Characteristics of intermittent mitochondrial transport in guinea pig enteric nerve fibers

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Vanden Berghe, Pieter, Grant W. Hennig, and Terence K. Smith. Characteristics of intermittent mitochondrial transport in guinea pig enteric nerve fibers, Am J Physiol Gastrointest Liver Physiol 286: G671–G682, 2004. First published October 30, 2003; 10.1152/ajpgi.00283.2003.—Enteric neurons controlling various gut functions are prone to oxidative insults that might damage mitochondria (e.g., intestinal inflammation). To resume local energy supply, mitochondria need to be transported. We used MitoTracker dyes and confocal microscopy to investigate basic characteristics of mitochondrial transport in guinea pig myenteric neurites. During a 10-s observation of 1 mm nerve fiber, on average, three mitochondria were transported at an average speed of 0.41 ± 0.02 μm/s. Movement patterns were clearly erratic, and velocities were independent of mitochondrial size. The velocity oscillated periodically (~6 s) but was not consistently affected by structures such as en route boutons, bifurcations, or stationary mitochondria. Also, mitochondria transported in opposite directions did not necessarily affect each others’ mobility. Transport was blocked by microtubule disruption (100 μM colchicine), and destabilization (1 μM cytochalasin-D) or stabilization (10 μM phalloidin) of actin filaments, respectively, decreased (0.22 ± 0.02 μm/s, P < 0.05) or increased (0.53 ± 0.02 μm/s, P < 0.05) transport speed. Transport was inhibited by TTX (1 μM), and removal of extracellular Ca2+ (plus 2 mM EGTA) had no effect. However, depletion of intracellular stores (thapsigargin) reduced (to 33%) and slowed the transport significantly (0.18 ± 0.02 μm/s, P < 0.05), suggesting an important role for stored Ca2+ in mitochondrial transport. Transport was also reduced (to 21%) by the mitochondrial uncoupler FCCP (1 μM) in a time-dependent fashion and slowed by oligomycin (10 μM). We conclude that mitochondrial transport is remarkably independent of structural nerve fiber properties. We also show that mitochondrial transport is TTX sensitive and speeds up by stabilizing actin and that functional Ca2+ stores are required for efficient transport.

myenteric neuron; axonal transport; intracellular calcium stores; MitoTracker

MITOCHONDRIA ARE VITAL ORGANELLES that generate the bulk of cellular energy by oxidative phosphorylation. Although the ATP produced is highly diffusible, diffusion can be the rate-limiting step in areas where ATP is consumed rapidly. Besides ATP production, mitochondria also play a crucial role in the regulation of intracellular Ca2+ concentration ([Ca2+]i) (7, 10, 34, 45), particularly in localized Ca2+ uptake and release (5, 35). To optimally perform these tasks, mitochondria need to be positioned precisely at those sites that require high amounts of energy or extensive Ca2+ buffering (33, 50). In highly polarized cells such as neurons, regions of high energy consumption (e.g., growth cones, synapses, etc.) can be located far away from the soma; therefore, the translocation of mitochondria along nerve fibers is crucial to maintain proper functioning.

Transport of mitochondria has been described in lobster, rat hippocampal, and chick sympathetic neurons (8, 16, 27, 28, 30, 32), and its regulation has been associated with the growth properties of nerve fibers (28).

Axonal transport is an ATP-dependent mechanism that involves at least three components: 1) a motor molecule, 2) a track, over which the motor molecule traffics, and 3) a cargo to transport. Two main groups of microtubule-associated motor molecules have been identified: the kinesins (generally microtubule plus-end-directed motors) and dyneins (minus-end-directed motors) (42). The kinesins consist of an extensive superfamily (Kif) of proteins (15, 40), of which some are specific for a certain cell type and others are specific for certain cargos in the cell. Many neurodegenerative diseases such as amyotrophic sclerosis, Huntington’s, Parkinson’s, Alzheimer’s, and Charcot-Marie-Tooth disease II have been linked to defects in either axonal transport in general or disabled mitochondrial function and transport (12, 22, 29).

Myenteric neurons, including intrinsic primary afferent, inter-, and motoneurons regulate different functions in the mammalian gastrointestinal tract. They are located in a ganglionated plexus between longitudinal and circular muscle layers of the intestine and convey information from sensory neurons via interneurons to motoneurons (23). Compared with the central nervous system, these neurons are organized as a relatively simple “two-dimensional” network. Myenteric neurons are well maintained in primary culture (48), can reform in networks reminiscent of the ganglionated plexus, and are ideally suited for optical recording methods. Due to their position in the gut wall, the mitochondria in these neurons are more prone to damaging insults, such as ischemia, anoxia, allergic reactions, or endotoxin exposure after intestinal infections. Re-population of the nerve fibers with healthy mitochondria is therefore crucial.

The aims of this study were 1) to visualize mitochondrial transport in myenteric neurons, 2) to study the basic properties, and 3) to analyze the mechanisms underlying this transport using confocal video microscopy combined with offline analysis tools.

MATERIALS AND METHODS

Myenteric Neuron Cultures

Guinea pigs (250–350 g) were euthanized by CO2 asphyxiation followed by exsanguination (a method approved by the Animal Ethics Committee at the University of Nevada, Reno). Primary neuron cultures were obtained from the small intestine (48) and were grown in dishes with coverglass bottoms. The culture medium consisted of...
M199 supplemented with 10% FBS, 28 mM glucose, 50 ng/ml nerve growth factor (NGF), 20 μg/ml streptomycin, and 20 U/ml penicillin. The cultures were kept in a 5% CO2 incubator (37°C), medium was changed every other day, and arabinose-C-furanoside (ARA-C; 10 μM) was added (days 5–7) to prevent proliferation of mitotic cells. All results are based on recordings from 36 culture dishes (from 8 animals) that were age matched (~10 days in vitro) and had a comparable cell density.

**Mitochondrial Stains**

MitoTracker green/red was used to visualize mitochondria. These dyes bind through their chloromethyl moiety to free sulphydryl groups in mitochondria (13). After incubation with MitoTracker green or red (50–100 nM, 30 min, 37°C), cultured neurons were washed twice with normal culture medium and left for at least 30 min (37°C) before imaging to permit further clearing of excessive dye.

**Rhod-2**

Cultured cells were exposed to 0.9 μM reduced (BH4) rhod-2 AM (1 h, 4°C), washed, and incubated in normal culture medium (37°C) for at least 8 h before the experiment. With this method, specific mitochondrial labeling was obtained, because rhod-2 is converted to its oxidized fluorescent form only in the actively respiring mitochondria.

Although MitoTracker facilitates identification of mitochondria and renders the study of mitochondrial transport more accurate (27), some putative caveats such as interference with mitochondrial metabolism, specificity, and phototoxicity need to be considered. Buckman et al. (3) addressed the metabolism issue by comparing mitochondrial respiration in the presence of several MitoTracker dyes. They found that micromolar concentrations of MitoTracker dye could interfere with respiration, whereas, especially for MitoTracker green, concentrations in the submicromolar range did not. They concluded that, especially in neurons, low concentrations of MitoTracker green can be used safely to assess mitochondrial size, location, and structure (3). The issues of specificity and phototoxicity are described in DISCUSSION. All data on travel distance and velocity were obtained from MitoTracker green stains, because the signal-to-noise ratio was superior compared with the rhod-2 signal.

**Experimental Setup**

All experiments were performed during constant perfusion of warm (35.5 ± 0.5°C) HEPES-buffered solution (pH 7.38) on a Nikon Diaphot microscope coupled to a Noran Odyssee confocal scanning unit (Middleton, WI). A ×60 (Nikon Plan APO) lens was used to record images (620 × 480 pixels, 53.4 × 41.3 μm²) either at 6 or 30 Hz. The 488- and 514-nm lines of a 50-mW Ar laser were used to excite MitoTracker green and MitoTracker red/rhod-2, respectively. We compared different MitoTracker loading protocols as well as laser intensities. An acceptable image quality without causing photodamage was obtained with laser powers of ~17% for 488-nm and ~25% for 514-nm excitation. The low laser power combined with the short pixel dwell time (inherent to the confocal scanning system) guaranteed negligible photodamage. Lower laser power did not reveal any improvement in cell condition but only resulted in lower image quality. We used relatively high sampling frequency to avoid confusion between individual mitochondria especially in fibers that transported mitochondria continuously. Moreover, some mitochondria moved quickly, and therefore, high sample rates were required to accurately assess speed and movement patterns.

**Analysis**

The spatiotemporal maps we developed are a compact and easy way to summarize long periods of movement and provide a comprehensive view on the position of mitochondria in myenteric nerve fibers. In combination with a time average of the recording, these maps hold all necessary information: parameters such as speed, travel distance, directions, interactions with other structures, and movement patterns can easily be derived from the maps.

**Generation of Spatiotemporal Maps**

Raw data files were imported in Scion Image with custom written routines and saved as TIFF image stacks (Fig. 1A). An averaged image was obtained by compressing the whole stack in one single picture (Fig. 1B). In this averaged image, line selections were made along a fiber of interest (Fig. 1C). For each image in the stack, the selected line and the adjacent perpendicular pixels (line width = 5 pixels) were averaged and plotted in a spatiotemporal map, in which each horizontal line represents the fluorescence along the fiber for each frame of the movie. Therefore, in such maps, particle positions along the fiber are found in the horizontal direction, and positions in time are found in the vertical direction (Fig. 1D).

**Calculation of Velocity**

Typically, in these maps, moving particles left an angled trace due to the change in position (δx) for a given period of time (δt), whereas static particles (δx = 0) generated a simple vertical line (Fig. 1D). In regions where particles traveled at a constant speed, the velocity was calculated by measuring δx and δt. To avoid erratic velocities induced by pixel jitter, particle rotation, or limitations in the accuracy by which the maps were generated, we only included velocities that were maintained over at least 2 μm or for at least 2 s. Continuous velocities were obtained by calculating the tangent of α (as indicated in Fig. 1D), which is the point velocity (tangent α = δx/δt) of a particle at a given point in time and space. The angle α was calculated from the coordinates of the filtered trace (9 pixel iterating moving average filter). To calculate the tangent, we used a sufficiently long intercept (13 pixels) to avoid inclusion of exaggerated velocities due to pixel jitter. Within the limits of the field of view and the duration of the recording, the travel distance and total travel time could be derived from these maps. To compare normal and drug conditions, we calculated an average from all velocities that were maintained for either 2 s or 2 μm; alternatively, velocities could also be weighted in time or space (see APPENDIX) emphasizing the slower and stationary periods.

**Background Calculation and Subtraction**

Compression of the maps in time generated an average line, from which the position of stationary mitochondria could easily be derived (Fig. 1E). The subtraction of this line from all horizontal lines which the position of stationary mitochondria could easily be derived (Fig. 1F).

**Variation in Velocity**

Point velocities could be plotted either for every pixel position along the fiber or for every point in time. To assess periodicity in the constantly changing velocity, inflection points (δx/δt² = 0) were counted for a fixed intercept of fiber or period of time. Furthermore, to analyze changes in velocity around some defined static structures (e.g., static mitochondria) the standard deviation of the velocity was calculated in the vicinity of such structures (Fig. 4G). All analysis routines were custom written in the macrolanguage provided by Scion Image and could also be useful to analyze transport of other organelles, neurofilaments (36), and eGFP-labeled proteins (19).

**Statistics**

Results are presented as means ± SE and were compared with ANOVA (with a Bonferroni post hoc test) in case of mitochondrial speed. The numbers of moving mitochondria were normalized to control, and changes were presented as percentages ± SE. Statistical analysis was performed on the original data with Mann-Whitney for
single and Kruskal-Wallis tests for multiple comparisons. A P value of 0.05 was considered the cutoff for statistical significance, unless mentioned otherwise. Statistical analysis was performed either with MS Excel or Graphpad Prism (San Diego, CA).

**Immunohistochemistry**

Cultured cells were fixed in 4% paraformaldehyde solution (pH 7.2), washed in 0.1 M PBS, and permeabilized with 0.5% Triton X-100. Nonspecific binding sites were blocked using 4% goat serum (pH 7.2), washed in 0.1 M PBS, and permeabilized with 0.5% Triton X-100. Confocal imaging was performed on a Nikon Diaphot/BioRad (Hercules, CA) MRC600 microscope. Confocal micrographs are digital composites of z-scans of four to eight optical sections (pinhole <20% of the maximum) through a depth of 1.9–3.8 μm.

**Drugs and Solutions**

M-199, FBS, and antibiotics were from Invitrogen (Grand Island, NY); TTX, thapsigargin (Tg), and NGF were from Alomone Laboratories (Jerusalem, Israel); protease, collagenase, ARA-C, FCCP, oligomycin, and the tubulin, actin, kinesin, and dynein antibodies were from Sigma (St. Louis, MO); secondary antibodies were from Vector (Burlingame, CA); anti-Kif5B was generously provided by Dr. R. Vale; MitoTracker green and red and Rhod-2 were from Molecular Probes (Eugene, OR).

The experimental solution contained (in mM) 150 NaCl, 6 KCl, 1.5 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES (pH 7.38). The 0-mM Ca²⁺ solution contained 2.5 mM MgCl₂ and 2 mM EGTA.

**RESULTS**

**Mitochondrial Staining in Myenteric Neurons**

MitoTracker green stained mitochondria in all cells that were present in the primary cultures. Neuronal somata were densely packed with mitochondria, which made it difficult to differentiate between and assess the dimensions of individual organelles. However, mitochondria could easily be identified at variable distances along the processes as well as in the variabilities of the myenteric nerve fibers (Fig. 2A). The mitochondria were oval in shape and were on average 1.5 ± 0.1 μm long (n = 46). The dimensions of mitochondria in neuronal somata were highly variable and could be as long as 10 μm.

To check the specificity of the MitoTracker dyes, we used MitoTracker red (50 nM) and rhod-2 AM (0.9 μM). MitoTracker red labeled exactly the same structures as MitoTracker green (data not shown). Rhod-2 AM, a Ca²⁺ indicator that is loaded in reduced form and only becomes fluorescent when oxidized in the actively respiring mitochondria, stained the same organelles as MitoTracker green (Fig. 2B), providing further support for the specificity of MitoTracker green. The

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1 Supplemental video for this article may be found at http://ajpgi.physiology.org/cgi/content/full/00283.2003/DC1
Tracker green (B 2) they appear yellow in the overlay image (green, we used reduced rhod-2 AM that only becomes fluorescent in actively respiring mitochondria (Fig. 3A). The neurons were double stained with MitoTracker green staining of guinea pig myenteric neurons. Whereas in neuronal somata the density of mitochondria was too high, individual mitochondria could easily appear yellow in the overlay image (green staining of guinea pig myenteric neurons. Whereas in neuronal somata the visualization of mitochondrial movement (Fig. 3B) allowing antero- and retrograde movement to be determined accurately. Periods of constant speed (up to −3 μm/s) were never maintained for a long time and were often followed by pauses. An average velocity was calculated by selecting all regions in which the velocity was constant for at least 2 s or over a distance of at least 2 μm. A frequency histogram of all velocities observed is shown in Fig. 3D. The average velocity (v), calculated from 73 moving mitochondria, was 0.41 ± 0.02 μm/s (Fig. 3D). The velocity could also be weighted (see Appendix) in space (v = 0.13 ± 0.01 μm/s) or in time (v = 0.09 ± 0.008 μm/s) These velocities were significantly lower than v, because slow and stationary periods contribute more significantly and reduce the average speed.

Properties of Mitochondrial Transport

Spatiotemporal maps (see Materials and Methods) were generated for each fiber in the field of view. These maps provide a succinct summary from which parameters such as travel distance, speed, and changes in speed can easily be extracted.

Velocity

Mitochondria moved through nerve fibers in an intermittent, saltatory manner. Periods of constant speed (up to −3 μm/s) were never maintained for a long time and were often followed by pauses. An average velocity was calculated by selecting all regions in which the velocity was constant for at least 2 s or over a distance of at least 2 μm. A frequency histogram of all velocities observed is shown in Fig. 3D. The average velocity (v), calculated from 73 moving mitochondria, was 0.41 ± 0.02 μm/s (Fig. 3D). The velocity could also be weighted (see Appendix) in space (v = 0.13 ± 0.01 μm/s) or in time (v = 0.09 ± 0.008 μm/s). These velocities were significantly lower than v, because slow and stationary periods contribute more significantly and reduce the average speed.

Size of The Transported Mitochondria

The average length of the moving mitochondria was 1.08 ± 0.05 μm (n = 82; Fig. 3H). To investigate whether the length of the particles, and therefore the load of the cargo, determined the velocity, a scatter plot was made between mitochondrial size and speed of transportation (Fig. 3H). No correlation (R² = 0.06) could be observed, indicating that velocity is independent of the size of the cargo. Furthermore, no relationship (R² = 0.03) was found between the distance traveled and mitochondrial size (Fig. 3I). Within the boundaries of the field of view, the average travel distance was 11.6 ± 1.5 μm (n = 47).

Temporal Events and Spatial Characteristics of the Track

We sought to investigate whether movement of mitochondria was dependent on specific spatial properties of the fiber in which they were transported. For example, in a narrow part of the fiber or in a region with possibly disabled tracks, transport might be hampered. If so, every mitochondrion passing through that region should experience an equivalent retardation. Similarly, with time, movement might be determined by events at specific times (e.g., bursts of action potentials), which would influence all particles. The point velocity of several pairs of mitochondria was calculated (Fig. 4A), either for one section of the fiber (14 pairs from 6 fibers) or for one period of time (10 pairs from 6 fibers). The point velocity varied substantially between two mitochondria moving in the same fiber section or at the same time (Fig. 4, B–C). Similarly, when the point velocity of each pair of mitochondria was plotted against each other (Fig. 4, D–E), no correlation either for fiber prop-
properties \((R^2 < 0.01)\) or for time events \((R^2 < 0.001)\) could be observed.

**Presence of Stationary Mitochondria**

In the same manner, we analyzed whether mitochondrial movement was affected by static mitochondria. Background images were generated to determine the position of the stationary mitochondria (Fig. 4F). To determine the change in velocity, we calculated standard deviations of the velocity over 1 \(\mu\)m for three different regions along the fiber (Fig. 4F): randomly at distances at least 2.5 \(\mu\)m away from \((\alpha)\), 0.5 \(\mu\)m at both sides of \((\beta_1\) and \(\beta_2)\), and at the precise location of the static mitochondrion \((\gamma)\). Standard deviations were similar for each of the four regions \((\alpha: 83 \pm 10 \text{ nm/s}; \beta_1: 90 \pm 19 \text{ nm/s}; \beta_2: 91 \pm 17 \text{ nm/s}; \gamma: 83 \pm 22 \text{ nm/s}; \text{ANOVA, } P > 0.9, \text{ Fig. 4G})\), indicating that stationary mitochondria did not consistently affect the transport.

**Periodicity**

We also determined whether the changes in velocity had a certain periodicity, by calculating inflection points \((\ddot{R}^2/\dot{R}^2 = 0)\) for a certain fiber section or a certain time. The mitochondria reached a peak velocity every \(3.5 \pm 0.2 \mu\)m \((n = 26, \text{ Fig. 4H})\) and every \(6.2 \pm 0.3 \text{ s} (n = 22, \text{ Fig. 4I})\), suggesting an inherent cyclical process of transportation.

**Structural Proteins: Microtubules and Actin Filaments**

To investigate the role of structural proteins, we incubated neurons 36–42 h before the experiment with colchicine (100 \(\mu\)M) to destabilize the microtubule network or cytochalasin D (1 \(\mu\)M) or phalloidin (10 \(\mu\)M) to depolymerize or stabilize the actin network, respectively.

Only few mitochondria \((19.1 \pm 7.0\% \text{ of control, } P < 0.05, n = 7)\) were transported in the nerve fibers after...
colchicine, and both speed (0.09 ± 0.02 μm/s, P < 0.001) and travel distance (2.6 ± 0.5 μm, P = 0.01) were significantly reduced (Fig. 5, A and B). Cytochalasin D and phallolidin did not significantly affect the number of moving mitochondria (74.4 ± 25.0 and 75.3 ± 19.5% of control, respectively; Fig. 5A). However, speed and travel distance was significantly reduced by cytochalasin D (0.22 ± 0.02 μm/s, P < 0.001 and 5.8 ± 1.2 μm, P < 0.01). Conversely, phallolidin sped up the mitochondria (0.53 ± 0.03 μm/s, P < 0.01) and increased their travel distance (18.1 ± 2.4 μm, P = 0.02; Fig. 5B).

We used antibodies raised against the anterograde and retrograde motor proteins kinesin and dynein to reveal their association with the structural proteins tubulin and actin. The actin staining was mainly observed in fibroblasts present in the culture. Kinesin and dynein seemed not to be colocalized with actin, although for dynein, some colocalization was observed in the neuronal cell bodies (Fig. 6, A and B). Microtubules were revealed in neurons and fibroblasts. Both kinesin and dynein were unequivocally colocalized with the microtubules in myenteric neuronal processes (Fig. 6, C and D).

Kif5B is one of the kinesin members that has been linked to mitochondrial transport. In cultures stained with MitoTracker red before fixation (Fig. 6E), we used an antibody against Kif5B to reveal its presence and position in the myenteric nerves. Although Kif5B was abundantly present in myenteric neurons (Fig. 6, F and G) and also at the edges of the underlying cells, there was no clear spatial relationship between the location of mitochondria and Kif5B. In nerve fibers, however, Kif5B was clearly colocalized with some mitochondria but not with others (Fig. 6, H–J). It remains to be elucidated whether this was indicative of their transport status at the moment of fixation.

**Depolarization, Na⁺ Action Potentials, and Ca²⁺ Concentration**

Depolarization and Na⁺ action potential blockade with 1 μM TTX. It has to be noted that these cultures are spontaneously active and that some fibers display Ca²⁺ transients at a frequency of up to 0.4 Hz (data not shown). Therefore, we used the Na⁺ channel blocker TTX (1 μM) to study whether mitochondrial transport was an action potential-dependent event (n = 4). TTX significantly reduced the number of moving mitochondria (6.5 ± 3.8% of control, P < 0.01, Fig. 7A); however, the average speed was not altered (0.47 ± 0.07 μm/s, P = 0.7, Fig. 7B). We did not observe any difference in the properties of mitochondrial transport when neurons were briefly (5–10 s) depolarized with high K⁺ (50–75 mM).

**Extracellular and intracellular Ca²⁺**. Ca²⁺ was suggested to be a potential signal controlling transport (16), and functional intracellular Ca²⁺ stores seem required for mitochondrial permeability transition pore opening (18). Also, in myenteric neurons, intracellular Ca²⁺ stores play a crucial role in the control of [Ca²⁺.] (14, 41, 45, 47). Therefore, we investigated the effect of Ca²⁺ on mitochondrial transport. Removal of extracellular Ca²⁺ (n = 7) did not affect (Fig. 7, A and B) the number (79.3 ± 19.3%, P > 0.05) and average speed of moving mitochondria (0.43 ± 0.03 μm/s, n = 38, P = 0.98). To test whether this also held true for intracellular Ca²⁺ sources, we depleted the endoplasmic reticulum Ca²⁺ stores by incubating the neurons in Tg (5 μM, 45 min) followed by a short exposure to caffeine (2 mM, 1 min). Mitochondrial transport was recorded either in normal or 0 mM (2 mM EGTA) extracellular Ca²⁺. The number of moving mitochondria in Tg-treated neurons was reduced (33.2 ± 9.1%, P <
0.05, \( n = 6 \)), and this number was not affected any further by removal of extracellular \( Ca^{2+} \) (40.4 ± 7.8\%, \( P < 0.05 \), \( n = 6; \) Fig. 7A). The speed of mitochondria in fibers with depleted stores was also reduced independently of the extracellular \( Ca^{2+} \) concentration \( [0.18 \pm 0.02 \text{ (} P < 0.001 \text{) } \text{ and } 0.15 \pm 0.02 \text{ \( \mu \)m/s } (P < 0.001) \text{ for normal } (n = 20) \text{ and } 0 \text{ mM } Ca^{2+} (n = 27) \text{, respectively; Fig. 7B}] \). These results indicate that intracellular store \( Ca^{2+} \) and not extracellular \( Ca^{2+} \) is important in controlling mitochondrial transport and is required to maintain the speed of moving mitochondria.

**Blockade of Mitochondrial Function: FCCP Oligomycin**

The question of whether mitochondria had to be functional to be transported was addressed in experiments with the protonophore FCCP (1 \( \mu \)M) and \( F_0/F_1 \) ATPase blocker oligomycin (10 \( \mu \)M). FCCP depolarizes mitochondria and therefore stops mitochondrial \( Ca^{2+} \) buffering and ATP production (6, 10, 45). FCCP reduced the number of transported mitochondria in a time-dependent fashion: 55.6 ± 10.5\% (\( P > 0.05 \), \( n = 8 \)) for exposures <5 min and 20.9 ± 14.5\% (\( P < 0.05 \), \( n = 4 \)) for longer exposures (Fig. 7C). The velocity was similar to control for short (0.37 ± 0.03 \( \mu \)m/s, \( P = 0.33 \)) and reduced for prolonged exposures (0.25 ± 0.06 \( \mu \)m/s, \( P = 0.08 \); Fig. 7C). This reduction was, however, not significant on the 5\% level, probably due to the low numbers of mitochondria that were still moving in FCCP. Oligomycin slowed the mitochondria (0.31 ± 0.01 \( \mu \)m/s, \( P < 0.01 \)) but did not significantly affect their number (93.2 ± 21\%, \( P > 0.05 \); Fig. 7, C and D).

**DISCUSSION**

Mitochondrial transport in nerve processes of myenteric neurons was investigated using fluorescent mitochondrial markers, video-rate confocal microscopy, and specialized analysis algorithms. We found that mitochondrial movement was saltatory and that velocities changed periodically. Transport was not affected by other structures such as varicosities or stationary mitochondria and was conducted over microtubuli, with which motor molecules were colocalized. Actin filaments had a regulatory role. Transport was blocked by TTX and inhibited by depletion of intracellular \( Ca^{2+} \) stores but not by removal of extracellular \( Ca^{2+} \).

**Mitochondrial Size and Movement**

In earlier reports on axonal transport (8, 32), organelles were identified based on morphology, which inevitably excluded some smaller mitochondria. MitoTracker facilitates the identification (4, 27), although some putative pitfalls such as unspecificity and “laser-induced effects” might need some attention. Dye specificity was assessed in double-labeling experiments with MitoTracker green/red and rhod-2. Due to the loading procedure, rhod-2 only fluoresces in actively respiring mitochondria, thereby confirming MitoTracker specificity. Our observations also argue against laser-induced events, because mitochondria enter and leave the field of view from and to

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**Fig. 6.** Immunohistochemical staining for structural and motor molecules in primary cultures of myenteric neurons. Antibodies against structural proteins (tubulin and actin) and against motor molecules (kinesin, dynein, and Kif5B) were used in combination with FITC/TXR secondary antibodies. A: actin-FITC and kinesin-TXR double staining. Actin filaments are not abundant in neurons but clearly visible in the underlying nonneuronal cells. Kinesin, present in fibers and neuronal cell bodies (arrows), is not colocalized with actin filaments. B: actin-FITC and dynein-TXR double staining. Dynein, present in the fibers and neuronal cell bodies (arrows), is not colocalized with actin filaments in nerve fibers. However, there seems to be colocalization of the 2 proteins in neuronal cell bodies. C: tubulin-FITC and kinesin-TXR double staining. Tubulin is present in neurons and other cells in culture. In neurons, kinesin and tubulin are colocalized in somata and fibers (arrows). D: tubulin-FITC and dynein-TXR double staining. Dynein and tubulin are colocalized in neuronal somata and fibers (arrows). E–G: immunohistochemical staining for Kif5B (antibody provided by Dr. R. Vale) in cultured neurons stained with MitoTracker red before fixation. Kif5B was abundant in myenteric neuronal cell bodies (arrow) and also present in fibers. Because cell bodies contain many mitochondria, they appear yellow in overlay images (G). H–J: detail of a myenteric nerve fiber stained with MitoTracker red and the Kif5B antibody (FITC). Note that some mitochondria are colocalized with Kif5B (arrowhead), whereas others are not (arrow; J). Bars: 25 \( \mu \)m for A–G and 10 \( \mu \)m for H–J.
nonexcited regions. Furthermore, we did not observe any launch or arrest after a certain illumination time, suggesting that the experimental conditions (dye concentration, laser settings, and recording time) did not impede or initiate mitochondrial transport.

The majority of mitochondria was stationary; most likely, these mitochondria were anchored to the cytoskeleton to operate locally. Brodin et al. (2) described two populations of mitochondria in lamprey axons: along the axon, they were longer than at the synapse (~1 μm). In myenteric nerve fibers, moving mitochondria were also smaller than stationary ones, suggesting that moving ones might have been meant for synapses.

Spatiotemporal maps offer the possibility to accurately assess velocity and patterned movement. The average velocity of mitochondria (~0.4 μm/s) was similar to velocities observed for organelle movement in other neurons (27, 38) and together with peak velocities was well in the range of fast axonal transport (~37–250 mm/day) (42). Interestingly, the time-weighted velocity (~0.09 μm/s = 7.8 mm/day) approximates the speed proposed for slow axonal transport, which corroborates the idea that slow axonal transport might be the summation of fast movement and prolonged pauses (36, 49). However, Almenar-Queralt and Goldstein (1) clearly describe the differences in fast and slow axonal transport and suggest that slow transport is not characteristic of mitochondria.

Moving mitochondria changed velocity periodically. Similar "saltatory" movement was described for organelles in Xenopus (21), lobster (8), and hippocampal axons (16). The reason why mitochondria are transported so erratically is unclear. We studied velocity in relationship to mitochondrial size: do larger, heavier mitochondria move slower? Vale et al. (44) already suggested that velocity was dependent on particle size. Kaether et al. (19) came to an opposite conclusion for axonal membrane proteins. In this study, we found no relationship between mitochondrial size and velocity, which might indicate that more motor molecules connect to longer mitochondria to maintain speed. This confirms the finding of Forman et al. (8), who suggest that there is no such relationship for long mitochondria moving in lobster giant axons.

We further investigated whether the velocity changes were due to properties of the track. By analyzing pairs of mitochondria, we found that changes do not consistently occur at specific loci, e.g., en route boutons. Likewise, changes in velocity were not simultaneous, which makes it unlikely that bursts of action potentials would cause these changes. The standard deviation was calculated to assess whether velocity changed approaching or passing stationary mitochondria. Again, speed was not consistently affected, indicating that gradients (e.g., ATP) that might exist around stationary mitochondria are not influential. It also shows that stationary mitochondria were not attached to the same track or were detached from the microtubuli and anchored to the cytoskeleton. Moreover, mitochondria could move in opposite directions without affecting each other, which again indicates that different tracks exist within one process. In these cultures, there are no obvious morphological differences between dendrites and axons. Although clear anterograde and retrograde movement was observed, a detailed comparison was not feasible in this...
model (32), because from which neuron processes were sprouting could not be assessed consistently.

Role of Microtubules and Actin Filaments

We used two parameters to compare mitochondrial transport in normal and drug conditions: 1) the number of moving mitochondria and 2) the average velocity. Due to the variability in fiber density, we chose to express the number of moving mitochondria per millimeter of fiber for a given observation period. We did not use a ratio between moving and static mitochondria (27), because the latter are often part of a network and therefore hard to count. Colchicine reduced and slowed transport significantly, indicating that most mitochondria traffic over microtubuli. Interestingly, destabilization of the actin cytoskeleton (cytochalasin D) reduced the speed without significantly changing the number of moving mitochondria. These data suggest that although it is not as crucial as tubulin, there is an actin component (~20%) to the mitochondrial transport. The presence of an actin-dependent mechanism for organelle movement was also described for other neurons (24, 28, 30).

In this study, we also show that mitochondrial transport can be sped up by stabilizing actin with phalloidin, indicating that actin plays a role in transport efficiency.

Two families of motor proteins are responsible for transport of cellular material (43). Immunohistochemical experiments revealed that kinesins and dyneins were colocalized with tubulin, further supporting the important role of microtubuli. Whether one family of proteins detaches from the cargo when directions change or there is a perpetual competition between all (plus- and minus-end-directed) motor molecules attached to the mitochondria remains to be elucidated. The lack of commercially available antibodies prevented us from investigating the specific kinesins (Kif) involved. In Kif5B-null mutations, mitochondria are abnormally clustered in the perinuclear region, pointing to a crucial role in mitochondrial transport (39). Kif5B was present in myenteric neurons but colocalized only with some mitochondria in the fibers. These might have been the mitochondria transported at the time of fixation.

Role of Na$^+$ and Neuronal Excitability

To assess what physiological events were driving mitochondrial transport we used high K$^+$ or TTX. A short depolarization had no effect, which suggested that either transport is already at its maximum or that depolarization is not local enough to affect transport. Alternatively, transport might also be relatively independent of membrane potential changes. The TTX data suggest, however, that mitochondrial transport is activity dependent or at least reliant on Na$^+$ influx. The latter was also suggested by Lavoie (26), who found that Na$^+$ was important for axonal transport, which was confirmed by Kanai et al. (20) who showed that axonal transport was blocked by lidocaine in a Ca$^{2+}$-dependent manner. In contrast to this, Forman and Shain (9) described that the open Na$^+$ channel stabilizer batrachotoxin stopped organelle movement, which could be reversed by TTX. All these data indicate that organelle transport is probably under subtle Na$^+$ control. TTX did, however, not slow the few moving mitochondria.

Role of Ca$^{2+}$

Action potential firing in myenteric neurons leads to Ca$^{2+}$ influx (11, 37), release from stores (14, 47), and uptake via mitochondria and Ca$^{2+}$ stores (45, 46). The subsequent increase in mitochondrial Ca$^{2+}$ links activity to cellular energy demand. We removed extracellular Ca$^{2+}$ to investigate whether transport required Ca$^{2+}$ and found that transport and speed were not altered. However, when Ca$^{2+}$ stores were depleted, transport and speed were significantly reduced (to ~35%). Removal of extracellular Ca$^{2+}$ from these neurons did not cause a further change. Functional Ca$^{2+}$ stores are important to maintain mitochondrial function (3, 25), and mitochondrial potential oscillations have been shown to depend on stored Ca$^{2+}$ (18). In the same line, our data indicate that specifically stored Ca$^{2+}$ and not extracellular Ca$^{2+}$ is required for initiation and maintenance of mitochondrial transport. A further study will be needed to clarify the exact role of Na$^+$ and Ca$^{2+}$ in the control of mitochondrial transport in these neurons.

Role of Mitochondrial Potential and ATP Production

What exactly triggers mitochondrial traffic is as yet unknown. Growth, elongation, and maturation of nerve fibers (51) are likely candidates. Some kinesins have been shown to be upregulated in growing axons (31). Mitochondria are likely transported to regions of high energy consumption or high Ca$^{2+}$ loads or to replace impaired mitochondria. We also used FCCP and oligomycin to interfere with mitochondrial function. Although FCCP depolarizes mitochondria at once, short applications (<5 min) did not affect transport or speed. During prolonged presence of FCCP (>5 min), moving mitochondria were rare and inclined to travel more slowly. Hence, mitochondrial depolarization did not affect transport directly, although negative effects become apparent over time. A slow effect of FCCP (30 min) on organelle movement in chick sensory neurons was also observed by Hollenbeck et al. (17). Although the ATPase blocker oligomycin did not reduce the number of moving mitochondria, velocity was slowed by 25%, which might reflect a lack of ATP to fuel transport quickly. What exactly triggers mitochondrial traffic is as yet unknown. Growth, elongation, and maturation of nerve fibers (51) are likely candidates. Some kinesins have been shown to be upregulated in growing axons (31). The cultures used in this study are between 7 and 10 days in vitro; from that age not much structural change in the nerve network is observed. However, it cannot be excluded that some fibers are still actively modified and that the observed transport reflects ongoing physiological processes, such as growth and network reorganization.

In conclusion, the recording and analysis tools in this study provide a method to investigate the patterns in axonal transport of mitochondria and other organelles. Here, we show for the first time that mitochondria are intermittently transported in enteric neurons and that they change velocity periodically. The detailed analysis allowed us also to conclude that transport is independent of track-specific properties, such as bifurcations or varicosities, and that stationary mitochondria do not influence the transport significantly. Although intact microtubules are required, there is also an actin component to this transport. We succeeded to speed up mitochondrial transport by stabilizing...
the filamentos actin with phalloidin and showed that the transport is abolished by TTX. Furthermore, we could show that, although extracellular Ca\(^{2+}\) is not essential, the transport was reduced and slower in neurons with depleted intracellular Ca\(^{2+}\) stores, suggesting an important role for these organelles in mitochondrial trafficking.

**APPENDIX**

Average velocity

\[
\bar{v} = \frac{\sum \Delta x_i}{\Delta t_i} \quad \text{with} \quad \Delta t_i \geq 2 \text{s} \quad \text{or} \quad \Delta x_i \geq 2 \mu m
\]

Average velocity weighted in space

\[
\bar{v}_s = \frac{\sum v_i \Delta x_i}{\sum \Delta x_i} \quad \text{with} \quad \Delta x_i \geq 2 \mu m
\]

Average velocity weighted in time

\[
\bar{v}_t = \frac{\sum v_i \Delta t_i}{\sum \Delta t_i} \quad \text{with} \quad \Delta t_i \geq 2 \text{s}
\]

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