

Nerve Growth Factor Signaling Regulates Motility and Docking of Axonal Mitochondria

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Summary

Axonal transport is thought to distribute mitochondria to regions of the neuron where their functions are required [1]. In cultured neurons, mitochondrial transport responds to growth cone activity, and this involves both a transition between motile and stationary states of mitochondria and modulation of their anterograde transport activity [2]. Although the exact cellular signals responsible for this regulation remain unknown, we recently showed that mitochondria accumulate in sensory neurons at regions of focal stimulation with NGF and suggested that this involves downstream kinase signaling [3]. Here, we demonstrate that NGF regulation of axonal organelle transport is specific to mitochondria. Quantitative analyses of motility show that the accumulation of axonal mitochondria near a focus of NGF stimulation is due to increased movement into bead regions followed by inhibition of movement out of these regions and that anterograde and retrograde movement are differentially affected. In axons made devoid of F-actin by latrunculin B treatment, bidirectional transport of mitochondria continues, but they can no longer accumulate in the region of NGF stimulation. These results indicate that intracellular signaling can specifically regulate mitochondrial transport in neurons, and they suggest that axonal mitochondria can respond to signals by locally altering their transport behavior and by undergoing docking interactions with the actin cytoskeleton.

Results and Discussion

In the long axonal processes of nerve cells, mitochondria are thought to be transported to regions where their functions are critical for cell survival [1]. In particular, they have been shown to accumulate in regions of intense ATP consumption, including motile growth cones [2], synapses [4, 5, 6], nodes of Ranvier [7], and myelination boundaries [8]. Because we have previously observed the pronounced influence of axonal growth on the behavior of mitochondria [2], in this study we have focused on signaling pathways that affect axonal outgrowth—particularly growth factor signaling. We have previously shown when the axon of a cultured chick sensory neuron is in contact with a nerve growth factor (NGF)-coupled bead, mitochondria accumulate in the immediate vicinity, indicating that NGF signaling can regulate the distribution of mitochondria within the axon

[3]. Although NGF can bind to both the high-affinity TrkA receptor and the pan-neurotrophin receptor p75 [9, 10], the ability of focal NGF stimulation to redistribute axonal mitochondria is eliminated by a low soluble (NGF) [3] or inhibition of receptor tyrosine kinase activity (Figure S1 available in the Supplemental Data available with this article online), indicating that the effect is mediated by the TrkA receptor. In addition, pharmacological evidence suggests that downstream of NGF-TrkA binding, the effect on mitochondria requires the activity of the PI3 kinase pathway, probably along with the MAP kinase pathway (Figure S1).

Accumulation in the immediate region of focal NGF stimulation could be specific to mitochondria, or it could reflect a more general effect on axonal organelle transport. To assess this, we labeled two other major classes of organelles with fluorescent vital dyes and quantified their distribution in live neurons in contact with NGF beads or control beads (Figure 1). Although mitochondria accumulated in the region of the axon adjacent to an NGF bead (Figures 1A–1D), neither organelles of the endocytic-lysosomal pathway stained with acridine orange (Figures 1E and 1F) nor anterograde-transported vesicles stained with DiO (Figures 1G and 1H) accumulated there. This is consistent with live-phase contrast observations that other axonal organelles moved continuously through a region of axon-NGF bead contact without stopping [3]. Thus, the traffic of organelles of the biosynthetic and degradative pathways is not discernibly affected by focal NGF stimulation.

The observed effect of NGF on the axonal distribution of mitochondria could result from at least two kinds of regulation of their movement. Mitochondria could accumulate in the region of an NGF bead as a result of the stimulation of their motor activity in nearby regions causing an increase in mitochondrial entry into the region of NGF stimulation, or they could accumulate as a result of inhibition of their motility in the immediate region of the bead, or both effects could occur. To differentiate among these possibilities, we followed and quantified mitochondrial movements in a 100 μ m-long region of the axon centered on a point of axon-bead contact and then compared the data for NGF beads versus heat-denatured control beads. Figure 2 shows representative plots of position versus time for individual mitochondria as they entered a region of an axon in contact with a bead. It was clear that mitochondria that enter the region of an NGF bead tend to remain there (Figure 2A), whereas those entering the region of a control bead tend to pass through (Figure 2B). Analysis of these data showed that the percent of mitochondria showing movement during a 100 s observation window decreased as they moved from the adjacent region (where 38% were moving) to the immediate region of the NGF bead (29% were moving). This suggested that mitochondria might be effectively captured in the immediate region of the NGF bead. Consistent with this possibility, when followed over the entire length of the observation period, mitochondria exited the region of an NGF-bead contact

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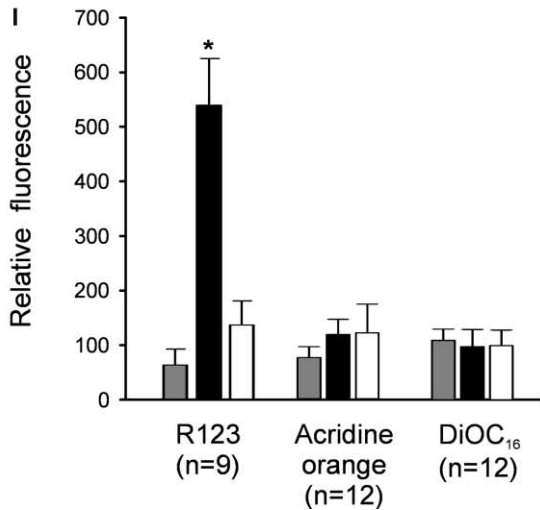
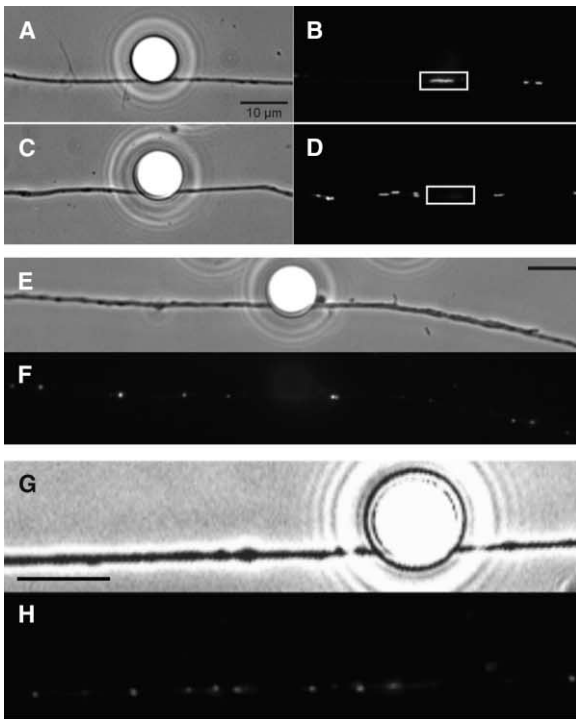


Figure 1. Unlike Mitochondria, Endosomes/Lysosomes and Vesicles Do Not Accumulate in Axons at Sites of Contact with NGF Beads

R123 fluorescence in live cells shows the accumulation of mitochondria near an NGF bead (A and B) but not near a heat-denatured NGF bead (C and D). AO staining of organelles of the endocytolysosomal pathway (E and F) and DiOC₁₆-staining of mainly anterograde-transported vesicles (G and H) shows that although both classes are numerous and distributed along the length of the axon, they do not accumulate in regions contacting an NGF bead. (I) Quantitative determination of density of three types of organelles in the axon adjacent to an NGF bead. The region near the bead is shown by the black bar, and the regions 100 μm away toward the cell body and toward the growth cone are shown as gray and white bars, respectively. Unlike R123-stained mitochondria, the densities of AO- and DiOC₁₆-positive organelles are not significantly different in the NGF-bead axon sites when compared to nonbead control regions of the axon. The scale bars represent 10 μm. Values for n

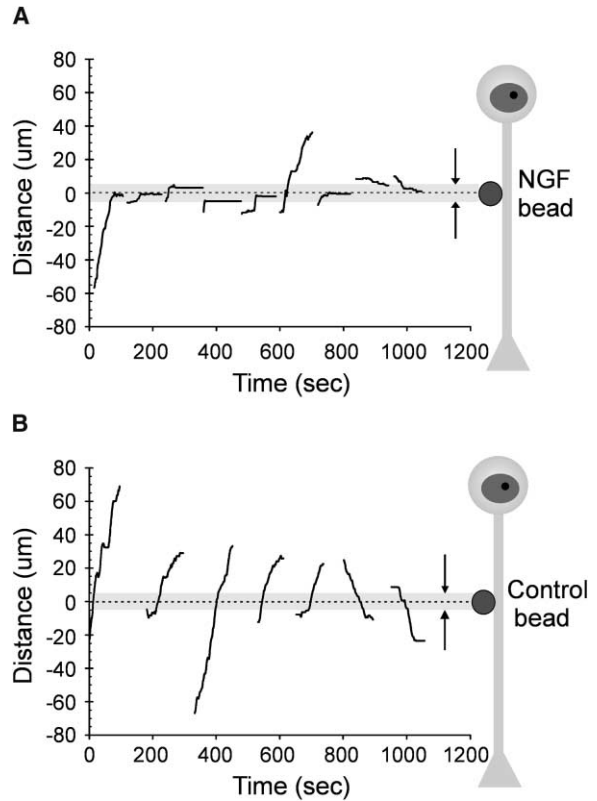


Figure 2. Mitochondrial Transport Behavior is Different Near NGF versus Control Beads

Displacement versus time for individual mitochondria in neurons treated with NGF beads (A) or control beads (B). Movement of mitochondria in axons is saltatory with frequent stops and starts and occasionally they reverse direction. Individual mitochondria halt more frequently when they enter axon regions contacting an NGF bead versus a control bead. Positive values indicate that the location of mitochondria is on the side of the bead toward the cell body, whereas negative values are designated to mitochondria located on the side of the bead toward the growth cone. Images of R123-labeled mitochondria were acquired every 2 s for 100 s by using a cooled CCD camera as described above. Mitochondria were categorized as “moving” if they traversed at least 5 μm in either direction along the axon during the observation period. The movement of individual mitochondria was tracked using Scion Image processing and analysis software, and the displacements of mitochondria were calculated in the region of axons adjacent to either an NGF bead or heat-denatured control bead. A repeated measures logistic regression test with a compound symmetric variance-covariance matrix and F test were used to compare the percent of mitochondria that exit from the region adjacent to an NGF bead versus a control bead.

less frequently than that of a control bead: 56% of mitochondria that entered a control-bead region exited within 100 s (n = 51), compared to only 21% for an NGF-bead region (n = 52), a significant difference (p = 0.004).

We then quantified how frequently mitochondria en-

indicate the number of axons examined. NGF was covalently coupled to 10 μm-diameter polystyrene carboxylate beads by using the carbodiimide method and stored up to 5 weeks at 4°C. Beads were applied to cultures of DRG neurons, and mitochondria were labeled and imaged as previously described [3].

Table 1. Numbers of Mitochondria Per Minute Entering and Leaving the 10 μm Bead-Axon Region in Control-Bead- and NGF-Bead-Treated Cells

Control Beads ^a		NGF Beads ^a	
entering 10 μm region	0.24 \pm .079	entering 10 μm region	0.92 \pm .137
anterograde	0.10 \pm .041	anterograde	0.32 \pm .091
retrograde	0.14 \pm .062	retrograde	0.60 \pm 0.16
exiting 10 μm region	0.58 \pm .12	exiting 10 μm region	0.39 \pm .16
anterograde	0.22 \pm .054	anterograde	0.035 \pm .035
retrograde	0.36 \pm .094	retrograde	0.35 \pm .14

Data are derived from tracking a total of 318 mitochondria in 30 neurons (control beads) or 146 mitochondria in 17 neurons (NGF beads). For illustrative purposes, the data are shown as mean rates \pm SEM. However, because of the small number of entries or exits in each sampling period, the data were more similar to counts than to continuously variable rates, thus they were analyzed using Poisson regression or Wilcoxon signed ranks tests. This revealed two significant differences in mitochondrial behavior: the rate of entry into an NGF-bead region was greater than entry onto a control bead region ($p = 0.001$), and the exit of mitochondria from an NGF-bead region was greater in the retrograde than in the anterograde direction ($p = 0.03$).

^aAll data are per min per axon.

tered and how frequently they exited the 10 μm region adjacent to a bead and in which direction they entered and exited the region (Table 1). NGF-bead-stimulated axons showed two further significant differences from controls in the patterns of mitochondrial motility near the bead. First, mitochondria entered the 10 μm region of an NGF-bead contact more frequently than that of a control bead (0.92 versus 0.24 mitochondria/min/axon, respectively; $p = 0.001$), and this held for mitochondria moving both anterogradely and retrogradely. Second, of the small population of mitochondria that succeeded in exiting the region of an NGF bead, the vast majority exited in the retrograde direction (0.35 mitochondria/min/axon) versus the anterograde direction (0.035 mitochondria/min/axon; $p = 0.03$). This indicates that the effects of signaling on motor protein activity are unequal for anterograde and retrograde motors, both in the immediate region of and at a short distance from the focal signal. Taken together, these data show that both motor activity and docking of axonal mitochondria are regulated to produce their accumulation in regions of NGF stimulation and that some of the effects on motor activity are selective for direction.

These data suggest a specific regulation of motor protein activity. However, the retention or docking of mitochondria in the immediate region of NGF stimulation might result from factors other than motor protein inhibition such as local steric obstruction of organelle movement by regulated changes in MAP conformation or abundance [11, 12] or such as the activation of docking proteins. We think the former possibility unlikely, because only mitochondria halt in this region (Figure 1). However, the accumulation of mitochondria could also be explained by an upregulation of specific, static docking interactions. Axonal mitochondria exhibit prolonged stationary phases [1], suggesting that they have static interactions with the cytoskeleton, and it seems likely that at least some of the crosslinks observed between mitochondria and the axonal cytoskeleton in ultrastructural studies represent static interactions by putative docking proteins. Some cytoskeletal proteins have been suggested to fill this function [13, 14], but little is known about relevant proteins in vivo [1]. Although mitochondria in chick peripheral neurons can undergo bidirectional movement along either MTs or F-actin alone [15,

16], persistent long-range movements are dependent on MTs and MT-based motor proteins [17], whereas F-actin-based transport has been proposed to serve mainly as a local distribution system [15, 18]. Instead, or in addition, we suggest that F-actin supports the more static cytoskeleton interactions that underlie the capacity of mitochondria to occupy relatively fixed positions in the cytoplasm.

To assess this hypothesis, we carried out the NGF-bead assay in axons that were first established for 24 hr in culture and then treated with latrunculin B to eliminate all F-actin while leaving the MT arrays intact [19]. In agreement with previous results using cytochalasin E [15], latrunculin-treated neurons lacked any detectable F-actin when stained with fluorescent phalloidin, while the MTs remained intact (data not shown). Mitochondria were motile and distributed throughout these axons, but they failed to accumulate in the region of the axon in contact with an NGF bead (Figure 3). These results indicate that an intact actin cytoskeleton is necessary for mitochondria to accumulate in response to focal NGF stimulation. We suggest that this is because mitochondria dock with the actin cytoskeleton, although it remains possible that F-actin-based movements over short distances are required to deliver mitochondria into a region where they can then be retained via an unknown mechanism, or that a subtle accumulation of F-actin in response to NGF impedes traffic in a mitochondrion-specific fashion. To examine whether MTs are also necessary for the mitochondrial docking, we treated established cultures with vinblastine to depolymerize MTs. Although bidirectional mitochondrial movement continued in axons without MTs [15], the axons contained varicosities that precluded accurate determination of mitochondrial densities. In addition, axonal mitochondria do not undergo long-range transport in the absence of MT tracks, making it very difficult to quantify their transport behavior in axons contacting NGF beads.

Motors, receptors, and docking proteins are all targets that could be regulated to produce the results we report here. But how might focal NGF stimulation, acting via the PI3 kinase and/or MAP kinase pathways, both increase mitochondrial movement into the region and inhibit mitochondrial escape from the region? Both pathways have been implicated in the control of organelle motility. MAP

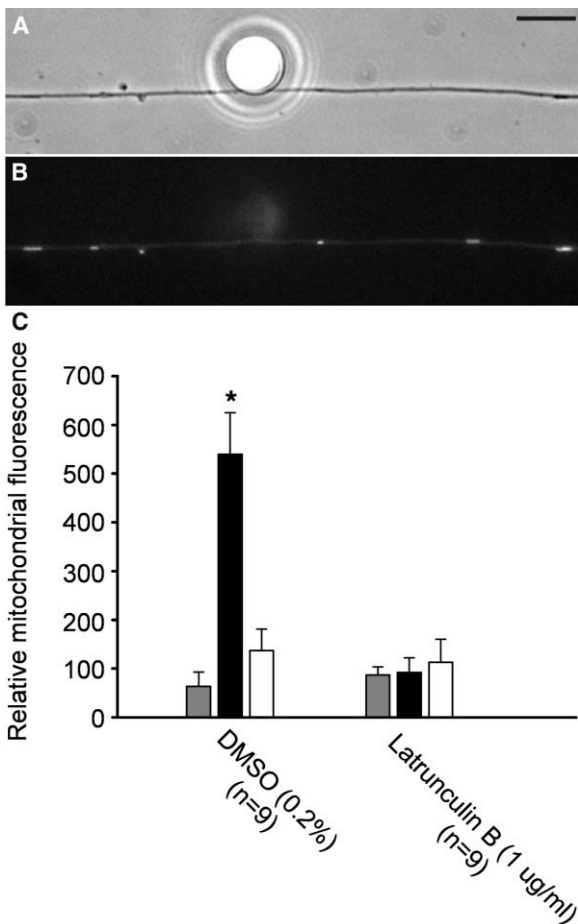


Figure 3. Accumulation of Mitochondria in the Region of NGF-bead Stimulation Does Not Occur in the Absence of F-actin

Phase (A) and R123 fluorescence (B) images of a live neuron treated with latrunculin B. No accumulation of mitochondria is observed in regions contacting an NGF bead. (C) Quantification of relative mitochondrial fluorescence shows that in latrunculin-treated neurons, the density of mitochondria is not significantly higher in the 10 μm region of the axon adjacent to an NGF bead (black bar) than in identically-sized nonbead control regions 100 μm away toward the cell body (gray bar) or growth cone (white bar) ($n = 9$; paired t tests; $p > 0.8$).

kinase signaling has been shown to result in hyperphosphorylation of the kinesin light chain and inhibition of mitochondrial movement [20], whereas increased binding of PI(4,5-P)2 by pleckstrin homology domains has been shown to increase anterograde transport of mitochondria without affecting other parameters [21]. However, glycogen synthase kinase 3, which can phosphorylate the kinesin light chain at site(s) that cause it to dissociate from organelle surfaces and thereby inhibit organelle traffic [22], can be inactivated downstream of PI-3 kinase [22, 23]. Thus, both motor activation and inactivation are potential results of stimulation of these two pathways, and different net effects at different distances from a focus of NGF stimulation might carry mitochondria into the region but inhibit their movement once they have arrived. In addition, downstream effectors of NGF signaling could regulate mitochondrial motility by

modifying the receptors for motor proteins [24, 25], and it is noteworthy that at least some receptors for motor proteins are themselves kinase-scaffolding proteins with an intimate connection to the cell signaling pathways [26]. It is also worth noting that a protein has been identified that is neither a motor protein nor a receptor but that nonetheless regulates the motility of bidirectionally-transported organelles [27]. Because, in addition to these, docking proteins for mitochondria remain to be identified, the range of possible targets of regulation on the mitochondrion for now remains broad.

Supplemental Data

Supplemental Data including Experimental Procedures and an additional figure are available at <http://www.current-biology.com/cgi/content/full/14/14/1272/DC1/>.

Acknowledgments

We thank Gordon Ruthel for helpful technical advice, Rebecca Hicks for technical assistance, and Jeffrey Lucas for help with statistical analyses. This work was supported by National Institutes of Health grant NS27073.

Received: December 12, 2003

Revised: May 24, 2004

Accepted: June 1, 2004

Published: July 27, 2004

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