Mitochondrial Swelling Impairs the Transport of Organelles in Cerebellar Granule Neurons*

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Organelle transport in neuronal processes is central to the organization, developmental fate, and functions of neurons. Organelles must be transported through the slender, highly branched neuronal processes, making the axonal transport vulnerable to any perturbation. However, some intracellular structures like mitochondria are able to considerably modify their volume. We therefore hypothesized that swollen mitochondria could impair the traffic of other organelles in neurite shafts. To test this hypothesis, we have investigated the effects of mitochondrial swellings on the organelle traffic. Our data demonstrate that treatment of neurons with potassium ionophore valinomycin led to the fast time-dependent inhibition of organelle movement in cerebellar granule neurons. Similar inhibition was observed in neurons treated with the inhibitors of the mitochondrial respiratory chain, sodium azide and antimycin, which also induced swelling. No decrease in the motility of organelles was observed in cultures treated with inhibitors of ATP production or transport, oligomycin or bongkrekic acid, suggesting that inhibition of the ATP-generating activity itself without swelling does not affect the motility of organelles. The effect of swellers on the traffic was more important in thin processes, thus indicating the role of steric hindrance of swollen mitochondria. We propose that the size and morphology of the transported cargo is also relevant for seamless axonal transport and speculate that mitochondrial swelling could be one of the reasons for impaired organelle transport in neuronal processes.

Organelle transport in neuronal processes is essential for neuron viability and function. Organelles move along microtubule and microfilament tracks powered by molecular motor proteins; plus-end-directed kinesins carry organelles anterogradely, whereas dynein propels them retrogradely. Golgi-derived transport vesicles, endocytic vesicles, lysosomes, and autophagosomes move at maximal rates of 1–5 μm/s, whereas mitochondria move at maximal rates of 0.3–0.7 μm/s (1). Axonal transport of mitochondria and lysosomes is bidirectional, sharing the same tracks and motor proteins (2). Organelles must be transported through the slender, highly branched neuronal processes; the axonal shafts between the varicosities are extremely thin, with an average diameter around 200–300 nm (3, 4), matching exactly with the size of the most sizable organelles, mitochondria and lysosomes (5, 6). All these factors make axonal transport vulnerable to any perturbation that could lead to axonal traffic jams and axonal degeneration and cause neuronal dysfunction (7). Axonal transport of organelles seems to be a highly orchestrated and well coordinated process, and under physiological conditions, the organelles could move and cross each other without difficulties (8). However, several lines of evidence suggest that the axonal traffic becomes compromised when the size of cargo increases or the axonal diameter decreases. Defects in axonal transport have been indirectly linked to a number of progressive human neurodegenerative diseases including Alzheimer disease, Huntington disease, and amyotrophic lateral sclerosis. All these diseases are characterized by perturbed axonal transport that could be related to accumulation of proteins within axons. On the other hand, Shaw and Goldstein’s group (9) has demonstrated dramatic impairment of mitochondrial transport in the neurites after internalization of fluorescent beads with diameters between 200 and 500 nm. In recent reports (5, 6), we have demonstrated that mitochondrial swelling, induced either by mitochondrial depolarization or by opening of K\textsubscript{ATP} channels, leads to a dramatic increase in the diameters of neuronal mitochondria (ranging between 300 and 800 nm). This raises a possibility that in living cells, various conditions affecting mitochondrial homeostasis are able to modify the geometry of these organelles.

We therefore hypothesized that swollen mitochondria could impair the traffic of other organelles in neurite shafts. To test this hypothesis, we have investigated the effects of mitochondrial swellers on the organelle traffic. The results obtained show that increase in mitochondrial volume is associated with a marked drop in the motility of mitochondria themselves and also impair the motility of lysosomes.

EXPERIMENTAL PROCEDURES

Sample Preparation—Primary cultures of cerebellar granule neurons were prepared according to the method of Gallo et al. (26), with slight modifications. Briefly, the cerebella from 8-day-old Wistar rat pups were dissociated by mild trypsinization (0.25% trypsin at 35 °C for 15 min) followed by trituration in a 0.004% DNase solution containing 0.05% soybean trypsin.
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inhibitor. The cells were resuspended in Eagle’s basal medium with Earle’s salts containing 10% heat-inactivated fetal bovine serum, 25 mM KCl, 2 mM glutamine, and 100 µg/ml gentamycin. The cell suspension was plated at a density of 1.0 × 10⁶ cells/ml on poly-L-lysine-coated LabTek II chambered cover-glass (0.3 ml/chamber; Nalge Nunc International, Rochester, NY) or at a density of 1.25 × 10⁶ cells/ml on poly-L-lysine-coated MatTek glass bottom culture dishes (2 ml; MatTek Corp., Ashland, MA). 10 µM cytosine arabinoside was added 24 h after plating to prevent the proliferation of glial cells. The cells were cultured for 5–7 days in a humidified 5% CO₂/95% air mixture at 37 °C.

Staining and Transfection—For mitochondrial tracking, intact neurons were loaded for 30 min at 37 °C with 100 nM MitoTracker Green or 200 nM MitoTracker Red in culture medium. To stain the cytoplasm, intact neurons were loaded for 30 min with 5 µM CellTracker Green 5-chloromethylfluorescein diacetate at 37 °C in Lockey’s solution that was replaced with conditioned medium after staining and incubated for a further 30 min before microscopy. For lysosomal staining, the intact cells were loaded for 30 min with 100 nM LysoTracker Red in culture medium. All dyes were purchased from Invitrogen.

In another set of experiments, the cerebellar neurons were transiently transfectected on second day in vitro using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Briefly, 100 µl of OPTI-MEM® (Invitrogen) containing 2% Lipofectamine 2000 (Invitrogen) and 1 µg of cytosolic-green fluorescent protein DNA (Clontech) mixed with either 1 µg of DNA for Lamp1-RFP² (Addgene, PubMed Identifier: 14617360) or mitochondria-targeted pDsRed2 (mtDsRed2, Clontech) was incubated with ~10⁶ cells in glass bottom microwell dishes (MatTek) for 4 h at 37 °C. On completion of incubation conditioned culture medium (Eagle’s basal medium, Invitrogen) was added to the cells, and cells were further grown in humidified 5% CO₂ at 37 °C. The transfected DNAs were allowed to express and accumulate respective proteins in targeted organelle for 3 days.

Three-dimensional Analysis—512 × 512 pixel digital optical sections were acquired by a confocal laser scanning microscope (MRC1024, Bio-Rad) equipped with an Ar-Kr laser (excitation wavelengths 488 and 568 nm) and a 100× oil immersion objective (1.35 numerical aperture, Olympus). Voxels were collected at 15-nm lateral and 100-nm axial intervals. Raw images were deconvolved by the AutoDeblur software package (Media Cybernetics, Bethesda, MD). Isosurface three-dimensional pictures were generated using the AutoVisualize software package (AutoQuant Imaging) after binarizing with a fixed 32% threshold value. Details are given in Safiulina et al. (6).

Quantitative Analysis of Organelle Motility—Organelle motility was measured in intact cultures of cerebellar granule neurons loaded with MitoTracker Green and LysoTracker Red or expressing Lamp1-RFP or mtDsRed2 by time course confocal microscopy. Temperature in the culture medium was kept at 37 °C with a temperature-controlled chamber system, and images were collected using a 40× or 63× spring objective. The pH of culture medium in the chamber was maintained at 7.1 with 20 mM HEPES. Time course analysis was performed using the LaserSharp 2000 software time course option by recording 30 frames with 5-s intervals for one time point. Coordinates of randomly chosen mitochondria were recorded by a computer and were tracked through the time series. Matrices of the data were further processed using a Microsoft Excel macro that calculated the percentage of time spent in motile state, distance of displacement, maximal velocity, average velocity, and average velocity in motile state for each mitochondrion.

Statistical Analysis—The data are presented as mean ± S.E. One-way analysis of variance followed by Bonferroni post hoc test was used for statistical analysis except for data presented in Fig. 4, where the Mann-Whitney test was used.

RESULTS

Our first aim was to validate the effect of mitochondrial modulators on mitochondrial morphology. We have shown previously that mitochondrial inhibitors depolarizing mitochondrial inner membrane increase considerably the mitochondrial size, and this is associated with impaired mitochondrial motility (6). However, compounds inducing mitochondrial depolarization and swelling also de-energize mitochondria and inhibit production of ATP, which might also account for the loss of mitochondrial motility. It was therefore not possible to differentiate whether the mitochondrial motility ceased because of mitochondrial swelling or due to decline in cytoplasmic ATP required to power cytoplasmic motor proteins. To distinguish between these effects, we decided to use in current experiments two groups of mitochondrial inhibitors. The first group, valinomycin (induces massive potassium influx into the mitochondrial matrix), antimycin (inhibits ubiquinone-cytochrome c reductase), and high concentrations of sodium azide (inhibits cytochrome c oxidase together with ATP synthetase), ceases the mitochondrial energy supply to cytoplasmic consumers and produces mitochondrial swelling due to dissipation of the mitochondrial potential. The second group of inhibitors, bongkrekic acid (inhibits mitochondrial adenine nucleotide translocase, and thus, the ATP transport from the mitochondrial matrix to the cytoplasm), and oligomycin (inhibits the ATP synthetase), inhibits mitochondrial energy supply to cytoplasm but does not induce mitochondrial swelling. As could be seen from Fig. 1, the inhibitors from the first group, valinomycin, azide and antimycin, induced marked mitochondrial swelling associated with increase in mitochondrial diameter from 200–300 to 400–600 nm. On the other hand, the inhibitors belonging to the second class, oligomycin and bongkrekic acid, had no effect on the mitochondrial morphology. Thus, although all inhibitors block the ATP-generating activity of mitochondria, only depolarizers induce mitochondrial swelling.

Next question addressed in the study was to know whether the decreased mitochondrial motility in response to mitochondrial depolarization is related to the mitochondrial swelling or to the impaired ATP production. We measured

² The abbreviation used is: RPF, red fluorescent protein.
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the mitochondrial motility in the presence of these different classes of mitochondrial inhibitors. Valinomycin, antimycin, and azide inhibit almost completely the mitochondrial motility as demonstrated (Fig. 2) by decline in the average velocity (average distance per second traveled during motile and stationary state) as well as by fallen duty percentage (percentage of time in motile state) of individual mitochondria. However, no statistically significant change in these motility parameters was observed when we treated the neurons with bongkrekic acid (25 μM) or oligomycin (50 μM). These results demonstrate that inhibition of mitochondrial energy production per se cannot be the reason for impaired mitochondrial traffic in neuronal processes. The latter event is instead related to mitochondrial morphology modification or mitochondrial membrane potential drop (it cannot be excluded that mitochondrial membrane potential regulates somehow its transportation of this organelle (6)).

Next, we designed an experiment to test whether the mitochondrial swellers affect the lysosome traffic. Although possibly energy-dependent, the lysosome traffic should be independent on mitochondrial membrane potential itself and could be impaired only in the case when swollen mitochondria block mechanically the lysosome passage. The individual experiment depicted in Fig. 3, A and C, demonstrates that treatment of neurons with valinomycin led to fast, time-dependent inhibition of lysosomal movement within a few minutes as estimated by drop in their average speed and duty percentage. At about 10 min after start of the treatment, the lysosomal movement almost completely ceased. No decrease in motility of lysosomes was observed either in control neurons or in bongkrekic acid-treated neurons at any time point after treatment. Fig. 3, B and D, showing summarized data of sodium azide (C, 25 mM), antimycin (D, 100 μM), bongkrekic acid (E, 25 μM), or oligomycin (F, 50 μM)-treated neurons. Each panel shows three-dimensional isosurface reconstruction of the deconvolved image series (frame size 2.5 × 1.0 × 1.0 μm, 1 × w × h). Note the increase in the maximal diameter of mitochondria treated with depolarizers (valinomycin, azide, and antimycin).

FIGURE 1. Effect of mitochondrial inhibitors on mitochondrial morphology. The figure shows three-dimensional reconstructions of a MitoTracker Green-stained single mitochondrion from control (A), valinomycin (B, 10 μM), sodium azide (C, 25 mM), antimycin (D, 100 μM), bongkrekic acid (E, 25 μM), or oligomycin (F, 50 μM)-treated neurons. Each panel shows three-dimensional isosurface reconstruction of the deconvolved image series (frame size 2.5 × 1.0 × 1.0 μm, 1 × w × h). Note the increase in the maximal diameter of mitochondria treated with depolarizers (valinomycin, azide, and antimycin).

FIGURE 2. Effect of mitochondrial inhibitors on mitochondrial motility. The figure depicts changes in the average velocity of mitochondria after a 15-min treatment with valinomycin (10 μM), antimycin (100 μM), azide (25 mM), bongkrekic acid (25 μM), or oligomycin (50 μM). The control column represents nontreated mitochondria. Each column summarizes data from 4–5 independent experiments and represents at least 100 mitochondria. *, different from control at p < 0.05, **, different from control at p < 0.005.
independent experiments, demonstrate that compounds inhibiting mitochondrial movement, valinomycin and azide, inhibited lysosomal movement almost completely when measured ∼15 min after the start of the drug treatment. Another mitochondrial inhibitor, antimycin, also inhibited the lysosomal traffic, although not as efficiently as others did. On the other hand, oligomycin (which does not change the mitochondrial geometry), similarly to bongkrekic acid, exerted no inhibitory effect. Rather, on the contrary, lysosomal motility tended to increase in bongkrekic acid-treated neurons. Thus, these data demonstrate that all mitochondrial modulators that induce mitochondrial swelling also inhibit the organelle transport in the processes of granule neurons and that this effect is not directly related to mitochondrial energy production.

We confirmed these findings by an additional set of experiments using mtDsRed2-labeled mitochondria and Lamp1-RFP-labeled lysosomes. Fig. 4 demonstrates that these fluorescently labeled organelles respond to mitochondrial sweller valinomycin similarly to MitoTracker Green- or LysoTracker Red-stained organelles. Thus, our previous findings could not be ascribed to the effect of valinomycin on fluorescent intensity. Moreover, in our experiments with Lamp1-RFP-labeled lysosomes, we failed to detect any effect of valinomycin or other swelling agents on the lysosomal morphology.

Several observations from our time lapse imaging of organelles support the “traffic jam” hypothesis. Fig. 5 demonstrates that under valinomycin treatment, lysosomes still move in the thick processes but not in small ones, although initially, the motility was similar. Swollen mitochondria tend to block mitochondrial movement, as shown in the pictures set in Fig. 6, where lysosome is trying to pass rounded mitochondrion but fails and turns back. Note that swollen mitochondria are often localized near lysosomes and that the effect of mitochondrial swelling on lysosome transport is dependent on the diameter of neurites. Fig. 5 shows that average velocity of lysosomes in thin processes is almost stopped when culture is treated with valinomycin, whereas in thick processes, the average is decreased only by about 50% of control value. Thus, these observations suggest that steric hindrance of swollen mitochondria rather than mitochondrial membrane potential itself or energy drop could be the major factor behind the ceased organelle movement.

FIGURE 3. Effect of mitochondrial inhibitors on lysosomal motility. A and B depict the data from single experiment where neurons were treated with valinomycin (10 μM, closed triangles) or bongkrekic acid (50 μM, closed circles) when compared with untreated control (open circles). The figure shows time-dependent changes in average velocity (A) and in duty percentage (B) of lysosomes (each data point represents 25–50 lysosomes). C and D demonstrate changes in average velocity and in duty percentage of lysosomes after a 15 min treatment with valinomycin, azide (25 mM), antimycin (100 μM), bongkrekic acid (25 μM), or oligomycin (50 μM). Each column summarizes data from 4–5 independent experiments and represents at least 100 lysosomes, *, different from control at p < 0.05, **, different from control at p < 0.005.

FIGURE 4. Effect of valinomycin on the motility of the mtDsRed2-labeled mitochondria or Lamp1-RFP-labeled lysosomes. The figure shows changes in average velocity and in duty percentage of mitochondria (A and B, respectively) and of lysosomes (C and D, respectively) after 5 and 15 min treatment with 10 μM valinomycin. Each column represents data from 5–6 independent experiments. *, different from control at p < 0.05, **, different from control at p < 0.005.
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DISCUSSION

It is well known that mitochondrial dysfunction, due to either environmental or genetic factors, is one of the primary events in several neurodegenerative diseases (10). Oxidative damage and dysfunction of mitochondria occur early in the Alzheimer disease brain, before the onset of significant plaque pathology (11). In Parkinson disease, several mutations in putative mitochondrial proteins leading to mitochondrial alterations have been described. The most frequent familial form of amyotrophic lateral sclerosis is related to mitochondrial damage; overexpression of the respective superoxide dismutase mutation in transgenic mice causes mitochondrial energy metabolism disturbances in brain (12). In Huntington disease, the mutant huntingtin seems to directly affect mitochondria as well. In general, mitochondrial dysfunction is related to mitochondrial depolarization, calcium accumulation, permeability transition pore opening, e.g. related to conditions that ultimately lead to mitochondrial swelling (5).

On the other hand, it is well known that the length, complexity, and slenderness of neuronal processes coupled with the amount of material that must be transported makes the axonal transport vulnerable to any kind of perturbation. Traffic jams are therefore thought to be involved in the pathogenesis of several neurodegenerative diseases. The molecular nature of these traffic jams is diverse and could involve transport proteins or aggregates blocking the axons. Recent findings of Goldstein and co-workers (13) suggest that disruption of axonal transport of vesicles, through lesions in APP or APP-interacting components, may result in axonal block and be a causative factor in the development of Alzheimer disease. Mutations in dynactin, a protein required for axonal transport, have been described in patients with motor neuron disease (14). Abnormalities in axonal transport have been suspected for some time in amyotrophic lateral sclerosis, and axonal transport defects have been observed in mouse models expressing mutant forms of superoxide dismutase 1 (SOD1) (15–17); in addition, large axonal swellings with neurofilament accumulations, consistent with a failure in axonal transport, are observed in patients with amyotrophic lateral sclerosis (18, 19). Recent data from several models of polyglutamine disease have suggested that abnormalities in axonal transport contribute to this pathology (20). Also, some other pathologies related to oversized and nonfunctional organelles (for example, paraplegia and drug-induced neuromopathies) are associated with impaired axonal transport (21).

Thus, here we propose that size and morphology of the transported cargo are also relevant for seamless axonal transport, and we speculate that mitochondrial swelling could be one of the reasons for impaired organelle transport in neuronal processes. Current data demonstrate that mitochondrial swellers increasing the diameter and volume of mitochondria inhibit mitochondrial as well as lysosomal traffic; all conditions leading to inhibition of organelle transport in processes also lead to a parallel increase in mitochondrial diameter. In all cases, the mitochondrial diameter increased from around 200 to 400–600 nm, which is larger than the average diameter of processes being around 200–300 nm. We demonstrate clearly that this inhibition is not related to cessation of the mitochondrial ATP production as proposed earlier (6, 22, 23); bongkrekic acid and oligomycin, inhibiting ATP transport from the mitochondrial matrix to cytosol and mitochondrial ATP synthetase, respectively, had no effect on mitochondrial or lysosomal traffic. The absence of their effect could be explained by earlier data demonstrating that glycolysis can support the function of cerebellar granule cells in vitro (24) and by the observations that molecular motors responsible for mitochondrial traffic have relatively high affinity to ATP (25). It is also unlikely that mitochondrial depolarization per se induced by swellers could affect the axonal traffic. Mitochondrial membrane potential could modify the mitochondrial binding to the motor proteins or motor protein activity but not lysosomal traffic directly. It is therefore most tempting to speculate that swollen mitochondria will physically block the passage of other sized organelles in neurites, and thus, inhibit the axonal organelle traffic. Several observations support that hypothesis; swollen mitochondria tend to block mitochondrial

FIGURE 5. Effect of valinomycin on lysosomal movement depends on neurite diameter. The figure shows average velocity (A) and duty percentage (B) of lysosomes after 15 min treatment with valinomycin (10 μM) in thin (340 ± 20 nm) and thick (730 ± 30 nm) neurites.

FIGURE 6. Depicted time series demonstrating lysosome-mitochondrion interaction. Note the LysoTraker Red-stained lysosome marked with an arrow (panel 0s, lower part), which starts to move and turns back after reaching to a swollen mitochondria in a neuron treated with antimycin (10 μM). Note also the lysosome (panel 0s, upper part) standing aside of swollen mitochondria.
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movement, and this interaction seemed to be less evident in bigger neurites.

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