cytosolic Ca^{2+} .

Mitochondria sequester cytosolic Ca^{2+} via a $\text{Ca}^{2+}/\text{H}^{+}$ antiporter (Sparagna et al., 1995; Jiang et al., 2009) and a Ca^{2+} uniporter (Gunter et al., 2004; Kirichok et al., 2004) using the

chemiosmotic gradient generated across the inner mitochondrial membrane by the respiratory chain. Mitochondria near the Ca^{2+} channels of the plasma membrane or endoplasmic reticulum (ER) can sequester Ca^{2+} during fast Ca^{2+} transients when Ca^{2+} reaches micromolar concentrations (Alonso et al., 2006; Rizzuto et al., 2009); and even when they are not close to these channels, they can sequester Ca^{2+} at ambient levels as low as 200 nM (Gunter and Sheu, 2009).

A major problem in determining the role of mitochondrial Ca^{2+} sequestration in Ca^{2+} regulation arises from the fact that mitochondrial ATP production is coupled to Ca^{2+} signaling (McCormack et al., 1990). Cytosolic Ca^{2+} spikes can activate at least four Ca^{2+} -sensitive mitochondrial dehydrogenases and the F_1F_0 -ATPase, increasing ATP production through oxidative phosphorylation (Balaban, 2009; Denton, 2009). Accordingly, mitochondrial Ca^{2+} sequestration in nerve terminals may constitute an effective cytosolic Ca^{2+} buffer, but it also may serve to integrate ATP supply with the demands of synaptic activity.

At nerve terminals, pharmacological inhibition of mitochondrial Ca^{2+} sequestration results in higher presynaptic Ca^{2+} levels than those seen in the presence of functional mitochondria during prolonged and/or high-frequency nerve stimulation (Tang and Zucker, 1997; David et al., 1998; Narita et al., 1998; David and Barrett, 2000; Billups and Forsythe, 2002; Suzuki et al., 2002; Bennett et al., 2007), suggesting that mitochondrial Ca^{2+} sequestration may limit presynaptic Ca^{2+} levels at least during

Received Sept. 21, 2009; revised Dec. 17, 2009; accepted Dec. 30, 2009.

This study was supported by grants from the National Institute of Neurological Disorders and Stroke to G.T.M. [National Institutes of Health (NIH) R01 NS061914] and K.E.Z. [NIH R21 NS055202]. We are grateful to Andrea Wellington for assistance in preparing cDNA for transforming animals, and to Mingshan Xue and James Fisher for assistance in collecting preliminary data. We thank Roger Tsien and Atsushi Miyawaki for permission to use Camgaroo (Tsien), Yellow Cameleon (Tsien), and Ratiometric Pericam (Miyawaki), and Tullio Pozzan and Nicolas Demarex for providing plasmids containing the cDNA. We thank Richard Axel, Hugo Bellen, Mary Lilly, and William Saxton for providing fly strains, Rosario Martinez for maintaining fly stocks, Harold Atwood for helpful discussions, and Joel Rawson for critical comments on the manuscript.

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DOI:10.1523/JNEUROSCI.4701-09.2010

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excessive stimulation. However, data from such experimental protocols can be difficult to interpret since ATP deficiencies may occur in the absence of Ca^{2+} -stimulated mitochondrial ATP production. ATP deficiencies will in turn compromise ATP-driven Ca^{2+} pumps (Blaustein et al., 2002; Guerini et al., 2005) and ATP-driven pumps that maintain Na^+ and K^+ gradients, which provide the driving force for Ca^{2+} removal by Na^+ - and $\text{Na}^+/\text{K}^+-\text{Ca}^{2+}$ exchangers (Lyttton, 2007). Accordingly, the tightly interrelated nature of mitochondrial Ca^{2+} sequestration, ATP production and nonmitochondrial Ca^{2+} clearance mechanisms makes it difficult to discern the physiological role of mitochondrial Ca^{2+} sequestration in nerve terminals, especially when stimulation protocols do not reflect physiological ranges of nerve activity.

In this study, we examined whether the sensitivity of mitochondrial Ca^{2+} sequestration differed between synaptic terminals of two functionally different motor neurons (MNs) in *Drosophila* larvae, and whether mitochondrial Ca^{2+} sequestration limits presynaptic Ca^{2+} levels within physiological rates of MN activity. Despite the very different activity levels of tonic and phasic MNs, mitochondria in the two terminal types showed the same affinity for taking up Ca^{2+} , and were similarly ineffective in limiting cytosolic Ca^{2+} levels within physiologically relevant ranges of neuronal activity.

Materials and Methods

Fly stocks. Flies were raised on standard medium with dry yeast at 25°C. The w^{1118} strain was used as a wild-type control for all experiments. We used the enhancer-trap strain $w^{1118}; P[w+, OK6::Gal4]$ to drive expression of each reporter transgene in MNs of third instar larvae (Aberle et al., 2002). Flies containing the $drp1^2$ mutant allele of the *dynammin related protein-1* gene (Verstreken et al., 2005) were obtained from H. Bellen (Baylor College of Medicine, Houston, TX). $drp1^2$ was kept balanced over *CyO*, *P{ActGFP}JMR1* allowing selection of $drp1^2$ homozygous mutant larvae. Flies containing the *dmiro*^{B682} mutant allele of the *dmiro* gene were kept balanced over *TM6, Tb, Sb* allowing selection of non-tubby *dmiro*^{B682} homozygous larvae (Guo et al., 2005). Flies containing a double insertion of the *GCamp1.3* transgene on the X-chromosome (Wang et al., 2003) were obtained from R. Axel (Columbia University, New York, NY). Flies carrying a UAS-DsRed transgene were obtained from the Bloomington Stock Center (Bloomington, IN). Transgenic UAS-Lyso-GFP-KDEL flies were a gift from M. Lilly (National Institutes of Health, Bethesda, MD). UAS-mito-GFP transgenic flies were a gift from W. Saxton (Indiana University, Bloomington, IN).

Solutions and chemicals. Unless indicated otherwise, chemicals were purchased from Sigma-Aldrich. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Cat. No. 0452) was from Tocris Bioscience. Thapsigargin (Cat. No. 586005) and Ryanodine (Cat. No. 559276) were from Calbiochem (EMD Biosciences). Stock solutions were made in Pluronic F-127 (20% Pluronic in DMSO; Cat. No. P3000MP; Invitrogen). Hemolymph-like solution No. 6 (HL6) containing 15 mM Mg^{2+} , pH 7.3, was used in all experiments (Macleod et al., 2002). Synthetic Ca^{2+} -indicators (rhod-2, rhod-FF, rhod-5N, fura-dextran and rhod-dextran) were obtained from Invitrogen (Cat. Nos. R1245MP, R23983, R14208, F3029, and R34677, respectively), as were AF647-dextran, Texas Red dextran and BAPTA-AM (Cat. Nos. D22914, D1828, and B6769, respectively).

Immunohistochemistry. Third instar *Drosophila* larvae were fillet-dissected in HL6 containing no Ca^{2+} , rinsed in HL6, and fixed with 4% paraformaldehyde for 60 min at room temperature. Fixed preparations were washed 3 times in PBS, pH 7.4, containing 0.1% Triton X-100 (PBST) before blocking for 1 h with 2% bovine serum albumin dissolved in PBST. Preparations were incubated overnight at 4°C in PBST containing rabbit anti-GFP (green fluorescent protein) Alexa Fluor 488-conjugated antibodies (1:500; Invitrogen, Cat. No. A21311), along with either mouse anti-CSP (cysteine-string protein) (1:100, ab49, Zinsmaier

et al., 1990) or mouse anti-Brp (Bruchpilot) antibodies (1:100, nc82; obtained from E. Buchner, University of Würzburg, Germany). After washing in PBS, the preparation was then incubated in PBST containing anti-mouse CY3-conjugated goat antibody (1:500; Jackson ImmunoResearch, Cat. No. 715-165-150) for 2 h at room temperature, washed in PBST, and incubated in PBS for image acquisition using an Olympus FV300 confocal laser-scanning microscope equipped with an Olympus 60× 0.9 numerical aperture (NA) water-immersion objective.

Electron microscopy. Dissected larvae were prefixed in a mixture of 3% glutaraldehyde and 1% formaldehyde in 0.1 M cacodylate buffer for 2 h at room temperature and then overnight at 4°C. After washing in 0.1 M cacodylate buffer, the tissue was postfixed in 2% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at room temperature, dehydrated in a series of ethanol dilutions (50%, 70%, 90%, 95%, and 100%) followed by propylene oxide, and embedded in Epon/Araldite. Ultrathin sections (60 nm) were poststained with 2% uranyl acetate and Reynold's lead citrate. Images were obtained on a JEOL (JEM-1200EX) electron microscope fitted with a Gatan BioScan digital camera.

Generation of flies transgenic for mitochondrially targeted Ca^{2+} indicators. Genetically encoded Ca^{2+} indicators (GECIs) were expressed in MNs under the control of the GAL4/UAS system (Brand and Perrimon, 1993). cDNAs for ratiometric pericam (RP), camgaroo-2 (CG2), and yellow cameleon-2 (YC2) were provided by Nicolas Demaurex. GECIs were targeted to matrices of mitochondria by fusing a tandem repeat of the first 36 aa of subunit VIII of human cytochrome oxidase (COX) (Filippin et al., 2005) to the N terminus of each GECI. The fused cDNAs were then cloned into a P-element vector (pUAST), and injected into w^{1118} *Drosophila* embryos by Rainbow Transgenic Flies (Newbury Park, CA). Strains containing homozygous transgenes were obtained after determining the chromosome harboring the transgene and outcrossing all other chromosomes. In Ca^{2+} -imaging experiments, a single copy of a UAS-GECI transgene was driven by a single copy of the MN driver *OK6-Gal4*.

Wide-field Ca^{2+} imaging. Imaging of fura-dextran, rhod-dextran, AF647-dextran and all mitochondrial GECIs, was performed using wide-field microscopy on an Olympus BX51WI microscope fitted with a 100× water-immersion objective (1.0 NA). A Sutter Instrument DG4 fluorescence excitation system was used to select excitation wavelengths, while a Sutter Instrument Lambda 10-B filter wheel, placed before an Andor Technology EMCCD camera (DV887), was used to filter emitted fluorescence. Filters and dichroic mirrors were obtained from Chroma Technology or Semrock. The imaging system was controlled through an Andor Technology PCU100 and Dell PC running Andor IQ software (ver.1.8).

To stimulate larval MN axons, the segmental nerve was cut close to the ventral ganglion and drawn into the lumen of a glass micropipette. Individual stimulus impulses were 300 μs in duration and 2.4 V in amplitude. Stimuli were delivered via a Master-8 stimulator (A.M.P.I.) and a Digitimer model DS2A Mk.II. All Ca^{2+} imaging was performed in HL6 solution containing 2 mM Ca^{2+} and 7 mM L-glutamic acid to desensitize postsynaptic glutamate receptors and eliminate muscle contraction (Macleod et al., 2004).

Fura-dextran, a high-affinity ($K_D = 594$ nM *in vitro*) Ca^{2+} -indicator conjugated to 10,000 MW dextran, was used to measure the concentration of free Ca^{2+} in the cytosol ($[\text{Ca}^{2+}]_c$). Fura was forward-filled into terminals by cutting the segmental nerve of a dissected larva and exposing the cut end to a mixture of HL6 and distilled-water containing the fluorophore (~2 mM). After ~40 min Fura was rinsed off and the dye was allowed to equilibrate in the axon for >60 min (Macleod et al., 2002; Rossano and Macleod, 2007).

Fura fluorescence was examined using alternating 340/26 and 387/11 nm exciter filters, a 409 nm dichroic mirror and a 510/84 nm emission (em) filter. Fluorescence signals were converted to $[\text{Ca}^{2+}]_c$ using Equation 5 of Grynkiewicz et al. (1985). Values of R_{max} were obtained *in situ* through incubation of preparations in HL6 containing 10 mM Ca^{2+} and 100 μM ionomycin (Sigma, Cat. No. I9657). Values of R_{min} were more difficult to obtain *in situ*. Despite long incubation times in Ca^{2+} -free HL6 with 5 mM EGTA and high concentrations of various permeabilization agents (ionomycin, digitonin and β -escin), the measured value of R

did not fall significantly below resting values of R in 2 mM Ca^{2+} HL6. Hence, $R_{\min}$ was obtained by incubating preparations in Ca^{2+} -free HL6 with 1 mM EGTA and 100 μM BAPTA-AM (1% DMSO) for 20 min (Hendel et al., 2008). The levels of $[\text{Ca}^{2+}]_c$ estimated in this study are sensitive to the quality of the estimate of K_D for fura-dextran *in vivo* and the estimate of $R_{\min}$. It is likely that the K_D value used here (865 nM; Tank et al., 1995) is an underestimate for fura-dextran (Thomas et al., 2000) and that the value of $R_{\min}$ reflects R in the presence of a low (but non-zero) concentration of Ca^{2+} . Both errors will result in an underestimate of the true $[\text{Ca}^{2+}]_c$ values. Hence, the obtained values are considered to define the lower bound for the true values of $[\text{Ca}^{2+}]_c$. Importantly, however, any errors in the conversion from fura ratio values to $[\text{Ca}^{2+}]_c$ will not void tests examining differences in $[\text{Ca}^{2+}]_c$ (see Figs. 7 and 8).

Rhod-dextran, a low-affinity 10,000 MW dextran-conjugated Ca^{2+} indicator ($K_D = 3.0 \mu\text{M}$), was forward-filled and examined using a 543/22 nm exciter filter, a 552 nm dichroic mirror and a 593/40 nm emission filter. AF647-dextran, a Ca^{2+} -insensitive dextran-conjugated fluorophore (10,000 MW), was forward-filled and examined using a 628/40 nm exciter filter, a 660 nm dichroic mirror and a 692/40 nm emission filter.

Confocal Ca^{2+} imaging. Imaging of rhod-2, rhod-FF, rhod-5N, Texas Red dextran (10,000 MW) and GCaMP1.3 was performed on an upright Olympus confocal line-scanning microscope (BX50WI/FluoView 300) using a 60 $\times$ water-immersion objective (0.9 NA). Rhod-2, -FF and -5N and Texas Red imaging settings were as follows: 543 nm laser excitation, 570 nm edge dichroic, BA605BP emission filter. GCaMP1.3, a high affinity GECI ($K_D \sim 250$ nM), was used to confirm the propagation of action potentials into nerve terminals, but was not used to quantify cytosolic Ca^{2+} levels. GCaMP and GFP imaging settings were: 488 nm laser excitation, 505 nm edge dichroic, BA510IF emission filter. When alternating between GCaMP imaging and rhod-FF imaging on the same terminal, an acoustic-optical tunable filter was used. Axons were stimulated as above.

Loading of synthetic mitochondrial Ca^{2+} indicators. Rhod-2, -FF and -5N were loaded by bath application of the acetyloxymethyl (AM) forms using a larval preparation with uncut segmental nerves. Briefly, a 2 mm stock was made in 100% DMSO/20% pluronic acid, diluted to 5 μM (0.25% DMSO/0.05% pluronic acid) in HL6, applied to the preparation at room temperature, and incubated in the dark at 4°C for 10 min. To clear the Ca^{2+} -indicator from the cytosol, the preparation was rinsed in Ca^{2+} -indicator-free chilled HL6 and incubated in the dark at room temperature for 90–300 min. HL6 was replaced every 30 min during this incubation and nerves were cut and drawn into a stimulating pipette at least 20 min before imaging.

Electrophysiology. Experiments were performed on fillet-dissected wandering third instar larvae pinned on Sylgard (Dow Corning, Midland). Segmental nerves were left intact and electrophysiological measurements during randomly occurring fictive locomotion were made within 30 min of dissection in HL6 with 2 mM CaCl_2 . Recordings were made using a 20 $\times$ water-immersion objective of a BX51WI Olympus microscope allowing unequivocal identification of body-wall muscles 6, 13 and 12 in abdominal segment 4 (Hoang and Chiba, 2001). Micropipettes were filled with a 1:1 mixture of 3 M KCl and 3 M K^+ -acetate (final resistance ~ 50 M Ω). Each electrode was connected to an Axoclamp 900A amplifier through 0.1 or 1.0 gain headstages (Molecular Devices). Data were digitized (model 4/30, PowerLab) and acquired with Chart 5.5.6 software (ADInstruments software and hardware). Data were analyzed using Chart software. It was often difficult to maintain long-lasting recordings during rhythmic activity. Hence, records were accepted for analysis even if resting membrane potentials fell as low as -30 mV. However, as we were quantifying a presynaptic phenomenon, and excitatory junction potentials (EJPs) at high extracellular $[\text{Ca}^{2+}]$ can be unambiguously resolved from the baseline (see Fig. 6), it is unlikely that accepting such data could compromise the conclusions.

To determine the identity of the MN responsible for an EJP during fictive locomotion, we simultaneously recorded from muscle 13 and a neighboring muscle fiber (see Fig. 6A); a recording configuration used in early studies on crayfish abdominal muscles (Kennedy and Takeda, 1965). Since MNSNb/d-Is forms terminals on muscles 6, 7, 13 and 12 while MN13-Ib forms terminals only on muscle 13 (Hoang and Chiba,

2001), the dual recordings allowed us to identify the MN responsible for each EJP in muscle 13. A similar technique has been used by Lnenicka and Keshishian (2000) and Dunn and Mercier (2005) to identify MNs giving rise to nerve stimulus-evoked EJPs in *Drosophila* muscles.

For example, if an EJP was recorded only in muscle 13 but no simultaneous EJP was recorded in the adjacent muscle, then MN13-Ib was responsible for the EJP, as this is the only MN that exclusively innervates muscle 13 (see Fig. 6B–D, left). If EJPs were recorded simultaneously in adjacent muscles, the EJP was most likely triggered by MNSNb/d-Is, as it innervates both muscles (see Fig. 6B–D, right). Simultaneous EJPs in muscle pairs can also be expected if an AP in MN6-Ib or MN12-Ib is simply coincident with an AP in MN13-Ib. However, these events are relatively easy to discriminate, since at physiological levels of Ca^{2+} (2 mM) the amplitudes of EJPs triggered by MNSNb/d-Is are larger than those that are triggered by MN13-Ib (Kurdyak et al., 1994; Cattaert and Birman, 2001) (see Fig. 6B–D, right).

Our analysis could be confounded if either MNSNb/d-II or MN12-III, which also form boutons on these muscles (muscle pair 13 and 12 and muscle 12, respectively), also generate EJPs in these muscles. Therefore, we examined the frequency of the simultaneous activity measured in muscle pair 13 and 6 versus 13 and 12. We might expect to estimate a higher average instantaneous frequency for MNSNb/d-Is when recording from muscle pair 13 and 12 (Ib: 44.2 ± 3.0 Hz, Is: 7.8 ± 0.1 Hz, mean and SEM, $n = 9$ and 5), relative to 13 and 6 (Ib: 40.8 ± 1.0 Hz, Is: 7.8 ± 0.6 Hz, $n = 9$ and 6), as MNSNb/d-II innervates muscle pair 13 and 12 but only muscle 13 in the 13 and 6 muscle pair, but no difference was detected. Therefore, any EJPs that result from APs in MNSNb/d-II are either very small or very infrequent. MN12-III is unlikely to confound our analysis since it only innervates muscle 12.

Results

Subcellular distribution of mitochondria and ER in motor neuron terminals

Each muscle fiber in the body wall of *Drosophila* larvae is typically innervated by two glutamatergic MNs that are morphologically and functionally different (Johansen et al., 1989; Atwood et al., 1993; Jia et al., 1993; Kurdyak et al., 1994; Lnenicka and Keshishian, 2000; Karunanithi et al., 2002). “Tonic-like” MNs form type Ib (big-bouton) terminals, while “phasic-like” MNs form type Is (small-bouton) terminals. Immunolabeling reveals an enrichment of mitochondria in most boutons of type Ib and Is terminals (Fig. 1A,B).

Within axon terminals in the rat CNS and crayfish MNs, mitochondria are typically localized to a central region of synaptic boutons, termed the mitochondrial domain, which contains numerous mitochondria but almost no synaptic vesicles (Gotow et al., 1991; King et al., 1996). Mitochondria in *Drosophila* MN terminals show a similar localization (Fig. 1A,B,D–F). Most mitochondria are found in a more central region of the bouton, showing little overlap with synaptic vesicles, which are mostly localized in the periphery of the bouton (Fig. 1A). Importantly, most mitochondria are localized well clear of active zones (Fig. 1B). Consistently, electron micrographs show that most mitochondria are found at the interface between a central synaptic vesicle-free region and a peripheral synaptic vesicle-rich region of type Is and Ib boutons (Fig. 1D–F). Sporadically, mitochondria localize close to the plasma membrane and active zones (see also Meinertzhagen et al., 1998).

Since mitochondria can sequester Ca^{2+} originating from Ca^{2+} channels in the plasma membrane or the ER membrane (Alonso et al., 2006; Rizzuto et al., 2009), we also tested whether ER is present within terminals of larval *Drosophila* MNs by expressing a transgenic ER-GFP marker (Lysozyme-GFP-KDEL) and subsequently filling the MN cytosol with Texas Red dextran. ER-GFP expression has previously been shown to reliably label

the ER in *Drosophila* cells (Snapp et al., 2004). Live imaging revealed a filamentous, likely continuous, GFP-positive network that invades every bouton in both terminal types (Fig. 1C). The extensive presynaptic distribution of the ER at larval *Drosophila* neuromuscular junction (NMJ) is consistent with the large number of presynaptic (ER-like) cisternae observed by EM (Guo et al., 2005), the presynaptic localization of ER-associated reticulum (Wakefield and Tear, 2006), and the effects of mutations in the sarco-ER Ca^{2+} -ATPase (SERCA) on neurotransmitter release (Sanyal et al., 2005). Considering the extensive distribution of the ER in type Is and Ib terminals (Fig. 1C), it is therefore possible that presynaptic mitochondria may sequester Ca^{2+} originating from the ER.

Targeting genetically encoded Ca^{2+} -indicators to presynaptic mitochondria

Using the GAL4/UAS system (Brand and Perrimon, 1993), we expressed genetically encoded Ca^{2+} -indicators (GECIs) that were targeted to the mitochondrial matrix in a tissue-specific manner. Targeting of ratiometric pericam (RP), camgaroo-2 (CG2) and yellow cameleon-2 (YC2) to the mitochondrial matrix (Fig. 2A, insets) was achieved by fusing a tandem repeat of the signal sequence from subunit VIII of cytochrome oxidase c (Filipin et al., 2005) to the N terminus of each GECI. Coexpression of each GECI with cytosolic DsRed in MNs (OK6-GAL4) revealed distinct, stationary punctae of mito-RP, mito-CG2 and mito-YC2 fluorescence in synaptic boutons of larval NMJs (Fig. 2A). These punctae were often mobile in axons, moving either antero- or retrogradely in a saltatory manner (data not shown). These observations on the distribution and motion of labeled punctae are similar in all respects to those made previously for mito-GFP fluorescence (Guo et al., 2005; Pilling et al., 2006). Although expression of DsRed was strongest in type Ib terminals, mitochondrial GECIs were expressed equally in both (Fig. 2A).

To test functionality and targeting specificity of each GECI, we compared the changes in fluorescence of each mitochondrially targeted GECI with those of a synthetic low-affinity cytosolic Ca^{2+} -indicator (rhod-dextran) before, during and after nerve stimulation (Fig. 2B). Since rhod-dextran is invisible at resting Ca^{2+} levels in the cytosol, we coloaded Ca^{2+} -insensitive AF647-dextran to visualize nerve terminals. All Ca^{2+} -imaging data were obtained from synaptic boutons of the two MNs innervating muscle 13, MN13-Ib and MNSNb/d-Is.

Nerve stimulation for 2 s at 80 Hz caused a rapid increase in cytosolic rhod-dextran fluorescence that decayed immediately after stimulation (Fig. 2C, purple trace). Mito-RP, mito-CG2 and mito-YC2 fluorescence also changed rapidly in response to nerve stimulation but, in contrast to the cytosolic rhod-dextran fluorescence, returned only slowly to baseline after stimulation (Fig. 2C). The slow decay of mito-GECI fluorescence cannot be explained by slower Ca^{2+} binding and release kinetics, as all 3 untagged indicators are capable of reporting the fast decay of

Figure 1. Distribution of mitochondria and the ER in larval *Drosophila* MN terminals. **A**, Confocal microscopy images of type Ib and Is terminals on muscle 6 immunolabeled for mito-GFP (green) and CSP of synaptic vesicles (SVs; magenta). Mito-GFP was expressed in MNs with the OK6-Gal4 driver. **B**, Confocal images of terminals immunolabeled for mito-GFP (green) and Brp (stained with antibody nc82) that is found in active zones (AZs; magenta). **C**, Confocal image of the ER in type Ib and Is terminals labeled by OK6-Gal4-driven expression of ER-GFP (Lysio-GFP-KDEL; green). The MN was visualized by filling the cytosol with Texas Red dextran (magenta). Note that the ER-GFP fluorescence suggests a continuous ER system throughout all boutons. Scale bars (**A–C**), 5 μm . **D–F**, Electron micrographs showing cross-sections of type Is and Ib boutons in overview (**D**) and in higher magnification (**E**, **F**). Mitochondria are mostly found at the interface between a central synaptic vesicle-free and a peripheral synaptic vesicle-rich region of Is and Ib boutons. Active zones with or without T-bars are indicated. Scale bars (**D**, **E**, **F**), 2, 2, and 1 μm , respectively.

cytosolic Ca^{2+} transients in *Drosophila* MN terminals (Reiff et al., 2005; Mank et al., 2006). To further test the specificity of GECI targeting, we used the protonophore CCCP, which permeabilizes the inner mitochondrial membrane to protons thereby eliminating the driving force for mitochondrial Ca^{2+} uptake (David et al., 1998). Application of 3 μM CCCP completely abolished stimulation-dependent changes in mito-RP, mito-CG2 and mito-YC2 fluorescence while changes in cytosolic rhod-dextran responses were undiminished (Fig. 2D). This confirms that all 3 GECIs are effectively targeted to mitochondria.

It is noteworthy that mito-RP and mito-CG2 responses differ slightly from mito-YC2 responses as both show a rapid, although partial, decrease in fluorescence immediately after stimulation (Fig. 2C). This feature is not due to small amounts of mito-RP and mito-CG2 in the cytosol since application of CCCP abolishes any stimulation-dependent response (Fig. 2D). Neither mito-YC2 (Fig. 2C) nor the synthetic Ca^{2+} indicators rhod-2, rhod-FF and rhod-5N show a similar characteristic (see Fig. 4). Since both RP and CG2 contain cpYFP, a confirmed reporter of the superoxide anion ($\text{O}_2^{\cdot-}$; Wang et al., 2008), it is likely that the transient feature is an artifact of mitochondrial $\text{O}_2^{\cdot-}$ production.

Stimulation-dependent mitochondrial Ca^{2+} uptake differs between type Is and Ib terminals

To determine whether mitochondrial Ca^{2+} uptake is different between functionally different MN terminals, we compared fluorescence responses of mito-RP between type Is and Ib terminals on muscle 13 (Fig. 3). To increase cytosolic Ca^{2+} in a systematic manner, we applied a series of 2 s long stimulus trains spaced at 7 s

Figure 2. GEICs targeted to mitochondria report mitochondrial Ca^{2+} uptake in response to nerve stimulation. **A**, Fluorescence images (wide-field microscopy) showing OK6-driven expression of ratiometric mito-RP, mito-CG2, mito-YC2, and mito-GFP in type Ia and Ib MN terminals on muscle 13 (top row) that were filled with cytosolic DsRed (second row). The inset below shows the basic design of each GEIC. **B**, Fluorescence images of mito-RP, mito-CG2, mito-YC2, and mito-GFP in terminals (two top rows) filled with AF647-dextran and rhod-dextran (bottom row). Mito-RP fluorescence (520 nm emission) was excited alternately at 420 and 470 nm, mito-YC2 fluorescence (485 and 535 nm emission) was excited at 440 nm, and CG2 fluorescence (520 nm emission) and mito-GFP fluorescence (520 nm emission) was excited at 470 nm. **C**, **D**, Changes in fluorescence (boxed regions in **B**) in response to nerve stimulation (80 Hz, 2 s) in the absence (**C**) and presence (**D**) of the protonophore CCCP. Changes in the fluorescence of mito-RP, mito-CG2, and mito-YC2 were only observed in the absence of 3 μM CCCP, and showed little delay relative to changes in fluorescence of the cytosolic Ca^{2+} indicator rhod-dextran. The maximum fluorescence responses of rhod-dextran and the GEICs were normalized to a value of 1.0 (**C**), and the same scaling factors were used after application of CCCP (**D**). As expected, mito-GFP showed no response to nerve stimulation.

Figure 3. GECIs reveal differences in mitochondrial Ca^{2+} uptake between terminals of MN13-Ib and MNSNb/d-IIs. **A**, Images of mito-RP fluorescence before and after successive nerve stimulus trials (left to right: stimulation frequency indicated between frames) from type Ib and Is terminals on muscle 13. Mito-RP fluorescence was collected through a 520/35 nm filter during sequential 420 nm (top panel) and 490 nm (bottom panel) excitation. **B**, Plot of nerve stimulation-induced changes in mito-RP fluorescence (from **A**). The 490ex/520em mito-RP fluorescence increased while 420ex/520em fluorescence decreased in response to 10, 20, 40, 60, and 160 Hz stimulus trains (2 s each). In contrast to 420ex/520em fluorescence, 490ex/520em fluorescence decreased when stimulation ceased, and the transient is likely due to changes in superoxide anion concentration (see Results). Images were captured at 2 frames per second for each excitation wavelength. **C**, Left panel: Plot of the mito-RP emission intensity ratios (420/490 nm excitation) for the terminals shown above (**A**, **B**). Right panel: Plot of the reciprocal mito-RP emission intensity ratio normalized to a value of 0 before the first train ($t = 3$ s) and a maximum response of 1 after the last train ($t = 38$ s), i.e., inverted relative to the left panel. **D**, Average mito-RP ratio responses plotted against the frequency of stimulation for type

long intervals. In addition, we doubled the frequency of stimulation with each stimulus train in the series; a protocol expected to double the concentration of free Ca^{2+} in the cytosol ($[\text{Ca}^{2+}]_c$) with each train in the progression (Macleod et al., 2002; Hendel et al., 2008). Surprisingly, mitochondria in type Is terminals were far more responsive to nerve stimulation than those in Ib terminals. While mitochondria in type Is terminals showed pronounced changes in mito-RP fluorescence at 20 Hz, mitochondria in Ib terminals were essentially unresponsive at the same stimulation frequency (Fig. 3B). The differential responses between terminal types were also observed with mito-CG2 and mito-YC2 (data not shown).

To quantify the mito-RP response, we took the ratio of RP fluorescence emitted at 520 nm when excited at 420 nm, relative to excitation at 490 nm (Fig. 3C, left), and inverted and normalized the ratio to the maximal response at 160 Hz (Fig. 3C, right). The average changes in the ratio for pairs of type Is and Ib terminals were then plotted against the frequency of stimulation (Fig. 3D). The difference in mitochondrial Ca^{2+} uptake between terminal types was highly significant at 20 and 40 Hz stimulation, with mitochondria in type Is terminals taking up Ca^{2+} at lower stimulus frequencies than in type Ib terminals (11/11 pairs; Fig. 3D). An analysis of only the 420ex/520em (excitation/emission) mito-RP response (excluding the influence of the purported $\text{O}_2^{\cdot-}$ transient in the 490 nm excitation response) revealed the same statistical differences between both terminal types (data not shown). Responses in the 420ex/520em mito-RP signal are considered to be very specific for Ca^{2+} changes whereas the 490ex/520em response is also pH-sensitive (Nagai et al., 2001; Jiang et al., 2009). Estimation of the average nerve stimulation rate at the half-maximal mitochondrial response revealed a severalfold difference between type Is and Ib terminals, i.e., 16 Hz versus 41 Hz, respectively (Fig. 3D).

Estimating the time course of decay of free Ca^{2+} concentration in the mitochondrial matrix ($[\text{Ca}^{2+}]_m$) is problematic for all three GECIs. For both mito-RP and mito-CG2, the purported $\text{O}_2^{\cdot-}$ transient (Fig. 2D) produces a biphasic decay that will lead to an overestimation of the initial rate of $[\text{Ca}^{2+}]_m$ decay. Estimates of $[\text{Ca}^{2+}]_m$ decay from mito-YC2 data are also problematic since EYFP of YC2 is pH-sensitive (Filippin et al., 2005) and susceptible to photochromism (Miyawaki et al., 1997; Abad et al., 2004). If mitochondria in type Is terminals sequester more Ca^{2+} under the same stimulus conditions, yet have the same Ca^{2+} release mechanism as mitochondria in Ib terminals, one would expect mitochondria in type Is terminals to release Ca^{2+} for a longer period of time after stimulation (García-Chacón et al., 2006). This trend was indeed observed with all three mitochondrial GECIs (data not shown), but the aforementioned limitations of the GECIs precluded a systematic quantitative comparison.

Synthetic Ca^{2+} -indicators confirm different nerve stimulus thresholds for mitochondrial Ca^{2+} uptake

The evoked responses from mito-RP (Fig. 3) seem to indicate that mitochondria in type Is and Ib terminals have different Ca^{2+} -handling properties. To test this further, we examined mitochondrial Ca^{2+} levels using synthetic Ca^{2+} indicators. We loaded the acetoxymethyl ester (AM) forms of rhod-2, rhod-FF or rhod-5N

Ib and Is terminals on muscle 13 ($n = 11$). Sigmoidal curve fits were made using the Hill equation (SigmaPlot 10, Systat Software Inc.), which was used to estimate half-maximal values shown. Asterisks indicate highly significant differences ($p < 0.001$) between type Ib and Is responses in pairwise comparisons (two-way ANOVA, $p < 0.05$ overall, Tukey *post hoc* test). Error bars indicate SEM.

Figure 4. Synthetic Ca^{2+} -indicators confirm differences in nerve stimulation-dependent mitochondrial Ca^{2+} uptake between terminal types. **A**, Fluorescence from mitochondrially localized rhod-FF (top panels) and cytosolic GCaMP1.3 (bottom panels), expression driven by OK6-Gal4) in type Is and Ib terminals on muscle 13 before, during, and after the indicated stimulus trains. **B**, Typical plots of nerve-stimulated (20 and 160 Hz) changes in rhod-FF and GCaMP fluorescence from the terminals in **A**. The $\Delta F/F$ response to 160 Hz was assigned a value of 1 and all other values of $\Delta F/F$ were scaled in proportion. **C**, Typical plots of changes in rhod-FF and GCaMP fluorescence in response to 80 and 160 Hz nerve stimulation. **D**, Typical plots of nerve-stimulated (40 and 160 Hz) changes in the fluorescence of mitochondrially targeted rhod-2, rhod-FF, and rhod-5N in type Is and Ib terminals. **E**, Average change in rhod-FF fluorescence from Is and Ib terminals on muscle 13 in response to various stimulation frequencies (10, 20, 40, and 80 Hz) after normalizing individual responses (as in **B** and **C**). Sigmoidal curve fits were made using the Hill equation, which was used to estimate half-maximal values as shown. Significant differences are indicated ($p < 0.001$, $N \geq 6$, two-way ANOVA, Holm-Sidak *post hoc* test). Error bars indicate SEM.

($K_D = 0.57$, 19, and 320 μM , respectively) into the mitochondria using a modified protocol of Guo et al. (2005). As rhod-FF and rhod-5N have lower affinities for Ca^{2+} than the mito-GECIs, they are unlikely to saturate at the previously estimated *in situ* values of $[\text{Ca}^{2+}]_m$ for presynaptic mitochondria ($\sim 1 \mu\text{M}$; David, 1999; David et al., 2003; $\geq 5 \mu\text{M}$; Billups and Forsythe, 2002). GCaMP was transgenically expressed in the same terminals to monitor cytosolic Ca^{2+} levels.

For these experiments, we used a modified nerve stimulation protocol to ensure that submaximal responses were both repeat-

able and additive. We first applied a stimulus train at a given frequency, repeated the same stimulus train after 30 s, and ended the series after a second 30 s rest period with a final high-frequency train at 160 Hz (320 pulses; Fig. 4A–C). Since all rhod-indicators are poorly visible at low levels of Ca^{2+} , $\Delta F/F$ measurements were normalized to the maximal response at 160 Hz to avoid the high degree of variance that would arise if responses were normalized to resting fluorescence levels. Stimulus-evoked transients of rhod-2 (Fig. 4D), rhod-FF (Fig. 4B–D) and rhod-5N (Fig. 4D) were qualitatively much different to those of GCaMP in the cytosol (Fig. 4B, C). In addition, application of the protonophore CCCP abolished changes in rhod-FF but not GCaMP fluorescence (data not shown), confirming a mitochondrial localization.

Like mitochondrial GECIs (Fig. 3), Rhod-2, -FF and -5N revealed pronounced differences in mitochondrial Ca^{2+} uptake between type Is and Ib terminals (Fig. 4B–D). A rhod-FF response was usually evident in type Is terminals at a stimulation frequency of just 10 Hz, while Ib terminals were usually not responsive below 40 Hz (Fig. 4E). The stimulus-dependent, half-maximal mitochondrial rhod-FF response was significantly different between type Is (15 Hz) and Ib (67 Hz) terminals (Fig. 4E). A similar relative difference between terminal types was seen with rhod-2 and rhod-5N (Fig. 4D). The rhod-FF-based half-maximal estimate for type Ib was higher than that based on mito-RP data (67 Hz vs 41 Hz, respectively). A possible explanation is that $[\text{Ca}^{2+}]_m$ approaches levels that saturate mito-RP, but not rhod-FF.

When the high-affinity Ca^{2+} indicator rhod-2 was substituted for rhod-FF, mitochondrial Ca^{2+} uptake in type Ib terminals was still not detectable during 10 Hz stimulation (data not shown). In addition, when the low-affinity Ca^{2+} indicator rhod-5N was substituted for rhod-FF, the fluorescent signal from type Is terminals still appeared to “peak” at a stimulation frequency of 40 Hz (Fig. 4D, bottom panel, and see Fig. 4E).

Since the ratio of the 40 Hz to the 160 Hz response was similar when using rhod-5N or rhod-FF (compare Fig. 4D bottom panel with middle panel), rhod-FF was likely to be within a linear response range (i.e., the 40 Hz response in either terminal type is well below the rhod-FF K_D of $\sim 19 \mu\text{M}$).

Notably, the time course of mitochondrial rhod-FF fluorescence decay was significantly different between terminal types. In type Is terminals it took 181 s to decay by $\sim 63\%$ after a 40 Hz 2 s stimulus train, while it took only 46 s in Ib terminals (monoexponential fit, median values, Mann–Whitney rank sum test; $p < 0.01$). The slower $[\text{Ca}^{2+}]_m$ decay in type Is terminals indicates

either a slower rate of Ca^{2+} release or a greater mitochondrial Ca^{2+} load. Considering that mitochondria in type Is terminals are more responsive to nerve stimulation it is likely that these mitochondria obtained a greater Ca^{2+} load. The slower rate of rhod-2 fluorescence decay in both terminal types (Fig. 4D), relative to rhod-FF or rhod-5N, may be due to a contribution of rhod-2 to the Ca^{2+} buffering capacity of the mitochondrial matrix, which can slow the kinetics of mitochondrial Ca^{2+} loss (Gaspers and Thomas, 2008).

Mitochondrial Ca^{2+} uptake does not rely on ER Ca^{2+} transfer

One possible explanation for the higher sensitivity of mitochondrial Ca^{2+} sequestration to nerve stimulation in type Is terminals is that mitochondria in these terminals are in close association with Ca^{2+} micro-domains. Since most mitochondria in both type Is and Ib boutons are found relatively distant from active zones harboring presynaptic Ca^{2+} channels (Fig. 1B,D–F), a close association between mitochondria and Ca^{2+} -channels in plasma membrane can be excluded. However, mitochondria in type Is and/or Ib terminals could be in close association with Ca^{2+} channels on the ER membrane due to the extensive nature of the ER network (Fig. 1C). However, mitochondrial Ca^{2+} uptake was undiminished in either terminal type in the presence of 20 μM thapsigargin (Fig. 5A,B), a SERCA inhibitor that mimics presynaptic Ca^{2+} clearance defects of *Drosophila* SERCA mutants at larval NMJs (Kuromi and Kidokoro, 2002; Sanyal et al., 2005). Similarly, in the presence of 100 μM ryanodine, which inhibits ryanodine receptors (Shakiryanova et al., 2007), mitochondrial Ca^{2+} uptake was also undiminished (Fig. 5C). Hence, the differential mitochondrial responses in type Is and Ib terminals cannot be explained by preferential ER Ca^{2+} transfer. Consequently, it seems likely that mitochondria mostly sequester Ca^{2+} from the undifferentiated cytosol, where $[\text{Ca}^{2+}]_c$ only rises to several hundred nM during comparable stimulus trains (Macleod et al., 2004; Guo et al., 2005, their Fig. 7C).

Motor neurons MNSNb/d-Is and MN13-Ib fire at different frequencies

To provide a physiological context for the observed differences in mitochondrial Ca^{2+} -uptake between type Is and Ib terminals, and to determine whether mitochondrial Ca^{2+} sequestration limits cytosolic Ca^{2+} within a physiological range of activity, we determined the native firing frequencies of the two MNs innervating muscle 13 (MNSNb/d-Is and MN13-Ib). Since MN13-Ib only innervates muscle 13 while MNSNb/d-Is also innervates neighboring muscles (Fig. 6A), simultaneous recordings of excitatory junctional potentials (EJPs) from muscle 13 and the neighboring muscle 6 can identify the MN that triggered a particular EJP (Fig. 6A–D; see Materials and Methods for a full description).

When the nerve was left intact, we observed rhythmic bursts of spontaneous synaptic activity that have been earlier described as fictive locomotion activity (Budnik et al., 1990; Cooper and Neckameyer, 1999; Cattaert and Birman, 2001; Barclay et al., 2002; Suster et al., 2004; Wang et al., 2004; Klose et al., 2005; Fox et al., 2006). After correlating each EJP with its originating MN (Fig. 6C), we calculated the instantaneous firing frequency for each MN (1/time interval in s between EJPs; Fig. 6D,E), from which we then determined the average peak firing frequency for a period of 2 s (Fig. 6F). This analysis revealed, on average, a maximum firing frequency of 7.8 ± 0.3 Hz (SEM; $n = 11$) for MNSNb/d-Is, and $42.4 \text{ Hz} \pm 1.6$ Hz ($n = 18$) for MN13-Ib ($p < 0.005$, Fig. 6F). The same significant difference in firing frequen-

Figure 5. Mitochondrial Ca^{2+} uptake is not dependent on Ca^{2+} transfer from the ER. **A–C**, Plots of the average mito-RP fluorescence in type Ib and Is terminals on muscle 13 in response to nerve stimulation (40 Hz, 2 s). The mito-RP ratio (420/470) is shown on an inverted scale for untreated terminals (**A**) and terminals treated with 20 μM thapsigargin (**B**) or 100 μM ryanodine (**C**). The records are truncated at the end of the stimulus train, as treated preparations (≥ 30 min) commonly contracted at the end of each train. The numbers of terminal pairs examined are shown at the bottom of each plot.

cies was observed for paired recordings from muscle pair 13 and 6 (Fig. 6F, left) and 13 and 12 (Fig. 6F, right).

The firing frequencies measured for both MNs provided an important foundation for considering the physiological significance of mitochondrial Ca^{2+} sequestration. In addition, comparing the average maximum firing frequencies with the observed sensitivity of mitochondrial Ca^{2+} sequestration to nerve stimulation (Figs. 3D, 4E) predicted that mitochondria in MN13-Ib terminals may sequester Ca^{2+} more actively than those of MNSNb/d-Is terminals.

Stimulus-dependent cytosolic Ca^{2+} levels are higher in type Is than Ib terminals

Why do mitochondria in type Is terminals take up Ca^{2+} at much lower axon excitation frequencies than those in Ib terminals? Mitochondria could have different thresholds (set points) for Ca^{2+} sequestration. Alternatively, $[\text{Ca}^{2+}]_c$ could rise higher in type Is than Ib terminals for a given stimulation frequency, although this was previously discounted (Karunanithi et al., 1997; Reiff et al., 2002; but see Rossano and Macleod, 2007, and He et al., 2009).

Since a reliable estimate of $[\text{Ca}^{2+}]_c$ is required for evaluating the role of mitochondrial Ca^{2+} sequestration, we measured $[\text{Ca}^{2+}]_c$ in type Is and Ib terminals using the ratiometric Ca^{2+} -indicator Fura. We applied a series of 2 s long stimulus trains spaced at 10 s intervals, doubling the stimulation frequency with each train (Fig. 7A). Unexpectedly, $[\text{Ca}^{2+}]_c$ reached much higher levels in type Is than in Ib terminals at all examined stimulation frequencies (Fig. 7A,B). Since type Is boutons are much smaller than Ib boutons, it was possible that the higher $[\text{Ca}^{2+}]_c$ in Is boutons is due to the difference in the surface area-to-volume ratio. However, this possibility could be excluded since no correlation was detected between the size of a bouton and its stimulated $[\text{Ca}^{2+}]_c$ level (Fig. 7D). The higher $[\text{Ca}^{2+}]_c$ in type Is boutons is consistent with a higher per-volume-density of active zones in phasic MN terminals, as reported previously in *Drosophila* (Meinertzhagen et al., 1998; He et al., 2009) and crayfish (Moghini et al., 1999).

The much higher cytosolic Ca^{2+} levels of type Is terminals provide a simple explanation for why mitochondria in type Is terminals sequester Ca^{2+} at lower stimulation frequencies than

Figure 6. Motor neurons MNSNb/d-Is and MN13-Ib fire at different frequencies during fictive locomotion. **A**, Left: Cross-sectional representation showing the location of larval body-wall muscles 7, 6, 13, and 12. Right: Representation of dual recording from muscles 13 and 6 and the pattern of MN innervation. MNSNb/d-Is forms type Is boutons on all muscle fibers while motor neurons MN6/7-Ib, MN13-Ib, and MN12-Ib form type Ib boutons on selected muscle fibers. The asterisk indicates another branch of the MNSNb/d-Is. **B**, Two example traces (left and right) of EJPs during fictive locomotion recorded simultaneously from adjacent muscles (6 and 13). Left: Traces showing synaptic activity from separate MNs (MN6/7-Ib and MN13-Ib) on the separate muscles. Right: Traces showing synaptic activity from MN6/7-Ib and MN13-Ib, in addition to periodic input from MNSNb/d-Is that innervates both muscles. Scale bars, 100 ms and 5 mV. **C**, EJP–MN relationship (from boxed sections in **B**). Open circles (in the boxes below the traces) denote the occurrence of each EJP according to the muscle in which it is detected. Color, filled circles (in the lowest boxes) identify the MN from which each EJP originates. Coincident events between traces can be attributed to chance, alone (*left), or a common innervating axon (MNSNb/d-Is, †right). **D**, Plots of instantaneous EJP frequencies for muscle 13 from the example traces in **B**. The identity of each contributing MN is indicated: open circles, all EJPs before identification; blue circles, MN13-Ib EJPs; red circles, MNSNb/d-Is EJPs. Events occurring < 3 ms apart could not be discriminated and were plotted with all other events with an instantaneous frequency of > 200 Hz. **E**, Firing frequency distributions for the MNs producing the EJPs shown in **D**. **F**, Average maximum firing frequencies for MN13-Ib and MNSNb/d-Is (calculated during the most active 2 s period in each record). Each pair of connected circles represents estimates made from a different pair of muscle fibers, either 13 and 6 (left) or 13 and 12 (right). Asterisks indicate significance ($p < 0.005$) in paired t tests.

in Ib terminals. In addition, relating stimulation-dependent $[\text{Ca}^{2+}]_c$ to mitochondrial Ca^{2+} sequestration revealed that mitochondrial Ca^{2+} sequestration in Is and Ib terminals has similar Ca^{2+} thresholds and affinities. At stimulation frequencies corresponding to the half-maximal mitochondrial response (Figs. 3D, 4E), the projected values of $[\text{Ca}^{2+}]_c$ were quite similar for both terminals, reaching 402 nM for MNSNb/d-Is and 422 nM for MN13-Ib (Fig. 7C). This suggests that mitochondria in different types of terminals exhibit a similar affinity for Ca^{2+} uptake. In addition, mitochondria in both terminal types start acquiring Ca^{2+} once it exceeds ~ 250 nM (i.e., mitochondria start acquiring Ca^{2+} at ~ 10 Hz in type Is terminals and soon after 20 Hz is exceeded in Ib terminals, Figs. 3D, 4E).

Stimulus-dependent cytosolic Ca^{2+} levels are not affected by the absence of functional mitochondria

As the Ca^{2+} affinity of mitochondrial Ca^{2+} sequestration is similar in type Is and Ib terminals, it seemed possible that a higher mitochondrial density may more effectively limit stimulus-dependent elevations in $[\text{Ca}^{2+}]_c$. Both immunolabelings (Fig. 1A,B) and 3D-EM reconstructions suggest that type Ib terminals may have more mitochondria than Is terminals (Atwood et al., 1993; Hill, 1996). Accordingly, the higher stimulus-dependent $[\text{Ca}^{2+}]_c$ in type Is terminals could be due to a reduced mitochondrial Ca^{2+} buffering capacity.

To determine the significance of mitochondrial Ca^{2+} sequestration for limiting $[\text{Ca}^{2+}]_c$, we measured the $[\text{Ca}^{2+}]_c$ in type Is and Ib terminals after acutely or chronically abolishing mitochondrial Ca^{2+} uptake (Fig. 8). Although application of the protonophore CCCP acutely eliminated mitochondrial Ca^{2+} uptake (Fig. 2D), it had little impact on the $[\text{Ca}^{2+}]_c$ in type Ib or Is terminals during stimulation (Fig. 8A) or at rest (Fig. 8B). In addition, stimulus-dependent or resting cytosolic Ca^{2+} levels were not affected by the chronic reduction of mitochondria (Fig. 8A,B) in terminals of *drpl²* mutants (Verstreken et al., 2005), or the chronic lack of mitochondria (Fig. 8A,B) in terminals of *dmiro^{B682}* mutants (Guo et al., 2005). Hence, mitochondrial Ca^{2+} sequestration does not limit stimulated Ca^{2+} levels in these MN terminals during short trains of up to 40 Hz stimulation, which mimics the upper range of MN13-Ib firing frequencies during fictive locomotion and exceeds those of MNSNb/d-Is by fivefold.

Figure 7. Nerve stimulation-dependent cytosolic Ca^{2+} levels are significantly different in type Is and Ib terminals. **A**, Changes in the ratio (340/380 nm) of fura-dextran fluorescence in response to nerve stimulation at 10, 20, 40, 80, and 160 Hz in MN13-Ib and MNSNb/d-Is terminals. Changes in the fura ratio can be calibrated and converted to changes in $[\text{Ca}^{2+}]_c$. **B**, Average $[\text{Ca}^{2+}]_c$ in type Is and Ib terminals for each stimulation frequency. Data were fitted with a cubic spline. Asterisks indicate significant differences in pairwise comparisons (two-way ANOVA, $p < 0.05$ overall, Holm-Sidak *post hoc* test). Error bars indicate SEM. **C**, Same plot as in **B** but overlaid with projections of the half-maximal response frequencies of mito-RP and rhod-FF (Figs. 3D, 4E) from the abscissa to the ordinate. The corresponding values of $[\text{Ca}^{2+}]_c$ are: MNSNb/d-Is, 412 nM (mito-RP), 391 nM (rhod-FF); MN13-Ib: 355 nM (mito-RP), 489 nM (rhod-FF). **D**, Plot of nerve stimulation-dependent (40 Hz, 2 s) changes in $[\text{Ca}^{2+}]_c$ ($\Delta[\text{Ca}^{2+}]_c$) for boutons of different sizes from type Is and Ib terminals (6 boutons from each terminal, as indicated in the image of fura-loaded MN terminals). There was no correlation between bouton size and $\Delta[\text{Ca}^{2+}]_c$ in either terminal (Spearman rank order correlation, $p > 0.2$).

Figure 8. Cytosolic Ca^{2+} levels ($[\text{Ca}^{2+}]_c$) in type Ib and Is terminals do not depend on the presence of functional mitochondria. **A**, Average $[\text{Ca}^{2+}]_c$ in type Ib (left) and Is terminals (right) on muscle 13 in response to a 2 s 40 Hz stimulation train. Average $[\text{Ca}^{2+}]_c$ is shown for the wild-type control (w^{1118}), wild-type control exposed to 3 μM CCCP, and mutant terminals homozygous for drp1^{12} or dmiro^{B682} . The numbers of type Ib and Is terminal pairs are indicated in the bars. No difference was found within terminal type between control and CCCP, drp1^{12} , or dmiro^{B682} . For each treatment, $[\text{Ca}^{2+}]_c$ responses to nerve stimulation are significantly different between type Ib and Is terminals (two-way ANOVA, $p < 0.05$ overall, Holm-Sidak *post hoc* test). **B**, Average $[\text{Ca}^{2+}]_c$ in the same terminals as in **A** before nerve stimulation. No significant differences were found within terminal type or between treatments (two-way ANOVA). Error bars indicate SEM.

Discussion

This study pursued two major goals: to test whether mitochondrial Ca^{2+} sequestration limits cytosolic Ca^{2+} in axon terminals within a physiological range of activity, and to test whether mitochondrial Ca^{2+} sequestration is tuned to the different Ca^{2+} environments and demands of two functionally different MN terminals. The arrangement of the *Drosophila* larval NMJ, where phasic-like and tonic-like MNs innervate the same muscle fiber, provides a convenient model for investigating both questions. Despite the striking differences in native firing rates and stimulus-dependent Ca^{2+} levels of the two terminals, their presynaptic mitochondria exhibit a similar affinity for Ca^{2+} uptake in response to transient, depolarization-dependent Ca^{2+} elevations without significantly constraining cytosolic Ca^{2+} levels.

Suitability of GECs for monitoring mitochondrial Ca^{2+} uptake in presynaptic terminals

Synthetic Ca^{2+} -indicators have excellent properties but cell-specific loading can be challenging. While GECs have been pre-

viously targeted to mitochondria in neurons (Núñez et al., 2007), their utility as reporters of $[\text{Ca}^{2+}]_m$ in the highly dynamic Ca^{2+} environment of a nerve terminal has not been tested. In our system, RP, YC2 and CG2 were very effectively targeted to the matrix of presynaptic mitochondria, as no fluorescence originating from the presynaptic cytosol could be detected. This was a fortunate result, as a similar targeting strategy resulted in significant fluorescence from the cytosol of HeLa cells (Filippin et al., 2005). Mitochondrial transport may take credit for this serendipitous result, since axon terminals are likely out of range for diffusion from the soma where the proteinaceous indicators are translated and likely imported into mitochondria.

All of the mitochondrial GECs examined are highly responsive to nerve stimulation. Synthetic Ca^{2+} -indicators covering a wide range of Ca^{2+} -affinities did not report any changes in $[\text{Ca}^{2+}]_m$ that went undetected by the mitochondrially targeted GECs, indicating that the Ca^{2+} -affinity of mito-RP, mito-YC2 and mito-CG2 are well suited for reporting changes in $[\text{Ca}^{2+}]_m$. Although the response time of GECs is often inferior to that of synthetic Ca^{2+} -indicators (Pologruto et al., 2004; Wallace et al., 2008), this did not limit their utility in the mitochondrial matrix.

Mitochondrial Ca^{2+} sequestration is not tuned to different presynaptic Ca^{2+} environments

Mitochondria isolated from different regions of the rat brain differ in their enzyme activities, fatty acid content, ROS content, and their Ca^{2+} dependence of mitochondrial depolarization and permeability transition pore opening (Lai et al., 1977, 1994; Battino et al., 1991; Friberg et al., 1999; Dubinsky, 2009). In the context of such functional heterogeneity, it seems possible, if not likely, that mitochondrial Ca^{2+} sequestration may be tuned to different presynaptic Ca^{2+} environments, like those of phasic and tonic neurons.

Our analysis of mitochondrial Ca^{2+} sequestration in terminals of tonic-like MN13-Ib and phasic-like MNSNb/d-Is MNs provided no evidence for differential regulation of mitochondrial Ca^{2+} sequestration in functionally different terminals. Presynaptic mitochondria in type Is and Ib terminals exhibited similar affinities (~ 410 nM) for Ca^{2+} sequestration, although the native firing rate and stimulated $[\text{Ca}^{2+}]_c$ differ significantly between the two terminal types. In addition, $[\text{Ca}^{2+}]_c$ needs to reach only ~ 250 nM for ≤ 2 s before mitochondria take up Ca^{2+} in each type of terminal. This threshold is similar to that estimated for lizard MN terminals (≤ 300 nM; David et al., 1998; David et al., 2003) and is consistent with the low values estimated in dissociated sympathetic neurons (Colegrove et al., 2000). Similar to findings in other terminals (Tang and Zucker, 1997; David, 1999; Billups and Forsythe, 2002; Kim et al., 2005), we also found that Ca^{2+} release from the ER is not required for presynaptic mitochondrial Ca^{2+} sequestration in response to presynaptic stimulation. Finally, mitochondrial Ca^{2+} sequestration had little or no impact on $[\text{Ca}^{2+}]_c$ in response to nerve stimulation in either terminal type.

Mitochondrial Ca^{2+} sequestration is unlikely to be a universal mechanism limiting presynaptic Ca^{2+} levels at native firing rates

A primary objective of this study was to test the ability of mitochondrial Ca^{2+} -sequestration to limit the $[\text{Ca}^{2+}]_c$ in presynaptic terminals of MNs during physiologically relevant firing rates and durations. Our data provide such a test for the following reasons: a) Both MN axons were stimulated at, or above, the maximal firing rate that was recorded *in situ* for each MN during fictive

locomotion. b) We used a 2 s long stimulation train, which is similar in duration to the period of rhythmic bursts of activity during fictive locomotion, and probably exceeds the duration of episodic activity during unrestrained locomotion (Klose et al., 2005). c) It seems unlikely that a single, 2 s long stimulation train will significantly deplete the ATP available to power Ca^{2+} clearance mechanisms. d) After inhibiting the mitochondrial proton gradient, MN terminals were only stimulated after a resting period of at least 10 min, a period over which it is reasonable to expect that the ATP/ADP ratio reaches equilibrium by glycolysis alone.

To comprehensively address the role of mitochondrial Ca^{2+} sequestration in buffering cytosolic Ca^{2+} levels, we used the protonophore CCCP to acutely inhibit mitochondrial Ca^{2+} sequestration. In addition, we examined the effects of chronically reducing or abolishing presynaptic mitochondria by examining $[\text{Ca}^{2+}]_c$ in mutant terminals that either contained a greatly reduced number of mitochondria (*drp1*²) or no mitochondria at all (*dmiro*^{B682}). Consistently, neither acute nor chronic manipulations had an impact on evoked $[\text{Ca}^{2+}]_c$ during short stimulus trains at 40 Hz. This lack of an effect indicates that mitochondrial Ca^{2+} sequestration is ineffective at buffering changes in presynaptic $[\text{Ca}^{2+}]_c$ at nerve firing rates close to or above native firing rates. Hence, mitochondrial Ca^{2+} sequestration is not universally required for buffering transient changes in presynaptic Ca^{2+} within physiological ranges of nerve activity.

Two other studies examining mitochondrial Ca^{2+} sequestration at MN terminals also found no evidence that mitochondrial Ca^{2+} sequestration limits $[\text{Ca}^{2+}]_c$ during lower frequencies of stimulation (David and Barrett, 2000; Lnenicka et al., 2006) but did not provide the physiological context of native firing frequencies. In addition, mitochondria at the Calyx of Held apparently participate in Ca^{2+} clearance only when the Ca^{2+} load is large or prolonged (Kim et al., 2005; Lee et al., 2007), although it is not clear whether these are physiological Ca^{2+} loads.

The design of our study, and consequently our findings, differ from those of earlier studies on MN terminals (Tang and Zucker, 1997; David et al., 1998; Narita et al., 1998; David and Barrett, 2000; Suzuki et al., 2002; Guo et al., 2005; Verstreken et al., 2005; Bennett et al., 2007). We specifically focused on the impact of mitochondrial Ca^{2+} sequestration in limiting $[\text{Ca}^{2+}]_c$ at physiological axon firing rates to avoid misleading or artifactual results. Specifically, we did not consider conditions of either prolonged or high-frequency nerve stimulation, where Ca^{2+} -induced mitochondrial ATP production is likely required to limit $[\text{Ca}^{2+}]_c$. In fact, prolonged stimulation (> 30 s at 10 Hz) of *Drosophila* type Ib terminals that lack a normal amount of mitochondria (*drp1* or *dmiro* mutants) causes an abnormal increase in $[\text{Ca}^{2+}]_c$ and a run-down of neurotransmitter release, which can be restored by providing ATP in the recording solution (Guo et al., 2005; Verstreken et al., 2005).

What is the consequence of mitochondrial Ca^{2+} uptake?

If Ca^{2+} -sequestration by presynaptic mitochondria is not required for buffering cytosolic Ca^{2+} transients within physiological ranges of stimulation, then what is its physiological role? One possibility is that it may serve to coordinate ATP supply with demand in axon terminals during synaptic transmission. Considering the high ATP demands of recycling synaptic vesicles, Ca^{2+} clearance mechanisms, and maintaining ion gradients, it is unlikely that glycolysis alone can meet a rapid increase in presynaptic ATP demand. Consistently, glycolysis alone is adequate to maintain ATP-dependent processes in synaptosomes at rest, but

not in response to challenges that accelerate ATP demand (Scott and Nicholls, 1980). Oxidative phosphorylation, and thereby ATP production, can be increased in non-neuronal cells by cytosolic Ca^{2+} spikes that stimulate up to four Ca^{2+} -sensitive mitochondrial dehydrogenases and the F_1F_0 -ATPase (for review, see Denton, 2009; Balaban, 2009). Mitochondrial ATP production in neurons and axon terminals may also be controlled by such a mechanism although experimental evidence is lacking (Kann and Kovacs, 2007).

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