

Mitostasis in Neurons: Maintaining Mitochondria in an Extended Cellular Architecture

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Neurons have more extended and complex shapes than other cells and consequently face a greater challenge in distributing and maintaining mitochondria throughout their arbors. Neurons can last a lifetime, but proteins turn over rapidly. Mitochondria, therefore, need constant rejuvenation no matter how far they are from the soma. Axonal transport of mitochondria and mitochondrial fission and fusion contribute to this rejuvenation, but local protein synthesis is also likely. Maintenance of a healthy mitochondrial population also requires the clearance of damaged proteins and organelles. This involves degradation of individual proteins, sequestration in mitochondria-derived vesicles, organelle degradation by mitophagy and macroautophagy, and in some cases transfer to glial cells. Both long-range transport and local processing are thus at work in achieving neuronal mitostasis—the maintenance of an appropriately distributed pool of healthy mitochondria for the duration of a neuron's life. Accordingly, defects in the processes that support mitostasis are significant contributors to neurodegenerative disorders.

Mitostasis in Neurons

Neurons face unique problems because of their exceptional architecture. To transmit electric signals rapidly between regions of the CNS and to and from peripheral organs, neurons have evolved long and highly branched processes. In humans, peripheral nerves and corticospinal tracts can have axons 1 m long. Moreover, because information processing requires extensive convergence and divergence of signals, the cytoplasmic volume of highly branched arbors easily dwarfs the volume of the neuronal cell body (Figure 1, center). In the human brain, individual neurons of the substantia nigra pars compacta are calculated to give rise to 4.5 m of axon, once all the branches are summed (Bolam and Pissadaki, 2012; Matsuda et al., 2009). A similar calculation for cholinergic basal forebrain and raphe neurons has predicted that over 100 m of axon can arise from a single soma (Wu et al., 2014).

However, a neuron is merely a cell and, like other cells, is constrained by the limitations and machinery of cells. Humans may live for 80 years, but most proteins survive only for days or weeks due to constant turnover by proteolytic digestion and ribosomal resynthesis (Goldberg, 2003). Because neurons are not dividing cells and need to last a human's lifetime, the discrepancy between cellular and protein turnover is particularly extreme. Moreover, as in every cell, nearly all neuronal proteins are encoded by genes in the nucleus, no matter how far that nucleus may be from the protein's site of action. Likewise, all cells need to transport components from one part of the cell to another by motor proteins that move along microtubule tracks, but the demands placed on those motors are more extreme in the context of highly extended and branched axons.

We focus here on one particular challenge for neuronal cell biology that we have termed neuronal mitostasis: how do neurons preserve a healthy, functioning pool of mitochondria throughout their arbor and throughout their lives? Mitostasis can be thought of as a specialized form of homeostasis, a mechanism by which mitochondrial number and quality are maintained over time in each compartment of the neuron. As mitochondrial proteins must, inevitably, be turned over and replaced, how is mitostasis accomplished? Vertebrate mtDNA encodes only 13 proteins (Alston et al., 2017), and the remainder of the >1,000 proteins found in mitochondria (Calvo et al., 2016) are encoded in the nucleus. How do those genes appropriately generate proteins that are required at the extremities of the cell? Because mitochondrial biogenesis involves mtDNA replication, mitochondria cannot be made de novo but must derive from other mitochondria. Where are mitochondria made, and where are they destroyed? What are the roles of mitochondrial movements, fusions, and fissions in this process? Neuronal mitostasis, though increasingly appreciated, still has many unsolved questions.

Neurons depend on mitochondria. ATP generation by mitochondrial respiration in the brain consumes 20% of the body's oxygen although the brain is only 2% of the mass of the body (Attwell and Laughlin, 2001). Maintaining resting potentials and firing action potentials are energetically expensive, as is neurotransmission on both the pre- and postsynaptic sides (Howarth et al., 2012). Although some of the ATP can be provided by glycolysis, mitochondrial respiration carries most of the burden (Rangaraju et al., 2014); this is why one rapidly loses consciousness if deprived of oxygen. Every part of the neuron requires ATP

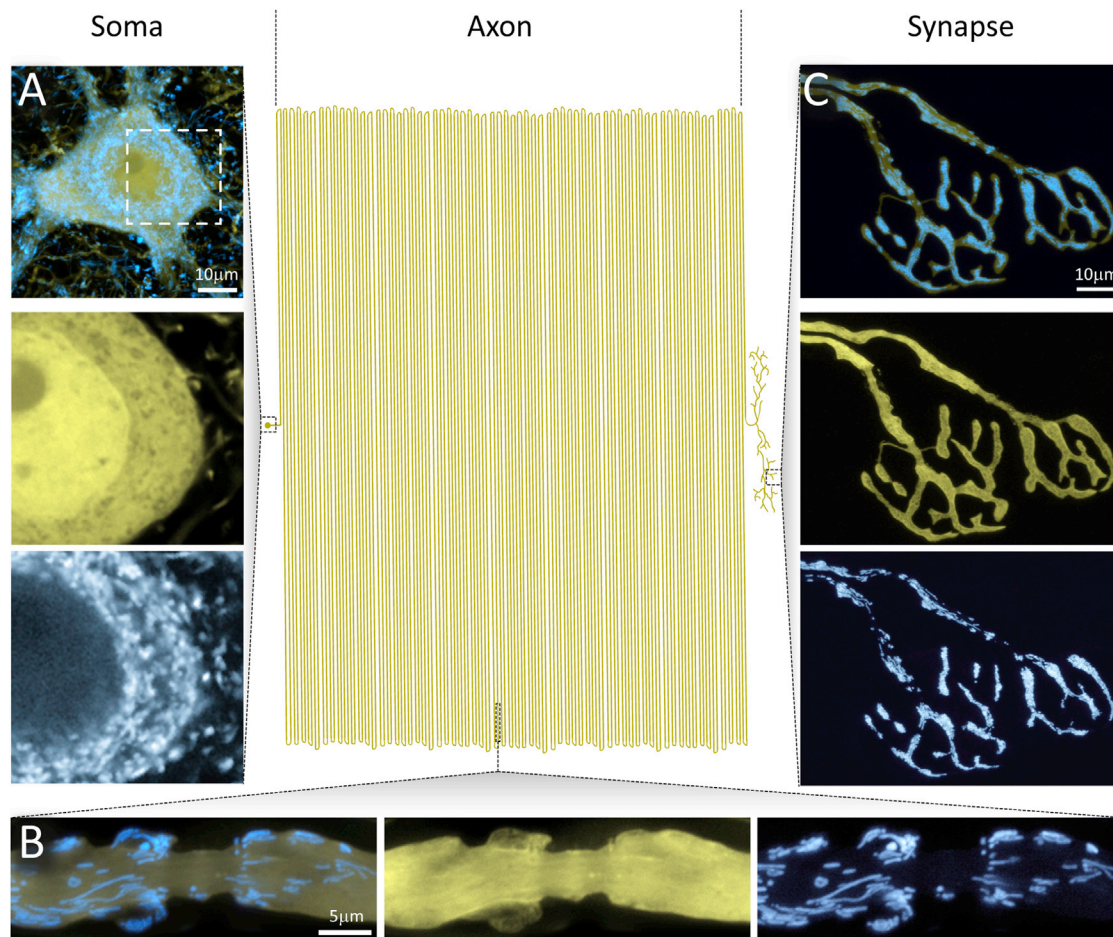


Figure 1. Neuronal Mitochondria in a Mouse Neuron

The extended geometry of neurons poses special challenges for maintaining mitostasis, here illustrated by a schematic attempt to draw a mammalian motor neuron to scale, with the soma to the left, the axon in the center, and the synapse-studded branches to the right (modified from Devor, 1999). A small soma (A) with a limited pool of mitochondria has to supply a vast axonal arbor that contains a far larger population of unevenly distributed mitochondria. At the same time, neuronal compartments with specific bioenergetic needs and hence accumulations of neuronal mitochondria, such as paranodes (B) and presynaptic terminals (C), need to be served.

and therefore requires mitochondria to be present. Therefore, some mitochondria need to be retained in the soma, while other mitochondria need to be trafficked to and positioned along axons and dendrites to be concentrated at energy-hungry sites, such as presynaptic terminals and near nodes of Ranvier (Figures 1A–1C and 2) (Berthold et al., 1993; Fabricius et al., 1993; Shepherd and Harris, 1998). In this regard, the transport of mitochondria poses a complex challenge for the neuron, because balancing energy supply and energy demand requires attention to the needs of each region of the neuron.

To understand mitostasis, therefore, it is necessary to understand the dynamics of mitochondria; their movements are crucial to both achieve an initial distribution in the cell and accomplish the resupply, as mitochondria and mitochondrial proteins are turned over. Mitochondria are governed by four dynamic processes: motility, anchoring, fission, and fusion (Figure 3). The movement of mitochondria is readily appreciated by video microscopy of mitochondria that have been fluorescently labeled either with a fluorescent dye, such as tetramethylrhodamine

(TMRM), or with a fluorescent protein targeted for import into mitochondria. As discussed in detail below, whether in tissue culture or *in vivo*, one population of mitochondria is in motion, while another is stationary (Plucińska and Misgeld, 2016; Saxton and Hollenbeck, 2012; Schwarz, 2013). The distinction between these two classes of mitochondria appears to be stable, reflecting two different pools; the motile mitochondria may temporarily pause but tend to continue onward and only infrequently alter their direction. Thus, some of the motile mitochondria are designated to move anterograde, down the axon, while others prefer retrograde movement; what creates this distinction is unknown. Mitochondria in the stationary pool, in contrast, remain in place often for as long as the experimenter can image (Faits et al., 2016; Lewis et al., 2016; Smit-Rigter et al., 2016). The movement of mitochondria is mediated by a motor adaptor complex that attaches the anterograde kinesin-1 motor (Kif5B in mammals) and retrograde dynein motor to the mitochondrial outer membrane (Figure 3A). The attachment is mediated by the membrane-anchored Miro (also called RhoT1/2) and its motor-binding

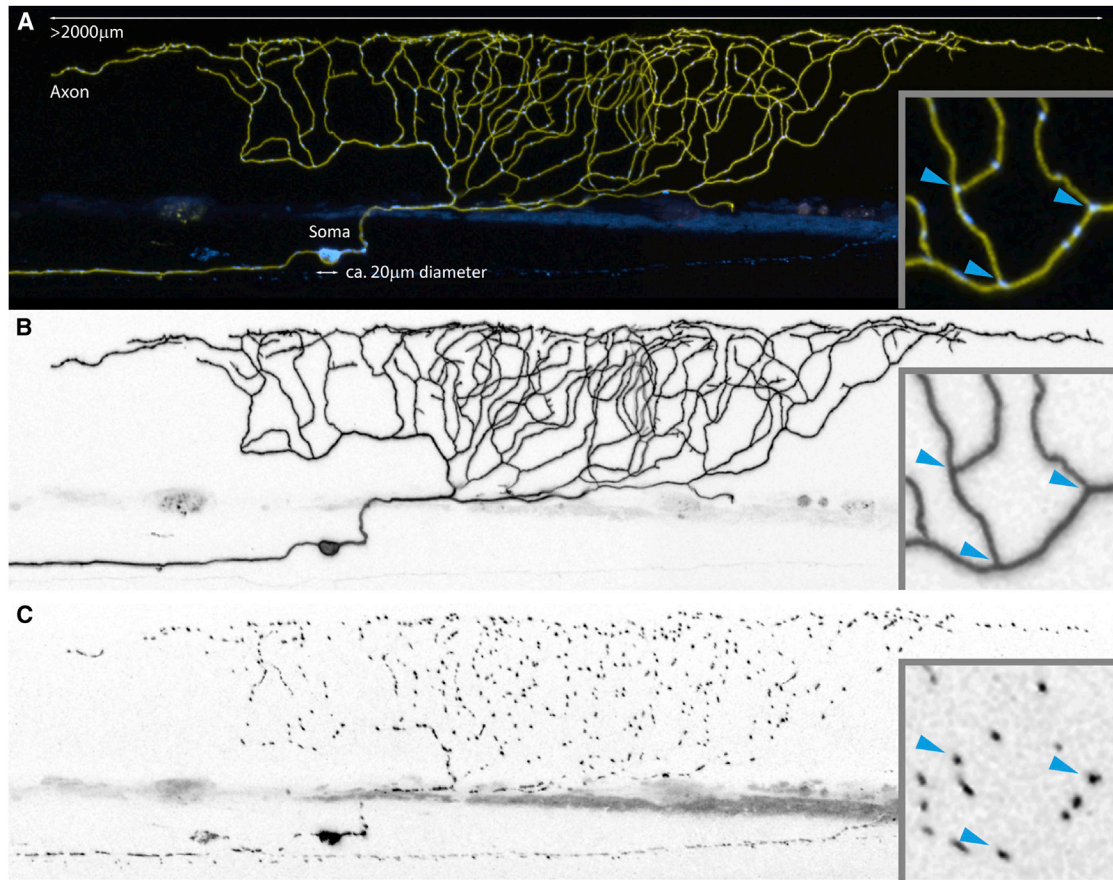


Figure 2. Neuronal Mitochondria in a Zebrafish Neuron

Smaller model organisms, such as flies, nematodes, and zebrafish larvae (shown here), allow for the study of mitostatic mechanisms in a slightly less complex *in vivo* environment than in mammals.

(A) A zebrafish sensory Rohon-Beard neuron (96 hr post-fertilization), which expresses a yellow fluorescent protein in the membrane and a cyan fluorescent protein in the mitochondrial matrix, providing an impression of the relative size of soma and axon (Plucińska et al., 2012).

(B and C) Optical accessibility allows entire axonal arbors (B) to be visualized and interrogated for mitochondrial distribution (C) and turnover. Even in these unmyelinated axons lacking synapses, mitochondria do not appear to be randomly distributed; for example, many mitochondria reside at branch points (blue arrowheads in inset).

partner, Milton (also called TRAK1/2) (Schwarz, 2013). This complex is present on all axonal mitochondria, even the stationary pool (Wang and Schwarz, 2009b). The stationary pool consists, at least in part, of mitochondria that are held in place by anchoring proteins. Syntaphilin, a protein attached to the outer mitochondrial membrane, is one such anchor and tethers the mitochondrion via a microtubule-binding domain (Kang et al., 2008). Mitochondria can also be anchored via the actin cytoskeleton (Pathak et al., 2010).

Fission and fusion are also likely to play an essential role in mitostasis (Figures 3B and 3C). In many cell types, mitochondria do not exist as small, distinct organelles but rather form a highly interconnected reticulum with contiguous inner and outer membranes and multiple nucleoids containing mtDNA (Braschi and McBride, 2010). This reticulum is highly dynamic, with ongoing divisions and reconnections. The balance of fission and fusion determines the length of mitochondria and is regulated by stress and nutrient availability (Wai and Langer, 2016). Interconnected mitochondria are also found in neuronal cell bodies, but in order

to enter the axon, a mitochondrion must undergo a fission reaction that frees it from the reticulum (Berthet et al., 2014; Fukumitsu et al., 2016; Verstreken et al., 2005). Fission is mediated by the GTPase Drp1 (dynamin-related protein 1), and in many cases, actin and contact with the endoplasmic reticulum (ER), together with the Drp1 receptor MFF (mitochondrial fission factor), initiate the process. The opposing fusion reaction is particularly likely to occur when mitochondria meet end to end (Labbé et al., 2014; Twig et al., 2010). Full fusion requires two separate events: outer membrane fusion mediated by mitofusins (MFN1/2) and inner membrane fusion mediated by Opa1 (optic atrophy 1). The importance of these processes for neurons is underscored by the neurological disorders caused by mutations in key components. Mitofusin 2 mutations cause Charcot-Marie-Tooth disease type 2A (Kijima et al., 2005; Züchner et al., 2004) and Opa1 mutations are responsible for autosomal-dominant optic atrophy (Alexander et al., 2000; Delettre et al., 2000; McFarland et al., 2010). Mutations of Drp1, MFN, and Trak1 (Milton) are very rare in humans but cause early lethality with broad

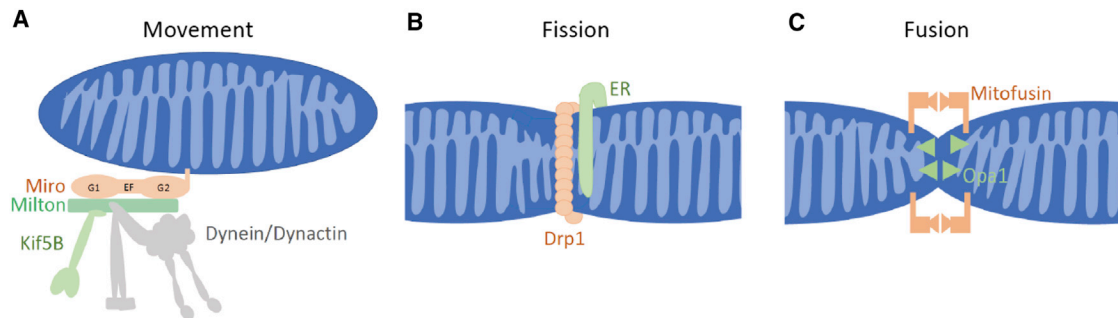


Figure 3. Mechanisms of Mitochondrial Dynamics in Neurons

(A) To move along microtubules in both the anterograde direction (toward the plus ends of microtubules) and the retrograde direction (toward minus ends), mitochondria employ a motor/adaptor complex that links Kif5b and the dynein/dynactin complex to the mitochondrial surface (Saxton and Hollenbeck, 2012; Schwarz, 2013).

(B) Fission of both the inner and outer mitochondrial membranes is mediated by a ring of the dynamin-like GTPase Drp1. In many cases, fission appears to be initiated by a contact with the endoplasmic reticulum (ER).

(C) Full fusion of mitochondria has two components. For the outer membranes to fuse, Mitofusins must be present on the membranes of both mitochondria, and their interaction initiates the fusion. The inner membranes undergo a separate fusion that is mediated by Opa1.

defects, including neurological conditions such as fetal encephalopathy and optic atrophy (Barel et al., 2017; McFarland et al., 2010).

The centrality of mitochondrial function, and consequently mitostasis, to neuronal health is further underscored by the prevalence of additional neurological disorders with a mitochondrial basis. These disorders fall in three categories: those that are directly due to defects in bioenergetics and metabolic functions, those that are quite closely linked to mitostatic processes such as mitochondrial clearance and dynamics, and those that indirectly influence mitostasis through broader defects in axonal transport or autophagy. The bioenergetic/metabolic disorders, which can arise from mutations in either mtDNA or nuclear DNA, are very heterogeneous in their presentation and often affect many organ systems; neurological syndromes are among the most frequent and serious (McFarland et al., 2010). Examples include many mutations in the complexes of the electron transport chain that result in encephalopathies, ataxia, and cerebellar atrophy. True mitostatic disorders, caused by mutations in the proteins that govern mitochondrial dynamics and mitophagy, result in diseases that range from severe developmental abnormalities to optic atrophy, peripheral neuropathy, and Parkinson's disease (Barel et al., 2017; Chen and Chan, 2006; Pickrell and Youle, 2015). Disorders that more broadly affect axonal transport, such as tauopathies and motor neuron disease, may include phenotypes with a significant mitochondrial etiology (De Vos et al., 2008; Millecamps and Julien, 2013). More recently, changes in mitostatic processes have also been described during neuroinflammation (Sadeghian et al., 2016; Sorbara et al., 2014; Witte et al., 2014) and after neurotrauma (Cartoni et al., 2016; Han et al., 2016; Sheng, 2017; Zhou et al., 2016). Such links to pathology rightly spur interest in understanding mitostasis.

The Problem of Mitostasis in Neurons

The challenge of maintaining a healthy and demand-matched pool of mitochondria in a neuron concerns the large number of neuronal mitochondria, as well as their residence at sites far

removed from the genomic information and biosynthetic hubs of the neuron's soma. The peripheral mass of mitochondria scales with the size of the neuron, and consequently with the size and developmental state of the organism. From our own measurements, we estimate that a typical sensory neuron in larval zebrafish maintains a pool of several hundred mitochondria in its periphery (Plucińska et al., 2012). Notably in an adult mouse, a single peripheral synapse—a neuromuscular junction—likely contains a similar number of mitochondria (Misgeld et al., 2007; T.M., unpublished data). This implies that the synaptic compartment of a moderately sized murine motor unit of 50 synapses (Keller-Peck et al., 2001; Lu et al., 2009) contains several tens of thousands of mitochondria. The length of the axon, which even in axial muscles of small rodents can add up to several centimeters (Lu et al., 2009) and typically contains 3 mitochondria per micrometer (S. Hanan and T.M., unpublished data), adds a similar mitochondrial mass on top. Finally, if one considers the numbers for diffuse projecting axons in the CNS, which might have added branch lengths of several tens of centimeters even in mice, and then scale corrections for larger organisms, such as humans, the number of mitochondria maintained by a <math><100\text{-}\mu\text{m}</math> neuron soma easily grows into the millions. This suggests that the mitostatic mechanism of a neuron must have substantial capacity and is scalable across orders of magnitude. A single neuron of the human substantia nigra, for example, has been estimated to form 1–2 million striatal synapses (Bolam and Pissadaki, 2012). If mitochondria are present at almost half the synapses (Shepherd and Harris, 1998) and throughout several meters of axon, then an estimated population of 2 million mitochondria per cell is reasonable.

Such geometrical considerations apply not only to organelle numbers but also to the distances that a mitostatic system in neurons has to cover. The number of 1 m of axon length from soma to synapse for human neurons is often cited. Even larger organisms, such as blue whales or giraffes, possess much longer projection neurons. Moreover, neurons are not only long but also branched, in a pattern that in its details at least in vertebrates is likely an imprint of the cell's developmental history

Table 1. *In Vivo* Parameters of Mitochondrial Transport

Species/preparation	% Mov	Flux a/r	Length a/r	Speed a	Speed r	Reference
<i>Homarus americanus</i> leg nerve	–	>1	0.80	0.72	1.33	Forman et al., 1987
<i>C. elegans</i> PLML or PLMR neurons	25%	–	–	–	–	Fatouros et al., 2012
<i>Drosophila</i> larva intersegmental nerve	43%	1.5	0.84	0.26	0.45	Pilling et al., 2006
<i>Drosophila</i> larva segmental nerve	–	1.1	–	0.08	0.07	Baqri et al., 2009
<i>Drosophila</i> larva segmental nerve	26%	1.3	–	–	–	Devireddy et al., 2015
<i>Drosophila</i> larva L3 peripheral nerve	35%	–	–	–	–	Avery et al., 2012
<i>D. rerio</i> Rohon Beard neurons	17%	1.7	0.96	0.68	0.73	Plucińska et al., 2012
<i>D. rerio</i> Rohon Beard neurons	23%	–	–	0.5	0.69	O'Donnell et al., 2013
<i>D. rerio</i> Rohon Beard neurons	27%	2.3	–	–	–	O'Donnell et al., 2014
<i>M. musculus</i> intercostal nerve	13%	1.9	0.87	1.02	1.41	Misgeld et al., 2007
<i>M. musculus</i> dorsal column axons	–	3.9	–	0.7	0.4	Sorbara et al., 2014
<i>M. musculus</i> tibialis nerve	–	3.2	–	0.6	0.5	Gilley et al., 2012
<i>M. musculus</i> retina	–	1.3	–	–	–	Takahara et al., 2015
<i>M. musculus</i> sciatic nerve	–	2.7	–	0.4	0.4	Bilsland et al., 2010
<i>M. musculus</i> sciatic nerve	22%	–	–	–	–	Magrané et al., 2014
Summary	26%	2.1	0.9	0.6	0.7	–

Compilation of *in vivo* values measured for basic mitochondrial transport parameter such as moving fraction (% mov), anterograde-to-retrograde ratio (flux a/r), length (length a/r), and anterograde and retrograde speed (speed a and speed r). Note, for comparison, corresponding average values as measured in rodent cell culture systems: motile fraction, 31% ± 8% (mean ± SD); anterograde-to-retrograde ratio: 1.3 ± 0.5.

A, anterograde; r, retrograde.

(Lu et al., 2009). Such developmental remodeling results in arbors that are highly irregular, not predicated in genomic information, and not easily captured by any idealized geometric description. The neuron needs to supply each of these branches, as well as the synapses that exist along their length, with an adequate number of organelles to ensure mitostatic stability. Together, these geometrical features (overall size, linear extent, and irregular branching) raise three conundrums that a comprehensive description of mitostatic regulation in neurons needs to account for the following three problems:

- (1) The problem of “sufficient supply.” The fact that mitochondria show substantial anterograde transport has led to the notion that most mitochondrial biosynthesis happens in the soma; after all, in vertebrates, the vast majority (~99%) of mitochondrial proteins are encoded in the nuclear genome. Are the biosynthetic capacity of the soma and the rate of mitochondrial export sufficient to maintain all peripheral mitochondrial compartments? If not, how are peripheral mitochondria supplied?
- (2) The problem of “long commutes.” The distance between the soma and the synapses of neurons in many species, together with the measured speed of mitochondrial transport, suggest that the journey of a mitochondrion from soma to synapse in the larger neurons of many species might exceed the available estimates of the lifetimes of mitochondrial proteins. How does a mitochondrion maintain its protein composition and stoichiometry as it travels out toward the neuronal periphery?
- (3) The problem of “local demand matching.” The number and size of synapses supplied by a given axonal sub-arbor is not easily predicted and probably subject to change

by synapse remodeling and long-term plasticity. How is the provision of mitochondria matched to these local and shifting demands?

The answers to these questions largely lie in the details of how mitochondrial dynamics, particularly the long-distance transport of mitochondria, work in a neurons.

How (Much) Do Mitochondria Move *In Vivo*?

In early studies, the size and shape of dynamic organelles seen by Nomarski contrast (e.g., in isolated frog axons; Cooper and Smith, 1974) suggested that many neuronal mitochondria are on the move. The specific dye (and later genetic) labeling of mitochondria then revealed in neuronal cell cultures the basic translocation behaviors of this organelle (Chang and Reynolds, 2006; Saxton and Hollenbeck, 2012), with a dominant pool of persistently stationary mitochondria and a smaller proportion that move (10%–40%; Table 1 for references). Moreover, mitochondrial transport was shown to be, on the one hand, dependent on the same motors and microtubules that characterize axonal transport of other organelles (Figure 3). On the other hand, the detailed characteristics of mitochondrial transport in neurons were found to be unique. First, there are mitochondria that move in either direction (anterograde and retrograde) with few apparent reversals of directionality mid-axon. Second, there are pauses interspersed in the phases of sustained translocation, resulting in average speeds (0.25–1 μm/s; Table 1 for references) that are slower than the classical fast transport speeds of other organelles, even though substantial variability exists in the reported parameters, likely due to technical differences in the methods used to measure transport (Figure 4). Third, in addition to transport, mitochondria show other dynamic behaviors, fusion

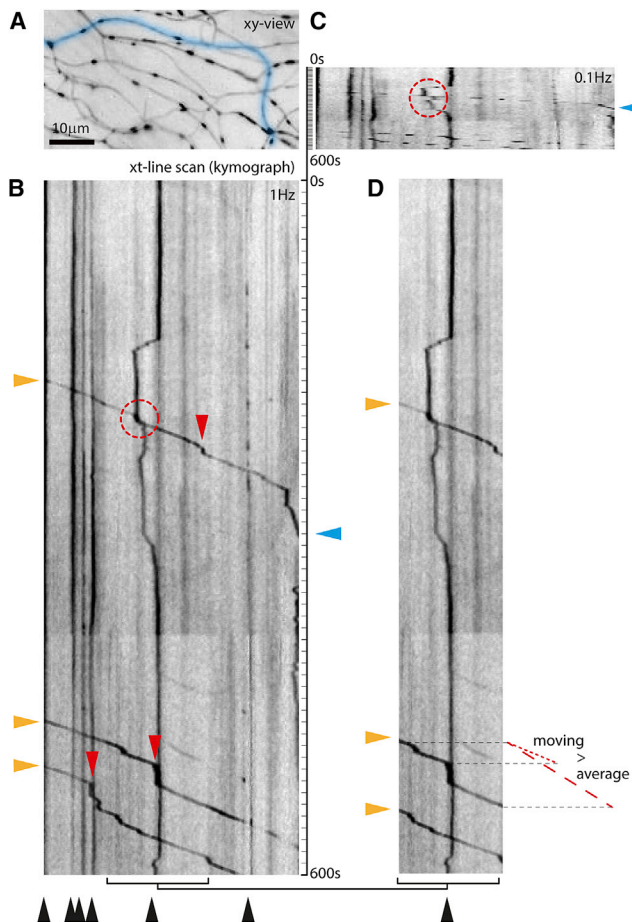


Figure 4. Challenges in Measuring Mitochondrial Movement

The parameters of motility are often difficult to compare, as different studies may choose to quantify the flux through a point, the percentage of mitochondria that move, or the fraction of time that mitochondria spend in motion. Moreover, parameters that would appear to be fundamental properties are in fact operationally defined. The imaging protocol can affect analysis of kymographs from sensory axons in the skin of larval zebrafish (Plucińska et al., 2012).

(A) xy view of the region of interest imaged at 1 Hz.

(B) A fully resolved kymograph along the blue line in (A) reveals different mitochondrial populations: stationary as vertical lines (black arrowheads) versus moving as angled lines (three anterograde mitochondria; orange arrowheads), with short vertical segments (i.e., pauses; red arrowheads). Such pauses explain the difference between average speed (dashed red line next to D) and “moving” speed (excluding pauses; dotted red line).

(C) Temporal under-sampling (0.1 Hz was mimicked) leads to broken tracks that are difficult to follow (red circle) and obscures small pauses (compare track with cyan triangle).

(D) The size of the field of view can affect the measured motile fraction (3 out of 9 mitochondria [33%] in B versus 3 out of 4 mitochondria [75%] in D), as can the recording length because the stationary count will stay constant as moving mitochondria continue to pass through the field (not depicted; consider the motile fraction in the upper or lower half of B [11% and 25%, respectively]). Together, these sampling discrepancies and signal-to-noise and movement artifacts explain some of the reported ranges of transport parameters (Table 1). Not illustrated here are the effects of axonal branching or fission and fusion, which further complicate such measurements.

and fission, that complicate analysis of mitochondrial transport, as a traveling organelle’s individuality can be abolished by interaction with other mitochondria (Owens and Walcott, 2012).

Finally, the existence of pools of mitochondria associated with specific neuronal compartments, such as presynaptic boutons and around nodes of Ranvier, suggests a unique layer of regulation related to a neuron’s activity state, a notion that was confirmed by manipulations that globally or locally alter electrical activity or membrane potential (Chang and Reynolds, 2006).

Thus, *in vitro* observations have been able to paint a consistent picture of how neuronal mitochondria can behave and have allowed fundamental cell biological and mechanistic analysis. However, such experiments are typically done in neurons of embryonic origin that exhibit a rather unrealistic geometry and are embedded in networks of unknown functional significance. Thus, how neuronal mitochondria behave *in vivo* has also required attention. The last decade has seen substantial progress to address this question, based on experimental paradigms that allow monitoring mitochondrial dynamics *in vivo* in the peripheral nervous system and CNS of a wide range of species, including nematodes (Fatouros et al., 2012; Rawson et al., 2014; Williams et al., 2013), fruit flies (Babic et al., 2015; Pilling et al., 2006; Vagnoni and Bullock, 2016; Wang and Schwarz, 2009a), zebrafish (O’Donnell et al., 2013; Plucińska et al., 2012), and mice (Chandrasekaran et al., 2006; Misgeld et al., 2007). Reassuringly, the basic tenets of mitochondrial dynamics derived from *in vitro* work have been confirmed *in vivo*. Beyond this, however, the *in vivo* work has refined many aspects of the canonical *in vitro* description, raised a number of new questions, and opened up studies in complex developmental and disease contexts that could not be modeled *in vitro*.

First, there have been seemingly conflicting reports on the degree to which mitochondria move *in vivo*. On the one hand, a substantial body of studies has shown that about 10%–40% of the mitochondria in axons near the neuronal soma are moving at any given time, even under conditions in which neuronal activity is either intrinsically absent (due to surgical isolation) or suppressed by anesthesia (Bilsland et al., 2010; Gilley et al., 2012; Misgeld et al., 2007; Sorbara et al., 2014; Takihara et al., 2015). However, some other studies have suggested that baseline mitochondrial flux in fully mature neurites would be low, with a steep developmental decline (Faits et al., 2016; Lewis et al., 2016; Smit-Rigter et al., 2016). However, a full appreciation of the effects of branched neuronal geometry on local flux rates reconciles these seemingly conflicting reports. In some systems, measurements are possible in proximal axonal locations, where flux reflects the totality of moving mitochondria needed to supply the arbor, while in others, due to imaging constraints, only very distal parts of the arbor can be visualized. Each time an axon bifurcates, the number of moving mitochondria per branch will be halved, and therefore, in each fine terminal branch, the moving pool will be a very small fraction of what once left the soma. Another methodological constraint has to do with the way in which the moving pool is determined, either as an absolute flux or as a percentage of all mitochondria. Clearly, the latter, as a relative measurement, depends on the size of the stationary pool. In developing arbors, this percentage might be dropping as a function of an increasing denominator as mitochondria accumulate, without a major change in the absolute numbers of mitochondria entering the axon. Similarly, in different neuronal compartments (e.g., with a gradient along the axon), the size of

the stationary pool might vary, again resulting in different values for the moving fraction despite consistent flux.

In parallel to determining the characteristics of baseline transport, a number of studies have attempted to determine the effect of *in vivo* activity on mitochondrial transport and positioning. Somewhat surprisingly, the majority of *in vivo* studies have failed to detect acute effects of activity (either spontaneous or imposed) and plasticity-inducing manipulations on bulk transport rates (Faits et al., 2016; Smit-Rigter et al., 2016). This questions the prevailing notion of neuronal activity as a major driver of baseline transport (but cf. Sajic et al., 2013; Zhang et al., 2010) and rather points to a model in which activity-related effects are subtle and chronic, with other factors more likely determining the predilection sites of mitochondrial residence in synapses and around nodes. The anchoring that creates the stationary pool is thus distinct from the calcium sensitivity of the Miro adaptor, which will temporarily arrest mitochondria *in vitro* (Macaskill et al., 2009; Wang and Schwarz, 2009b), but neither contributes to the distinction between stationary and motile pools nor appears to be readily activated by variations in firing rate *in vivo*.

Taken together, it is probably fair to say that work in intact preparations has confirmed the presence of a consistent and steady stream of mitochondria into and from the axonal arbor. Regarding the “sufficient supply” problem, for cell types where measurements near or at the axon hillock were possible, rough calculations suggest that the measured flux could sustain a turnover rate of the distal stationary mitochondria on the order of several days (Misgeld et al., 2007; Plucińska et al., 2012). Such a lifetime for mitochondria seems consistent with previous estimates based on proteome dynamics in the brain (Price et al., 2010; Vincow et al., 2013), even though the ability of mitochondria to fuse and divide, probably in a manner that does not evenly distribute molecular content, complicates the notion of a single “lifetime” for the entire organelle. There remains the puzzle, however, how neurons with more extreme geometries (such as the diffusely projecting neuromodulatory neurons mentioned above) or in species larger than small experimental animals establish flux sufficient to sustain their distal mitochondrial population. Their estimated arbor size suggests flux rates in the order of several tens of mitochondria per minute, a value well above the 5–10 mitochondria that enter a murine motor neuron axon every minute. It will be informative to see such measurements from more cell types (e.g., with endoscopic imaging probes) to reveal whether the same transport rules apply across all arbor sizes (but scaled up for enormous mitochondrial flux). Alternatively, extreme neurons could have found “work-arounds,” such as by altering other parameters, including mitochondrial lifetimes or dependence on long-distance replacement.

Similar considerations apply regarding the long-commute problem. For a small model organism, the observed average transport speed ($\sim 0.5 \mu\text{m/s}$) leads to reasonable travel times (e.g., ~ 1 hr from soma to synapse for fly segmental nerves but ~ 10 hr for the same trip for mouse intercostal nerves). For larger neurons and species, however, such calculations yield less reasonable results, as they exceed the lifetimes of short-lived nuclear-encoded mitochondrial proteins (Price et al., 2010; Vincow et al., 2013). One solution to this conundrum can be found in the ample evidence for the axonal presence of transcripts for mito-

chondrial proteins not only *in vitro* (Aschrafi et al., 2016) but also in mature neurons *in vivo* (Shigeoka et al., 2016), which is compatible with a model of peripheral mitochondrial maintenance by local protein synthesis. Such a model essentially shifts the cell biological problem toward the transport, lifetime, and persistence of mRNAs and axonal ribosomes. Indeed, the biosynthetic machinery needed to make mitochondrial proteins is relatively simple compared to, for example, transmembrane proteins (which in addition to ribosomes require rough ER and Golgi outposts). The amplification inherent in translational outposts eases the concern that the soma alone could sustain such a vast distal organelle population. In addition, some mitochondrial proteins are imported into the organelle prior to their folding, and therefore, import needs to follow rapidly after their synthesis on cytoplasmic ribosomes or the unfolded state needs to be stabilized by chaperones (Ahmed and Fisher, 2009). Transport of mRNA for mitochondrial proteins may hold advantages for the cell over transport of the protein itself because of the amplification afforded, the possibility of co-translational import, and the ability to deliver proteins with half-lives too short to survive transport down axons. Local protein synthesis also raises the possibility of the generation of entire fresh mitochondria in distal axon locations, which would require not only the availability and assembly of all mitochondrial proteins but also the machinery for mitochondrial DNA replication and lipid biogenesis. Indeed, at least *in vitro*, metabolic labeling with nucleotide homologs confirmed the peripheral replication of mtDNA (Amiri and Hollenbeck, 2008). Moreover, the presence of smooth ER in axons (Wu et al., 2017; Yalçın et al., 2017) signifies that the capacity for lipid synthesis is also locally available.

Another pathway of supplying the neuronal periphery with mitochondria is organelle transfer from other cells. It is becoming clear that many metabolic pathways are shared between axons and glia by exchange of specific metabolites across the extracellular space (Saab et al., 2013). This concept has been expanded to the exchange of entire organelles, such as ribosomes (Court et al., 2008). There is a growing body of evidence that mitochondria can also be exchanged between cells, either via local cell fusions and “nanotubes” or via microvesicles that contain organelles (Sinha et al., 2016). While there are first reports that this might have functional implications in disease settings, such as stroke (Berridge et al., 2016), and could contribute to mitochondria clearance in specific anatomical locations (Davis et al., 2014), it appears unlikely that mitochondrial exchange is a general feature that significantly contributes to mitostasis. Numerous experiments have taken advantage of cell-type-specific expression of fluorescent mitochondrial proteins, and the fluorescent mitochondria are not seen spreading out from the cells in which they are expressed (Misgeld et al., 2007; Plucińska et al., 2012), suggesting that such intercellular transfer is rare, restricted to specific situations, or followed by swift removal.

The supply of mitochondria to axons, therefore, can be either a canonical source (synthesis in the soma and transport) or a non-canonical source (local synthesis or transfer from an adjacent cell). How do these mechanisms relate to the majority of axonal and synaptic mitochondria, which appear to be completely stationary and yet must somehow be turned over or rejuvenated? *In vivo* observations have shown that stationary mitochondria

are so sparse that lateral fusion is not happening in axons on the timescale characteristic of other cells, suggesting that they are turned over individually. At least three models could explain the rejuvenation of the stationary pool. (1) In the “changing of the guard” model, stationary mitochondria may pick up and leave their guard stations, perhaps once every several weeks, to undergo mitophagy and then be replaced by a new arrival from the motile pool. This would be such an infrequent event that it would be rarely observed in most imaging protocols. (2) In the “space station” model, they are undergoing rejuvenation by fusion with the motile pool in much the same way that deliveries by rockets from earth can keep the space station resupplied. This would require a low fusion probability for motile mitochondria as they move through the axon, because otherwise, the most proximal mitochondria would get all the deliveries, leaving none to arrive at the periphery. In imaging experiments of axonal mitochondria, some fusion events are observed, but they are indeed infrequent, as expected. (3) In the “locally grown produce” model, mitochondria undergo constant low-level rejuvenation from local sources, such as axonal protein synthesis. Of course, all of these mechanisms may operate in parallel, and only careful (and difficult) quantification of rates of re-mobilization, fusion, and protein synthesis will sort out their contributions.

The non-canonical mechanisms of mitochondrial provision to axonal arbors also suggest that a straightforward accounting approach to mitostasis, by counting and measuring mitochondria as they come and go from the cell body, is too simplistic a model of the life cycle of a mitochondrion and of mitostasis. Not all mitochondrial mass needs to arise in the soma, and, as reviewed in the next section, not all mitochondrial mass needs to be degraded in the soma. Nevertheless, if one focuses on transport to and from the soma, an important difference emerges. Almost all *in vivo* studies have noted that more mitochondria are delivered to, then retrieved from, the axon, even if size differences are taken into consideration (ratio mean 2.1; ranges 1.1–3.9 *in vivo* and intact preparations; notably, this effect is less consistent *in vitro*; see Table 1 for details and references). Although, in some cases, this could be due to axonal growth requiring a net addition of axonal mitochondria, measurements in fully grown organisms find a similar discrepancy. If, as now seems likely, there is also substantial mitochondrial biogenesis in axons, the discrepancy between their supply and retrieval in the soma is yet greater and implies that the peripheral removal of mitochondria from axons and synapses is substantial.

How Are Mitochondria Cleared?

Though the question of mitochondrial supply to axons is critical, the “how” and “where” of mitochondrial clearance and protein turnover are no less significant. As Fred Goldberg has pointed out, cells are not a safe place for a protein; no competent biochemist would choose to store a protein at 37°C instead of the freezer or leave it in the presence of potentially harmful cellular components (Goldberg, 2003). Proteins are prone to misfolding, denaturation, oxidation, and glycation and therefore must be regularly degraded and replaced to preserve the quality of the cellular machinery. Mitochondrial proteins are no exception, and mitochondrial proteins may be at greater risk of damage, because the electron transport chain and other mitochon-

drial processes are probably producing most of the reactive oxygen species in the cell (Andreyev et al., 2015). Therefore, mitochondrial proteins need to be degraded as neuronal mitochondria are refreshed. In non-neuronal cells, many pathways have been identified for the turnover of mitochondria and their proteins. Although less work has been done in neurons, they are likely to use the same pathways. These pathways differ in their capacity; they can remove proteins individually, remove a small portion of a mitochondrion, remove a large fragment or an entire mitochondrion, or sweep up mitochondria together with other cellular components as part of a broad macroautophagic process (Figure 5).

Individual mitochondrial proteins can be degraded by proteases resident within the mitochondrion. The most significant class for the removal of misfolded and denatured proteins is the mitochondrial AAA+ proteases (Glynn, 2017). These barrel-like proteases are present in the matrix and also in the inner mitochondrial membrane, facing both matrix and intermembrane space. They will recognize the hydrophobic domains that are exposed on misfolded and oxidized proteins and efficiently unfold and degrade them. The significance of these proteases for mitochondrial quality control in neurons is clear from the neurologic phenotypes associated with mutations in their genes. These phenotypes include non-syndromic mental retardation, spastic paraplegia, and spastic and spinocerebellar ataxias (Levytsky et al., 2017). When oxidative damage has occurred, this mode of protein turnover may be considered the mitochondrion’s first line of defense, capable of removing a potentially problematic protein as soon as it forms. Indeed, the mitochondrial proteases in neurons may be commandeered for non-mitochondrial quality control; misfolded cytosolic proteins can also be imported into mitochondria for degradation (Ruan et al., 2017).

Mitochondria-derived vesicles (MDVs) represent a degradative pathway with the potential to remove large assemblies of protein and lipid rather than individual molecules (Roberts et al., 2016; Shlevkov and Schwarz, 2014) and, as such, may become particularly important when a stressor causes a local accumulation of damaged proteins. MDVs are particles that bud off from the mitochondrion and can then be targeted to either peroxisomes (Neuspiel et al., 2008), lysosomes (McLelland et al., 2014; Soubannier et al., 2012), or multivesicular bodies and exocytosis (Matheoud et al., 2016). They are a diverse population whose subtypes are still being clarified. As they form, they can select for distinct components of the outer mitochondrial membrane or include inner membrane and matrix proteins as well. Different forms of oxidative or heat stress can induce different subtypes of MDVs (Matheoud et al., 2016; McLelland et al., 2014; Soubannier et al., 2012). Much remains to be learned about the triggers of MDV formation, the mechanisms by which they form, and how they select their cargo. Formation of at least some MDVs depends on the Parkinson’s disease proteins PINK1 and Parkin (discussed further below) and also Drp1, the GTPase that mediates mitochondrial fission (Figure 3). Other MDVs are Drp1 independent but require Rab9 and the sorting nexin SNX-9. However, MDV production has been studied almost exclusively in cell lines, and its importance for neurons, though likely, remains to be demonstrated.

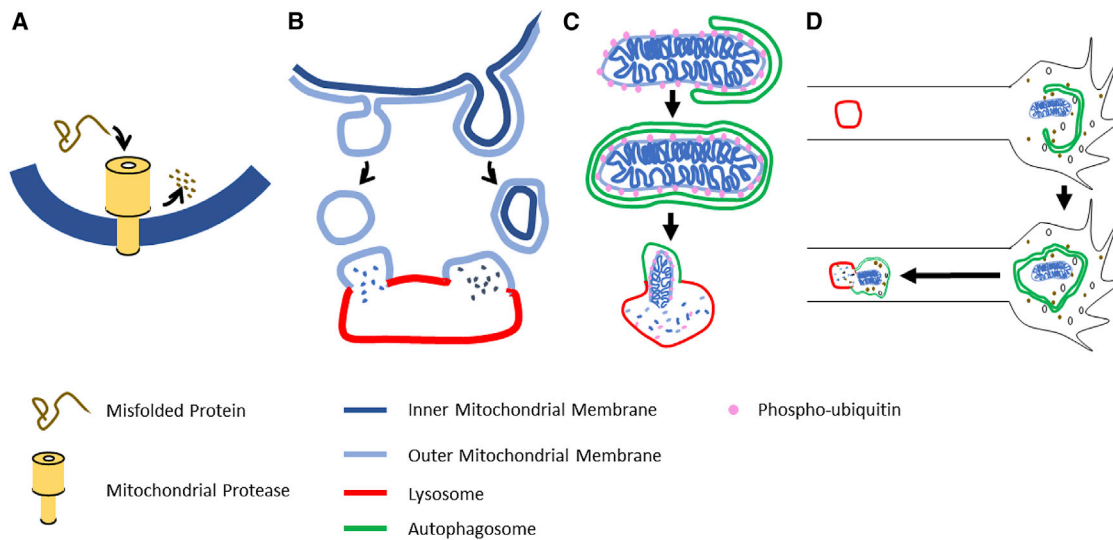


Figure 5. Mechanisms of Mitochondrial Protein Turnover

Mitochondrial quality is preserved by four mechanisms illustrated in order of their increasing scale.

(A) Individual misfolded or damaged proteins are degraded by any of several mitochondrial proteases.

(B) Mitochondrial components can be shed from the organelle in MDVs that bud from the organelle and can contain either only outer membrane components or also components of the inner membrane and matrix.

(C) Mitophagy can engulf mitochondria, whose membrane potential or protein misfolding has triggered the PINK1/Parkin pathway to add phospho-ubiquitin to the outer membrane and thereby induce the formation of autophagosomal membranes. The autophagosome will subsequently fuse with a lysosome.

(D) Macroautophagy occurs in growth cones and can sweep up mitochondria along with other cytoplasmic components. Once formed, the autophagosome moves retrograde and fuses with lysosomes in the axon.

In contrast to the pathways mentioned above, mitophagy targets an entire mitochondrion or fragmented mitochondrion for destruction. Mitophagy is frequently studied in the context of severe damage to mitochondria (such as application of agents that dissipate the mitochondrial membrane potential) or an exceptional need to reduce mitochondrial content (such as hypoxia or erythrocyte maturation). The extent to which mitophagy figures in the normal, on-going turnover of mitochondria is less certain. Mitophagy is a highly specialized form of autophagy and has three necessary stages: recognition of the mitochondrion to be targeted, formation of an autophagic membrane around the organelle, and fusion of the resulting mitoautophagosome with a lysosome for degradation. The formation of autophagic membranes and trafficking to lysosomes employs mechanisms shared by all forms of autophagy and is reviewed elsewhere (Yin et al., 2016). Mitophagy, however, requires specialized mechanisms for the targeting stage that identify a mitochondrion that requires clearance and then recruit to it the autophagosomal membranes. One key pathway involves PINK1 and Parkin, proteins whose importance in mitochondrial quality control was first demonstrated in *Drosophila* and provided a strong indication that mitochondria play a key role in the etiology of Parkinson's disease (Clark et al., 2006; Park et al., 2006; Poole et al., 2008; Yang et al., 2008). Subsequent studies from many labs have illuminated a pathway by which PINK1, a protein kinase, serves as a sensor of mitochondrial quality and can trigger subsequent Parkin-dependent recruitment of LC-3 (Greene et al., 2012; Heo et al., 2015; Lazarou et al., 2015; McWilliams and Muqit, 2017; Pickrell and Youle, 2015; Wong and Holzbaur, 2014). In this schema, PINK1 un-

dergoes constant synthesis, import into mitochondria, and proteolysis by mitochondrial enzymes that keep the pathway in an "off" state. Because import across the inner mitochondrial membrane is driven by the mitochondrial membrane potential, and because misfolding of proteins can also block import, the ability to import (and thereby disable) PINK1 depends on mitochondrial health. If the mitochondrion loses its membrane potential or builds up misfolded proteins, PINK1 will instead be stabilized on the outer surface of the mitochondrion and trigger mitophagy. The events downstream of PINK1 stabilization include phosphorylation of Miro and mitofusin (Figure 3), phosphorylation (and activation) of Parkin, an E3 ubiquitin ligase, and phosphorylation of ubiquitin. Parkin binds to both phospho-Miro and phospho-ubiquitin and is thereby recruited to the mitochondrial surface (Heo et al., 2015; Kane et al., 2014; Lazarou et al., 2015; McWilliams and Muqit, 2017; Shlevkov et al., 2016; Wong and Holzbaur, 2014). The resulting positive feedback loop results in extensive addition of phospho-ubiquitin to the mitochondrial surface. Phospho-ubiquitin in turn binds one of several mitophagy adaptors that recruit the autophagosomal membrane so that engulfment can proceed. It should be noted that this pathway is heavily dependent on the presence of ATP, due to the constant need for PINK1 synthesis and for phosphorylation of Parkin and ubiquitin. Experimental conditions that poison all the mitochondria in a neuron will therefore also likely shut down the PINK1/Parkin pathway to mitophagy (Cai et al., 2012; Van Laar et al., 2011). There are additional routes to mitophagy that are not dependent on either PINK1 or Parkin or both (Roberts et al., 2016), and the relative contribution and extent of redundancy of these alternatives is not clear (Devireddy et al.,

2015; Sung et al., 2016), nor are the actions of PINK1 and Parkin necessarily restricted to triggering mitophagy (Johnson et al., 2012; Morais et al., 2014; Müller-Rischart et al., 2013). In particular, as mentioned above, they can also mediate formation of MDVs, and it is not known what determines the choice between MDV formation and full-fledged mitophagy.

In parallel with mitophagy, cells have an ongoing process of macroautophagy in which sections of cytoplasm are engulfed by autophagosomes. A basal rate of macroautophagy is present in all cells, but it is also subject to regulation, including by amino acid availability and the mTOR pathway (Yin et al., 2016). Macroautophagy is not an intrinsically selective process, and therefore no special receptors for mitochondria are needed; however, mitochondria, or fragments of mitochondria, can be swept up alongside other cytoplasmic components. Macroautophagy is thus another process by which mitochondrial proteins can be delivered to lysosomes and degraded. Though not selective for oxidized proteins or depolarized mitochondria, degradation upon macroautophagy can nonetheless contribute to protein turnover and thereby enhance mitochondrial quality.

To what extent do mitophagy and macroautophagy figure in neurons? The relevance of the PINK1/Parkin pathway in neurons is apparent from the neurodegenerative phenotype of early-onset Parkinson's caused by mutations in those genes. Moreover, when mitophagy is triggered, either by application of antimycin A to axons or by the production of reactive oxygen species by photo-bleaching of mito-KillerRed, autophagosomes are recruited to the axonal mitochondria in a manner dependent on both PINK1 and Parkin (Ashrafi et al., 2014). In growth cones of cultured neurons, macroautophagy is very active and can include engulfment of mitochondria (Fu et al., 2014; Maday and Holzbaur, 2014). Less clear at present is the contribution of PINK1, Parkin, and autophagy to the overall maintenance of mitochondrial quality. In *Drosophila*, loss of PINK1 decreases the membrane potential of axonal mitochondria and causes structural abnormalities to the organelles (Devireddy et al., 2015), but knockout of PINK1 and Parkin in rodent models causes little or no neurodegeneration. Patients carrying these mutations generally do not have the onset of symptoms for three or four decades. Taken at face value, the relatively greater severity of neurologic phenotypes when the mitochondrial AAA proteases are mutated suggests that mitophagy is the less important pathway, at least in mammals, and potentially triggered only by exceptional levels of mitochondrial damage. Defects in macroautophagy can produce neurological disorders (Yin et al., 2016), but to what extent these arise from altered mitostasis rather than aggregation of misfolded proteins remains unclear.

Further evidence that mitophagy and autophagy are not the major route of turnover for mitochondrial proteins was obtained in a study of the half-lives of mitochondrial proteins in *Drosophila* neurons (Vincow et al., 2013). This study found that half-lives varied widely from protein to protein, and this was not consistent with a mechanism in which all of the contents of a mitochondrion were turned over simultaneously by engulfment and fusion with a lysosome. In addition, mutations in *atg7*, a key autophagy protein, and in Parkin, while altering half-lives, differed in their influence from protein to protein and for most mitochondrial proteins caused less than a 50% change in half-life. Thus at least four

pathways lead to clearance of mitochondrial proteins: mitochondrial proteases, MDVs, the PINK1/Parkin mitophagic pathway, and macroautophagy. Each of these pathways likely contributes to neuronal mitostasis, maintaining a correctly sized pool of high-quality mitochondria.

Where Are Mitochondria Cleared?

In considering the location of mitochondrial clearance, the special problems posed by axons raise issues that small cells do not face. In particular, can mitochondria be degraded wherever they reside? It has often been presumed that they need to return to the soma for removal, like salmon returning to the stream where they were spawned. The logic behind this presumption is 3-fold: (1) mitochondria need to be degraded by lysosomes, (2) lysosomes are most abundant in the soma, and (3) the presence of a retrogradely moving population of mitochondria is best explained by the need for mitochondria to return to the soma at the end of their useful life. Early evidence for this model came from a study of the voltage-sensitive dye JC-1 in axons, which found that retrogradely moving mitochondria had lower membrane potentials than anterogradely moving mitochondria and that inhibition of the electron transport chain (ETC) with antimycin A increased retrograde movement (Miller and Sheetz, 2004). A subsequent study, however, that used TMRM to assay mitochondrial membrane potential found no distinction between anterograde and retrograde populations (Verburg and Hollenbeck, 2008), and others have observed the arrest of mitochondrial movement in response to inhibitors of the ETC, an arrest mediated by the proteolytic degradation of Miro via the PINK1/Parkin pathway (Hsieh et al., 2016; Liu et al., 2012; Shlevkov et al., 2016; Wang et al., 2011). Most recently, a study employed exposure of dorsal root ganglion neurons for 6 hr or longer to very low levels of antimycin A to mimic a long-term mild stress, followed by 1 hr of recovery. In those conditions, membrane potential and directionality were correlated with better membrane potentials preferentially moving anterograde (Lin et al., 2017). One further complication needs be mentioned: if retrogradely moving mitochondria are destined for degradation, once they enter the soma, they should be kept apart for mitophagy. Instead, our unpublished observations suggest that mitochondria that move from the axon into the soma undergo fusion with the resident somatic pool and mix their components in with the resident and presumably younger pool of mitochondria. One set of experiments with the slowly oxidizing indicator mito-Timer (Ferree et al., 2013) found that distal axonal mitochondria were, on average, older than somatic mitochondria, but one did not see intact, old mitochondria as a distinct population returning to the soma. Thus, although studies of retrograde transport, differing in their approaches, have also diverged in their findings, there seems to be little evidence to support the idea that the majority of retrogradely moving mitochondria in a normal, healthy neuron are older or of worse quality than the rest of the mitochondrial pool; they are not all salmon returning home to die.

In light of the paucity of evidence for the "dying salmon" model, it is worth examining the presumptions behind it. As noted in the previous section, not all forms of mitochondrial turnover require lysosomes. Many misfolded or oxidized proteins can be degraded by the proteases in mitochondria, and this

may represent the primary means of protein turnover in mitochondria. MDVs that are shed by mitochondria may be transported to lysosomes independent of the mitochondrion from which they arose. Furthermore, although lysosomes are most abundant in the soma, there is evidence for lysosomes in axons as well (Ashrafi et al., 2014; Maday and Holzbaur, 2014) and for late endosomes arising in distal axons that mature into lysosomes and increase in their proteolytic capacity en route as they move retrograde (Gowrishankar et al., 2017). The axonal pool of lysosomes may be sufficient to mediate clearance, when needed, in an otherwise healthy neuron. Given the abundance of stationary mitochondria and the amount of time necessary for transport from distal regions of an axon to the soma, a different set of considerations would favor the logic of local clearance as preferable for efficiency and preventing damaged mitochondria from releasing reactive oxygen species throughout the cell while journeying back to the soma.

The locus of mitochondrial protein turnover may therefore vary depending on the method of degradation of the components and the conditions that drive it. Mitochondrial proteases are presumably present in all neuronal mitochondria and may be continuously preserving mitochondrial quality in distal axons. The proteins removed in this fashion would, however, need to be replaced either by local protein synthesis or by fusion with the motile pool. Similarly, for MDVs, there is at present no information about either their generation or fate in axons, but they can presumably be generated anywhere in the neuron. If a mitochondrion is diminished by shedding MDVs, it too will need replacement proteins from local synthesis or the motile pool. At least in cultured neurons, macroautophagy that incorporates mitochondria appears to be a very active process at axon tips and much less frequent in the soma or along the length of the axon (Maday and Holzbaur, 2014). Whether macroautophagy has a very particular role in the tip is less clear, but it may be necessary to clear the large volume of material that is shipped down growing axons (Maday and Holzbaur, 2016). What is clear is that the autophagosome, once formed, recruits the retrograde dynein motor and moves retrograde in a highly processive fashion and with increasing acidity consistent with its maturation into an autophagolysosome (Maday et al., 2012). When retrograde mitochondrial transport is measured, it is unlikely that mitochondrial fragments within autophagosomes are a significant contributor to the calculated flux because the fragments are small and degradation en route will remove the markers. If macroautophagy is also active at mature synapses, it may thus account for some of the imbalance between delivery of mitochondrial material to the distal reaches and the amount that returns.

Mitophagy via the PINK1/Parkin pathway presents the most complex issues regarding the location of clearance and necessarily involves two questions: where are PINK1 and Parkin first activated to engage autophagosome formation, and where does the autophagosome undergo fusion with a lysosome and degradation? Ashrafi et al. (2014) observed the local recruitment of Parkin to damaged axonal mitochondria followed by the recruitment of autophagosomal markers and eventual fusion with lysosomes and loss of the mitochondrial markers. This was consistent with the degradation of Miro by this pathway (Liu et al., 2012; Wang et al., 2011); the consequent arrest of

mitochondrial movement made it unlikely that this process required damaged mitochondria to be translocated to the soma. That said, the observation of local mitophagy does not exclude the possibility that, under some circumstances (Lin et al., 2017), particularly those where there is unlikely to be enough ATP to support the PINK1/Parkin pathway, older or slightly damaged mitochondria will not be arrested and will preferentially move retrograde.

In conclusion, local clearance of mitochondria, whether by mitochondrial proteases, MDVs, mitophagy, or macroautophagy, is likely to be a major component of mitochondrial protein turnover in neurons and may account for much of the discrepancy in the amount of mitochondria transported anterograde down axons compared to that which moves retrograde. Moreover, whereas this review has focused entirely on cell-autonomous means of mitochondrial clearance, there is also evidence that axonal mitochondria can be shed and engulfed by glia, a pathway that provides a further alternative for turnover without retrograde transport (Bishop et al., 2004; Davis et al., 2014; Melentijevic et al., 2017). Additional clearance in the soma and dendrites will also contribute, but there is little reason to invoke an obligatory return to the soma. Moreover, because returning mitochondria fuse with other somatic mitochondria, the majority of retrogradely transported mitochondria do not appear to be sequestered and destined for degradation as isolated organelles.

Mitostatic Diseases

The literature on the involvement of mitochondria in neurological diseases is exploding in volume, and at this point, it is difficult to name a neurological disorder in which this organelle has not been implicated. This poses the challenge to differentiate epiphenomenological damage to a vulnerable organelle from a true pathomechanistic role of mitochondria in a disease process. We believe that the concept of mitostatic neurological disorders can help to organize the plethora of roles that mitochondria play in the diseased nervous system. Such conditions would be defined as diseases in which clear-cut disturbances in aspects of mitostasis have been demonstrated, such as altered provision of mitochondria by biosynthesis and transport, disrupted fusion/fission kinetics, or disturbed clearance. This definition would exclude conditions in which bioenergetic failure, depolarization, altered shape, or ultrastructure of mitochondria are observed as the sole argument for mitochondrial involvement.

In some diseases, genetics provides a strong indicator for a mitostatic origin. This is certainly true for hereditary disorders with disruptions of genes with well-defined functions in the mitostatic pathway. One example includes neuropathies of the optic and peripheral nerves that emerge because of disruptions in mitochondrial fission and fusion genes. Their manifestation as degeneration of specific axonal projections underscores the special role that mitochondrial dynamics play in neurons. Indeed, the number of individually rare mutations discovered in the pathways illustrated in Figure 3 is growing. For instance, mutations in DRP1 can cause lethal encephalopathies, even though the relative role of disrupted homeostasis of mitochondria or peroxisomes is not clear here (Waterham et al., 2007). Mutations in mitofusin 2 cause the neuropathic degeneration of motor and sensory axons known as Charcot-Marie-Tooth disease type 2A (Kijima et al., 2005;

Züchner et al., 2004). Mutations in Opa1 cause autosomal-dominant optic atrophy, a disease of progressive blindness due to degeneration of retinal ganglion cells, but which can also include more widespread neuronal degeneration (Alexander et al., 2000; Chen and Chan, 2006; Delettre et al., 2000; McFarland et al., 2010). Truncations of the human miton isoform TRAK1 cause fatal encephalopathy (Barel et al., 2017). As indicated above, the most common mitostatic disease identified to date is Parkinson's disease. Some forms of familial Parkinson's disease are due to mutations in PINK1 and Parkin that alter mitochondrial clearance (Pickrell et al., 2015; Pickrell and Youle, 2015; Vincov et al., 2013). At the same time, the toxicology of Parkinsonism-inducing toxins, such as MPTP+ and rotenone, points to a mitochondrial origin of the disease (Sherer et al., 2001). Most recently, a study of mitophagy in neurons from patient-derived iPSCs found that slowed degradation of Miro, and consequent failure to arrest mitochondrial movement, was a characteristic of many cell lines derived from Parkinson's disease patients, not just those with PINK1 and Parkin mutations. This included LRRK2 mutations and sporadic cases without a known genetic link (Hsieh et al., 2016). Parkinson's illustrates the complexity of the interrelationship of mitochondrial movement and mitochondrial clearance. In healthy cells, adequate mitochondrial movement helps to keep the axonal pool healthy, but if axonal transport is disrupted or mitochondria are compromised, the movement of mitochondria will be diminished and mitochondrial health can further suffer. Persistent movement of damaged mitochondria in Parkinson's disease neurons, and the consequent spreading of reactive oxygen species (ROS), may be as deleterious to the cell as insufficient movement. Moreover, spurious activation of the PINK1/Parkin pathway in an otherwise healthy context, by arresting mitochondrial movement, may prove as deleterious as loss of the pathway when PINK1 or Parkin are mutated. This may explain why kinetin, a PINK1 activator, failed to have the beneficial effects that were hoped for in a rodent model of synuclein Parkinson's disease (Orr et al., 2017).

Mitochondria have also been implicated in many other diseases, such as Alzheimer's disease, amyotrophic lateral sclerosis (ALS), and other motor neuron diseases, and of course in conditions where bioenergetic substrates are missing, such as hypoxia and stroke. In all these cases, mitochondrial pathology is a conspicuous, and even an early, pathological change, and potential roles in pathogenesis have been put forward as reviewed previously (Schon and Przedborski, 2011; Sheng and Cai, 2012). It remains unclear, however, whether there is a genuine mitostatic component in these diseases. In another category of diseases, mutations in motors or microtubule-associated proteins may impact the movement of many types of organelle. It is likely, but mostly unproven, that the disruption of mitostasis is among the serious pathogenic consequences (De Vos et al., 2008).

There are also "new kids on the block," or conditions in which a role for mitochondria has been suggested more recently. These include demyelinating and neuroinflammatory diseases, such as multiple sclerosis, and the response of neurons after trauma. In multiple sclerosis, mitochondrial damage is attracting increasing attention as a potential contributor to axonal injury, which is an important hallmark of the disease and a central driver of lasting disability. In neuroinflammatory lesions, altered mito-

chondria are present (Nikić et al., 2011; Witte et al., 2014). *In vivo* imaging in disease models has revealed reduced mitochondrial transport, which results in accumulations of dysfunctional mitochondria locally and a lack of mitochondria in the distal axonal arbor (Sadeghian et al., 2016; Sorbara et al., 2014). These processes seem largely driven by the inflammatory milieu, which appears to impair transport and prevent normal function of mitochondria, possibly via reactive oxygen or nitrogen species. Notably, demyelination, another hallmark of multiple sclerosis lesions, influences mitochondrial trafficking and density, in part via the anchoring protein syntaphilin. A knockout of syntaphilin, intriguingly, prolonged the survival of Purkinje cells in the shiverer mouse model of chronic demyelination (Joshi et al., 2015), but it had the opposite effect in a model of acute demyelination (Ohno et al., 2014) and did not enhance motor neuron survival in a SOD1 mutant ALS model (Zhu and Sheng, 2011). The inflammation- and demyelination-mediated effects intersect in a complex fashion, in part augmenting each other (regarding local accumulations), in part by counterbalancing (transport speed). Hence, the role of mitochondria in multiple sclerosis, and in converse the effect of neuroinflammatory demyelination on mitochondrial dynamics, is complex, and a clear mechanistic role in the pathology cannot be deduced from the currently available data. Still, this string of work by several labs illustrates how new technical approaches to mitochondrial morphology and dynamics *in situ* can begin to shed light on the specific contributions of this neuronal organelle to nervous system disease.

Another area of recent progress is in the field of neuroregeneration. Parallel work in invertebrate and rodent model organisms has shown that mitochondria are likely to influence axonal growth patterns not only during development (Courchet et al., 2013; Spillane et al., 2013) but also after axotomy during regeneration (Cartoni et al., 2016; Han et al., 2016; Knowlton et al., 2017; Zhou et al., 2016). In this case, most data point to a classical bioenergetic role, where axonal mitochondria at or near the growing axon tip are simply needed for sustained axon growth. Importantly, an axotomized neuron, ranging from *C. elegans* to mice, seems to endogenously upregulate the export of mitochondria into the axonal stump (Han et al., 2016; Misgeld et al., 2007). These observations underscore the evolutionary persistence of higher-order regulation of mitostatic processes and open the opportunity to use the power of genetic screens to understand the role of neuronal mitochondria in disease.

Despite much progress in our understanding of mitostatic processes and their importance to neuronal survival, many open questions remain. (1) Regarding mitochondrial populations, are mobile and stationary mitochondria metabolically equivalent? Do interorganelle contacts play a role in creating mitochondrial diversity? How different are mitochondria among cell types in the nervous system? (2) Regarding mitochondrial biogenesis, which modes of mitochondrial rejuvenation are most important, and how are they tied to transport, fusion, and fission? What is the relative contribution of somatic versus peripheral biogenesis? What is the contribution of axonal and dendritic translation, and is there a dedicated translational machinery in neurites that serves mitochondria? (3) Regarding mitochondrial transport, what determines the direction of movement? How far do neuronal mitochondria typically travel? How long do they

maintain identity as isolated organelles? How is exchange with the stationary pool regulated? What is the function of retrograde mitochondrial transport? (4) Regarding mitochondrial distribution, what holds mitochondria at synapses? How are specific compartments enriched in mitochondria, and how is spacing ensured in areas of low density? How is flow into branches matched to axon arbor geometry, and how are axonal versus dendritic fates specified? (5) Regarding mitochondrial clearance, do all of the described pathways operate in neurons, and if so, where in the cell? (6) Regarding mitochondrial transfer, what is the extent to which mitochondria move between cells in the nervous system? How much does this movement contribute to the removal of neuronal mitochondria in health and disease? Is this process important for glial support of axons? (7) Regarding axon to nucleus communication, is transcription of nuclear genes coupled to changes in energy needs or mitochondrial status in distant regions of the cell? (8) Regarding neuropathology, why do mitostatic disorders differ in their cell-type-specific effects? How can mitostasis be enhanced? Will enhancing mitostasis help to preserve neurons?

In conclusion, neurons have met the complexity of their mitostatic challenge with a diverse set of mechanisms to drive biogenesis, transport, fission, fusion, and clearance. Although much remains unknown about the interplay of these mechanisms and their relative contributions, the centrality of mitostasis to neuronal survival is clear. The importance of understanding the mitostatic apparatus transcends those disorders that are most directly caused by disruption of that apparatus. In many pathological states, improving mitostasis may improve mitochondrial health and benefit neuronal survival. Cell biologists still have much to explore to understand the fundamentals of mitochondrial dynamics and turnover in neurons.

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