

Mitochondrial trafficking and anchoring in neurons: New insight and implications

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Mitochondria are essential organelles for neuronal growth, survival, and function. Neurons use specialized mechanisms to drive mitochondria transport and to anchor them in axons and at synapses. Stationary mitochondria buffer intracellular Ca^{2+} and serve as a local energy source by supplying ATP. The balance between motile and stationary mitochondria responds quickly to changes in axonal and synaptic physiology. Defects in mitochondrial transport are implicated in the pathogenesis of several major neurological disorders. Recent work has provided new insight in the regulation of microtubule-based mitochondrial trafficking and anchoring, and on how mitochondrial motility influences neuron growth, synaptic function, and mitophagy.

Introduction

Mitochondria are cellular power plants that convert chemicals into ATP through coupled electron transport chain and oxidative phosphorylation. A cortical neuron in human brain utilizes up to ~ 4.7 billion ATP molecules per second to power various biological functions (Zhu et al., 2012). Constant ATP supply is essential for nerve cell survival and function (Nicholls and Budd, 2000). Mitochondrial ATP production supports synapse assembly (Lee and Peng, 2008), generation of action potentials (Attwell and Laughlin, 2001), and synaptic transmission (Verstreken et al., 2005). Synaptic mitochondria maintain and regulate neurotransmission by buffering Ca^{2+} (Medler and Gleason, 2002; David and Barrett, 2003). In addition, mitochondria sequester presynaptic Ca^{2+} transients elicited by trains of action potentials and release Ca^{2+} after stimulation, thus inducing certain forms of short-term synaptic plasticity (Werth and Thayer, 1994; Tang and Zucker, 1997; Billups and Forsythe, 2002; Levy et al., 2003; Kang et al., 2008). Removing mitochondria from axon terminals results in aberrant synaptic transmission likely due to insufficient ATP supply or altered Ca^{2+} transients (Stowers et al., 2002; Guo et al., 2005; Ma et al., 2009).

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Abbreviations used in this paper: KIF5, kinesin-1 family; MT, microtubule.

Neurons are polarized cells consisting of a relatively small cell body, dendrites with multiple branches and elaborate arbors, and a thin axon that can extend up to a meter long in some peripheral nerves. Due to these extremely varied morphological features, neurons face exceptional challenges to maintain energy homeostasis. Neurons require specialized mechanisms to efficiently distribute mitochondria to far distal areas where energy is in high demand, such as synaptic terminals, active growth cones, and axonal branches (Fig. 1; Morris and Hollenbeck, 1993; Ruthel and Hollenbeck, 2003). Axonal branches and synapses undergo dynamic remodeling during neuronal development and in response to synaptic activity, thereby changing mitochondrial trafficking and distribution. Neurons are postmitotic cells surviving for the lifetime of the organism. A mitochondrion needs to be removed when it becomes aged or dysfunctional. Mitochondria also alter their motility and distribution under certain stress conditions or when their integrity is impaired (Miller and Sheetz, 2004; Chang and Reynolds, 2006; Cai et al., 2012). Therefore, efficient regulation of mitochondrial trafficking and anchoring is essential to: (1) recruit and redistribute mitochondria to meet altered metabolic requirements; and (2) remove aged and damaged mitochondria and replenish healthy ones at distal terminals. Research into neuronal regulation of mitochondrial trafficking and anchoring is thus a very important frontier in neurobiology. This review article focuses on new mechanistic insight into the regulation of microtubule (MT)-based mitochondrial trafficking and anchoring and provides an updated overview of how mitochondrial motility influences neuronal growth, synaptic function, and mitochondrial quality control. Additional insight and overviews from different perspectives can be found in other in-depth reviews (Frederick and Shaw, 2007; Morfini et al., 2009; Hirokawa et al., 2010; MacAskill and Kittler, 2010; Schon and Przedborski, 2011; Court and Coleman, 2012; Saxton and Hollenbeck, 2012; Sheng and Cai, 2012; Birsa et al., 2013; Lovas and Wang, 2013; Schwarz, 2013).

Neuronal mitochondria display complex motility patterns

The complex motility patterns of mitochondrial transport in neurons can be visualized by applying time-lapse imaging in

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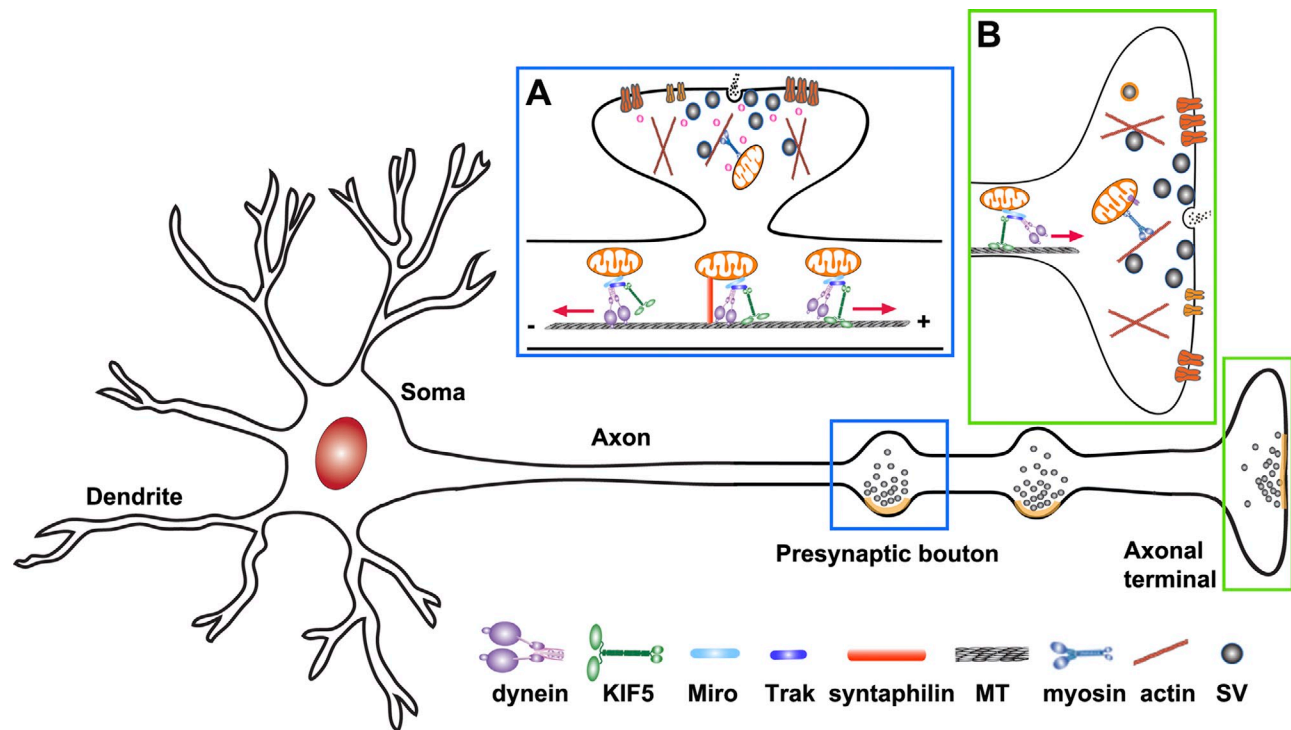


Figure 1. Mitochondrial trafficking and anchoring in neurons. Due to complex structural features, neurons require specialized mechanisms trafficking mitochondria to their distal destinations and anchoring them in regions where metabolic and calcium homeostatic capacity is in a high demand. The figure highlights transporting mitochondria to a presynaptic bouton (A) and an axonal terminal (B). MT-based long-distance mitochondrial transport relies on MT polarity. In axons, the MT's plus ends (+) are oriented toward axonal terminals whereas minus ends (–) are directed toward the soma. Thus, KIF5 motors are responsible for anterograde transport to distal synaptic terminals whereas dynein motors return mitochondria to the soma. The motor adaptor Trak proteins can mediate both KIF5- and dynein-driven bi-directional transport of axonal mitochondria (van Spronsen et al., 2013). Myosin motors likely drive short-range mitochondrial movement at presynaptic terminals where enriched actin filaments constitute cytoskeletal architecture. Motile mitochondria can be recruited into stationary pools via dynamic anchoring interactions between syntaphilin and MTs (Kang et al., 2008) or via an unidentified actin-based anchoring receptor (Chada and Hollenbeck, 2004). Such anchoring mechanisms ensure neuronal mitochondria are adequately distributed along axons and at synapses, where constant energy supply is crucial (figure courtesy of Qian Cai, Department of Cell Biology and Neuroscience, Rutgers University, Piscataway, NJ).

live primary neurons. Mitochondria are labeled by expressing DsRed-Mito, a mitochondria-targeted fluorescent protein, or by loading fluorescent dye MitoTracker Green or MitoTracker Red CMXRos, which stains mitochondria in live cells dependent upon membrane potential. Mitochondria move bi-directionally over long distances along processes, pause briefly, and move again, frequently changing direction. Motile mitochondria can become stationary, and stationary ones can be remobilized and redistributed in response to changes in metabolic status and synaptic activity. Given frequent pauses and bi-directional movements, the mean velocities of neuronal mitochondrial transport are highly variable, ranging from 0.32 to 0.91 $\mu\text{m/s}$ (Morris and Hollenbeck, 1995; MacAskill et al., 2009a). In mature neurons, $\sim 20\text{--}30\%$ of axonal mitochondria are motile, some of which pass by presynaptic terminals while the remaining two thirds are stationary (Kang et al., 2008). Using dual-channel imaging of mitochondria and synaptic terminals in hippocampal neurons, a recent study identified five motility patterns of axonal mitochondria including: stationary mitochondria sitting out of synapses ($54.07 \pm 2.53\%$) or docking at synapses ($16.29 \pm 1.66\%$); motile mitochondria passing through synapses ($14.77 \pm 1.58\%$); or pausing

at synapses for a short (<200 s, $7.01 \pm 1.29\%$) or a longer period of time (>200 s, $8.30 \pm 1.52\%$; Sun et al., 2013). These findings are consistent with a previous study in primary cortical neurons (Chang et al., 2006).

Altered mitochondrial transport is one of the pathogenic changes in major adult-onset neurodegenerative diseases (Sheng and Cai, 2012). Although in vitro live imaging in primary neurons prepared from embryonic or newborn mice provides an important tool to study mechanisms regulating mitochondrial transport, it has obvious limitations for investigating adult-onset disease mechanisms. For example, reduced transport of axonal mitochondria was consistently reported in amyotrophic lateral sclerosis-linked hSOD1^{G93A} mice (De Vos et al., 2007; Magrané and Manfredi, 2009; Bilsland et al., 2010; Zhu and Sheng, 2011; Marinkovic et al., 2012; Song et al., 2013). Therefore, it is critical to establish spinal motor neuron cultures directly isolated from adult mice, which will provide more reliable models to investigate mitochondrial transport defects underlying adult-onset pathogenesis in motor neurons. Given that the morphology and conditions of neurons grown in culture dishes differ from that of neurons in vivo, an in vivo transport study would better reflect physiological environments. An ex vivo study of mitochondrial

transport in acute nerve explants was developed in transgenic mice, in which neuronal mitochondria are labeled with mito-CFP or mito-YFP (Misgeld et al., 2007).

Moving mitochondria along MTs

Mitochondrial transport between the soma and distal processes or synapses depends upon MT-based motors, which drive their cargos via mechanisms requiring ATP hydrolysis (Martin et al., 1999; Hirokawa et al., 2010). MTs are uniformly arranged in axons: their plus ends are oriented distally and minus ends are directed toward the soma. Such a uniform polarity has made axons particularly useful for exploring mechanisms regulating bi-directional transport: dynein motors drive retrograde movement, whereas kinesin motors mediate anterograde transport. Of the 45 kinesin motor genes identified, the kinesin-1 family (KIF5) is the key motor driving mitochondrial transport in neurons (Hurd and Saxton, 1996; Tanaka et al., 1998; Górska-Andrzejak et al., 2003; Cai et al., 2005; Pilling et al., 2006). KIF5 heavy chain (KHC) contains a motor domain with ATPase at the N terminus and a C-terminal tail for binding cargo directly or indirectly via a cargo adaptor. Three isoforms (KIF5A, KIF5B, and KIF5C) of the KIF5 family are found in mammals, of which KIF5B is expressed ubiquitously whereas KIF5A and KIF5C are expressed selectively in neurons (Kanai et al., 2000).

KIF5 motors attach to mitochondria through adaptor proteins. The motor–adaptor coupling ensures targeted trafficking and effective regulation of mitochondrial transport. *Drosophila* protein Milton acts as a KIF5 motor adaptor by binding the KIF5 cargo-binding domain and mitochondrial outer membrane receptor Miro (Stowers et al., 2002; Glater et al., 2006). The *Drosophila* mutation in Milton reduces mitochondrial trafficking to synapses. Milton appears specific for mitochondrial trafficking because the trafficking of other cargoes such as synaptic vesicles is not affected. Two Milton orthologues, Trak1 and Trak2, are found in mammals and are required for mitochondrial trafficking (Smith et al., 2006; MacAskill et al., 2009b; Koutsopoulos et al., 2010). Elevated Trak2 expression in hippocampal neurons robustly enhances axonal mitochondrial motility (Chen and Sheng, 2013). Conversely, depleting Trak1 or expressing its mutants results in impaired mitochondrial motility in axons (Brickley and Stephenson, 2011). A recent study revealed that mammalian Trak1 and 2 contain one N-terminal KIF5B-binding site and two dynein/dynactin-binding sites, one at the N-terminal domain and the other at the C-terminal domain (van Spronsen et al., 2013). Thus, the Trak proteins can mediate both KIF5- and dynein-driving bi-directional transport of axonal mitochondria (Fig. 1). In contrast to the specific role of *Drosophila* Milton in mitochondrial transport, mammalian Trak proteins are also recruited to endosomal compartments and associate with GABAA (γ -aminobutyric acid A) receptors and K⁺ channels, highlighting their role in neuronal cargo transport (Grishin et al., 2006; Kirk et al., 2006; Webber et al., 2008).

The mitochondria outer membrane receptor Miro is a Rho-GTPase with two Ca²⁺-binding EF-hand motifs and two GTPase domains (Frederick et al., 2004; Fransson et al., 2006). Mutation of the *Drosophila miro* gene impairs mitochondrial anterograde transport, thus depleting mitochondria at distal synaptic

terminals (Guo et al., 2005). The first crystal structures of *Drosophila melanogaster* Miro comprising the tandem EF-hands and GTPase domains were recently resolved (Klosowiak et al., 2013). From these structures, two additional hidden EF-hands were identified, each one pairing with a canonical EF-hand. These structures will help our understanding of the conformational changes in Miro upon Ca²⁺ binding and nucleotide sensing in regulating mitochondrial motility, dynamics, and function. There are two mammalian Miro isoforms, Miro1 and Miro2. The Miro1–Trak2 adaptor complex regulates mitochondrial transport in hippocampal neurons (MacAskill et al., 2009b). Elevated Miro1 expression increases mitochondrial transport, likely by recruiting more Trak2 and motors to the mitochondria (Chen and Sheng, 2013). Thus, KIF5, Milton (Trak), and Miro assemble into the transport machinery that drives mitochondrial anterograde movement (Fig. 2 A). An alternative KIF5 adaptor for neuronal mitochondria is syntabulin (Fig. 2 B; Cai et al., 2005). Depleting syntabulin or blocking the KIF5–syntabulin coupling reduces mitochondrial anterograde transport in axons. Several other proteins are also suggested as candidate KIF5 motor adaptors for mitochondrial transport, including FEZ1 (fasciculation and elongation protein zeta-1) and RanBP2 (Ran-binding protein 2; Cho et al., 2007; Fujita et al., 2007), although their role in neuronal mitochondrial transport requires further investigation. The existence of multiple motor adaptors may highlight the complex regulation of mitochondrial motility in response to various physiological and pathological signals.

Dynein motors are composed of multiple chains including heavy chains (DHC) as the motor domain, intermediate (DIC), light intermediate (DLIC), and light chains (DLC), which function by associating with cargos and regulating motility. Dynein associates with *Drosophila* mitochondria, and mutations of DHC alter velocity and run length of mitochondrial retrograde transport in axons (Pilling et al., 2006). Miro may serve as an adaptor for both KIF5 and dynein motors in *Drosophila* (Guo et al., 2005; Russo et al., 2009), as loss of *dMiro* impairs both kinesin- and dynein-driven transport and overexpressing *dMiro* also alters mitochondrial transport in both directions. These findings support an attractive model in which the relative activity of the opposite-moving motors is regulated by their cargo–adaptor proteins.

Mitochondrial motility and membrane dynamics

Mitochondrial trafficking, anchoring, and membrane dynamics are coordinated, thus controlling their shape, integrity, and distribution in neurons. It is expected that the size of a mitochondrion may have a direct impact on its mobility. Indeed, interconnected mitochondrial tubules found in fission-deficient neurons are less efficiently transported to distal synapses. Hippocampal neurons expressing defective Drp1, a mitochondrial fission protein, display accumulated mitochondria within the soma and reduced mitochondrial density in dendrites (Li et al., 2004). *Drosophila* Drp1 is required for delivering mitochondria to neuromuscular junctions (Verstreken et al., 2005). Conversely, mutation of Miro results in mitochondrial fragmentation in addition to impaired motility, whereas overexpressing

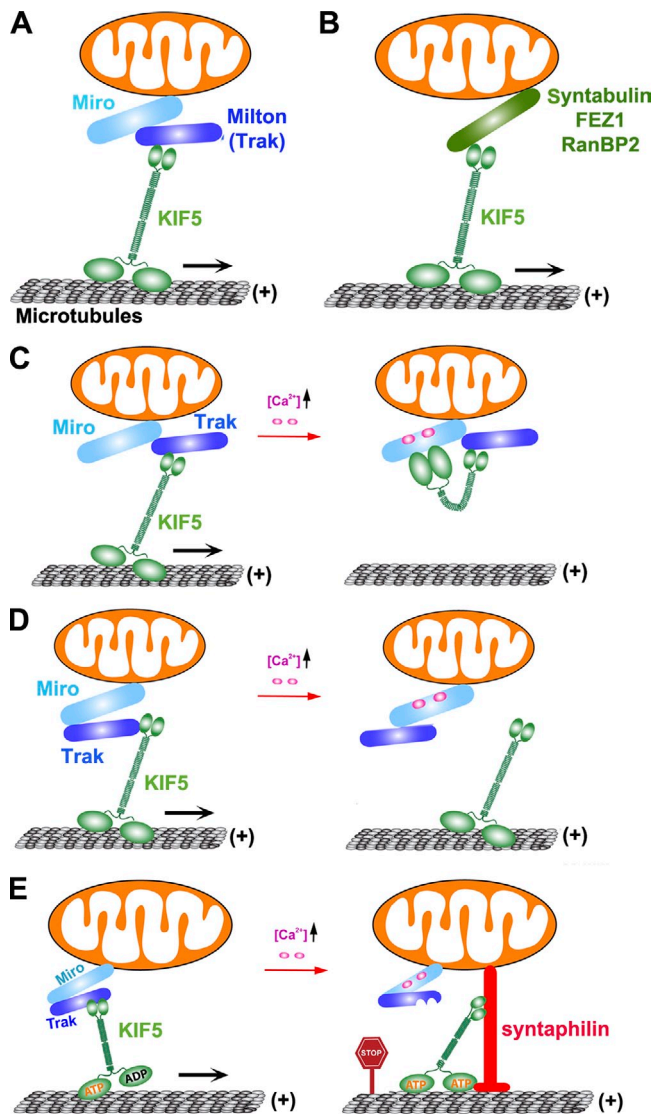


Figure 2. Activity-dependent regulation of mitochondrial transport. (A) The Miro–Milton (or Miro–Trak) adaptor complex mediates KIF5-driven mitochondrial transport. (B) Syntabulin, FEZ1, and RanBP2 serve as an alternative KIF5 motor adaptor in driving mitochondrial anterograde transport. (C and D) Miro- Ca^{2+} models in regulating mitochondrial motility. Miro contains Ca^{2+} -binding EF-hand motifs. The C-terminal cargo-binding domain of KIF5 motors binds to the Miro–Trak adaptor complex. (C) Ca^{2+} binding to Miro’s EF-hands induces the motor domain to disconnect with MTs and thus prevents motor–MT engagement (Wang and Schwarz, 2009). (D) Alternatively, Ca^{2+} binding releases KIF5 motors from mitochondria (MacAskill et al., 2009a). Thus, Ca^{2+} influx after synaptic activity arrests motile mitochondria at activated synapses. (E) Syntaphilin-mediated “engine-switch and brake” model. A Miro- Ca^{2+} -sensing pathway triggers the binding switch of KIF5 motors from the Miro–Trak adaptor complex to anchoring protein syntaphilin, which immobilizes axonal mitochondria via inhibiting motor ATPase activity. Thus, syntaphilin turns off the “KIF5 motor engine” by sensing a “stop sign” (elevated Ca^{2+}) and putting a brake on mitochondria, thereby anchoring them in place on MTs. When in their stationary status, KIF5 motor-loaded mitochondria remain associated with the MT tracks while KIF5 ATPase is in an inactive state (Chen and Sheng, 2013; Figure courtesy of Qian Cai).

Miro not only enhances mitochondrial transport but also induces their interconnection (Fransson et al., 2006; Saotome et al., 2008; Liu and Hajnóczky, 2009; MacAskill et al., 2009b). Additionally, yeast Miro, Gem1p, regulates mitochondrial morphology;

cells lacking Gem1p contain collapsed, globular, or grape-like mitochondria (Frederick et al., 2004).

Mitochondrial fusion events require two organelles to move closer. A “kiss-and-run” interplay is proposed for driving mitochondrial fusion (Liu et al., 2009). Thus, motile mitochondria, particularly in distal axons and at synapses, undergo fusion events at a higher rate than anchored stationary mitochondria. Recent studies highlight the cross talk between the mitochondrial fusion protein Mfn2 and a motor adaptor complex. Mfn2 associates with Miro2 and regulates axonal mitochondrial transport by affecting kinesin motor processivity or by coordinating the motor switch between kinesin and dynein (Misko et al., 2010). Neurons defective in Mfn2 display reduced mitochondrial motility and altered mitochondrial distribution (Chen et al., 2003, 2007; Baloh et al., 2007). Therefore, regulating mitochondrial trafficking directly affects their morphology. Conversely, changing mitochondrial fusion–fission dynamics also impacts their transport and distribution.

Anchoring mitochondria at MTs

Proper axonal and synaptic function requires stationary mitochondria docking in regions where energy is in high demand. The diffusion capacity of intracellular ATP is rather limited (Hubley et al., 1996), particularly within long axonal processes (Sun et al., 2013). Therefore, docked mitochondria ideally serve as stationary power plants for stable and continuous ATP supply necessary to maintain the activity of $\text{Na}^+ - \text{K}^+$ ATPase, fast spike propagation, and synaptic transmission. In addition, mitochondria maintain calcium homeostasis at synapses by sequestering intracellular $[\text{Ca}^{2+}]$ (Billups and Forsythe, 2002; Kang et al., 2008). Given the fact that two thirds of the mitochondria are stationary for an extended period along axons, neurons require mechanisms dissociating mitochondria from motor proteins or anchoring mitochondria to the cytoskeleton. One intriguing mitochondrial-anchoring protein is syntaphilin, which is a “static anchor” for immobilizing mitochondria specifically in axons (Kang et al., 2008; Chen et al., 2009). Syntaphilin targets axonal mitochondria through its C-terminal mitochondria-targeting domain and axon-sorting sequence. Syntaphilin immobilizes axonal mitochondria by anchoring to MTs. Deleting *syntaphilin* results in a robust increase of axonal mitochondria in motile pools. Conversely, overexpressing syntaphilin abolishes axonal mitochondrial transport. These findings provide new mechanistic insight that motile mitochondria can be recruited into stationary pools via anchoring interactions between mitochondria-targeted syntaphilin and MTs. Thus, syntaphilin serves as an attractive molecular target for investigations into mechanisms recruiting motile mitochondria into activated synapses. The *syntaphilin* knockout mouse is an ideal genetic model to examine the impact of enhanced motility of axonal mitochondria on presynaptic function and mitochondrial quality control in distal axons.

Synaptic activity-dependent regulation

Mitochondrial transport and distribution in axons and at synapses are correlated with synaptic activity. Mitochondria are recruited to synapses in response to elevated intracellular

Ca²⁺ during sustained synaptic activity. Elevated Ca²⁺ influx, either by activating voltage-dependent calcium channels or NMDA receptors, arrests mitochondrial movement (Rintoul et al., 2003; Yi et al., 2004; Chang et al., 2006; Szabadkai et al., 2006; Ohno et al., 2011). These studies raise a fundamental question: how are mitochondria recruited to synapses in response to Ca²⁺ influx? Recent work from three groups identified Miro as a Ca²⁺ sensor in regulating mitochondrial motility (Saotome et al., 2008; MacAskill et al., 2009a; Wang and Schwarz, 2009). Miro has four Ca²⁺-binding EF-hands (Klosowiak et al., 2013). Elevated cytosolic Ca²⁺ levels, by firing action potentials or activating glutamate receptors, arrest mitochondrial transport through a Miro-Ca²⁺-sensing pathway that inactivates or disassembles the KIF5-Miro-Trak transport machineries. By this mechanism, mitochondria are recruited to activated synapses. A Miro-Ca²⁺-sensing model was proposed in which KIF5 is loaded onto mitochondria through physical coupling via the motor adaptor complex Miro-Milton (or Miro-Trak) at resting Ca²⁺ levels. When motile mitochondria pass by an active synaptic terminal, the local elevated intracellular Ca²⁺ disrupts or inactivates the Miro-Trak-kinesin transport complex by binding to the EF-hand and changing Miro's conformation; thus, mitochondria are immobilized at activated synapses (Fig. 2, C and D; MacAskill et al., 2009a; Wang and Schwarz, 2009). The two studies disagreed, however, as to whether kinesin remained associated with stationary mitochondria or whether the motor was completely decoupled from the organelle upon immobilization. In addition, Miro-Ca²⁺-dependent regulation affects both anterograde and retrograde transport. When KIF5 transport machinery is disrupted by the Miro-Ca²⁺-sensing mechanism, dynein-driven retrograde transport does not necessarily take over. It is unclear how the Miro-Ca²⁺-sensing pathway inactivates dynein transport machineries. Thus, the role of the Miro-Ca²⁺-sensing pathway in immobilizing mitochondria likely occurs through an anchoring mechanism.

A recent study provides evidence to support the above-described hypothesis. Activating the Miro-Ca²⁺ pathway fails to arrest axonal mitochondria in *syntaphilin*-null hippocampal neurons: axonal mitochondria keep moving during neuronal firing while dendritic mitochondria are effectively immobilized (Chen and Sheng, 2013). This study suggests that syntaphilin-mediated anchoring plays a central role in the activity-dependent immobilization of axonal mitochondria. Syntaphilin competes with Trak2 for binding KIF5 in cells and inhibits the KIF5 motor ATPase activity in vitro, thus suggesting that syntaphilin functions to prevent KIF5 from moving along MTs. Synaptic activity and elevated calcium levels favor the anchoring interaction between syntaphilin and KIF5 by disrupting the Miro-Trak-kinesin transport complex. In addition, sustained neuronal activity induces syntaphilin translocation to axonal mitochondria, although the underlying mechanisms remain elusive. Expressing the syntaphilin mutant lacking this axon-sorting domain results in its distribution into all mitochondria, including those in the soma and dendrites (Kang et al., 2008). These studies collectively suggest a possibility that syntaphilin is delivered to axons before its translocation into mitochondria. One interesting question is how syntaphilin-anchored mitochondria are

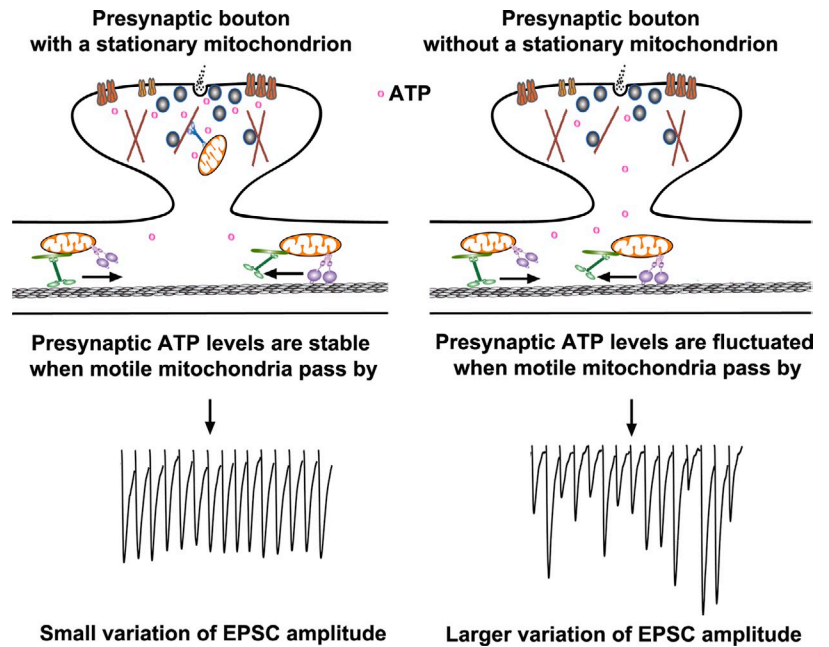
remobilized. Given the fact that syntaphilin is a cellular target of the E3 ubiquitin ligase Cullin 1 (Yen and Elledge, 2008), syntaphilin may be degraded by ubiquitin-mediated pathways. It is necessary to further investigate the mechanisms underlying activity-induced syntaphilin translocation and its subsequent removal from mitochondria.

A new “engine-switch and brake” model (Fig. 2 E) was recently proposed (Chen and Sheng 2013). In response to a “stop” sign (elevated Ca²⁺) at active synapses, syntaphilin switches off the kinesin motor and puts a brake on mitochondria, thereby anchoring them in place on MTs. Thus, KIF5-loaded mitochondria remain associated with the axonal MT track while in their stationary phase. This model may help reconcile the standing disagreements regarding how Miro-Ca²⁺ sensing immobilizes mitochondria in neurons. MacAskill et al. (2009a) propose that Ca²⁺ binding to Miro disconnects KIF5 motors from mitochondria in dendrites by activating glutamate receptors (Fig. 2 D), whereas Wang and Schwarz (2009) suggest that KIF5 remains associated with immobilized mitochondria within axons during elevated Ca²⁺ conditions (Fig. 2 C). These discrepant results are likely due to their selective observations of mitochondrial motility in dendrites versus axons. Upon Miro-Ca²⁺ sensing, KIF5 motors remain associated with axonal mitochondria by binding syntaphilin, which is absent from dendritic mitochondria (Fig. 2 E). This difference may help axonal mitochondria be more responsive to changes in synaptic activity. If the Ca²⁺ signal is removed, the cargo-loaded motor proteins can be quickly reactivated to move mitochondria to new active synapses. This model also suggests that motor loading on mitochondria is insufficient for transport. The release of the anchoring interaction is also required. The latter is supported by a study that both KIF5 and dynein motors remain bound on prion protein vesicles regardless of whether they are motile or stationary (Encalada et al., 2011). A newly reported Alex3 protein, organized in a genomic *Armcx* cluster encoding mitochondria-targeted proteins, regulates mitochondrial trafficking in neurons by interacting with the KIF5-Miro-Trak complex in a Ca²⁺-dependent manner (López-Doménech et al., 2012). Although the underlying mechanism is unknown, the study highlights the complexity in controlling mitochondrial motility in response to various physiological signals in neurons.

Mitochondrial motility and synaptic variability

Mitochondria are commonly found within synaptic terminals (Shepherd and Harris, 1998), where they power neurotransmission by supplying ATP. A stationary mitochondrion within presynaptic terminals provides a stable and continuous ATP supply and maintains energy homeostasis at synapses. Conversely, a motile mitochondrion passing by presynaptic boutons dynamically alters local ATP levels, thus influencing various ATP-dependent functions at synapses such as: (1) establishing the proton gradient necessary for neurotransmitter loading; (2) removing Ca²⁺ from nerve terminals; and (3) driving synaptic vesicle transport from reserve pools to release sites. For example, *Drosophila* photoreceptors expressing mutant Milton display impaired synaptic transmission due to reduced mitochondrial trafficking to synapses (Stowers et al., 2002). Mutation of *Drosophila* Drp1

Figure 3. Motile mitochondria passing by synapses contribute to presynaptic variation. A stationary mitochondrion within presynaptic terminals powers neurotransmission by stable and continuous ATP supply (left). Conversely, in the absence of a mitochondrion within a presynaptic bouton (right), there is no stable on-site ATP supply; a motile mitochondrion passing through this bouton temporarily supplies ATP, thus changing synaptic energy levels and influencing ATP-dependent synaptic functions over time when mitochondrial distribution and motility are altered. Therefore, the fluctuation of synaptic ATP levels resulting from mitochondrial movement is one of the primary sources for the wide variability of synaptic vesicle release and amplitudes of excitatory postsynaptic currents (EPSCs; Sun et al., 2013).



reduces synaptic localization of mitochondria and results in faster depletion of synaptic vesicles during prolonged stimulation (Verstreken et al., 2005). Addition of ATP to terminals partially rescues these defects, indicating that mitochondrial ATP production is required for maintaining sustained synaptic transmission. Syntabulin loss of function reduces mitochondrial density in axonal terminals, which is accompanied by accelerated synaptic depression and slowed synapse recovery during high-frequency firing (Ma et al., 2009).

One of the most notable characteristics of synaptic transmission is the wide variation in synaptic strength in response to repeated stimulations. The effects of synaptic variability on neuronal circuit activity are increasingly recognized. Some degree of synaptic variability may be necessary for signal processing in flexible or adaptive systems (Murthy et al., 1997). A long-standing fundamental question is how the variation in synaptic strength arises. There is general agreement that some structural and molecular stochastic alternations at synapses are the basis for heterogeneity in synaptic transmission from neuron to neuron or from synapse to synapse (Atwood and Karunanithi, 2002; Stein et al., 2005; Marder and Goaillard, 2006; Branco and Staras, 2009; Ribault et al., 2011). However, it is unknown whether mitochondrial motility at axonal terminals contributes to pulse-to-pulse variability of presynaptic strength at single-bouton levels. This is particularly relevant in hippocampal neurons, where approximately one third of axonal mitochondria are highly motile, some of which dynamically pass through presynaptic boutons.

We recently solved this puzzle by combining dual-channel imaging in live neurons and electrophysiological analysis in *syntaphilin* knockout hippocampal neurons and acute hippocampal slices in which axonal mitochondrial motility is selectively manipulated (Sun et al., 2013). Our study revealed that the motility of axonal mitochondria is one of the primary mechanisms underlying the variability of presynaptic strength. Enhanced

motility of axonal mitochondria increases the pulse-to-pulse variability of EPSC amplitudes, whereas immobilizing axonal mitochondria effectively diminishes the variability. The data raise two assumptions: (1) EPSC amplitudes are averaged through summation from multiple synapses; and (2) release at individual boutons changes over time when mitochondrial distribution and motility are dynamically altered. Thus, at any given time these boutons display different patterns of mitochondrial distribution and motility; mitochondria move either in or out of boutons or pass through boutons during stimulation, thus contributing to the pulse-to-pulse variability. By applying dual-channel live imaging of mitochondria and synaptic vesicle release at single-bouton levels, the study further shows that mitochondrial movement, either into or out of presynaptic boutons, significantly influences synaptic vesicle release due to fluctuation of synaptic ATP levels. In the absence of a stationary mitochondrion within an axonal terminal, there is no constant on-site ATP supply. A motile mitochondrion passing through could temporarily supply ATP, thus changing synaptic energy levels and influencing ATP-dependent processes including synaptic vesicle cycling, mobilization, and replenishment (Fig. 3). This is further supported by a recent study applying a new quantitative optical presynaptic ATP reporter. Synaptic activity drives large ATP consumption at nerve terminals and synaptic vesicle cycling consumes most presynaptic ATP (Rangaraju et al., 2014). It is possible that all of these ATP-dependent processes collectively contribute to presynaptic variability. Therefore, the fluctuation of synaptic ATP levels resulting from mitochondrial movement is one of the primary sources for the wide variability of EPSC amplitudes. This study revealed, for the first time, that the dynamic movement of axonal mitochondria is one of the primary mechanisms underlying the presynaptic variation in the CNS, thus providing new insight into the fundamental properties of the CNS to ensure the plasticity and reliability of synaptic transmission (Sun et al., 2013).

Mitochondrial motility, neuronal morphology, and energy consumption

Proper mitochondrial transport into neurites in developing neurons is tightly regulated to ensure that metabolically active areas are adequately supplied with ATP (Morris and Hollenbeck, 1993). A recent study showed the differential functions of two mitochondrial motor adaptors, Trak1 and Trak2, in driving polarized mitochondrial transport (van Spronsen et al., 2013). Trak1 is prominently localized in axons and is required for axonal mitochondria transport by binding to both kinesin-1 and dynein/dynactin, whereas Trak2 is present in dendrites and mainly transports mitochondria into dendrites by interacting with dynein/dynactin. The folded configuration of Trak2 preferentially binds dynein motors rather than kinesin-1. Consistently, depleting Trak1 inhibits axonal outgrowth, whereas suppressing Trak2 impairs dendrite morphology. Thus, this study established a new role of Trak proteins in polarized mitochondrial targeting necessary to maintain neuronal morphology.

Anchored stationary mitochondria ideally serve as local energy power plants. Increased intracellular $[ADP]_i$ slows down mitochondrial movement and recruits mitochondria to subcellular regions correlated with local $[ATP]_i$ depletion (Mironov, 2007). However, the underlying mechanisms coordinating mitochondria transport and energy consumption remain elusive. The balance between motile and stationary mitochondria responds quickly to changes in axonal growth status. Two recent studies demonstrated that generating and maintaining axonal branching depend upon stable and continuous ATP supply by stationary mitochondria captured at branching points. Courchet et al. (2013) revealed that LKB1, the serine/threonine kinase B1, regulates terminal axon branching of cortical neurons in both in vitro and in vivo systems through activating downstream kinase NUAK1, an AMPK-like kinase. AMPK is an AMP-activated protein kinase, which is a master regulator of cellular energy homeostasis and is activated in response to stresses that deplete cellular ATP supplies. Deleting LKB1 or NUAK1 results in a threefold decrease in stationary pools of axonal mitochondria, whereas overexpressing LKB1 or NUAK1 conversely increases the proportion of immobilized mitochondria along axons, thus establishing a causal correlation between stationary mitochondria and axonal branching. To test the hypothesis, Courchet et al. (2013) altered the motility of axonal mitochondria by changing expression levels of syntaphilin. Intriguingly, suppressing syntaphilin expression in cortical progenitors at E15.5 enhances axonal mitochondrial motility accompanied by decreased axon branching. Conversely, overexpressing syntaphilin immobilizes axonal mitochondria and increases axon branching, thus highlighting a critical role for syntaphilin-mediated mitochondrial anchoring in AMPK-induced axonal branching. It is possible that LKB1 and NUAK1-mediated signaling pathways regulate axon branch formation and stabilization by recruiting mitochondria to branch points. However, this study raises one mechanistic question: does syntaphilin or the mitochondrial transport motor–adaptor complex serve as a downstream effector of this signaling pathway? Syntaphilin is strictly developmentally regulated in mouse brains, with low expression before postnatal day 7 and peaking two weeks after

birth; thus, its expression is hardly detectable in early embryonic stages (Kang et al., 2008). It remains unclear how siRNA application at E15.5 significantly impacts endogenous syntaphilin levels in the brain. Furthermore, it is unknown whether local ATP homeostasis maintained by stationary but not motile mitochondria contributes to the formation and stabilization of axonal branching.

As a cellular energy sensor for detecting increased intracellular $[AMP]_i$ or ATP consumption, AMPK activation positively regulates signaling pathways that replenish cellular ATP supplies. Tao et al. (2013) provide further evidence that sustained neuronal depolarization for 20 min depletes cellular ATP and activates AMPK. Activation of AMPK increases anterograde flux of mitochondria into axons and induces axonal branching in regions where mitochondria are docked in an ATP-dependent manner. The mitochondrial uncoupler FCCP blocks axonal branching, even when mitochondria are localized nearby. These studies provide evidence for how the AMPK signaling pathway regulates axon branch formation and stabilization. These studies collectively highlight a critical role for syntaphilin-mediated mitochondrial anchoring in axon branching. One possible mechanism underlying mitochondrial recruiting and anchoring in support of axon branching is through localized intra-axonal protein synthesis (Spillane et al., 2013). Inhibiting mitochondrial respiration and protein synthesis in axons impairs axonal branching. Thus, stationary mitochondria coordinate maturation of axonal filopodia into axon branching likely by supporting active mRNA translation in axonal hot spots. Activity-induced mitochondrial recruitment in axons is further supported by an in vivo study in mouse saphenous nerve axons. Sustained electrical stimulation (50 Hz) enhances anterograde flux of mitochondria into myelinated axons and redistributes them at the peripheral terminals (Sajic et al., 2013).

Mitochondrial motility and mitophagy

Cumulative evidence indicates mitochondrial dysfunction, impaired transport, altered dynamics, and perturbation of mitochondrial turnover are associated with the pathology of major neurodegenerative disorders (Chen and Chan, 2009; Sheng and Cai, 2012). Defective mitochondrial transport accompanied by mitochondrial pathology is one of the most notable pathological changes in several aging-associated neurodegenerative diseases, including Alzheimer's disease (Rui et al., 2006; Du et al., 2010; Calkins et al., 2011) and amyotrophic lateral sclerosis (De Vos et al., 2007; Magrané and Manfredi, 2009; Bilsland et al., 2010; Shi et al., 2010; Martin, 2011; Zhu and Sheng, 2011; Marinkovic et al., 2012). Dysfunctional mitochondria not only supply less ATP and maintain Ca^{2+} buffering capacity less efficiently, but also release harmful reactive oxygen species (ROS). Defects in mitochondrial transport reduce the delivery of healthy mitochondria to distal processes and also impair removal of damaged mitochondria from synapses, thus causing energy depletion and altered Ca^{2+} transient at synapses. In addition, toxic ROS may further trigger synaptic stress, thereby contributing to neurodegeneration. Mitochondrial quality control involves multiple levels of surveillance to ensure mitochondrial integrity, including repairing dysfunctional mitochondria by fusion with healthy

ones (Chen and Chan 2009; Westermann, 2010) and segregating irreversibly damaged mitochondria for mitophagy, a cargo-specific autophagy to remove damaged mitochondria. Effective sequestration of damaged mitochondria into autophagosomes and subsequent clearance within the autophagy–lysosomal system constitute a key cellular pathway in mitochondrial quality control mechanisms. Recent studies indicate that PINK1/Parkin-mediated pathways ensure mitochondrial integrity and function (Clark et al., 2006; Gautier et al., 2008; Narendra et al., 2008). Loss-of-function mutations in PINK1 and Parkin are associated with recessive forms of Parkinson's disease. When mitochondria are depolarized or their integrity is damaged, PINK1 accumulates on the outer mitochondrial membrane and recruits the E3 ubiquitin ligase Parkin from the cytosol. Parkin ubiquitinates mitochondrial proteins (Geisler et al., 2010; Poole et al., 2010; Ziviani et al., 2010; Yoshii et al., 2011). This process is essential for damaged mitochondria to be engulfed by isolation membranes that then fuse with lysosomes for degradation.

Investigating those mechanisms coordinating mitochondrial motility and mitophagy represents an important emerging area for disease-oriented research. Mature acidic lysosomes are mainly located in the soma (Overly and Hollenbeck, 1996; Cai et al., 2010; Lee et al., 2011). Thus, a long-standing question remains: how are aged and damaged mitochondria at distal terminals efficiently eliminated via the autophagy–lysosomal pathway? Cai et al. (2012) recently reported the unique features of spatial and dynamic Parkin-mediated mitophagy in mature cortical neurons, in which mitochondria remain motile after prolonged and chronic dissipation of the mitochondrial membrane potential ($\Delta\psi_m$) for 24 h. Given the fact that in vitro mitochondrial depolarization will quickly trigger neuron apoptosis, a high-quality culturing system is essential so that the neurons survive long enough to exhibit altered mitochondrial transport and Parkin-mediated mitophagy. Chronically dissipating $\Delta\psi_m$ by prolonged treatment of low concentrations of $\Delta\psi_m$ -uncoupling reagent CCCP (10 μ M) results in accumulation of Parkin-targeted mitochondria in the soma and proximal regions. Such compartmental restriction for Parkin-targeted mitochondria is consistently observed in neurons treated with low concentrations of another $\Delta\psi_m$ -dissipating reagent, antimycin A (1 μ M), or mitochondrial ATP synthase inhibitor oligomycin (1 μ M). This distribution pattern is attributable to altered motility of chronically depolarized mitochondria with reduced anterograde and relatively enhanced retrograde transport, thus reducing anterograde flux of damaged mitochondria into distal processes. Intriguingly, when anchored by syntaphilin before the dissipation of $\Delta\psi_m$, mitochondria in distal axons also recruit Parkin for mitophagy. Altered motility may be protective under chronic mitochondrial stress conditions; healthy mitochondria remain distally while aged and damaged ones return to the soma for degradation (Fig. 4). This spatial distribution allows neurons to efficiently remove dysfunctional mitochondria distally and then eliminate them via the autophagy–lysosomal pathway in the soma.

Reduced anterograde transport of depolarized mitochondria is consistent with recent studies showing Parkin-mediated

degradation of Miro on the mitochondrial surface upon $\Delta\psi_m$ dissipation-induced mitophagy. In addition to binding to the KIF5–Trak motor complex, the mitochondrial outer membrane protein Miro also interacts with PINK1 and Parkin and is ubiquitinated by Parkin while mitochondria are depolarized (Weihofen et al., 2009; Chan et al., 2011; Wang et al., 2011; Liu et al., 2012; Sarraf et al., 2013). These studies support the current model that altered mitochondrial transport is a critical aspect of the mechanisms by which the PINK1/Parkin pathway governs mitochondrial quality control. Turnover of Miro on the mitochondrial surface may favor their retrograde transport to the soma or immobilize damaged organelles at distal regions for mitophagy (Fig. 4). A recent study reports the predominant retrograde transport of autophagosomes including those engulfing damaged mitochondria from distal axons to the soma (Maday et al., 2012). The retrograde movement is critical for autophagosome maturation and degradation within acidic lysosomes in the proximal regions of neurons. This study highlights the possibility that distal damaged mitochondria, which are either anchored by syntaphilin or immobilized by turning over Miro, may be also degraded in the soma after their retrograde transport in engulfed autophagosomes. Thus, a functional interplay is proposed between mitochondrial motility and mitophagy to ensure proper removal of aged and dysfunctional mitochondria from distal processes. The correlation between mitochondrial $\Delta\psi_m$ and motility is controversial, as demonstrated in two previous studies by acutely treating neurons with high concentrations of $\Delta\psi_m$ -dissipating reagents (Miller and Sheetz, 2004; Verburg and Hollenbeck, 2008). Instead, Cai et al. (2012) examined mitochondrial motility and mitophagy in mature cortical neurons after a 24-h treatment with a much lower concentration of CCCP (10 μ M) or antimycin A (1 μ M) combined with applying the pan-caspase inhibitor Z-VAD. The majority of neurons survive and mitochondria remain highly motile under such prolonged stress conditions, thus allowing detection of the altered transport event. Therefore, prolonged $\Delta\psi_m$ dissipation in cultured neurons better reflects chronic mitochondria stress under in vivo physiological or pathological conditions.

Moving forward

The recent discoveries of proteins involved in mitochondrial transport and anchoring boosted our understanding of the molecular mechanisms that regulate mitochondrial motility and distribution in response to physiological and pathological signals. However, there are still many mechanistic questions to be addressed in the near future. In particular, how does a Miro- Ca^{2+} -sensing pathway arrest both anterograde and retrograde movement of mitochondria at active synapses? How do dynein and KIF5 motors physically or mechanistically interplay to coordinate mitochondrial bi-directional transport? How are stationary mitochondria remobilized, recruited, and redistributed (and vice versa) to sense, integrate, and respond to changes in mitochondrial membrane potential, cellular metabolic status, neuronal growth signals, and pathological stress? Investigating how mitochondrial motility coordinates their function and integrity in neurons represents an important emerging frontier in neurobiology. Mitochondria undergo dynamic membrane fission/

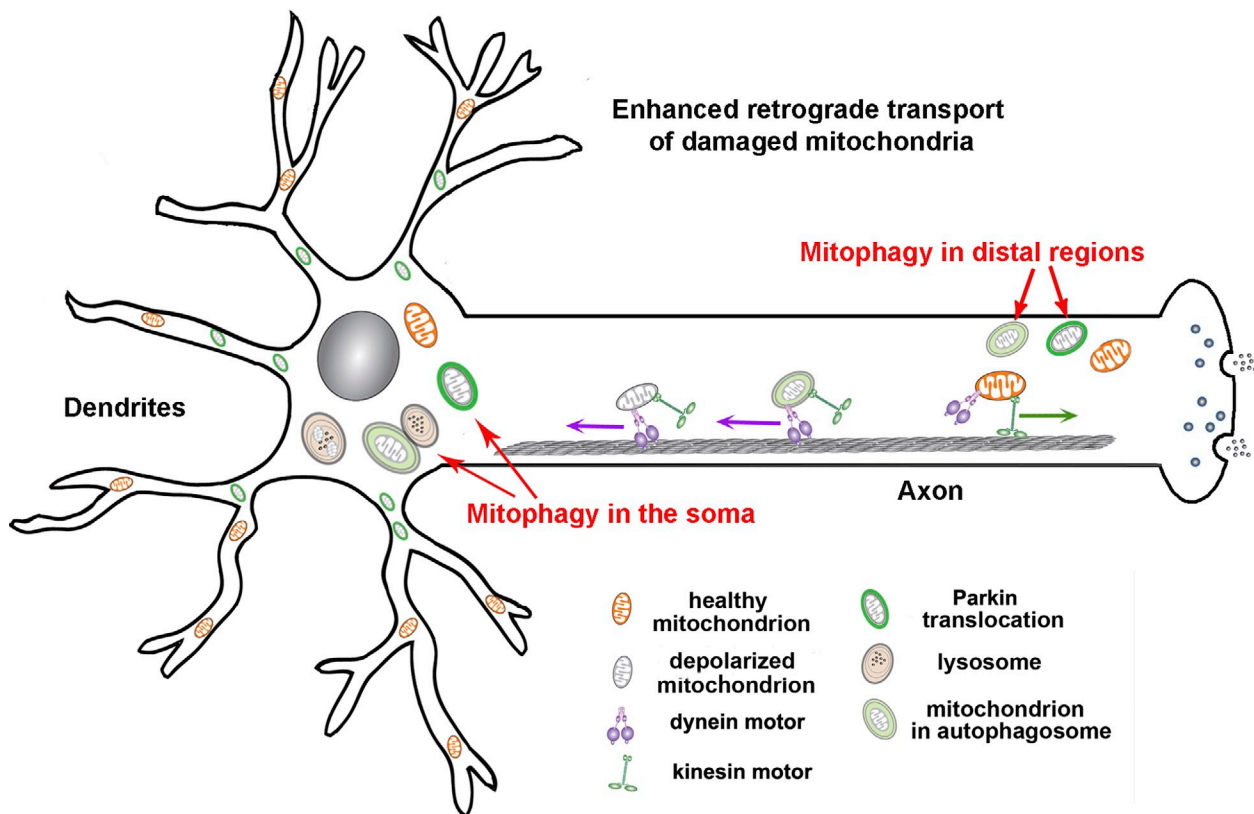


Figure 4. Functional interplay between mitochondrial transport and mitophagy. Chronically dissipating mitochondrial membrane potential ($\Delta\psi_m$) by prolonged treatment of low concentrations of $\Delta\psi_m$ -uncoupling reagents accumulates Parkin-targeted mitochondria in the soma and proximal regions. Such compartmental restriction is a result of altered motility of depolarized mitochondria with reduced anterograde and relatively enhanced retrograde transport, thus reducing anterograde flux of damaged mitochondria into distal processes (Cai et al., 2012). This spatial process allows neurons to efficiently remove dysfunctional mitochondria from distal axons via the autophagy–lysosomal pathway in the soma, where mature lysosomes are relatively enriched. Damaged mitochondria at axonal terminals can also recruit Parkin for mitophagy once they are anchored by syntaphilin (Cai et al., 2012) or immobilized by turnover of the motor adaptor Miro on the mitochondrial surface (Weihofen et al., 2009; Chan et al., 2011; Wang et al., 2011; Yoshii et al., 2011; Liu et al., 2012; Sarraf et al., 2013). Autophagosomes including those engulfing damaged mitochondria at axonal terminals transport predominantly to the soma for maturation and more efficient degradation of cargoes within acidic lysosomes (Maday et al., 2012).

fusion and mitophagy to maintain their quality during the neuronal lifetime. Mitochondrial pathology is one of the most notable hallmarks in several major aging-associated neurodegenerative diseases (Chen and Chan, 2009; Schon and Przedborski, 2011; Court and Coleman, 2012; Sheng and Cai, 2012; Itoh et al., 2013). The proper and efficient elimination of damaged mitochondria via mitophagy may serve as an early neuroprotective mechanism. Studying these dynamic processes in live neurons directly isolated from disease-stage adult mice will provide more reliable models for delineating defects in mitochondrial transport and mitophagy underlying adult-onset pathogenesis. Mechanistic insight into these fundamental processes in coordinating mitochondrial trafficking and anchoring, membrane dynamics, and mitophagy will advance our understanding of human neurodegenerative diseases.

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