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Review

Mitochondrial movement and positioning in axons: the role of growth factor signaling

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Summary

The extreme length of axonal processes requires that aerobic ATP production and Ca^{2+} homeostasis are non-uniformly organized in the cytoplasm. As a result, the transport and positioning of mitochondria along axons is essential for neuronal homeostasis. Mitochondria undergo rapid but intermittent transport in both the anterograde and retrograde directions in axons. We have shown that in chick embryonic sensory neurons, the transport of mitochondria responds to physiological changes in the cell and, particularly, to growth cone activity. When an axon is actively elongating, mitochondria move preferentially anterograde and then become stationary, accumulating in the region of the active growth cone. When axonal elongation ceases, mitochondria in the distal axon resume movement but undergo net retrograde transport and become uniformly distributed along the axon. This redistribution of mitochondria is achieved in two ways: there is a transition between motile and stationary mitochondria and a large up- and downregulation of their anterograde, but not retrograde, motor activity. Mitochondrial transport does not respond to the experimentally induced elongation of axons in the absence of an active growth cone, implying that signals from the active growth cone regulate transport. To determine the nature of these signals, we have focally stimulated the shafts of sensory axons in culture with nerve growth factor (NGF) covalently conjugated to polystyrene beads. We find that mitochondria accumulate at regions of focal NGF stimulation. This response is specific to mitochondria and does not result from general disruption of the cytoskeleton in the region of stimulation. Disruption of the phosphoinositide 3-kinase (PI 3-kinase) pathway, one of the signaling pathways downstream from NGF–receptor binding, completely eliminates NGF effects on mitochondrial behavior in axons. We propose that mitochondrial transport and/or docking are regulated in part *via* NGF/TrkA/PI 3-kinase signaling.

Key words: mitochondrial movement, sensory axon, nerve growth factor, NGF, phosphoinositide 3-kinase, neuronal homeostasis, growth cone, signaling.

Introduction

The structural and functional asymmetry of the neuron is determined and maintained by the specific positioning of its organelles. As a consequence, in highly polarized axonal processes, the variety, volume and destination of organelle traffic must be tightly regulated. Because the different classes of organelles transported within vertebrate axons behave differently (Overly et al., 1996; Ligon and Steward, 2000), the signals that control organelle traffic are likely to be diverse. Mitochondria, in particular, have attracted our attention because of their unique metabolic functions and their unique pattern of motility. Their roles in aerobic ATP production, Ca^{2+} homeostasis and the production of reactive oxygen species make it clear that the correct localization of mitochondria is essential for the life of the neuron. But the manner in which their distribution is achieved differs from that of other organelles: mitochondria undergo movement in both directions within axons and spend a large but variable part of their time stationary (Hollenbeck, 1996). In the axons of embryonic peripheral neurons in culture, mitochondria undergo net anterograde movement and then halt in the region of an active growth cone but move retrogradely and retreat from the distal axon when growth cone activity ceases (Morris and Hollenbeck, 1993). This modulation of mitochondrial motility in concert with axonal growth occurs *via* two mechanisms: (1) the up- and downregulation of anterograde motor activity and (2) the recruitment of mitochondria between persistently motile and stationary states (Morris and Hollenbeck, 1993). It is possible that similar events occur in adult animals to modulate mitochondrial distribution in response to synaptic activity

(Wong-Riley and Welt, 1980; Kageyama and Wong-Riley, 1982; Bindokas et al., 1998).

But what are the specific molecular signals that control mitochondrial motility in the axon? Since the activity of the growth cone has a pronounced influence on the behavior of mitochondria (Morris and Hollenbeck, 1993), we have focused on signals that affect axonal outgrowth, particularly neurotrophins. Neurotrophins are trophic factors that act *via* the Trk family of receptor tyrosine kinases and several downstream intracellular signaling pathways to support the growth, survival, differentiation and maintenance of neurons (Huang and Reichardt, 2001; Lewin and Barde, 1996). One member of the neurotrophin family, nerve growth factor (NGF), supports the survival of sympathetic and neural crest-derived sensory neurons such as those in which we have analyzed mitochondrial transport (Otten et al., 1980; Davies, 1994; Kaplan and Stevens, 1994; Farinas, 1999; Verge et al., 1989). But in addition to supporting survival and differentiation, NGF specifically promotes axonal outgrowth and collateral branch formation (Yasuda et al., 1990; Diamond et al., 1992; Gallo and Letourneau, 1998), at least in part, because it can promote growth cone motility (Campenot, 1994) and serve as a guidance cue for the active growth cone (Letourneau, 1978; Gundersen and Barrett, 1979; Gallo et al., 1997; Paves and Saarma, 1997).

Because it can induce local changes in axons over a relatively short time scale, NGF is an attractive candidate for regulating the movement and distribution of axonal mitochondria. We have tested this hypothesis in chick embryonic sensory neurons in culture by exposing undistinguished regions of the axon focally to NGF, thus separating this one signaling pathway from the complex events of the growth cone. We report here that focal NGF stimulation causes a local accumulation of mitochondria, but not other organelles, in axons. The accumulation of mitochondria is not due to local disruption of the cytoskeleton by NGF. Furthermore, NGF-mediated regulation of mitochondrial transport is completely abolished by inhibiting the phosphoinositide 3-kinase (PI 3-kinase) pathway, indicating that the regulation of mitochondrial transport by NGF requires activation of PI 3-kinase.

Materials and methods

Materials

Rhodamine 123 (R123), Alexa Fluor 568–phalloidin and Alexa 488-conjugated goat anti-mouse IgG were obtained from Molecular Probes, Inc. (Eugene, OR, USA). DM1A antibody was obtained from Amersham (Arlington Heights, IL, USA). LY294002 was obtained from Calbiochem (La Jolla, CA, USA), prepared as a stock in dimethylsulfoxide (DMSO), and stored at –20°C.

Neuronal cell culture

Dorsal root ganglia (DRG) were dissected from E9-E11 chicken embryos, dissociated into individual neurons and grown at low density in 90 ng ml^{-1} nerve growth factor (NGF; Alomone Labs Ltd, Jerusalem, Israel) in F-12H medium (Life Technologies, Grand Island, NY, USA) on glass cover slips as previously described (Hollenbeck and Bray, 1987). Cover slips were treated with 50 μg ml^{-1} fibronectin (Life Technologies) overnight at 4°C before use. Prior to bead experiments, neurons were changed to F-12H medium containing a reduced concentration of NGF (0.05 ng ml^{-1}), as described by Gallo et al. (1997), and were maintained at this low NGF concentration throughout the experiments, up to a maximum of 5 h exposure.

Preparation of NGF-coated beads

NGF-coated beads were prepared using the carbodiimide method (Polysciences, Warrington, PA, USA) for covalently coupling proteins to 10 μm-diameter polystyrene carboxylate beads (Gallo et al., 1997). NGF for coupling was obtained from R & D Systems (Minneapolis, MN, USA). The prepared NGF-coated beads were stored at 4°C for up to five weeks. Cytochrome *c*-coated beads were prepared using identical conditions.

Image acquisition and quantitative analysis of mitochondrial distribution

To fluorescently label mitochondria in live neurons, 0.5–1.0 μg ml^{-1} R123 was added to culture medium for 20 min before adding the NGF-coated beads and was washed out before observation. Under our culture conditions, this concentration of R123 is at or near saturation and results in relatively uniform fluorescence intensity from all mitochondria, making it an equivalent but more easily quantified measure of mitochondrial location than linear or area projections of mitochondrion images. Cultures were secured into sealed chambers 25 mm in diameter and 1 mm thick between two cover slips and placed on a Nikon inverted microscope equipped with a 37°C air curtain stage heater for observation. R123-labeled mitochondria were viewed by epifluorescent illumination with a Nikon 60× objective (NA 1.4) and images were acquired using a Hamamatsu cooled CCD camera and MetaMorph imaging software (Universal Imaging, West Chester, PA, USA). Addition of NGF-coated beads to cultures was performed as previously described (Gallo et al., 1997) with some modifications. To determine the mitochondrial response to NGF-coated beads, we applied three selection criteria: (1) only unbranched sufficiently long axons (>300 μm) were considered for data collection; (2) only beads contacting the axon that did not move relative to the substratum during the observation period were considered. Stable NGF-coated bead–axon contact was characterized by the transient presence of one or more filopodia at the site of bead–axon contact, and beads that never induced any filopodia were not considered; (3) only neurons that had a single bead contacting the axon at least 100 μm away from both the growth cone and cell body were considered. Images were gathered 1 h after bead addition, which is sufficient time to detect large shifts in mitochondrial

distribution in these axons (Morris and Hollenbeck, 1993). Images were collected and morphometry was performed using MetaMorph image acquisition and analysis software. Densities of mitochondria based on the mitochondrial R123 fluorescence intensities were quantified in three discrete regions: a 10 μm region of the axon adjacent to an NGF-coated bead and the 10 μm regions located 100 μm in either direction along the axon from the bead. This distance from the site of the bead was chosen as 100 μm since previous results have demonstrated that the growth cone did not have a significant effect on mitochondrial density at ≥100 μm from the base of the growth cone (Morris and Hollenbeck, 1993). Quantification of total mitochondrial fluorescence intensity was facilitated by applying a fluorescence threshold binary mask to each image, to distinguish objectively between the objects being measured and other parts of the image.

Statistical analysis

Quantified mitochondrial fluorescence intensities were analyzed statistically by paired sample *t*-test (or non-parametric equivalents, as dictated by the normality of the distribution) to determine whether mitochondrial densities were significantly different between the region of bead–axon contact and control regions. In all cases, $t > t_{0.05,\,(2),\,n-1}$ was the criterion for rejection of H_o.

Immunocytochemistry

Neurons were fixed with 4% paraformaldehyde plus 0.05% glutaraldehyde in warm phosphate-buffered saline (PBS) for 20 min at room temperature, then blocked in 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature or overnight at 4°C. For double-labeling of microfilaments (MFs) and microtubules (MTs), Alexa 568-conjugated phalloidin (1 U μl^{-1}) and DM1A anti-tubulin primary antibody (1:15 000) were applied simultaneously for 1 h at room temperature. Neurons were then washed with PBS, incubated with Alexa 488-conjugated goat anti-mouse IgG (1:3000 dilution) for 1 h at room temperature, washed with PBS and mounted in Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) for analysis. A Nikon inverted microscope equipped with epifluorescence illumination was used to examine the samples. Images were obtained with a Hamamatsu cooled CCD camera and with MetaMorph software.

Video-enhanced phase-contrast microscopy and motility analysis

The movements of vesicular organelles in axons were analyzed by phase-contrast microscopy as previously described (Hollenbeck, 1993). Organelle movement was observed in the axon region that was in contact with an NGF-coated bead. We noted all detectable anterogradely and retrogradely moving organelles that passed through the region of axon–bead contact and scored whether each organelle passed through the region of the bead without stopping, halted in the region or changed direction.

Results

Mitochondria accumulate at sites of NGF stimulation

To determine whether local NGF stimulation had any effect on the transport and positioning of mitochondria, we applied NGF-coated beads to neuronal cultures in which mitochondria were stained with R123 (Johnson et al., 1981), immediately identified appropriate axons that were in contact with beads and returned to these axons after 1 h. At that time, we compared the density of mitochondria in the axon adjacent to NGF-coated beads with similar non-bead control regions 100 μm along the axon in either direction from the point of bead contact (see Materials and methods). Mitochondria clearly accumulated in regions of the axon in contact with an NGF-coated bead but not in a region of the axon in contact with an NGF-coated bead that had been subjected to mild heat denaturation prior to application (Fig. 1) or with a cytochrome *c*-coated bead (not shown). In addition, the mitochondrial response to NGF-coated beads was prevented by adding to the culture medium soluble NGF at concentrations much higher than the 0.05 ng ml^{-1} background level: either 90 ng ml^{-1}, which saturates both the high-affinity TrkA and the p75 receptor for NGF, or 10 ng ml^{-1}, which saturates only the TrkA receptor (Gallo et al., 1997; Fig. 2).

Quantification of the fluorescence signals confirmed that mitochondria accumulated preferentially in the region of NGF-coated bead contact; there was a significantly higher density of mitochondria in the 10 μm region of the axon adjacent to an NGF-coated bead (Fig. 2; Table 1) than in a 10 μm region located 100 μm away in either direction from the point of bead contact, reaching 2–3-fold higher density in the region of NGF-coated bead contact within 1 h (Fig. 2; Table 1). Significant but less dramatic accumulation of mitochondria in the region of bead contact occurred in as little as 15 min, and the

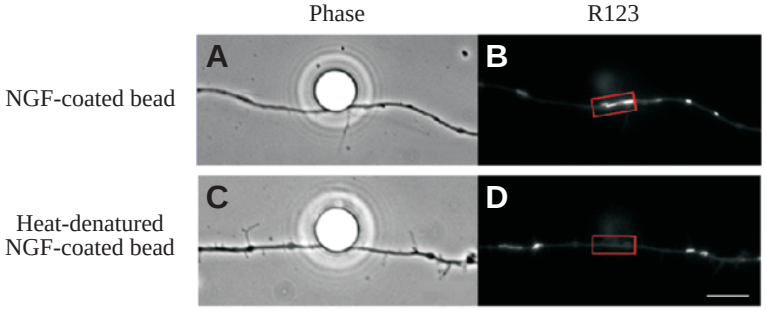

Fig. 1. Mitochondria accumulate at sites of nerve growth factor (NGF) stimulation. Phase-contrast micrographs (A,C) and epifluorescent images of mitochondria stained with rhodamine 123 (R123) in living neurons (B,D) were used to compare the distribution of mitochondria in the axon adjacent to an NGF-coated bead (A,B) and a heat-denatured NGF-coated bead (C,D). Mitochondria accumulate at sites of bead–axon contact within 1 h. No mitochondrial accumulation is observed using the control heat-denatured NGF-coated beads. Scale bar, 10 μm.

Table 1. *Comparison of total mitochondrial fluorescent intensities in different 10 μm regions of the axon by paired* t*-tests (two-tailed)*

	P value						
Regions compared	NGF-coated bead (*N*=10)	Control (heat-denatured NGF-coated bead; *N*=8)	90 ng ml^{-1} NGF (*N*=9)	10 ng ml^{-1} NGF (*N*=8)	DMSO (0.2%; *N*=9)	LY294002 (10 μmol l^{-1}; *N*=10)	LY294002 (100 μmol l^{-1}; *N*=12)
Bead *vs* proximal	0.002*****	0.994	0.563	0.102	0.001*****	0.992	0.331
Bead *vs* distal	0.007*****	0.305	0.117	0.589	<0.001*****	0.217	0.262
Proximal *vs* distal	0.361	0.293	0.474	0.233	0.239	0.295	0.954

This comparison indicates that the nerve growth factor (NGF)-coated bead, but not the heat-denatured control bead, was associated with a significantly greater density of mitochondria compared with the proximal and distal non-bead regions of the axon. Furthermore, the NGF-coated bead under control dimethylsulfoxide (DMSO) conditions, but not in the presence of the phosphoinositide 3-kinase (PI 3-kinase) inhibitor LY294002 at 100 μmol l^{-1} and 10 μmol l^{-1}, was associated with a significantly greater density of mitochondria compared with the proximal and distal non-bead regions.

Asterisks represent statistical significance.

accumulation continued to progress for at least 2 h. When NGF-coated beads were denatured prior to application or applied in the presence of saturating concentrations of soluble NGF, contact with the axons did not produce any difference in the density of mitochondria at the site of bead contact compared with other regions along the axon (Fig. 2; Table 1). These results indicate that NGF signaling *via* the TrkA receptor locally regulates mitochondrial transport and distribution in neurons.

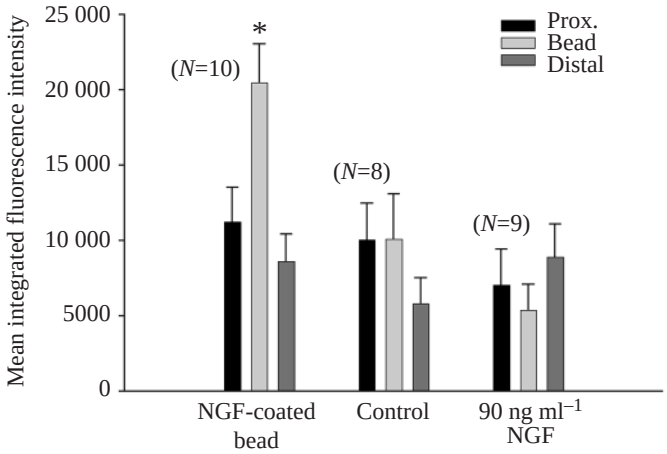

Fig. 2. Local nerve growth factor (NGF) activation regulates mitochondrial transport and distribution in neurons. Histograms of mitochondrial rhodamine 123 (R123) fluorescence intensities in the 10 μm region immediately adjacent to an NGF-coated bead *vs* the 10 μm control regions located 100 μm away in either direction along the axon from the point of bead contact. The mitochondrial fluorescence intensity is significantly higher (shown by the asterisk) in the 10 μm region of the axon adjacent to an NGF-coated bead than in similar proximal and distal non-bead control regions (*N*=10; paired *t*-test). No difference in mitochondrial densities was observed when using the control heat-denatured NGF-coated beads (*N*=8; paired *t*-test) or with a high background concentration of NGF. Adding 10 ng ml^{-1} NGF to the culture medium, which is above the K_d of the high affinity TrkA receptor (approximately 1 ng ml^{-1}) but below the K_d of the lower affinity p75 receptor (approximately 40 ng ml^{-1}), significantly reduced the mitochondrial response to NGF-coated beads (*N*=8; paired *t*-test). The mitochondrial accumulation was also blocked in the presence of 90 ng ml^{-1} NGF (*N*=9; paired *t*-test).

Effect of NGF signaling on axonal organelle traffic does not involve disruption of the cytoskeleton and is specific to mitochondria

Although we found that local NGF stimulation regulates the transport of mitochondria in the axon, this effect could be relatively non-specific. Neurotrophin signaling has been implicated in regulating neuronal morphology and cytoskeletal dynamics (Gallo and Letourneau, 2000), so it was possible that the accumulation of mitochondria reflected local gross disruption of the cytoskeletal tracks for mitochondrial transport – for instance, an accumulation of actin filaments or depolymerization of MTs in the region of NGF stimulation. We assessed the state of the cytoskeleton in axons interacting with NGF-coated beads using fluorescence detection of MTs and actin filaments. We detected no difference in the appearance of the MT or F-actin arrays in regions of the axon contacting an NGF-coated bead for 1 h, as compared with regions along the axon not in contact with NGF-coated beads (Fig. 3). MT staining showed the usual solid, bright signal throughout the axons, while F-actin staining showed the typical flocculent signal (Morris and Hollenbeck, 1995). These results indicate that the effects of NGF signaling on the axonal transport and distribution of mitochondria are not due to the local disruption of the cytoskeletal tracks for movement.

It remained possible that the effect on organelle traffic was more general and that other organelles were also accumulating in the region of bead–axon contact. We examined the transport of other organelles visible in these axons using phase-contrast microscopy; these include endosomes, autophagic vacuoles

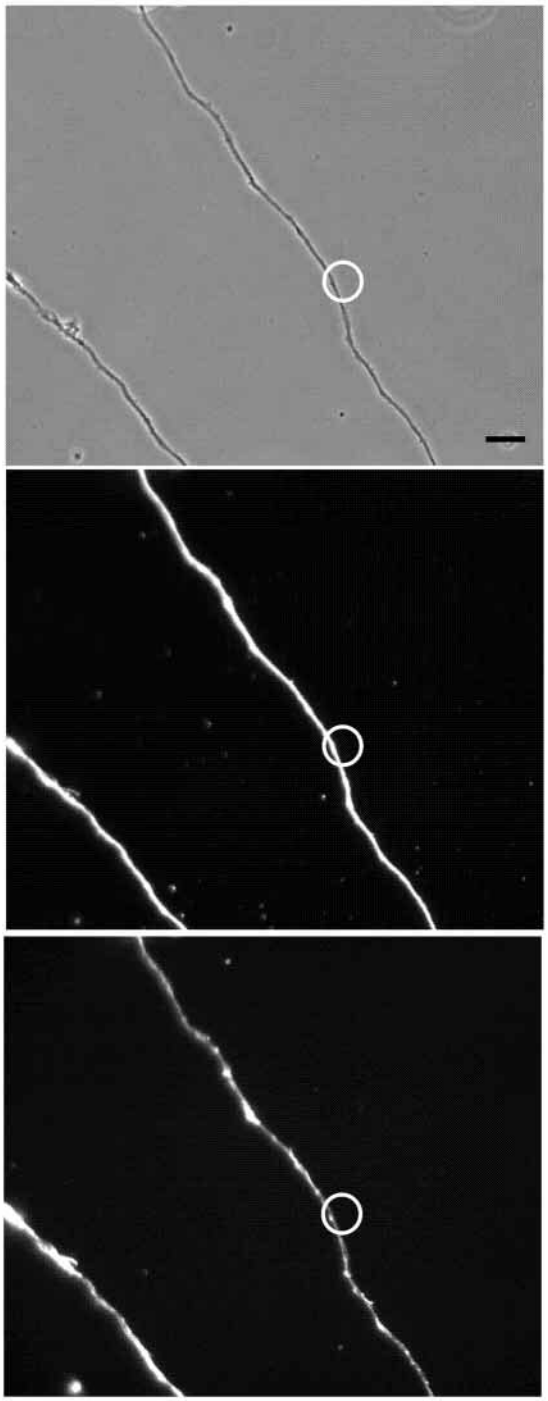

Fig. 3. Nerve growth factor (NGF)-coated beads do not affect the cytoskeleton. Primary cultured DRG neurons were double immunostained for microtubules (MTs) and F-actin (MFs). In all panels, the positions of the NGF-coated beads prior to fixation are indicated by circles. The regions of the axons in contact with NGF-coated beads have MT arrays and MF density essentially identical to control regions of the axon not contacting a bead. Scale bar, 10 μm.

and anterogradely transported vesicles, all of which are easily discerned from mitochondria (Overly et al., 1996). We observed 53 organelles moving in either the anterograde or retrograde direction past 83 NGF-coated bead contact sites and recorded whether these non-mitochondrial organelles stopped adjacent to a bead. With the exception of a single organelle that reversed direction, none of the non-mitochondrial organelles that we observed halted in the region of an NGF-coated bead (Table 2), indicating that the effect on organelle transport is specific to mitochondria.

Table 2. *Organelles other than mitochondria do not halt in the axon adjacent to an NGF-coated bead*

Direction of movement	Number of organelles	Number of bead encounters	Number of stops
Anterograde	5	7	1
Retrograde	48	76	0

Individual movements of large phase-dense vesicular organelles were tracked by video-enhanced phase-contrast light microscopy and pooled for all neurons as moving either in the anterograde or retrograde direction past a nerve growth factor (NGF)-coated bead, and the total number of stops near a bead was counted. Organelles other than mitochondria do not halt in the region of an NGF-coated bead. Data are derived from analysis of 83 bead encounters by 53 organelles in neurons. A single anterogradely moving organelle stopped near an NGF-coated bead and resumed movement in the retrograde direction.

Inhibition of PI 3-kinase eliminates NGF-mediated regulation of mitochondrial transport

We sought to determine which signaling pathway(s) downstream of the NGF–receptor interaction was responsible for regulating the axonal transport of mitochondria. Since the PI 3-kinase pathway has been shown previously to be involved in relatively short-term responses to NGF (Kimura et al., 1994; Gallo and Letourneau, 1998), we examined the effects of inhibiting this pathway on the response of axonal mitochondria to NGF-coated bead contact. The neuronal cultures were treated with LY294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one], a cell-permeant, specific, competitive inhibitor of PI 3-kinases (Vlahos et al., 1994; Walker et al., 2000). LY294002 abolishes PI 3-kinase activity *in vitro* and *in vivo* at low micromolar concentrations but has no inhibitory effect on a range of other protein kinases, including PI 4-kinase, mitogen-activated protein kinase (MAPK), protein kinase C (PKC), epidermal growth factor (EGF) receptor kinase, cAMP-dependent protein kinase and c-Src (Vlahos et al., 1994; Cheatham et al., 1994; Walker et al., 2000). LY294002 inhibited the mitochondrial response to NGF stimulation: treatment of neurons with 10 μmol l^{-1} or 100 μmol l^{-1} LY294002 completely eliminated the ability of NGF-coated beads to induce an accumulation of mitochondria in the region of bead–axon contact (Fig. 4; Table 1). In neurons treated with the drug vehicle alone (0.2% DMSO), NGF-coated beads induced a threefold increase in mitochondrial density in the region of bead–axon contact relative to control regions on the same axons (Fig. 4; Table 1). Hence, our data indicate that

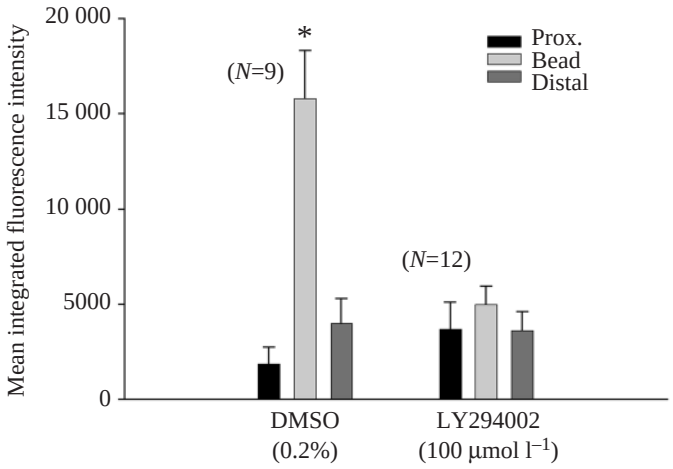

Fig. 4. LY294002 inhibits mitochondrial accumulation at sites of nerve growth factor (NGF) stimulation. LY294002 treatment prevented the accumulation of mitochondria in the region of NGF stimulation (N=12; paired t-test). Dimethylsulfoxide (DMSO; vehicle) alone did not affect the mitochondrial response to NGF stimulation. The density of mitochondria is 3-fold higher in the 10 μm region of the axon adjacent to the NGF-coated bead than in similar proximal and distal non-bead control regions (N=9; paired t-test). * represents statistical significance at $P \leqslant 0.001$.

transport and distribution of mitochondria in axons respond specifically to NGF binding to the cell surface *via* the PI 3-kinase pathway.

Discussion

Because mitochondria play critical roles in aerobic ATP production, calcium homeostasis and apoptosis, it is no surprise that their transport and distribution are tightly regulated in cells that are as large and functionally asymmetric as neurons. Although the molecular mechanism of regulation has not been demonstrated, the behavior of mitochondria in growing neurons shows that both their balance between motile and persistently stationary states and their anterograde motor activity are modulated by the physiological state of the axon and their proximity to the growth cone (Morris and Hollenbeck, 1993). Both of these features of mitochondrial movement are possible targets of regulation by intracellular signals. Based on the results presented here, we propose that signaling events initiated by NGF–receptor binding and mediated by the PI 3-kinase pathway operate to concentrate mitochondria in specific domains in neurons.

In this study, we used a neuronal cell culture system to investigate directly the intracellular signaling mechanisms that regulate mitochondrial transport. We were able to spatially isolate NGF signaling by stimulating axons with NGF-coated beads at points distant from their cell bodies or growth cones. It was clear that the accumulation of mitochondria in the region of bead–axon contact resulted specifically from the interaction of NGF with the axon: it did not occur when the NGF on the beads was gently denatured prior to application to the axons nor when it was replaced by cytochrome *c* nor when the NGF-coated bead application was carried out in the presence of a concentration of soluble NGF sufficient to saturate the TrkA receptors (Fig. 2). Furthermore, the NGF-dependent accumulation of mitochondria was not caused by a local disruption of the cytoskeleton, since neither microtubules nor actin filaments showed any discernible differences between bead regions and the rest of the axon (Fig. 3). In addition, other organelles moved through regions of the axon in contact with NGF-coated beads without delay, indicating that the cytoskeletal tracks were intact and could support organelle movement and also demonstrating that the regulatory effect was specific to mitochondria (Table 2). Previous studies have shown that prolonged contact of NGF-coated beads with axons of peripheral neurons will cause cytoskeletal changes leading to collateral branch formation (Gallo and Letourneau, 1998) but these changes occur only after treatment for several hours longer than in our experiments.

In addition to such short-term changes, NGF stimulation of peripheral neurons also causes a well-characterized set of long-term changes, including changes in gene expression and protein deployment that support neuronal differentiation, survival and maintenance (Sofroniew et al., 2001). These effects are thought to be mediated by at least three major signaling pathways that are activated by NGF–TrkA receptor binding: the phospholipase C-γ1 (PLC-γ1) pathway, the Ras/MAPK pathway and the PI 3-kinase pathway (Patapoutian and Reichardt, 2001). Signaling by PI 3-kinase is essential for survival and outgrowth of many populations of neurons by NGF (Kimura et al., 1994; Brunet et al., 2001). Several lines of evidence have also identified a requirement of PI 3-kinase in membrane vesicular trafficking (Schu et al., 1993; Yano et al., 1993; Kuruvilla et al., 2000). We found that the inhibition of the PI 3-kinase pathway alone was sufficient to eliminate completely the local regulation of mitochondrial transport by NGF.

Many NGF-stimulated effects on neurons are reported to require the internalization and retrograde transport of the receptor–ligand complex (Heerssen and Segal, 2002). However, at least some changes in function at the cell body (MacInnis and Campenot, 2002) as well as local events in the axon (Gallo and Letourneau, 1998) can occur in the absence of NGF–receptor internalization. The latter was the case in our studies, since NGF was immobilized on beads that could not be internalized by the axons. Although even signaling from soluble, freely internalized NGF shows a spatial gradient (Toma et al., 1997), it seems likely that the absence of both internalization and lateral diffusion of NGF-bound receptors in our experiments further limits the range of the intracellular signal generated by NGF. This could explain the spatial limitations of the regulation of mitochondrial transport that we observed: 100 μm away from the site of NGF stimulation, the mitochondrial density of the axon was unchanged. We previously reported a comparable limitation of the effects on mitochondrial transport of proximity to the growth cone (Morris and Hollenbeck, 1993). This may be significant *in*

vivo, since specific target mechanisms must exist by which neurons regulate the transport and delivery of mitochondria to their proper locations.

The specific regulation of mitochondrial transport by NGF stimulation and PI 3-kinase signaling suggests that mitochondria are a target for kinase or phosphatase activity. Phosphorylation and dephosphorylation are thought to regulate the activity and/or cargo binding of many organelle motor proteins (Reilein et al., 2001), so a reasonable possibility is that the PI 3-kinase pathway directly affects the activity of the mitochondrial motor proteins or their receptors on the organelle surface. Mitochondria offer many potential substrates, since their axonal transport is complex, involving bidirectional movement (Morris and Hollenbeck, 1993; Hollenbeck, 1996) and interaction with both microtubules and actin filaments (Morris and Hollenbeck, 1995). However, another example of regulated bidirectional organelle movement – pigment granule transport in melanosomes – not only shares these motility properties but also, like axonal mitochondrial transport, responds to stimulation by uninternalized extracellular ligands. In pigment cells, organelle transport is regulated globally by specific phosphorylation and dephosphorylation events that probably act both directly and indirectly on motor proteins (Tuma and Gelfand, 1999). The local effects of NGF stimulation on mitochondrial motility in neurons could result from a series of signal transduction events that are similar to those in melanophores but constrained to a portion of the axon by diffusion limits or by a different organization of signal transduction molecules.

More than one aspect of mitochondrial motility could be modulated to give the results that we have seen here. For example, when mitochondrial transport responds to growth cone activity, both the amount of anterograde motor activity and the balance between stationary and motile mitochondria change (Morris and Hollenbeck, 1993; Hollenbeck, 1996). Accumulation of mitochondria in a region of NGF stimulation could result from the stimulation of mitochondrial motor activity in nearby regions, inhibition of motor activity in the immediate region, or both. For instance, recent work has shown that phosphorylation of kinesin light chains *in vitro* and *in vivo* by glycogen synthase kinase 3 reduces the binding of kinesin to membrane-bounded organelles and inhibits anterograde axonal transport (Morfini et al., 2002). If such an effect on anterograde mitochondrial motor protein activity occurred *via* the PI 3-kinase pathway in the region of NGF stimulation, it would result in an accumulation of mitochondria as they moved anterogradely into the region.

Accumulation of mitochondria in a region of NGF stimulation could also be explained without resort to the direct regulation of motor proteins. Axonal mitochondria spend long periods persistently stationary (Morris and Hollenbeck, 1993, 1995; Hollenbeck, 1996), suggesting that they have static docking interactions with the cytoskeleton. Although proteins that could serve this function for mitochondria have not been clearly identified, numerous ultrastructural studies show the presence of cross-links between mitochondria and the axonal cytoskeleton (Smith et al., 1977; Hirokawa, 1982; Schnapp and Reese, 1982; Benshalom and Reese, 1985), and it is not known what fraction of these represents transient interactions by motor proteins *vs* more static interactions by putative docking proteins. In addition, studies of organelle–cytoskeleton interactions suggest a possible role for microtubule-associated proteins cross-linking mitochondria and microtubules (Linden et al., 1989; Jung et al., 1993; Leterrier et al., 1994). If regulated by NGF and PI 3-kinase signaling, such interactions could control the distribution of axonal mitochondria instead of, or together with, the regulation of mitochondrial motor protein activity.

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