Mitochondrial Clustering at the Vertebrate Neuromuscular Junction during Presynaptic Differentiation

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ABSTRACT: During vertebrate neuromuscular junction (NMJ) development, presynaptic motor axons differentiate into nerve terminals enriched in synaptic vesicles (SVs). At the nerve terminal, mitochondria are also concentrated, but how mitochondria become localized at these specialized domains is poorly understood. This process was studied in cultured Xenopus spinal neurons with mitochondrion-specific probe MitoTracker and SV markers. In nerve-muscle cocultures, mitochondria were concentrated stably at sites where neurites and muscle cells formed NMJs, and mitochondria co-clustered with SVs where neurites were focally stimulated by beads coated with growth factors. Labeling with a mitochondrial membrane potential-dependent probe JC-1 revealed that these synaptic mitochondria were with higher membrane potential than the extrasynaptic ones. At early stages of bead-stimulation, actin-based protrusions and microtubule fragmentation were observed in neurites at bead contact sites, suggesting the involvement of cytoskeletal dynamics and rearrangement during presynaptic differentiation. Treating the cultures with an actin polymerization blocker, latrunculin A (Ltn A), almost completely abolished the formation of actin-based protrusions and partially inhibited bead-induced mitochondrial and SV clustering, whereas the microtubule disrupting agent nocodazole was ineffective in inhibiting the clustering of mitochondria and SVs. Lastly, in contrast to Ltn A, which blocked bead-induced clustering of both mitochondria and SVs, the ser/thr phosphatase inhibitor okadaic acid inhibited SV clustering but not mitochondrial clustering. These results suggest that at developing NMJs, synaptogenic stimuli induce the clustering of mitochondria together with SVs at presynaptic terminals in an actin cytoskeleton-dependent manner and involving different intracellular signaling molecules.

Keywords: neuromuscular junction; synaptogenesis; presynaptic differentiation; mitochondria; synaptic vesicle; cytoskeleton

INTRODUCTION

During the development of the vertebrate neuromuscular junction (NMJ), the nerve-muscle contact induces the development of synaptic specializations. On the postsynaptic side, acetylcholine receptors (AChRs) become highly concentrated in the subsynaptic membrane. Presynaptic specializations include the clustering of synaptic vesicles (SVs) that transform the growing motor axon into a nerve terminal. Despite our extensive knowledge on the assembly of the postsynaptic apparatus (Hall and Sanes, 1993; Sanes and Lichtman, 1999), much less is known about the presynaptic development at the NMJ. To understand the latter aspect of NMJ formation, we have used cultured embryonic Xenopus spinal neu-
rons as a model. It was previously shown that in these neurons, presynaptic differentiation can be induced by coculturing with muscle cells and also by beads coated with heparin-binding growth factors (HBGFs), heparin-binding growth-associated molecule (HB-GAM), and basic fibroblast growth factor (bFGF). Both muscle and beads effect SV clustering, local transmitter release, the organization of Ca$^{2+}$ microdomains that reflect the clustering of calcium channels (Dai and Peng, 1995, 1996a,b, 1998; Rauvala and Peng, 1997; DiGregorio et al., 2001), and the assembly of an F-actin cytoskeleton (Dai and Peng, 1996a; Peng et al., 1997). HB-GAM and bFGF are muscle-intrinsic factors and are localized at the NMJ (Peng et al., 1991a, 1995). Thus, HBGF-coated bead mimics the target muscle to focally induce the development of presynaptic specializations in cultured spinal neurons. Additionally, a recent study has identified another HBGF, FGF22, as the principal organizer of presynaptic differentiation in the central synapses in vivo (Umemori et al., 2004), suggesting an important role of the HBGFs in presynaptic differentiation at the NMJ as well as at CNS synapses.

To further understand presynaptic development associated with NMJ development, the targeting and localization of mitochondria in relation to SVs in *Xenopus* spinal neurons were examined in this study. At mature presynaptic terminals, mitochondria, like SVs, are also concentrated, but, unlike SVs, these organelles are abundant in the region of nerve terminal away from the active zone where SVs are clustered (Robitaille and Tremblay, 1987). Using nerve-muscle cocultures and bead-induced presynaptic specializations, we sought to understand the mechanisms in SV and mitochondrial localization. We report here the coclustering of these two types of organelles in response to synaptogenic signals in relationship to cytoskeletal assembly and the contrasting signaling mechanisms that may govern their differential localization within the developing nerve terminal.

**MATERIALS AND METHODS**

**Materials**

MitoTracker Red CMXRos, JC-1, FM1-43, and latrunculin A (Lin A) were obtained from Molecular Probes (Eugene, OR). Nocodazole (Noc) was obtained from Sigma (St. Louis, MO). Okadaic acid (OA) was obtained from Calbiochem (San Diego, CA).

**Xenopus Primary Cultures**

Spinal neurons and muscle cells were cultured from *Xenopus* embryos as previously described (Peng et al., 1991b). In short, neural tubes or myotomes of embryos at stage 19–22 were isolated and dissociated in a Ca$^{2+}$, Mg$^{2+}$-free solution. Dissociated cells were plated on glass coverslips coated with the cell attachment matrix ECL (entactin, collagen IV, and laminin; Upstate Biotechnology, Waltham, MA). To prepare nerve-muscle cocultures, neurons were seeded onto 3-day-old muscle cultures.

**Live Cell Staining**

To visualize mitochondria and SVs, MitoTracker and FM1-43 were used. MitoTracker is a cell-permeant probe that passively diffuses across membranes and accumulates in active mitochondria (Poot et al., 1996); FM1-43 is a styryl dye that is trapped in recycling vesicles by endocytosis during active nerve stimulation (Betz and Bewick, 1992). To label mitochondria, neurons were stained for 5 min with 5 mM MitoTracker in culture medium [60 mM NaCl, 0.7 mM KCl, 0.4 mM Ca(NO$_3$)$_2$, 0.8 mM MgSO$_4$, 10 mM HEPES, 10% L-15, 1% fetal bovine serum, and 0.1 mg/mL gentamicin]. To label releasable pools of SVs, cells were stained with 2 mM FM1-43 by a 2 min exposure to high-potassium solution (30 mM NaCl, 51 mM KCl, 2 mM CaCl$_2$, and 5 mM HEPES, pH 7.4). Labeled cells were washed twice with culture medium or modified Ringer solution (80 mM NaCl, 1 mM KCl, 2 mM CaCl$_2$, and 5 mM HEPES, pH 7.4), respectively, before examination by fluorescence microscopy.

Muscle cells were labeled with rhodamine-conjugated α-bungarotoxin (R-BTX; Molecular Probes) to follow the clustering of AChRs at nerve-muscle contacts, which were identified in live cultures by phase-contrast microscopy.

To study the relative membrane potential of mitochondria, muscle- or bead-neuron cocultures were stained with 0.25 µg/mL JC-1 for 10 min, followed by washing twice with culture medium. The fluorescence intensity at emission wavelength of 525 and 590 nm was measured for ratiometric analysis.

**Induction of Presynaptic Specializations by Beads**

To induce focal presynaptic differentiation, we stimulated cultured spinal neurons with beads coated with HB-GAM. Polystyrene latex beads 4 or 10 µm in diameter (Polysciences, Warrington, PA) were coated with HB-GAM (a gift of Dr. Heikki Rauvala, University of Helsinki) using previously described procedures (Dai and Peng, 1995). Beads were washed with culture medium and blocked with medium containing bovine serum albumin (BSA) before being added to 1-day-old neuronal cultures. In some experiments, cultures were incubated with Lin A, Noc, or OA before bead addition. Bead-neurite contacts were scored positive for mitochondrial and SV clustering if the mean fluorescence intensity of the corresponding marker was at least twofold over the noncontact region.

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**Immunocytochemical Labeling**

For staining cells with antibodies or F-actin markers, neuronal cultures were fixed with 2% paraformaldehyde in PBS, followed by permeabilization with 0.5% Triton X-100. Fixed cells were blocked with phosphate buffered saline (PBS) containing 5% BSA for 1 h and, unless specified otherwise, labeled with primary antibodies for 2 h followed by fluorescent secondary antibodies (Zymed Laboratories, South San Francisco, CA). To label F-actin in neurons, fixed and permeabilized cells were incubated with either rhodamine- or fluorescein-conjugated phalloidin (Molecular Probes) at 1:500 dilution for 45 min.

**RESULTS**

**Distribution and Dynamics of Mitochondria in Naive and Muscle-Contacted Neurons**

In 1-day-old Xenopus spinal neuron cultures, mitochondria were visualized by MitoTracker labeling and SVs by FM1-43 loaded in Ringer solution containing high K+. Mitochondria were localized at varicosities along the length of the neurites [Fig. 1(A,B)] and at growth cones [panels (D,E)]. At these sites, FM1-43-labeled SVs were also concentrated [panels (C,F)] and these two organelles appeared to be colocalized. Mitochondria in cultured spinal neurons were either globular or filamentous in shape and highly variable in size. They moved along the neurite bidirectionally with intermittent pauses, consistent with previous findings (Morris and Hollenbeck, 1993). To quantify the mobility of mitochondria in naive neurites before target contact, eight morphologically distinct mitochondria in a segment of the neurite were imaged by time-lapse recording. During the 100 s recording period, three out of these were transported at an average rate of >0.15 μm/s [panels (G–L), #2, 5, 8; designated as “fast moving”]; one was transported at an intermediate rate of 0.1–0.15 μm/s [panels (G–L), #7; designated as “slow moving”] and the remainder were essentially immobile [panels (G–L), #1, 3, 4, 6; designated as “stationary”]. <0.1 μm/s, including the one located at the varicosity [panels (G–L), #1]. Because mitochondria have been shown to be transported bidirectionally in neurons (Morris and Hollenbeck, 1995), we used MSD analyses to further quantify the rate of this bidirectional movement by calculating the “diffusion coefficient” as an indication of this rate, although their movement is not truly diffusion in nature. The “fast moving” mitochondria had a diffusion coefficient 2–3 μm²/s, “slow moving” ones had values below 2 μm²/s, and “stationary” ones had coefficients smaller than 0.1 μm²/s. The slope of this plot is linearly related to the diffusion coefficient. This analysis shows that mitochondria vary considerably in their speed of movement within the naive neurite.

Mitochondria were closely associated with postsynaptic AChR clusters in 1-day-old nerve-muscle cocultures [Fig. 2(A–C)]. To examine the source of synaptic mitochondria, neurons were first labeled with MitoTracker and then cocultured with unlabeled muscle cells for 1 day. Mitochondria of neuronal origin were observed within the nerve terminal opposite to AChR clusters in muscle [panels (E,F)]. Again, the mobility of mitochondria at a segment of the neurite in contact with target muscle cell was selected for time-lapse imaging [panels (G–L)]. In contrast to those in naive neurites, all mitochondria in Figure 2 were synaptic because they were located at the sites of nerve-muscle contact; they were generally rather immobile as shown by the small MSD values [panel (M)]. Although #2, #6, and #8 showed some movement, it was on a much smaller scale [compare vertical axis of panel (M) with that in Fig. 1(L)]. Because SVs are also highly enriched and immobilized within the nerve terminal in these cocultures (Dai and Peng, 1996a), these results suggest that mitochondria appeared to be colocalized with SVs at developing presynaptic specializations during NMJ formation.

**Induction of Mitochondrial Clustering by HB-GAM Beads**

We previously showed that beads coated with certain growth factors such as bFGF or HB-GAM can induce
focal SV clustering and localized Ca\(^{2+}\) influx in neurites (Dai and Peng, 1996a, 1998; DiGregorio et al., 2001). Here, by stimulating spinal neurons with beads coated with HB-GAM, we found that mitochondria became clustered at bead-neurite contacts together with SVs visualized by labeling with an antisynaptotagmin antibody (Fig. 3). Mitochondrial and SV clusters were detectable at the bead-neurite contact site as early as 20 min after bead stimulation [Fig. 3(A–D)], and their size increased with time, as shown in the example of a 16 h cluster [Fig. 3(E–H)]. Synaptotagmin antibody staining showed that SV clusters induced by beads appeared to be colocalized with mitochondria at both early and late time points, but they were in adjacent but nonoverlapping domains as observed under high magnification [Fig. 3(D,H)]. Demonstrating specificity, beads coated with BSA instead of HB-GAM did not cluster mitochondria or SVs [Fig. 3(I–L)]. Quantification of bead-neurite contacts with staining for mitochondria and SVs revealed that these organelles were clustered in a temporally correlated manner. SV and mitochondrial clusters were detectable as early as 20 min at \(\approx 20\) and \(\approx 30\%\) of bead-neurite contacts, respectively, and with maximal clustering occurring 4 h after bead addition to cells [Fig. 3(M)]. The strong correlation between the number of bead-neurite contacts and the mitochondrial clusters was shown in Figure 3(N). These results suggest that in response to a postsynaptic target, mitochondria and SVs become clustered within the neurites over the same time frame but in adjacent subcellular domains.

To further investigate the dynamic targeting of mitochondria in response to focal synaptogenic stimulation, time-lapse imaging was carried out. Bead-neurite contacts were followed before the onset of mitochondrial clustering until 4 h after the bead stimulation. An example is shown in Figure 4 [note that the inter-
Figure 2  Localization and dynamics of neuronal mitochondria in nerve-muscle cocultures. (A–C) In 1 day nerve-muscle cocultures, mitochondria were localized at the synaptic sites as shown by close association of MitoTracker [(B), arrows] and R-BTX [(C), arrows] signals. (D–F) To determine if the synaptic mitochondria were neuron- or muscle-derived, mitochondria in spinal neurons were prelabeled with MitoTracker before muscle cells were plated. Newly formed NMJs were examined 1 day later by labeling muscle AChRs with R-BTX. Neuronal mitochondria [(E), arrows] were concentrated on the presynaptic side apposed to sites of AChR clustering in muscle [(F), arrows]. Asterisk (E) indicates nonspecific auto-fluorescence by yolk platelets in muscle. (G–L) The mobility of eight morphologically distinct mitochondria [(H–L), #1–8] at a neuritic segment in contact with a muscle cell (G) was followed by time lapse recordings (20 s interval between frames). These clusters were immobile at this NMJ. (M) MSD of the corresponding mitochondria, marked in (H–L), showed mitochondria at nerve-muscle contact were relatively immobile.
Figure 3  Clustering of mitochondria and SVs in neurons by HB-GAM beads. MitoTracker-labeled neuronal cultures were stimulated with HB-GAM beads for 20 min (A–D) or 16 h (E–H), and then fixed, permeabilized, and immunostained with the antisynaptotagmin antibody SV48. At bead-neurite contacts [(A,E), asterisks], SV [(C,G), boxed regions] and mitochondrial [(B,F), boxed regions] clusters appeared to be colocalized with each other. The boxed regions were magnified and merged in (D) and (H). At high magnification, mitochondria and SVs were resolved into adjacent but nonoverlapping domains at the bead-neurite contacts. (I–L) BSA control beads induced neither mitochondrial nor SV clustering (arrowheads). (M) Pooled data from three separate time course experiments showing that HB-GAM beads induced the clustering of mitochondria and SVs in a temporally correlated manner. Data are means ± SEM from three independent experiments, n > 150 bead-neurite contacts. (N) The number of mitochondrial clusters induced by HB-GAM beads was proportional to the number of bead-neurite contacts. A total of 30 neurites were represented in this graph. Each point represents one or more neurites because of data overlap. The regression line has a slope of 0.7. The correlation coefficient is $R^2 = 0.85$. 

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val between frames is 5 min in (B–F), different from that in Fig. 1(G–K)]. In this case, mitochondrial clusters were not yet detectable [(B), arrow] 3 h after bead stimulation. Over the next several minutes, mitochondria became clustered at the contact site apparently from anterogradely transported organelles [direction pointed by an arrow in (A)]. The interval between panels (B) to (F) was 5 min. The bead-induced mitochondrial cluster was stable at the end of 4 h time-lapse recording [(G), arrow]. Mitochondria were also stably associated with varicosities present on the neurites during the course of the bead-stimulation [(B–G), open arrowheads]. (H–M) Mitochondria located at the bead-neurite contact [(I–M), #1–3] were relatively immobile. Transport of mitochondria along neurite [(I–M), arrows and arrowheads] through bead contact site was unaffected. The interval between adjacent frames was 20 s. (N) MSD plots of three corresponding mitochondria at the bead-contacted site.

Figure 4 Time-lapse imaging of bead-induced mitochondrial clustering. A bead-neurite contact [(A), asterisk] on a MitoTracker-loaded neurite was recorded for several hours. At this site, mitochondrial clusters were not yet detectable [(B), arrow] 3 h after bead stimulation. Over the next several minutes, mitochondria became clustered at the contact site apparently from anterogradely transported organelles [direction pointed by an arrow in (A)]. The interval between panels (B) to (F) was 5 min. The bead-induced mitochondrial cluster was stable at the end of 4 h time-lapse recording [(G), arrow]. Mitochondria were also stably associated with varicosities present on the neurites during the course of the bead-stimulation [(B–G), open arrowheads]. (H–M) Mitochondria located at the bead-neurite contact [(I–M), #1–3] were relatively immobile. Transport of mitochondria along neurite [(I–M), arrows and arrowheads] through bead contact site was unaffected. The interval between adjacent frames was 20 s. (N) MSD plots of three corresponding mitochondria at the bead-contacted site.

Before bead stimulation, mitochondria were also enriched at varicosities along the neurite [panels (B–G), open arrowheads]. Unlike free mitochondria, the varicosity-associated ones were stationary and remained clustered there despite the bead stimulation. The mobility of individual mitochondria at bead-neurite contacts was further examined by time-lapse imaging at higher magnification [panels (H–M)]. Mitochondria within the clusters induced by beads were quite immobile [panels (I–M), #1–3]. The nonclustered mitochondria outside the bead-neurite contact
remained mobile and could even move across the clusters as shown by time-lapse recording [panels (I–M), arrows and arrowheads]. Like mitochondrial clusters at nerve-muscle contact, the MSD calculation on those located at the bead-neurite contact also reflected the greatly diminished mobility [panel (N)].

Relative Membrane Potential of Synaptic and Extrasynaptic Mitochondria

The membrane potential of mitochondria was studied by using another mitochondrial dye, JC-1, whose fluorescence is sensitive to mitochondrial membrane potential. JC-1 exists as a monomer at low membrane potential, exhibiting green fluorescence. However, at higher membrane potential, JC-1 forms aggregates that fluoresce red at ≈590 nm (Smiley et al., 1991). It has been extensively used to examine the change in mitochondrial membrane potential during transient depolarizations of individual mitochondria (Buckman and Reynolds, 2001) or in response to the mitochondrial function enhancer creatine in cultured neurons (Li et al., 2004). In this study, neuronal mitochondria exhibited higher membrane potential than muscle mitochondria [Fig. 5(A–D)]. Interestingly, among neuronal mitochondria, the ones located at sites of muscle contact [panels (B–D), arrows] showed slightly higher membrane potential than those at the noncontact site [panels (B–D), open arrowheads]. Similar results were observed in mitochondrial clusters induced by beads [panels (E–H)]. Mitochondria at the bead-neurite contact [panels (F–H), arrows] had higher membrane potential than those at noncontacted regions [panels (F–H), arrowheads]. These results were quantified by ratiometric analyses of fluorescence intensity at 525 and 590 nm [panel (I)]. The difference in mitochondrial membrane potential within nerve and muscle is highly significant and that at bead-contacted versus noncontacted sites is also significant.

Cytoskeletal Dynamics and Modification at Early Bead-Neurite Contacts

Previous studies have suggested the role of the actin cytoskeleton in the formation of SV clusters (Dai and Peng, 1996a; Peng et al., 1997; Bloom et al., 2003). Thus, its role in the clustering of mitochondria was investigated in this study. At mature motor nerve terminals, actin-based protrusions that extend beyond the borders of postsynaptic receptor clusters have been previously described and they are implicated in the establishment and refinement of neuronal connections (Robbins and Polak, 1988). Local application of neurotrophins via bead induces actin-based collateral sprouting along the axonal shafts (Gallop and Letourneau, 1998). When neurites were labeled with the F-actin probe phalloidin following stimulation by HB-GAM beads, we found that actin-based protrusions were induced at bead-neurite contacts [Fig. 6(B,E), arrows]. Microtubule debundling and fragmentation were occasionally observed in these protrusions [pan-
els (C,F), arrows]. Examination of multiple bead-neurite contacts further revealed that more protrusions were generated during the early stages of bead-stimulation. Treating cultures with 25 nM Ltn A, which interferes with actin polymerization through G-actin sequestration, abolished the formation of these axonal protrusions [panel (G)]. In contrast, microtubule fragmentation was insensitive to microtubule polymerization inhibition by 100 nM Noc. These results suggest that actin and/or microtubule remodeling induced by synaptogenic signals sets up cytoskeletal scaffolds for the clustering of mitochondria and SVs.

**Involvement of Cytoskeletal Assembly in the Clustering of Mitochondria and SVs**

To test whether actin polymerization is needed for mitochondrial clustering in addition to its involvement in SV clustering, we examined the effect of Ltn A on the former process. In control cultures, about half of the bead-induced mitochondrial clusters were highly colocalized with an F-actin accumulation [Fig. 7(D–F); 54.13 ± 4.63%, n = 150 bead-neurite contacts from three experiments]. When cultured neurons were treated for 1 h with 50 nM Ltn A before stimulation by HB-GAM beads, no appreciable actin localization and mitochondrial signal were visible at this bead contact [panels (G–I)]. SV and mitochondria clustering were both inhibited in a dose-dependent manner [panels (A–C) and (J)]. Maximal inhibition was reached with 25 nM Ltn A, at which concentration mitochondrial and SV clustering induced by beads was reduced to approximately 55% and 65%, respectively. However, the axonal transport of mitochondria in Ltn A-treated cultures was unaffected as shown by MSD of individual mitochondria [panel (K)].

In contrast to the effect of Ltn A, Noc had no significant effect on bead-induced mitochondrial and SV clustering at concentrations up to 100 nM [Fig. 8(A–D), arrows]. These results were also quantified in panel (M). To determine the effectiveness of Noc on microtubule polymerization inhibition, we examined the appearance of microtubules in the neuronal growth cone, where active assembly of microtubules takes place, in response to Noc treatment. In control growth cone, many individual microtubules penetrated to the P-domain of the growth cone [panel (F), arrow]. A large reduction in numbers of individual microtubules [panel (H), arrow] and an accumulation of tubulin background staining [panel (H), arrowhead] were observed at the growth cone in Noc-treated cultures. However, the diminishing in microtubule assembly with the growth cone under Noc had no effect on mitochondrial clustering induced by bead [panels (I–L)]. MSD plots in Noc-treated cultures showed that despite the lack of new microtubule assembly, mitochondrial transport along the neurite remained intact [panel (N)]. These observations suggest that the clustering of mitochondria in neurites, like that of SVs, is in part dependent on actin polymerization but is independent of microtubule assembly.

**Differential Signaling in Mitochondrial and SV Clustering**

The adjacent but nonoverlapping localization of mitochondria and SVs within bead-induced specializations suggests there is a difference in their assembly process. As a first step toward identifying molecules that selectively participate in mitochondrial or SV clustering, we examined the involvement of phosphatases through the use of an inhibitor. Previous works
have demonstrated that OA, which inhibits the ser/thr phosphatases PP1 and PP2A, disperses SV clusters in motor terminals in vivo and in nerve-muscle cocultures (Betz and Henkel, 1994; Dai and Peng, 1996a). We thus treated the cultured neurons with different concentrations of OA before or after bead stimulation and examined its effect on the formation and maintenance of SV and mitochondrial clusters. By pretreating the cultures with OA for 1 h and keeping it in the medium, the bead-induced formation of SV clusters was examined. OA at 30 nM attenuated this process in a time-dependent manner [Fig. 9(D), left]. On the other hand, the maintenance of bead-induced SV clusters was studied by post-treatment of OA. Overnight bead-neuron cocultures were treated with OA for 4 or 16 h. As shown in Figure 9(D) (right), this phosphatase inhibitor also affected the stability of established SV clusters, leading to their dispersal. In contrast, neither the formation nor maintenance of mitochondrial clusters induced by beads was affected by these OA treatments [panel (E)]. Together with our previous results showing OA disperses SVs but leaves actin polymerization intact (Dai and Peng, 1996a), these results suggest that while the SV cluster formation and maintenance are phosphatase-dependent, the mechanisms are different for the synaptic localization of mitochondria.

**DISCUSSION**

Before the nerve establishes a synapse on its target, functional packets of SVs, called exocytic hotspots...
(Goda and Davis, 2003), can be found in its growing axon. As the synapse develops, SVs become clustered at the nerve terminal apposed to the postsynaptic membrane of the target cell. In this study we found that mitochondria appeared to be colocalized with SV clusters in varicosities and growth cones of naive

![Figure 8](image)

Figure 8  Microtubule-independent clustering of mitochondria and SVs by beads. (A–D) Neither mitochondrial [(B), arrows] nor SV clustering [(C), arrows] induced by beads was affected by 100 nM Noc. (E–H) The effect of Noc on microtubule patterns and dynamics was assessed by examining the neuronal growth cones. In untreated growth cone (E,F), individual microtubules were frequently observed to reach the leading edge of the growth cone [(F), arrow]. In 100 nM Noc-treated culture (G,H), the presence of individual microtubules was largely reduced [(H), arrow]. Increase in background staining of tubulin [(H), arrowhead] was also observed. (I–L) Bead-induced mitochondrial clustering [(K), arrow] was formed in microtubule-absent region [(J), arrowhead] of the growth cone in Noc-treated cultures. (M) Incubation of neurons with up to 100 nM Noc affected neither mitochondrial nor SV clustering. Data are means ± SEM from three independent experiments, n > 150 bead-neurite contacts. (N) MSD plots of mitochondria in 100 nM Noc-treated cultures showed the general transport of mitochondria along neurites was intact.
neurites and were aggregated at nerve-muscle contacts as well as at presynaptic specializations induced by growth factor-coated beads that mimic the muscle target, and at these sites, their movement along the axon becomes highly restricted. Concomitantly, these target-localized mitochondria achieve a higher membrane potential. Through pharmacological studies we showed that the actin cytoskeleton plays a common role in the clustering of SVs and mitochondria but that ser/thr phosphatase signaling influences SV but not mitochondrial clustering. In culture, the neuritic contact with the bead target triggered the development of SV and mitochondrial clusters within minutes [Fig. 3(A–D,M)], consistent with the previous demonstration of neurotransmitter release from nerve terminals shortly after their coming into contact with muscle cells in cocultures (Xie and Poo, 1986; Dai and Peng, 1993). Thus, our results support the conclusion that mitochondrial clustering is an integral and rapid event associated with presynaptic differentiation during early stages of NMJ formation.

Potential Functions of Presynaptic Mitochondria at NMJ

Mitochondria within the nerve terminal perform a number of specific presynaptic functions in addition to energetic activities of transmitter release. Previous studies have shown that mitochondria participate in SV biogenesis by driving ATP-dependent vacuolar proton pumps in the SV membrane in the refilling of neurotransmitters into its lumen (Nelson, 1993). A recent study has shown that Ca$^{2+}$ sequestered in mitochondria can provide a source for the potentiation of neurotransmitter release (Yang et al., 2003). The ATP produced by mitochondria is also copackaged into SVs and coreleased with ACh to serve as modulators for transmitter release at the adult NMJ (Silinsky and Redman, 1996) and as inductive signals during postsynaptic development (Tsim and Barnard, 2002). Because the mitochondrion is an important mediator of apoptosis, its presence within the nerve terminal allows it to regulate neuronal apoptosis under conditions such as trophic factor deprivation (Ellerby et al., 1997). The rapid targeting and clustering of mitochondria during presynaptic development reported in this study are indicative of the important functions that this organelle plays at the NMJ.

Axonal Transport of Mitochondria to Synaptic Sites

Mitochondrial transport in polarized cells, such as neurons, is tightly regulated and mitochondria are usually found accumulated at regions of high metabolic consumption. In various types of cultured neurons, axonal transport of mitochondria is bidirectional (Morris and Hollenbeck, 1993). A recent study has suggested that approximately 90% of mitochondria...
with high potential are transported anterogradely towards the growth cone and approximately 80% of mitochondria with low potential are transported retrogradely towards the cell body (Miller and Sheetz, 2004). In this study, time-lapse imaging revealed that bead-induced mitochondrial clusters were largely formed from anterogradely transported mitochondria, suggesting mitochondria of high membrane potential accumulate during presynaptic differentiation at the NMJ. Consistent with this premise is our observation that synaptic, neuronal mitochondria have higher membrane potential than postsynaptic ones in muscle or even extrasynaptic ones within the axon (Fig. 5). The molecules responsible for regulating mitochondrial transport at vertebrate NMJ are unknown, but at the Drosophila NMJ, a novel adapter protein named Milton has recently been shown to be involved in kinesin-mediated axonal transport of mitochondria to terminals (Stowers et al., 2002). Interestingly, mitochondrial clustering is also stimulated by beads coated with nerve growth factor (NGF) in chick sensory neurons (Chada and Hollenbeck, 2003, 2004). In these cells, NGF-dependent Trk activation triggered downstream signaling by phosphoinositide 3-kinase (PI 3-kinase) and actin polymerization, which mediated mitochondrial clustering (Chada and Hollenbeck, 2004). Whether mitochondrial clustering induced by local, bead-mediated application of HB-GAM or NGF shares a common signaling pathway remains to be determined. The mobility of mitochondria has recently been shown to be regulated by intracellular Ca$^{2+}$ level in the physiological range (Yi et al., 2004). Our previous study showed that Ca$^{2+}$ elevation can be elicited in the neurite contacted by growth factor-coated beads (Dai and Peng, 1995). This Ca$^{2+}$ signal may serve to reduce mitochondrial mobility and facilitate the subsequent clustering process.

**Cytoskeletal Dynamics and Presynaptic Differentiation**

Actin-rich protrusions, lamellipodia and filopodia, are motile structures present in neuronal growth cones that play important roles in axonal outgrowth and guidance. Collateral branches along quiescent axonal shafts can be induced by application of neurotrophins either locally or globally (Gallo and Letourneau, 1998; Gibney and Zheng, 2003). Local fragmentation of existing microtubule is required for branch formation (Yu et al., 1994). These collateral branches are likely mediated by concerted actions of actin and microtubules (Dent and Kalil, 2001). In this study, we observed actin-based protrusions and microtubule fragmentation at bead contacts along neurites (Fig. 6). These protrusions were frequently found at early time points after bead stimulation, consistent with the rapid formation of collateral sprouting by NGF beads previously reported (Gallo and Letourneau, 1998). The formation of these protrusions was mediated by the assembly of new actin filaments. These actin-rich motile structures may be consequential to the Ca$^{2+}$ elevation induced by postsynaptic contact (Dai and Peng, 1993, 1998; Lau et al., 1999). On the other hand, microtubule fragmentation is not affected by Noc, suggesting the lack of *de novo* assembly of new microtubules at the bead contact site.

Recent studies have shown that the actin cytoskeleton is involved in SV transport and serves as a molecular scaffold for regulatory molecules at nerve terminals (Shupliaukov et al., 2002; Sankaranarayanan et al., 2003). At the frog motor nerve terminal, inhibition of actin polymerization with Ltn A blocks vesicle reformation from endosomes after tetanic stimulation, but not the spontaneous clustering of SVs at resting terminals (Richards et al., 2004). Consistent with these findings, we have observed that inhibition of actin polymerization with Ltn A attenuated bead-induced SV and mitochondrial clustering by 35 and 45%, respectively [Fig. 7(J)]. In contrast to the effects of Ltn A, the microtubule polymerization inhibition by Noc did not affect SV and mitochondrial clustering, suggesting a specific involvement of actin polymerization in the clustering of these organelles. It has been reported that targeting of mitochondria to nerve terminals of Drosophila NMJs is mediated by kinesin (Stowers et al., 2002), which interacts with microtubules. Our study has shown that Noc, at concentrations up to 100 nM, had no effect on the staining patterns of pre-existing microtubule at the neuritic shafts [Fig. 8(G,H)] and the mobility of mitochondria along the neurite [Fig. 8(N)], despite its effect on the inhibition of microtubule assembly. Thus, the roles of kinesin and microtubules in the clustering of mitochondria in vertebrate nerve terminals remain to be determined.

In this study we found that mitochondrial clusters induced by beads in naïve neurons remained stably associated with F-actin, as shown by phalloidin staining [Fig. 7(D–F)]. Thus, in addition to participating in the transport of mitochondria and SVs, the actin cytoskeleton may form the scaffold on which these organelles become anchored. How mitochondria are stably clustered and anchored to the cytoskeleton at sites of presynaptic differentiation is not currently understood. In the case of SVs, association with the actin cytoskeleton is mediated at least in part by a
neuron-specific protein synapsin I. Phosphorylation and dephosphorylation of synapsin by ser/thr protein kinases and phosphatases negatively and positively regulate its ability to cluster and link SVs to the cytoskeleton, respectively (Bahler and Greengard, 1987; Bloom et al., 2003). Consistent with these results, we observed that 30 nM OA inhibited bead-induced SV clustering and dispersed clustered SVs. At low concentrations (3–30 nM), OA potently inhibits the ser/thr phosphatases PP2A and PP1 (MacKintosh and MacKintosh, 1994), and PP2A has been shown to dephosphorylate specific sites (P-sites 1, 2, and 3) in synapsin that regulate its binding to SVs and actin (Jovanovic et al., 2001). At these concentrations, however, OA had no effect on bead-induced mitochondrial clustering. Thus, a different mechanism mediates mitochondria’s association with the cytoskeleton in neurons.

REFERENCES


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