MITOCHONDRIAL TRAFFICKING

IN HEALTHY AND INJURED NEURONS

by

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Mitochondria are the primary generators of ATP and are important regulators of intracellular calcium homeostasis. These organelles are dynamically transported along lengthy neuronal processes, presumably for appropriate distribution to cellular regions of increased need such as synapses. The removal of damaged mitochondria that produce harmful reactive oxygen species and promote apoptosis is also thought to be mediated by mitochondrial transport to autophagosomes. Mitochondrial trafficking is therefore important for maintaining neuronal and mitochondrial health while cessation of movement may lead to neuronal and mitochondrial dysfunctions.

The demands for mitochondria differ between developing and mature neurons, and separate mitochondrial recruitment signals have been identified in each case. In the first aim, we examined how mitochondrial dynamics are affected by the development of synaptic connections in cortical neurons. We revealed reduced mitochondrial movement and elongated morphology in mature neurons which probably serve to optimize mitochondrial contact with synaptic sites.

Synapses require mitochondria to supply ATP and regulate local $[Ca^{2+}]_i$ for neurotransmission. The second aim investigated mitochondrial trafficking patterns relative to synaptic sites on axons and dendrites. We demonstrated that synapses are targets for long-term

mitochondrial localization and dynamic recruitment of moving mitochondria, and that trafficking patterns are influenced by changes in synaptic activity. We also found that mitochondrial movement in dendrites is more severely impaired by neurotoxic glutamate and zinc exposures than in axons. These findings suggest a mechanism for postsynaptic dysfunction and dendritic degeneration in excitotoxicity.

The third aim examined impaired mitochondrial transport as an early pathogenic mechanism in Huntington's disease. Recent studies indicate that aggregates composed of mutant huntingtin fragments hinder axonal transport by sequestering wildtype huntingtin, cytoskeletal components and molecular motors. Our studies in cortical neurons demonstrated reduced mitochondrial trafficking specifically to sites of aggregates and impeded passage of moving mitochondria by aggregates resulting in discrete regions of mitochondrial accumulation and immobilization.

In summary, this dissertation provides new insight into our understanding of mitochondrial trafficking, morphology and distribution in cortical neurons that are developing, synaptically mature, acutely injured, and diseased. We conclude that mitochondrial movement is dynamic in healthy neurons and that injured neurons exhibit different manifestations of impaired movement.

FORWARD

Dedicated to my parents

Peter and Lai Ping Chang

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The accomplishment of this dissertation is the accomplishment of many individuals through the course of my life. It is with an enlightened perspective that I understand how opportunities for the development of creative thinking, analytical scrutiny, intellectual freedom, and professional and personal maturity were provided for me at home, in school, at work and in my everyday life. More importantly, these experiences were afforded by my family, my teachers, my mentors, my advisors, my colleagues and my friends. I have been incredibly fortunate to have these generous people in my life and I am deeply appreciative of all the ways in which they have helped me. My journey still has a long and unknown future, and I hope I will be as successful in providing for others that which has been provided for me.

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1.0 INTRODUCTION

1.1 PREFACE

The dissertation consists of a multi-faceted examination of mitochondrial trafficking and morphology in neurons. Special interest was given to trafficking aberrations as they relate to injury and disease. However, because fundamental regulatory mechanisms involved in mitochondrial trafficking are sparsely understood, we also focused a large part of this work on investigating mitochondrial movement in healthy neurons. The introduction broadly covers topics in normal mitochondrial physiology, the importance of mitochondrial trafficking and morphology in neuronal health, our current understanding of mechanisms that regulate mitochondrial movement and morphology, how mitochondrial dysfunction is fundamentally harmful to cells, the relevance of mitochondrial defects to neuronal injury and disease, and how impairments in mitochondrial trafficking can contribute to pathophysiology. Thus, when one considers the importance of mitochondria in supporting cellular functions and in rendering cell death, one can also appreciate the necessity for appropriate mitochondrial trafficking to maintain mitochondrial and neuronal health. By the same reasoning, consequences of impaired mitochondrial movement have great breadth and depth in their ability to cause injuries that are acute and chronic, severe and subtle, mitochondrial and cellular, and probably relevant to nearly all forms of neuronal death.

1.2 MITOCHONDRIA: MORE THAN JUST ENERGY POWERHOUSES OF THE CELL

1.2.1 ATP Production

Mitochondria are vital organelles for cell survival. They are the primary generators of cellular energy and they accomplish this with great efficiency. By coupling electron transport to the generation of proton gradients for oxidative phosphorylation, mitochondria produce 15 times more ATP from glucose than the glycolytic pathway in eukaryotic cells. Highly metabolic cells are therefore particularly dependent on mitochondria. For example, relatively inactive lymphocytes contain ~300 mitochondria whereas muscle and liver cells contain 2,000-3,000 mitochondria (Alberts et al., 1994). Furthermore, mitochondria can localize to regions of high ATP consumption in cells. They are densely arranged in the clefts between cardiac muscle myofibrils that utilize ATP for contraction and are coiled around the base of sperm flagella to power motility, implying that mitochondrial distribution serves a functional purpose (Segretain et al., 1981; Cardullo and Baltz, 1991).

Mitochondria are membranous organelles composed of a relatively permeable outer membrane, an intermembrane space, and a relatively impermeable, highly folded inner membrane that encapsulates the mitochondrial matrix. The locations of respiratory processes on mitochondrial membranes and compartments are depicted in Fig. 1. Mitochondria generate ATP by oxidative phosphorylation. First, pyruvate and fatty acids enter the mitochondrial matrix and are converted to acetyl CoA by pyruvate dehydrogenase and the fatty acid oxidation cycle, respectively. The carbon atoms of acetyl CoA are then oxidized by the citric acid cycle, producing NADH and $FADH_2$ as end products. NADH and $FADH_2$ serve as donors of high

energy electrons which are passed down four respiratory enzyme complexes composing the electron transport chain on the inner mitochondrial membrane (Fig. 2). Molecular oxygen is the final recipient of low energy electrons. The energy generated by electron transport is harnessed to translocate H^+ ions from the matrix to the intermembrane space so that a H^+ gradient is formed across the inner membrane. This protonmotive force drives H^+ flow back into the matrix through ATP synthase to form ATP from ADP and phosphate (Alberts et al., 1994).

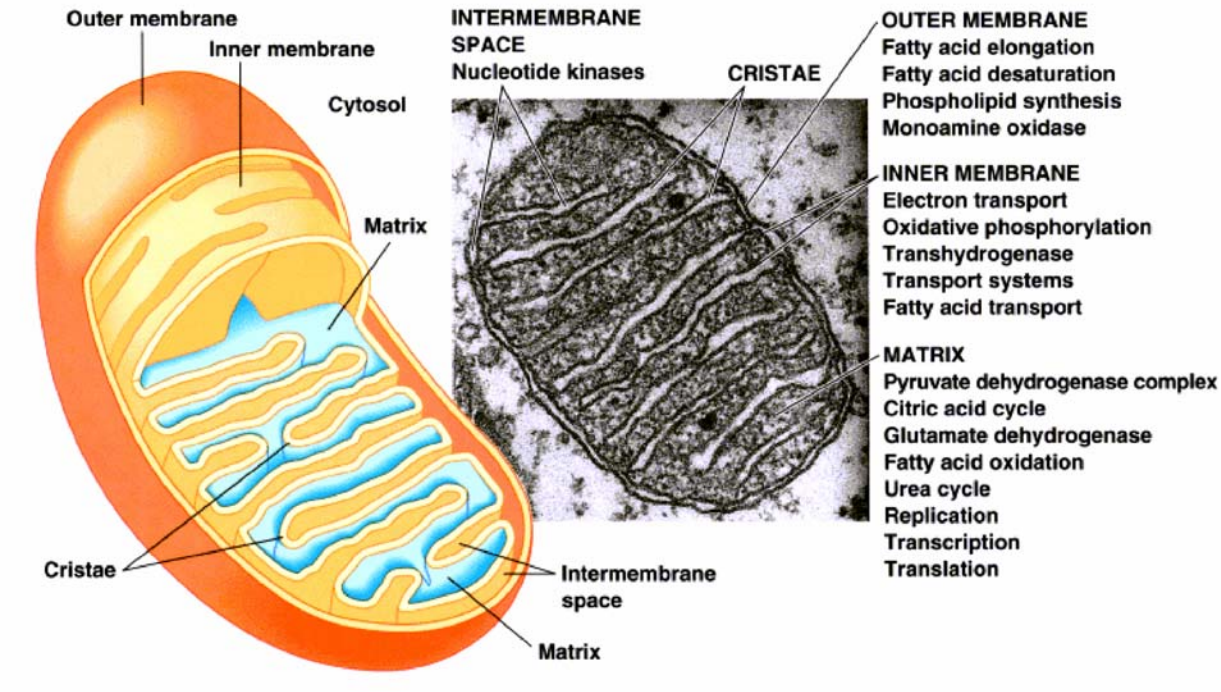

Figure 1.1 Localization of respiratory processes in the mitochondrion.
Adapted from Mathews and van Hold (1996).

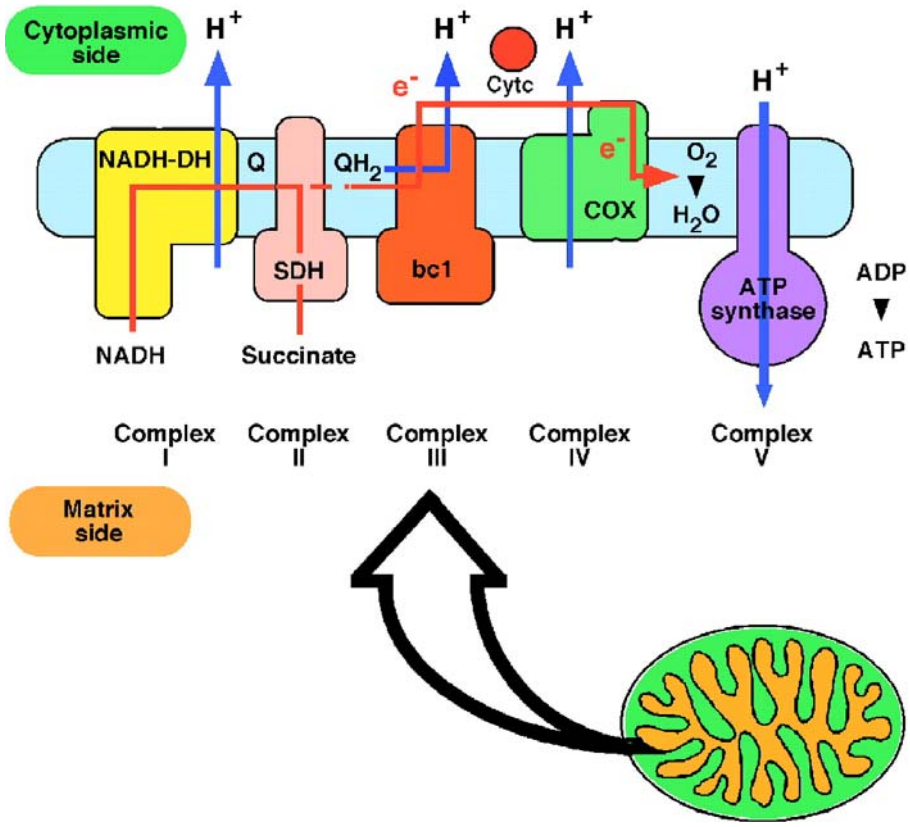

Figure 1.2 Electron transport down respiratory chain enzymes during oxidative phosphorylation.
Adapted from Sarsate (1999).

The central nervous system has an intense demand for mitochondria, as the human brain consumes 20% of resting metabolic energy while only comprising 2% of total body mass (Laughlin, 2001). Mitochondria produce over 95% of the ATP utilized by the brain (Erecinska and Silver, 1994). The generation, processing and transmission of neural impulses relies on Na^+, K^+ and Ca^{2+} ion gradients across the plasma membrane. In fact, 50-60% of total brain ATP is used to maintain these gradients, especially through Na^+/K^+ pumps. Within neurons, mitochondria are again distributed to regions of high metabolic demand, including synapses, nodes of Ranvier and myelination/demyelination interfaces (Kageyama and Wong-Riley, 1982; Berthold et al., 1993; Rowland et al., 2000; Bristow et al., 2002). The abundance of synapses is remarkable; for example, one study reports 2.4 x 10^4 neurons/mm^3 in the human temporal cortex

with each neuron containing 3.0 x 10^4 synapses (DeFelipe et al., 2002). Therefore, mitochondrial ATP generation is important for supporting dense networks of synaptically connected neurons.

1.2.2 $[Ca^{2+}]_i$ buffering

In addition to the generation of cellular energy, mitochondria also play an important role in regulating calcium homeostasis. Calcium serves as a regulator of kinases, phosphatases, proteases, transcription factors and ion channels as well as an intracellular messenger for membrane excitability, exocytosis, vesicle trafficking, muscle contraction, cell proliferation, fertilization, metabolism, crosstalk between other signaling pathways, and apoptosis (Carafoli et al., 2001). These diverse Ca^{2+}-mediated processes, which occur over the course of microseconds to hours, are highly dependent on the spatiotemporal distribution of $[Ca^{2+}]_i$ (Berridge et al., 2000; Berridge et al., 2003). Indeed, microdomains of high $[Ca^{2+}]_{cyto}$ have been identified near Ca^{2+} channels on the plasma membrane and endoplasmic reticulum (Brini, 2003). Additionally, Ca^{2+}-sensitive dehydrogenases can regulate oxidative phosphorylation and ATP synthesis during times of high cellular demand in a manner that is dependent on the relationship between $[Ca^{2+}]_{cyto}$ and $[Ca^{2+}]_{mito}$ (McCormack et al., 1990). Therefore, $[Ca^{2+}]_i$ must be tightly controlled. By regulating Ca^{2+} influx, efflux, buffering and storage, cells maintain $[Ca^{2+}]_i$ at 100 nM, ten times lower than $[Ca^{2+}]_o$, so that small changes in $[Ca^{2+}]_i$ are sufficient to activate physiological processes (Sattler and Tymianski, 2000).

Mitochondria play an important role in regulating $[Ca^{2+}]_i$, in concert with the sarco-endoplasmic reticulum Ca^{2+}-ATPase and the plasma membrane Ca^{2+}-ATPase and Na^+/Ca^{2+} exchanger. In particular, mitochondrial Ca^{2+} uptake becomes important at $[Ca^{2+}]_i$ above 400-500

nM (Nicholls and Scott, 1980). Mitochondria are able to buffer $[Ca^{2+}]_i$ by virtue of their electrochemical gradient for Ca^{2+}. This gradient is comprised of (i) the negatively charged mitochondrial membrane potential, ψ_m, which is -150-200 mV relative to the -70 mV potential across the neuronal plasma membrane, and (ii) a low $[Ca^{2+}]_{mito}$ relative to the $[Ca^{2+}]_{cyto}$. Thus, uptake of Ca^{2+} into the matrix is electrochemically favored, and is largely accomplished through the Ca^{2+}-uniporter. Mitochondrial Ca^{2+} sequestration is reversible, with Ca^{2+} efflux back into the cytosol occurring through the mitochondrial Na^+/Ca^{2+} exchanger (Duchen, 2004). Ca^{2+} release from mitochondria also has important physiological functions for prolonging elevated $[Ca^{2+}]_i$ which can mediate exocytosis and post-tetanic potentiation at presynaptic terminals (Tang and Zucker, 1997; Nicholls, 2005).

1.3 MITOCHONDRIAL LIFE CYCLE IN NEURONS

1.3.1 Evolution and biogenesis of mitochondria

Mitochondria in eukaryotic organisms are thought to be the result of engulfment of aerobically respiring prokaryotic organisms 1.5 x 10^9 years ago. A symbiotic relationship was then forged whereby mitochondria were able to rely on the host cell for transcription and protein synthesis and the host adopted efficient energy production (Alberts et al., 1994). Therefore, while present-day mitochondria still have their own circular double-stranded genomes and protein synthetic machinery, evolution probably removed a fair amount of redundancy. In fact, mammalian mitochondrial DNA (mtDNA) encodes only 37 genes: 22 tRNAs, 2 rRNAs and 13 structural proteins for oxidative phosphorylation (Garesse and Vallejo, 2001). All remaining

mitochondrial proteins, approximately 850 in total, are encoded by nuclear DNA and imported into the appropriate mitochondrial compartments (Scheffler, 2001; Schon and Manfredi, 2003).

Mitochondria have a limited lifespan, but our observations indicate that the number and function of mitochondria seems well preserved through the healthy lifetime of neurons. Although *de novo* synthesis of mitochondria has not been shown explicitly, mtDNA clearly replicates, repairs and recombines during the lifespan of a cell (Shadel and Clayton, 1997). Mitochondria are also known to arise from fission of preexisting mitochondria, both in dividing cells and post-mitotic neurons (Bereiter-Hahn and Voth, 1994; Rube and van der Bliek, 2004). Mitochondria seem to proliferate in response to increased energetic demands of cells, such as in resting skeletal muscle after prolonged contractile stimulation (Moyes and Hood, 2003). A reasonable proposition is that mitochondrial biogenesis occurs near the cell body in close proximity to the transcriptional and translational machinery for nuclear DNA on which mtDNA replication depends. In support of this, mtDNA replication has been shown to occur perinuclearly in mammalian cells and distribute peripherally with time (Davis and Clayton, 1996). Furthermore, 90% of mitochondria with relatively high membrane potentials were shown to move anterogradely toward growth cones in dorsal root ganglia neurons, implying that healthy mitochondria are trafficked away from their origins near the cell body (Miller and Sheetz, 2004). An important consideration therefore is how the appropriate distribution of mitochondria can be achieved in cells, and particularly neurons that have extensive neuronal processes.

1.3.2 Mitochondrial autophagy

Like all living things, mitochondria have a limited lifespan. The half-life of rat brain mitochondria has been reported to be ~24-30 days, in contrast to ~9-10 days for liver

mitochondria (Gross et al., 1969; Menzies and Gold, 1971). Mitochondria represent an interesting paradox because while they provide critical functions for cell survival, their dysfunction can be a key determinant of cell death, as discussed in Chapter 1.7. It is therefore imperative for cells to regulate the proper removal of damaged mitochondria, presumably when they are functionally impaired or have accumulated mtDNA mutations that generate reactive oxygen species (De Grey, 2005). This is accomplished through autophagy, a process in which autophagosomes engulf cytosolic contents and then fuse with lysosomes for proteolytic degradation (Lemasters, 2005). Autophagy can be targeted directly toward mitochondria, which is mediated by the outer mitochondrial membrane protein Uth1p (Kissova et al., 2004). Mitochondrial autophagy has been shown to occur in cells when apoptosis is induced, in hepatocytes with mitochondrial permeability transition pore formation, and in yeast with depolarized mitochondria (Elmore et al., 2001; Tolkovsky et al., 2002; Priault et al., 2005). Much like the biogenesis of mitochondria, the degradation of mitochondria may occur near the cell soma where lysosomes are more prevalent. Indeed, 80% of low-potential mitochondria in dorsal root ganglia neurons were demonstrated to move retrogradely, implying less healthy mitochondria may return to the cell body for repair or removal (Miller and Sheetz, 2004). Again, this raises the challenge of retrieving distant mitochondria from axons and dendrites for processing in the cell soma.

1.4 MITOCHONDRIAL TRAFFICKING IN NEURONS

The movement of mitochondria has been described for many years but only with the recent advent of fluorescent labels for mitochondria in live cells, time-lapse microscopy and genetic

mutants with abnormal trafficking patterns have we started to understand the phenomenon more clearly (Bereiter-Hahn, 1990). Properties studied include patterns of mitochondrial movement, mechanisms of mitochondrial transport, and perhaps most importantly, cellular and mitochondrial signals that govern mitochondrial trafficking. Stationary evidence in neurons, muscles cells and sperm cells support the idea that mitochondria are localized to regions of high metabolic demand and elevated $[Ca^{2+}]_i$ microdomains (Segretain et al., 1981; Cardullo and Baltz, 1991; Rowland et al., 2000; Brini, 2003). Only recently are we beginning to elucidate how moving mitochondria are dynamically influenced by cellular signals.

1.4.1 Significance of mitochondrial transport in neurons

While mitochondrial transport in mature neurons is of great interest as it relates to neurodegeneration and aging, it is also important to consider the relevance of mitochondrial transport in neurodevelopment. Neurons develop from undifferentiated neuroblasts that lack any sort of axonal or dendritic specializations. Mitochondria are therefore localized to the cell soma. Neuronal morphology then changes dramatically as neuronal processes rapidly elongate. An axon is designated, axonal and dendritic branches form and remodel, and synaptic connections are created and matured (Ramoa et al., 1988; Antonini and Stryker, 1993). These are highly dynamic processes marked by an intense need for ATP. Indeed, mitochondria have been shown to localize to growth cones of actively growing axons by chemoattraction to nerve growth factor (Morris and Hollenbeck, 1993; Chada and Hollenbeck, 2003, 2004). Furthermore, neuronal growth dictates that energetic demands extend greater distances from the cell body. Thus, appropriate trafficking of mitochondria is a necessary task beginning in the earliest moments of neuronal development that becomes increasingly important as development proceeds.

Neurons are one of the most specialized cell types and they have an enormous capacity to support complex neurotransmission communications with other cells. They represent a particular anatomical and physiological challenge for mitochondrial trafficking because (i) neuronal processes are highly branched and extensive, as long as several feet in some cases, and (ii) sites with large demands for ATP and local $[Ca^{2+}]_i$ regulation, such as synapses, are distributed throughout the length of axons and dendrites. Furthermore, synaptic connections are highly plastic, undergoing continuous, spontaneous and directed remodeling to modulate information processing (Konur and Yuste, 2004; Hayashi and Majewska, 2005). Remodeling of presynaptic terminals and postsynaptic sites involves not only local cytoskeletal rearrangements and protein synthesis, but also recruitment of vesicles and receptors as well as removal and degradation of synaptic proteins (Rao and Craig, 1997; Zito et al., 2004; Wiersma-Meems et al., 2005). Thus, neuronal processes present many sites of dynamically changing demand for mitochondria, and these sites are likely regulators of mitochondrial trafficking.

Anterograde and retrograde trafficking of mitochondria are not only necessary to support active processes throughout the cellular lifetime, but are also required throughout organellar lifetime. Mitochondrial movement facilitates fusion and fission events between individual organelles that likely maintain mitochondrial health and homeostasis, as discussed in Chapter 1.5. Furthermore, retrieval of damaged mitochondria from distal processes for degradation by autophagy in the perinuclear region poses as great a challenge as trafficking newly synthesized mitochondria out into neuronal processes.

1.4.2 Mitochondrial movement patterns

It is clear from our and others' observations that mitochondrial movement in neurons is extremely diverse and complex (Morris and Hollenbeck, 1993; Morris and Hollenbeck, 1995; Overly et al., 1996; Ligon and Steward, 2000a). Some mitochondria appear stationary whereas others are motile. The duration that different mitochondria remain stationary has not been well described. Motile mitochondria not only move with different speeds and in different directions, but they exhibit saltatory movement, making stops along their trajectory. These stops are also variable. Mitochondria can stop for seconds, minutes, or much longer time periods. They often stop where other mitochondria already reside, but can also pause along processes where no mitochondria are present. These organelles spontaneously change direction, sometimes appearing to shuffle between several specific locations and other times continuing to travel consistently in the new direction. Not only are mitochondrial movement patterns highly variable between axons, dendrites and among individual mitochondria in a single neuron, but also in different types of neurons at different developmental stages.

The intra- and intercellular diversity of mitochondrial movement patterns complicates the ability to describe such properties with absolute values. Automated data analysis is often unfeasible because it cannot account for the variability in movement patterns. Consequently, some of the most detailed data analysis has been performed manually, which can be complicated by subjectivity and labor intensiveness. Nevertheless, it is useful to review what has been quantitatively reported thus far. Studies in cultured hippocampal neurons found the percentage of mitochondria that move > 0.5 μm/sec to be 20% in axons and 7% in dendrites (Overly et al., 1996). When mitochondrial movement was defined as > 0.5 μm/15 sec, 47% of axonal mitochondria and 35% of dendritic mitochondria were considered mobile (Ligon and Steward,

2000a). These findings consistently indicate that a greater fraction of mitochondria move in axons than in dendrites. Mean mitochondrial speeds are similar in the anterograde and retrograde direction, but have been reported as 0.09 or 0.51 μm/sec in axons and 0.007, 0.04 or 0.42 μm/sec in dendrites (Overly et al., 1996; Ligon and Steward, 2000a; Li et al., 2004). Mitochondria seem to have an equal probability of moving anterogradely and retrogradely in axons in the range of 30-50%, but at least a 20% increase in probability of moving anterogradely in dendrites (Overly et al., 1996; Ligon and Steward, 2000a). Lastly, paused mitochondria resume moving in the opposite direction ~30% of the time in both axons and dendrites, but sustained movement in the new direction over at least a 3 μm distance was only observed by dendritic mitochondria (Overly et al., 1996).

1.4.3 Mechanisms of mitochondrial movement

Three major protein groups mediate mitochondrial movement in neurons: (1) the cytoskeleton on which mitochondria are transported, (2) the molecular motors that carry mitochondria as their cargos, and (3) a host of adaptor/scaffolding proteins that mediate interactions between motors and the cytoskeleton. Several proteins outside of these classes have also been implicated as modulators of movement, including plekstrin homology domain proteins and transiently expressed developmental proteins (De Vos et al., 2003; Gross et al., 2003).

Microtubules and actin microfilaments serve as cytoskeletal substrates on which mitochondria are transported. How exactly these different structural proteins mediate the dynamics of saltatory mitochondrial movement is not clear as different studies often report different effects of cytoskeletal disruption. For example, depolymerization of microtubules by nocodazole or vinblastine results in fewer moving mitochondria and slower velocities while

bidirectionality is preserved (Morris and Hollenbeck, 1995; Ligon and Steward, 2000b). When actin filaments are depolymerized or disorganized by latrunculin B, cytochalasin D or E, the results include fewer moving mitochondria, increased velocity bidirectionally, or no effect (Morris and Hollenbeck, 1995; Ligon and Steward, 2000b). In neurons, microtubules are likely to be tracks for transport over long-distances while actin microfilaments mediate travel over short-distances. Since mitochondrial transport on microtubules is bidirectional, the polarity orientation of tubulin filaments may also affect mitochondrial trafficking patterns. In axons, microtubules are uniformly arranged with (+)-ends directed toward the growth cones. Distal dendritic segments display the same orientation, but at distances > 15 μm from the growth cone, (+)-ends of microtubules are oriented in both directions with equal frequency (Baas et al., 1989). Different microtubule-associated proteins (MAPs) localize to axons, dendrites or throughout cells, where they help stabilize and promote oriented microtubule polymerization. Phosphorylation of MAPs results in microtubule disassembly (Kandel et al., 1991). Therefore, regulation of MAPs might be another method of modulating mitochondrial trafficking patterns.

Kinesins and cytoplasmic dynein are ATPases that transport mitochondria toward (+)- and (-)-ends of microtubules respectively (Hirokawa and Takemura, 2005). Generally, kinesin superfamily proteins (KIFs) consist of (i) a globular motor domain containing microtubule- and ATP-binding sequences, and (ii) a cargo-binding domain that imparts selectivity to different isoforms. Each KIF has an intrinsic velocity, ranging from 0.2-1.5 μm/sec. Monomeric KIF1Bα and homodimeric KIF5 have been shown to transport mitochondria in axons and dendrites (Nangaku et al., 1994; Tanaka et al., 1998; Kanai et al., 2000). Cytoplasmic dynein has a globular head from which a stalk and stem extend. The former binds microtubules and the latter binds cargo through association with the dynactin protein complex. Dynein has been

shown to transport organelles retrogradely although it can also localize to those that are anterogradely transported (Hirokawa et al., 1990). Phosphorylation states, conformational changes, and abundances of motor proteins and MAPs are thought to regulate aspects of motor activity, directionality and association with cargo (Sheetz, 1999; Reilein et al., 2001). Furthermore, motor proteins on mitochondria are distributed in discrete clusters that seem to generate force independently of each other, suggesting that differential regulation of individual motor protein clusters may mediate changes in velocity and direction (Hollenbeck, 1996). Interestingly, motor proteins are fully functional at ATP concentrations below normal $[ATP]_i$, suggesting that transport of mitochondria is an efficient method of energy dispersal in cells.

The interaction between motor proteins and their cargos are mediated by adaptor/scaffolding proteins. Milton and syntabulin are two proteins that were recently implicated as scaffolding proteins for mitochondria to kinesin heavy chain (Stowers et al., 2002; Cai et al., 2005). When expression of these proteins is reduced, mitochondria exhibit an abnormal distribution in agreement with disrupted anterograde transport. In summary, complex mitochondrial movement patterns are likely mediated by complex and dynamic interactions between a cohort of regulatable motor proteins, adaptor proteins, and cytoskeletal elements.

1.4.4 Mitochondrial docking signals

An important issue is not only what drives mitochondria to move, but also what signals them to stop. A large fraction of mitochondria are stationary for extended time periods and moving mitochondria often make stops along their trajectory. It can be speculated that some pauses may be incidental based on the structure and length of cytoskeletal filaments. However, many stops

are likely to occur for functional reasons. An important physiological question is what signals cause mitochondria to stop and how these stops are mechanically executed.

The rapid dynamics of mitochondrial movement have complicated the ability to study specific cellular influences in an isolated manner. However, the clearest evidence for a chemoattractive signal for mitochondria in neurons is nerve growth factor (NGF) (Chada and Hollenbeck, 2003, 2004). Time-lapse microscopy demonstrated more mitochondria moving toward and being retained at the focus of NGF-coated beads compared to control beads. Furthermore, these "docking" events involved PI 3-kinase-mediated signaling and intact actin microfilaments, neither of which were necessary for mitochondrial movement. One resulting theory is that phosphorylation events regulate dislodgement of mitochondria carried by kinesin or dynein from microtubule tracks in exchange for a longer-term static interaction of actin with myosin or other docking proteins (Reynolds and Rintoul, 2004). In a more correlational study, Li et al. (2004) demonstrated mitochondrial localization at morphogenic spines in dendrites of hippocampal neurons over 30 min to hour-long time intervals after synaptic excitation. Although not discussed by the authors, the high concentration of actin in dendritic spines suggests that this may be another actin-mediated docking event. Lastly, interactions between mitochondria and neurofilaments, promoted by neurofilament phosphorylation, may also mediate mitochondrial stationing (Leterrier et al., 1994; Wagner et al., 2003).

1.5 MITOCHONDRIAL MORPHOLOGY

1.5.1 Morphology is diverse and dynamic

Mitochondrial morphology varies between different cell types, within individual cells, and under different cellular environments. Mitochondria also undergo fission and fusion with each other and elongate or shorten under normal physiological conditions as well as during injurious stimuli. Our laboratory has analogized the shapes of mitochondria in neurons to different foods, ranging from discrete spherical bodies ("hamburgers"), to elongated string-like entities ("hotdogs"), and even to circular structures ("doughnuts"). Studies reveal instances where mitochondria are electrically isolated from each other, where they form large interconnected filamentous networks, and where both morphologies are found in a single cell (Skulachev, 2001; Collins and Bootman, 2003). Progress is being made in identifying the protein mediators and mechanistic properties of mitochondrial fusion and fission. Pharmacologic manipulations are also revealing how mitochondrial morphology may relate to mitochondrial function and cell survival.

1.5.2 Regulation of mitochondrial morphology

Mitochondrial fission and fusion are directly responsible for altering mitochondrial morphology in a rapid and dynamic manner in addition to changing the number of independently operating organelles. Fission and fusion are complex processes, involving separate reactions of the inner and outer mitochondrial membranes. Various proteins have been identified that mediate these reactions. Additionally, different physiologic conditions are known to influence the two reactions.

Dynamin-related GTPases have been shown to mediate division of the outer mitochondrial membrane (Rube and van der Bliek, 2004). Drp1 and its homologues can assemble into spiral structures thought to wrap around mitochondrial scission sites. Studies in yeast indicate that outer membrane fission is accomplished in conjunction with other proteins, including Mdv1/Fis2/Gag3, Dnm1, and Fis1/Mdv2, that either form or help to assemble a division apparatus. Very little is known about the nature of inner membrane division, which is a separate process thought to be mediated by inner mitochondrial membrane proteins. Electron microscopy has revealed that septation of the inner membrane divides the mitochondrial matrix prior to outer membrane division (Griparic and van der bliek, 2001). Inner membrane fission is often but not always coupled to outer membrane division.

Mitochondrial fusion also involves reactions between the outer and inner membranes that are mediated by mitochondrial membrane proteins (Griparic and van der bliek, 2001; Malka et al., 2005; Scorrano, 2005). In mammals, the outer membrane GTPases, mfn1 and mfn2, and the inner membrane dynamin-related protein, OPA1, interact to effect fusion events. However, how fusion of the separate membranes is coordinated is still unknown. Interesting evidence that inhibition of glycolysis and ψ_m depolarization preferentially impaired fusion of the inner membrane over the outer membrane supports the idea that fusion of the two membranes is separate and differentially regulated (Malka et al., 2005).

1.5.3 Functional implications of mitochondrial morphology

There is very little evidence directly relating mitochondrial morphology to mitochondrial function. Experiments in yeast indicate that mitochondria fuse after conjugation and the integrity of mtDNA relies on exchanges that occur when mitochondria fuse and divide (Nunnari et al.,

1997). For example, when fission and fusion are blocked, mitochondrial morphology appears normal but the organelles become less capable of respiration and lose their mtDNA more rapidly than wildtype mitochondria (Okamoto and Shaw, 2005). Furthermore, the respiratory defects of mutation-harboring mammalian mitochondria were shown to be protected by mitochondrial fusion and mtDNA complementation with wildtype mitochondria (Nakada et al., 2001; Ono et al., 2001). The recombination and repair of mtDNA between organelles is important because mtDNA is vulnerable to mutations from mitochondrially-generated reactive oxygen species and mitochondria have inefficient mtDNA repair mechanisms (Linnane et al., 1989). However, beyond the role of mitochondrial fusion in mtDNA exchange, little is known about why mitochondria fuse, divide, elongate and shorten. Much speculation has been generated about the purpose of different mitochondrial morphologies and these theories are discussed in the remainder of this section.

Different mitochondrial morphologies and the ability to rapidly alter morphology during normal physiological conditions are speculated to confer certain advantages to the organelles. For example, mitochondrial morphology might influence energy distribution. ATP dispersal over a more extensive cytoplasmic area could be facilitated by increasing mitochondrial length and thus reducing diffusion distance. By the same reasoning, longer mitochondria may sequester ions over a given surface area more effectively. This advantage might be particularly relevant to Ca^{2+}, which can be cytotoxic in large quantities (Stout et al., 1998; Szabadkai et al., 2004). Another bioenergetic advantage can be derived from the electric cable equation, which predicts that the energy loss associated with respiration is minimized over longer mitochondrial lengths (Skulachev, 2001). Therefore, mitochondria-requiring sites that are in close proximity to each other might be efficiently served by longer mitochondria. This could be an adaptive response

since mitochondria in yeast and plant cells transform from isolated organelles into extended networks under conditions of hypoxia and impaired energy production (Foissner, 1983; Bereiter-Hahn, 1990).

On the other hand, it is thought, though not experimentally proven, that discrete mitochondrial bodies harbor unique benefits over long filamentous mitochondrial structures. Mitochondrial mass is limited in cells, so division of mitochondria may be an effective method of generating "new" organelles (Posakony et al., 1977). Mitochondrial fission may also be an important response for addressing different energetic demands in postmitotic cells, as newly divided mitochondria have been anecdotally reported to adopt different motility patterns from each other. Considering the mechanisms of organellar transport, smaller cargos might require fewer motor proteins, and thus less ATP to power motility (Hollenbeck, 1996). Discrete mitochondria could additionally have the advantage of being electrochemically protected from mitochondrial damage or depolarization which would spread through an extensive mitochondrial network (Szabadkai et al., 2004). If different morphologies do prove to confer functional advantages to mitochondria, then a balance is probably achieved between mitochondrial morphology, the functional consequences of altered morphology, and the demands of the cellular environment.

1.6 ALTERED MITOCHONDRIAL MORPHOLOGY IN NEUROTOXICITY

Not only is mitochondrial morphology regulated for maintaining normal cell health, but it also undergoes remodeling during mitochondrial and cellular injury, death and disease. Typically, the morphology change takes the form of fragmentation. This is likely to be a response with

both protective and toxic consequences. For example, the spread of mitochondrial injury through extensive organellar networks can be restricted by physically dividing mitochondria into isolated entities (Szabadkai et al., 2004). Experiments with uncouplers and inhibitors of electron transport and ATP synthase in fibroblasts also suggest that intact oxidative phosphorylation is necessary to maintain mitochondrial networks (Skulachev, 2001). Yet drp1 seems to associate with proapoptotic Bax proteins on even relatively punctate mitochondria to mediate fission during programmed cell death (Karbowski et al., 2002). Mitochondrial division therefore has dual roles in cell survival.

1.6.1 Mitochondrial rounding after glutamate excitotoxicity

Our laboratory has a special interest in the role of mitochondria in glutamate excitotoxicity. We previously showed that mitochondria uptake large Ca^{2+} loads after acute excitotoxic glutamate exposure, and that this uptake by mitochondria rather than just elevated cytosolic [Ca^{2+}] has direct consequences for cell death (White and Reynolds, 1997; Stout et al., 1998). However, our investigation of the effects of moderately excitotoxic glutamate treatment on mitochondrial movement cessation and morphologic remodeling revealed a dependence on cytosolic Ca^{2+} (Rintoul et al., 2003b). We found that remodeling of mitochondrial morphology, which consisted of shortening and rounding of the organelles, was specific to elevated Ca^{2+} influx through N-methyl-D-asparate (NMDA) receptors separately from ψ_m depolarization and inhibition of mitochondrial ATP synthesis. Glutamate treatment actually caused swollen varicosities to form in the cytosol, which could very well influence mitochondrial morphology. However, the mechanistic details and functional consequences of mitochondrial remodeling remain to be clarified. It is possible that mitochondrial fragmentation contributes to the punctate

morphology of some mitochondria after glutamate exposure, but our limited imaging resolution precludes the differentiation of true membrane fission from independent rounding of closely apposed mitochondria.

1.6.2 Mitochondrial fragmentation in neuronal injury and apoptosis

In contrast to glutamate-induced morphologic remodeling which seems to preserve the continuity of individual mitochondria to a large degree, induction of apoptosis by staurosporine clearly produces fragmentation of mitochondria. Frank et al. (2001) demonstrated Drp1 mobilization from the cytosol to mitochondria when apoptosis was induced in COS-7 and HeLa cells. The proapoptotic protein Bax localized to discrete mitochondrial foci containing Drp1 during the initial stages of apoptosis (Karbowski et al., 2002). In fact, inhibition of Drp1 prevented not only mitochondrial fission, but also ψ_m depolarization, cytochrome c release, and cell death. Similar findings were also reported in yeast, where the anti-apoptotic proteins Bcl-2 and Bcl-xL inhibited Dnm1-mediated mitochondrial fission and cell death (Fannjiang et al., 2004). It should be noted that mitochondrial fragmentation is again difficult to distinguish from mitochondrial rounding by fluorescence imaging. Mitochondrial fission is thought to be related to mitochondrial outer membrane permeabilization and release of proteins involved in the cell death cascade, but the sequence of events are debatable (Perfettini et al., 2005).

1.7 MITOCHONDRIAL INJURY WREAKS HAVOC IN CELLS

Mitochondria provide critical functions for cells, most notably ATP production and intracellular Ca^{2+} regulation. Loss of mitochondrial function during injury would obviously compromise

these abilities. However, the consequences of mitochondrial damage are not just limited to energy deprivation and disrupted $[Ca^{2+}]_i$ homeostasis. Defects in electron transport can increase production of harmful reactive oxygen species (ROS) which have a variety of detrimental effects on cells and on mitochondria. Activation of mitochondrial permeability transition is stimulated by pathological conditions and is also involved in necrotic and apoptotic cell death (Crompton, 1999). Cell survival therefore relies vitally on mitochondrial health.

1.7.1 Energy deprivation and cell death

One of the most important consequences of extensive disruption of mitochondrial function is loss of ATP. This causes gross bioenergetic failure that results in necrotic cell death when insufficiently compensated for by glycolytic ATP synthesis. Necrosis is characterized by plasma membrane blebbing, mitochondrial membrane permeabilization, lysosomal disruption, leakage of cations and anions across the cell membrane, cell swelling, plasma membrane rupture, release of cytosolic contents and collapse of electrochemical gradients across the cell membrane (Lemasters et al., 2002). This is most relevant to severe anoxic and ischemic injuries where cells are deprived of oxygen for oxidative phosphorylation. ATP levels are an important determinant of whether cells die by apoptosis or necrosis, as ATP is necessary for events preceding apoptotic cell death such as apoptosome formation and caspase activation. Indeed, cells can be rescued from hypoxia-induced necrosis when supplemented with glycolytic substrates, and cells induced to undergo apoptosis instead die by necrosis when ATP synthase is inhibited (Lemasters et al., 2002; Bras et al., 2005). ATP regeneration during reperfusion of ischemic tissues then, is important in promoting apoptotic cell death over necrotic cell death.

1.7.2 Dysregulation of $[Ca^{2+}]_i$ homeostasis

Consequences of insufficient mitochondrial Ca^{2+} sequestration in cells are not only disruption of normal $[Ca^{2+}]_i$ cycling needed to maintain appropriate Ca^{2+}-dependent signaling pathways and Ca^{2+}-dependent enzyme activities, but also pathological accumulation of $[Ca^{2+}]_i$. Ca^{2+} influx into cells is an important event in ischemic injury, glutamate excitotoxicity, epilepsy and trauma. Our laboratory and others previously demonstrated the importance of mitochondrial Ca^{2+} uptake in mediating excitotoxic cell death of neurons after glutamate treatment (Budd and Nicholls, 1996; Stout et al., 1998). However, grossly elevated $[Ca^{2+}]_{cyto}$ still has important implications for cell disruption if not for cell death. These include the activation of (1) Ca^{2+}-sensitive proteases such as calpain that can cause cytoskeletal breakdown, (2) phospholipases that can disrupt membranes, activate the arachidonic acid cascade and lead to ROS production, (3) endonucleases that cause DNA degradation and (4) Ca^{2+}-binding proteins such as calmodulin which activates nitric oxide synthase for creation of reactive nitrogen species (Sattler and Tymianski, 2000; Farooqui et al., 2004).

Chronic conditions of mild mitochondrial injury such as those found in neurodegenerative diseases may impart a different type of disrupted Ca^{2+} homeostasis in cells and in mitochondria. For example, damaged mitochondria likely have reduced ATP synthesis, which is exacerbated by the ψ_m depolarization that accompanies normal Ca^{2+} cycling between cytosol and mitochondria. Consequently, ATP-dependent Ca^{2+} extrusion mechanisms from the cell may be compromised, causing prolonged periods of harmful elevations in $[Ca^{2+}]_{cyto}$ and $[Ca^{2+}]_{mito}$ (Murphy, 1999). Another possible outcome of damaged mitochondria and their reduced ability to uptake Ca^{2+} is the inability of Ca^{2+}-sensitive proteins in mitochondria to upregulate ATP synthesis appropriately during times of high demand (Brini, 2003). In summary,

mitochondrial injury can cause disrupted Ca^{2+} homeostasis in the cytosolic and mitochondrial compartments that lead to separate and interrelated cellular dysfunctions.

1.7.3 Production of reactive oxygen species

Oxidative phosphorylation is a key generator of ROS in cells (Duchen, 2004; Andreyev et al., 2005; Bras et al., 2005). High-throughput electron transport down the respiratory chain inevitably results in the escape of unpaired electrons, largely at complexes I and III. Reaction of electrons with O_2 yields the highly reactive superoxide anion, which can convert to other ROS such as hydrogen peroxide and hydroxyl radicals. Mitochondria have a high capacity for scavenging ROS by enzymes such as a superoxide dismutase, which converts superoxide anions to hydrogen peroxide, catalase, which converts hydrogen peroxide to water and oxygen, and glutathione, which can scavenge ROS nonenzymatically as well as donate electrons to other ROS-detoxifying enzymes. However, when ROS generation surpasses these protective mechanisms, multiple destructive redox reactions can ensue: (1) lipid peroxidation can compromise the integrity of membranes, including mitochondrial membranes, (2) protein oxidation can disrupt structures and activities of enzymes and structural proteins, and (3) oxidative damage to DNA, including mtDNA. ROS are therefore harmful to both cells and mitochondria.

Mitochondrial defects can increase ROS production as well as reduce ROS removal. Electron leak is heightened during complex I inhibition by rotenone, ψ_m hyperpolarization, and complex IV poisoning by cyanide. On the other hand, when mitochondrial permeability transition is activated in pathological conditions as discussed in the next section, loss of glutathione from the matrix can severely compromise ROS removal (Savage et al., 1991).

Mitochondria are important normal generators of ROS for cell signaling and because mitochondrial damage can alter the balance between ROS production and removal, they can be important contributors to oxidative damage. Chronic oxidative stress is implicated in many neurodegenerative conditions, including Parkinson's disease (PD), Alzheimer's disease (AD), Friedreich's ataxia, amyotrophic lateral sclerosis (ALS) and aging (Andersen, 2004). Additionally, ROS production is promoted by reperfusion after ischemic injury (Lemasters et al., 2002).

1.7.4 Mitochondrial release of apoptogenic proteins

Apoptosis is a highly regulated process of cell killing that is characterized by cell shrinkage, caspase activation, DNA fragmentation, chromatin condensation and nuclear fragmentation. Proteins released from mitochondria into the cytosol are important inducers of apoptosis. The first molecule to be released is cytochrome c, which forms an apoptosome complex in association with apoptosis protease-activating factor 1 (Apaf-1), ATP or dATP, and procaspase-9 (Liu et al., 1996; Li et al., 1997; Cain et al., 2000). ATP-dependent activation of procaspase-9 then occurs, followed by a proteolytic caspase cascade leading to cell death. The mitochondrial intermembrane space proteins Smac/DIABLO and Omi/HtrA2 are also released and promote apoptosis by antagonizing inhibitors of caspases (Du et al., 2000; Verhagen et al., 2000; Suzuki et al., 2001; van Loo et al., 2002). Additionally, apoptosis inducing factor and endonuclease G are mitochondrial proteins that can exit and mediate caspase-independent DNA fragmentation and chromatin condensation (Susin et al., 1999; Li et al., 2001b). Thus, mitochondria harness a cohort of proteins that can lead to the demise of cells.

The release of proapoptotic proteins from mitochondria is a critical event that probably involves permeabilization of mitochondrial membranes. Various mechanisms for achieving permeability have been suggested although the specific sequence of events and regulations thereof are still quite unclear (Lemasters et al., 2002; Duchen, 2004; Bras et al., 2005). One involves activation of the mitochondrial permeability transition pore (mPTP), a large conductance channel of solutes up to 1.5 kDa that spans the outer and inner mitochondrial membranes. It is composed of the inner membrane adenine nucleotide translocator (ANT), the outer membrane voltage-dependent anion channel (VDAC), and matrix cyclophilin D. mPTP can be opened in pathological conditions of high $[Ca^{2+}]_{mito}$, high levels of inorganic phosphate, oxidative stress, ATP depletion and ψ_m depolarization (Crompton, 1999). A second possibility is that proapoptotic proteins Bax and tBid interact with ANT and VDAC to cause mitochondrial permeability transition. Lastly, activated Bax or Bak may directly form channels that permit release of apoptogenic mitochondrial proteins (Bras et al., 2005). Ultimately, permeability transition of mitochondrial membranes leads to ψ_m depolarization, uncoupling of oxidative phosphorylation, mitochondrial swelling, ATP depletion and cell death.

1.8 MITOCHONDRIAL DYSFUNCTION IN NEURONAL INJURY AND DEGENERATION

Since mitochondrial damage clearly has multiple severe consequences on cell survival, it is important to consider pathological conditions under which mitochondrial injury occur in more detail. These can be both acute insults that cause immediate disruption of mitochondrial function

leading to rapid or delayed cell death, or chronic mitochondrial defects that manifest slowly but sufficiently to cause neurodegeneration.

The clinical situations that are most relevant to the dissertation are reviewed here, notably ischemic and excitotoxic injury and PD. Huntington's disease (HD) is discussed in detail in Chapter 4 and so it is omitted in this section. Additionally, mitochondrial dysfunctions involved in the common neurodegenerative disorders of AD and ALS are summarized. The relevance of less common neurodegenerative diseases with mitochondrial defects, including Leigh syndrome, Wilson's disease, Friedreich's ataxia, hereditary spastic paraplegia, and those associated with mtDNA mutations, are discussed in Chapter 5 (Schon and Manfredi, 2003).

1.8.1 Ischemia and excitotoxicity

Ischemic events, such as stroke, are characterized by significant reduction in blood flow to target tissues. Depending on the severity and duration of oxygen deprivation, hypoxic ATP loss and reduced electron transport chain complex activities can lead to necrosis and/or apoptosis through multiple concurrent and interrelated pathways. These include abnormal ion permeabilities, reduced oxidative phosphorylation, increased glycolysis, acidosis, ψ_m depolarization, mitogen-activated protein kinase (MAPK) activation, generation of reactive oxygen and nitrogen species, oxidation of mitochondrial lipids and proteins, increased glutamate release and increased $[Ca^{2+}]_i$ (Fiskum et al., 1999; Lipton, 1999). The damage wrought by free radicals and high $[Ca^{2+}]_{cyto}$ are described in Chapter 1.7. Here we will focus on the role of Ca^{2+}-mediated mitochondrial dysfunction in excitotoxicity.

Glutamate, the major excitatory neurotransmitter, is known to be released during ischemic injury *in vivo* and *in vitro* (Benveniste et al., 1984; Lobner and Lipton, 1990). This

results in a large Ca^{2+} influx into cells through glutamate receptors, of which the NMDA receptor mediates the most toxic effects. This may be related to neurotoxic second messengers such as neuronal nitric oxide synthase (nNOS) that are coupled to NMDA receptors, as nitric oxide (NO) has been shown to depolarize mitochondria and inhibit ATP synthesis (Sattler and Tymianski, 2000). A substantial amount of the incoming Ca^{2+} is sequestered by mitochondria, which could be particularly important during reperfusion-dependent injury as ATP synthesis resumes and ψ_m repolarizes (White and Reynolds, 1997). Elevated Na^+ influx into cells promotes some Ca^{2+} release from mitochondria through the mitochondrial Na^+/Ca^{2+} exchanger, although this also prolongs $[Ca^{2+}]_{cyto}$ elevation (Zhang and Lipton, 1999). Mitochondria become overloaded at $[Ca^{2+}]_{cyto}$ of 1-3 μM, and consequences include ψ_m depolarization, impaired ATP synthesis, ROS generation, permeability transition and cell death (Nicholls and Crompton, 1980; Murphy et al., 1999). Importantly, while elevated $[Ca^{2+}]_{cyto}$ has disruptive effects, neurotoxicity can be prevented when mitochondrial Ca^{2+} uptake is prevented by ψ_m depolarization (Budd and Nicholls, 1996; Stout et al., 1998). The neuroprotective effects were independent of glutamate-induced changes in intracellular pH, NOS activity and MAPK activity (Stout et al., 1998). These findings point to the specific importance of mitochondrial Ca^{2+}-sequestration in mediating glutamate-induced cell death.

1.8.2 Parkinson's disease

PD is a progressive movement disorder characterized by rigidity, bradykinesia and tremor. Hallmarks of PD are selective degeneration of dopaminergic neurons of the substantia nigra and the formation of Lewy bodies, which are cytoplasmic aggregates composed of ubiquitin, α-synuclein and other proteins (Baba et al., 1998). Inhibition of electron transport chain complex I

has been identified as a key pathogenic cause of sporadic PD (Betarbet et al., 2000). Behavioral, neurochemical and neuropathological features were recapitulated in rats treated chronically and systemically with the specific complex I inhibitor, rotenone. Thus, while complex I activity was impaired in all brain cells, only nigrostriatal dopaminergic neurons showed degeneration. Interestingly, only modest complex I inhibition, typically about 25%, is found in PD (Parker et al., 1989; Schapira et al., 1989). In animal models, this partial impairment did not significantly impair mitochondrial respiration, nor presumably ATP synthesis (Betarbet et al., 2000). Instead, chronic rotenone exposure *in vitro* was shown to reduce glutathione levels in cells, cause oxidative damage to proteins and DNA, and increase vulnerability to ROS-induced caspase-dependent cell death (Sherer et al., 2002).

1.8.3 Alzheimer's disease

AD is the most common form of dementia and is characterized clinically by progressive memory loss, language disturbance, visuospatial impairment, and behavioral and psychiatric symptoms. At the microscopic level, pathologic hallmarks are extracellular amyloid-β (Aβ) plaques and intracellular neurofibrillary tangles (NFT) composed of the microtubule-associated protein, tau. Familial cases of AD account for 5% of diagnoses and are caused by mutations in amyloid precursor protein (APP) or presenilins, which are involved in APP processing (Brunkan and Goate, 2005). The disease has an incompletely understood pathogenesis that probably includes mutual and perpetuating harm between injured mitochondria and amyloid- β. Mitochondrial abnormalities include reduction in mtDNA content and mitochondrial mass prior to NFT formation, reduced cerebral glucose utilization, reduced activity of mitochondrial enzymes α-ketoglutarate dehydrogenase, pyruvate dehydrogenase and cytochrome oxidase, and decreased

Ca2+ buffering capabilities. Chronic mitochondrial dysfunction can contribute to ongoing oxidative stress in AD, which further damages mitochondria, generates mutations in mtDNA, increases production of Aβ from APP and causes oxidative alterations in Aβ that promote plaque formation. Additionally, APP targets and causes injury to mitochondria, and Aβ suppresses mitochondrial succinate dehydrogenase activity, impairs respiration, depolarizes mitochondria, promotes cytochrome c release, and potentiates Ca^{2+}-induced mPTP opening and mitochondrial swelling (Fiskum et al., 1999; Andersen, 2004; Beal, 2004; Duchen, 2004).

1.8.4 Amyotrophic lateral sclerosis

ALS is an adult-onset degenerative disease of motor neurons that results in progressive skeletal muscle atrophy, paralysis and death. Mutations in Cu/Zn superoxide dismutase (SOD1) were identified as a causal factor in 20% of familial cases. Mitochondrial vacuolization by intermembrane space expansion and subsequent inner membrane disintegration was found to be an early pathologic event in SOD1 mutant mice. Other *in vivo* and *in vitro* studies of SOD1 mutants and ALS tissues demonstrated depolarized ψ_m, reduced mitochondrial mass, decreased activity of the mtDNA-encoded cytochrome c oxidase enzyme, increased toxicity with mitochondrial toxins, and impaired calcium homeostasis. Interestingly, complexes I and II-III were shown to have increased activity in various brain regions, although this may be a compensatory rather than pathologic effect. Mutant SOD1 also aggregates in mitochondria and associates with mitochondrial membranes, possibly causing direct damage and permeabilization. Lastly, evidence exists for slowed axonal transport in ALS motor neurons, leading to accumulation of neurofilaments and possibly mitochondria in proximal axons (Collard et al., 1995; Fiskum et al., 1999; Julien, 2001; Xu et al., 2004).

1.9 CESSATION OF MITOCHONDRIAL MOVEMENT AS A MECHANISM OF NEUROTOXICITY

Impaired axonal transport is considered a pathologic process in several neurodegenerative diseases, but evidence for specific involvement of mitochondrial trafficking is only beginning to emerge. Examples include HD, spinobulbar muscular atrophy, ALS and AD (Sasaki and Iwata, 1996; Ebneth et al., 1998; Piccioni et al., 2002; Trushina et al., 2004; Stokin et al., 2005). General mechanisms for impaired transport in neurons include mutations in motor proteins, defects in proteins that regulate or interact with motor proteins, and nonspecific disease processes such as protein aggregation that may affect transport (Goldstein, 2003). Mitochondria represent a special case because transport is also tied to mitochondrial function. In this section, we review how cessation of mitochondrial transport can harm cells and also the high susceptibility of mitochondrial movement to impairment. Thus, while impaired mitochondrial movement is only implicated in a few diseases thus far, it is likely to be a pathologic mechanism that can be generalized to many situations of neuronal injury and toxicity.

1.9.1 Immobilized mitochondria can harm cells

Mitochondria are crucial for energy production and Ca^{2+} buffering in cells, and dynamic distribution of these functions throughout neuronal processes is accomplished by mitochondrial trafficking. Furthermore, transport is necessary to bring unhealthy and healthy mitochondria together for fusion-mediated repair and to target damaged mitochondria to sites of regulated autophagic degradation. Impairment of mitochondrial transport would have multiple consequences, and the effects probably worsen substantially with longer periods of impaired movement. Maldistribution of ATP and inefficient Ca^{2+}-sequestration are likely to be initial

consequences. Since mitochondria are distributed throughout cells, these effects would probably be limited by the diffusional properties of ATP and Ca^{2+}, and the ability of other ATP sources and Ca^{2+}-regulators to compensate for ineffective mitochondrial delivery. The cellular processes that are most likely to be at a disadvantage are those that require mitochondria in a rapid and dynamic manner. These processes which remain to be identified but probably include specific synaptic sites undergoing morphogenesis or synaptic potentiation, are likely to recruit mitochondria actively under normal conditions (Tang and Zucker, 1997; Yang et al., 2003; Li et al., 2004).

Prolonged impairment of mitochondrial movement can impede the repair and removal of damaged mitochondria. As a result, mtDNA mutations can accumulate that cause electron transport defects and associated ROS production. This can not only cause more mtDNA mutations but also lead to lipid peroxidation and protein oxidation, as described in Chapter 1.7. Therefore, cell death could very well be the ultimate outcome of impaired mitochondrial movement. This could be an acute pathophysiological process if mitochondrial movement stops abruptly, as in the case glutamate excitotoxicity which also compromises mitochondrial function independently of transport. Alternatively, cell death could be an insidious process if mitochondrial movement is gradually impaired. Cellular and mitochondrial dysfunctions would then develop slowly as is suspected in neurodegenerative diseases.

1.9.2 Injured mitochondria stop moving

Our laboratory and others have shown that mitochondria stop moving under a wide variety of stimuli that affect mitochondrial and cellular function. These studies reveal the sensitivity and

broad impact of acute and chronic neurotoxic conditions on mitochondrial movement as well as possible mechanisms that govern mitochondrial movement.

First, intact mitochondrial function is an important contributor to mitochondrial movement. Depolarization of ψ_m with FCCP causes mitochondria to stop moving in a manner that is slowly reversible upon repolarization. FCCP is a protonophore that uncouples oxidative phosphorylation. Dissipation of the proton gradient across the inner mitochondrial membrane consequently removes the drive for ATP synthesis and in fact causes reversal of proton flow through ATP synthase, resulting in consumption rather than production of ATP (Nicholls and Ward, 2000). If ATP synthase is inhibited selectively with oligomycin, ψ_m is preserved or slightly increased, yet mitochondrial movement still stops (Rintoul et al., 2003b). Therefore, ψ_m seems important for mitochondrial movement, perhaps because it is a direct requirement for mitochondrial ATP synthesis. These findings are further supported by evidence that other drugs that impair respiration, including NO and rotenone, also impair mitochondrial movement (Rintoul et al., 2004; Reynolds and Santos, 2005).

Our studies in models of neuronal injury such as ischemia revealed that mitochondrial movement can also be impaired both independently of and probably in cooperation with ψ_m depolarization. In the first instance, we described the ability of neurotoxic zinc concentrations to inhibit movement while maintaining an intact ψ_m (Malaiyandi et al., 2005). This proved to be mediated by a rapidly activated PI 3-kinase dependent signaling cascade. On the other hand, we showed that more complicated disruption mechanisms accounted for cessation of mitochondrial movement after acute exposures to glutamate concentrations that cause excitotoxic cell death (Rintoul et al., 2003b). Here, mitochondrial movement could be affected by cytosolic remodeling caused by elevated Ca^{2+} influx in addition to mitochondrial Ca^{2+}-uptake, ψ_m

depolarization, and inhibition of ATP synthesis. Therefore, multiple mechanisms are clearly able to stop mitochondrial movement, and these mechanisms are relevant to mitochondrial dysfunction, neuronal injury and neurodegeneration. In fact, there may be convergence of several injury pathways on mitochondrial movement, suggesting that impairment of movement could be a common event in a variety of neuropathological processes.

1.9.3 Perpetuation of the vicious cycle

The intertwined relationship between mitochondrial movement, mitochondrial health and cellular health dictates that impairment of any one of these properties can negatively impact the other two properties. Mitochondrial movement seems quite sensitive to a variety of changes in the physiological state of mitochondria themselves, but also to changes in the cellular environment such as shifts in ion concentrations and alterations in cytoskeletal structure. Impairment of mitochondrial movement may have several phenotypes – slow and progressive as with subtoxic rotenone treatment, rapid and irreversible as with zinc, or rapid and reversible as with NO and glutamate (Rintoul et al., 2003b; Rintoul et al., 2004; Malaiyandi et al., 2005; Reynolds and Santos, 2005). The course of neuronal dysfunction and cell death is likely impacted by the timeframes over which movement is impaired and recovers, and any concurrent cellular and mitochondrial defects. Initial consequences such as inappropriate ATP distribution and Ca^{2+} sequestration would have more severe consequences in cells that are already injured and have compromised mitochondrial function. In turn, reduced ATP supply and disrupted $[Ca^{2+}]_i$ homeostasis compromise the ability of mitochondria to maintain ψ_m. Furthermore, worsening mitochondrial damage and increased ROS production leads to a cascade of further mitochondrial damage coupled to inappropriate repair or removal of the damaged organelles, and activation of

cell death pathways. Therefore, impaired mitochondrial movement has the capability to be a highly debilitating pathophysiological process that activates a vicious cycle of injury to the cell and to mitochondria.

1.10 THE DISSERTATION

The broad aims of the dissertation were to identify cellular signals that control mitochondrial trafficking, determine how those signals can contribute to dynamic mitochondrial movements in whole neurons, and to examine how impaired mitochondrial movement can cause or be caused by neuronal injury and neurodegenerative disease. As an inevitably related property, we also studied mitochondrial morphology in relation to these questions. Such ambitious goals in a field where so little is understood required that these aims be relatively focused in scope. As such, we decided to explore synapses as a target for mitochondrial trafficking. This rapidly became a more complicated task than initially anticipated as dynamic mitochondrial qualities were being studied in response to equally complex dynamic synaptic structures. This aim evolved into two chapters, one from the perspective of a synaptic development model and one addressing the synaptic requirements in mature neurons. In addition to answering basic physiological questions about mitochondrial trafficking, it was also important for us to understand how trafficking aberrations relate to neuronal injury and disease. It is only then that we can attempt to derive pharmacologic solutions to pathophysiological processes. With such a goal in mind, we examined mitochondrial movement in axons and dendrites under conditions of neuronal injury which could relate to synaptic dysfunctions, and mitochondrial impairment as a pathogenic event in HD.

In the first chapter, we investigated how mitochondrial movement and morphology change as neurons develop mature synaptic connections. The mitochondrial recruitment properties of NGF in developing neurons and morphogenic spines in mature neurons strengthen the idea that younger and older neurons impose very different cellular demands on mitochondria (Chada and Hollenbeck, 2003, 2004; Li et al., 2004). In addition to the synthesis of organelles and proteins needed for cell growth, mitochondrial ATP generation must support the dynamic and rapid process of synaptogenesis during neuronal development. In mature neurons, mitochondria probably adopt a different role – one of cellular maintenance and repair and synaptic support and remodeling. Not only do mitochondria sustain different processes as neurons mature, but the cellular locations and the requirements of these sites of need also change. Moreover, as neurons mature, their exposures and responses to extracellular signals may vary and differentially impact mitochondrial homeostasis. We demonstrated differences in mitochondrial movement, morphology and distribution that are probably optimized to best provide for the changing energetic needs of synaptically immature and mature neurons.

The second chapter consists of an in-depth analysis of mitochondrial trafficking relative to synaptic sites on axons and dendrites in mature cortical neurons. We described for the first time how populations of mitochondria with different motility patterns are targeted to synaptic structures under conditions of normal and altered synaptic activity. We also demonstrated how different synaptic distributions on axons and dendrites correlate with compartmentalized differences in mitochondrial trafficking and morphology. With greatest relevance to neuronal injury, we found clear evidence of differential alterations in mitochondrial movement and morphology between axons and dendrites under conditions pertinent to ischemia and excitotoxicity. These results implicate impaired mitochondrial trafficking as an effecter of

selective dendritic dysfunction, abnormal postsynaptic signal transmission and dendritic degeneration.

Lastly, we examined impaired mitochondrial trafficking as an early pathogenic event in HD. Bioenergetic defects characterize diseased neurons and a growing body of literature supports a physical relationship between huntingtin (Htt), motor proteins and accessory proteins that can impair protein and vesicle transport (Ludolph et al., 1991; Gutekunst et al., 1998; Li et al., 2001a; Gunawardena et al., 2003; Trushina et al., 2004). Recently, inhibition of mitochondrial transport was reported in striatal neurons (Trushina et al., 2004). Our work examined the ability of mutant Htt to impair mitochondrial trafficking, cause mitochondrial defects, and increase excitotoxic vulnerability in cortical neurons, which are relatively spared compared to striatal neurons. We revealed a novel mechanism whereby mutant Htt aggregates specifically immobilize moving mitochondria in their immediate vicinity. Furthermore, this proved to be an early pathogenic event that probably initiates a prolonged degenerative process of mitochondria and neuronal processes as would be expected in HD. In summary, the dissertation work contributes new understanding to multiple facets of mitochondrial movement through the lifetime of healthy and injured neurons and paves the way for more sophisticated investigation of regulatory mechanisms in the future.

2.0 DIFFERENCES IN MITOCHONDRIAL MOVEMENT AND MORPHOLOGY IN YOUNG AND MATURE PRIMARY CORTICAL NEURONS IN CULTURE

2.1 ABSTRACT

Mitochondria have many roles critical to the function of neurons including the generation of ATP and regulation of intracellular Ca^{2+}. Mitochondrial movement is highly dynamic in neurons and is thought to direct mitochondria to specific cellular regions of increased need and to transport damaged or old mitochondria to autophagosomes. Morphology also varies between individual mitochondria and is rapidly modulated by fusion and fission. Although mitochondrial movement and morphology are thought to be modulated to best meet cellular demands, few regulatory signals have been identified. In this study, we examined how the different cellular environments of synaptically immature and mature cortical neurons affect mitochondrial movement, morphology, distribution and function. In younger cells, mitochondria were more mobile, were shorter and occupied a smaller percentage of neuronal processes compared to older cells. However, the number of mitochondria per μm of neuronal process, mitochondrial membrane potential and the amount of basally sequestered mitochondrial Ca^{2+} were similar. Our results suggest that while mitochondria in young neurons are functionally similar to mature neurons, their enhanced motility may permit faster energy dispersal for cellular demands, such as

synaptogenesis. As cells mature, mitochondria in the processes may then elongate and reduce their motility for long-term support of synaptic structures.

2.2 INTRODUCTION

Mitochondria are vital to the function of cells not only because they are the main source of energy but also because they regulate intracellular Ca^{2+} homeostasis. Since mitochondria are thought to be synthesized in the perinuclear region, they must be trafficked appropriately to meet demands throughout the cell (Wong-Riley, 1989; Miller, 1992; Davis and Clayton, 1996). This has particular relevance to neurons where axons and dendrites can extend well beyond the cell body. In addition to supporting cellular functions, mitochondrial trafficking may be important for transporting damaged mitochondria to cellular locations where they can be repaired or degraded (Hollenbeck, 1993; De Vos et al., 2000; Tolkovsky et al., 2002; Miller and Sheetz, 2004). Mitochondrial movement is dynamic in neurons, and can vary in both velocity and motility patterns between individual organelles and between axons and dendrites (Overly et al., 1996; Ligon and Steward, 2000a). Microtubules and actin filaments serve as cytoskeletal substrates on which kinesins and dyneins transport the organelles (Hirokawa et al., 1990; Nangaku et al., 1994; Morris and Hollenbeck, 1995; Tanaka et al., 1998; Ligon and Steward, 2000b). Interestingly, mitochondrial morphology is also dynamic and can be regulated (reviewed by Rube and van der Bliek, 2004). For example, mitofusin-1 (mfn-1) mediates mitochondrial fusion while dynamin-related protein-1 (drp-1, also referred to as dlp-1) controls fission (Pitts et al., 1999; Santel and Fuller, 2001; Smirnova et al., 2001; Legros et al., 2002; Chen et al., 2003; Pitts et al., 2004). Mitochondrial fusion is thought to promote a

bioenergetically favorable morphology and to assist in the sharing of membrane and matrix proteins and mitochondrial DNA (Nakada et al., 2001; Chen et al., 2003). Fission, on the other hand, may contribute to mitochondrial proliferation as well as apoptosis in neurons (Frank et al., 2001). The dynamic processes of mitochondrial trafficking and morphology regulation that occur throughout the lifetime of a cell defines a process we term "mitochondrial homeostasis".

Recent studies on mitochondrial movement and morphology have been performed largely in the context of neuronal injury and cell death. It is thought that impairment of mitochondrial transport could result in inadequate distribution of ATP and sequestration of intracellular Ca^{2+}. Accumulation of injured mitochondria due to inefficient removal could also have severe consequences, as damaged mitochondria may promote apoptosis by producing reactive oxygen species and releasing cytochrome c (reviewed by Lee and Wei, 2000). Our laboratory and others have demonstrated that mitochondrial movement in neurons is inhibited by agents that depolarize mitochondria, including glutamate, FCCP and zinc (Rintoul et al., 2003b; Vanden Berghe et al., 2004; Malaiyandi et al., 2005). Miller and Sheetz (2004) also showed that mitochondria with high membrane potentials ($\Delta\psi_m$) move anterogradely while depolarized mitochondria moved back toward the cell body. Furthermore, mitochondrial morphology undergoes (i) shortening and rounding in neurons after excitotoxic glutamate exposure and (ii) drp-1 mediated fragmentation in COS-7 cells after induction of apoptosis (Frank et al., 2001; Karbowski et al., 2002; Rintoul et al., 2003b), suggesting an important relationship between morphology and cell viability.

The signals that govern mitochondrial movement and morphology in healthy cells are even less well understood than those that operate in injured cells. While mitochondria are thought to be trafficked in response to cellular signals such as reduced ATP or elevated $[Ca^{2+}]_i$,

few have been unambiguously identified. For example, some evidence exists for mitochondrial recruitment to dendritic spines during synaptic excitation in hippocampal neurons (Li et al., 2004). Nerve growth factor in active growth cones was also recently identified as a chemoattractive signal for mitochondria in developing neurons (Chada and Hollenbeck, 2003, 2004). In the present study we have investigated neuronal maturity as a variable affecting mitochondrial morphology and trafficking in healthy neurons, and show significant changes in mitochondrial characteristics associated with the development of mature synaptic connections.

2.3 EXPERIMENTAL PROCEDURES

Cell culture

All animals used for this study were maintained in accordance with the University of Pittsburgh *Guidelines for the Care and Use of Animals*. Primary neurons were isolated for culture from embryonic day 17 Sprague Dawley rat pups and grown in a 37˚C incubator containing 5% CO_2. Cortices were removed, trypsinized at 37˚C for 30 minutes, and plated on poly-D-lysine-coated 31 mm glass coverslips. Cells were plated in medium containing Dulbecco's Modified Essential Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Five hours after plating, plating medium was completely removed and replaced with N2-NB media (Neurobasal media with 0.5% penicillin/streptomycin and 1% N2 supplement). Four days after plating, approximately one-third of the media was removed and replaced with an equal volume of fresh N2-NB media. Eight and 11 days after plating, approximately one-third of the media was removed and replaced with B27-NB media (Neurobasal media with 0.5 %

penicillin/streptomycin and 2% B27 Supplement Minus antioxidants). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA).

Transfection of cortical cultures

The mito-eYFP construct obtained from Dr. Roger Tsien (Llopis et al., 1998) was produced by insertion of the gene for eYFP into the mammalian expression vector pcDNA3 (Invitrogen) and containing the cytochrome c oxidase subunit IV mitochondrial localization sequence. The plasmid was purified and amplified using the Qiagen Plasmid Maxi Kit according to manufacturer's instructions. Transfections were performed on neurons from the same culture after 3 or 12 days *in vitro* (DIV), using 2 µg mito-eYFP and 5 µl Lipofectamine 2000 reagent (Invitrogen) in an added volume of 250 µl DMEM. Media was completely replaced with conditioned media 24 hours after transfection and neurons were imaged 48 hours after transfection.

Immunocytochemistry

The primary antibodies used were mouse anti-PSD-95 IgG (Affinity Bioreagents, Golden, CO) and mouse anti-synaptophysin monoclonal antibody (Chemicon, Temecula, CA). Alexa 546 goat anti-mouse polyclonal antibody (Molecular Probes, Eugene, OR) was used as the secondary antibody. Coverslips containing transfected neurons were removed from culture plates and rinsed once with Dulbecco's PBS (Invitrogen) for 1-3 min. Cells were then fixed for 10 min at room temperature (RT) with 4% formaldehyde/PBS. Coverslips were rinsed, permeabilized for 30 sec with -20°C acetone, re-rinsed, and blocked with 10% goat serum/PBS for 1 hr at RT. Cells were incubated with anti-PSD-95 diluted 1:500 or anti-synaptophysin antibody diluted 1:100 in 3% bovine serum albumin (BSA)/PBS for 2 hr at RT. Cells were then rinsed, incubated

with secondary antibody diluted 1:500 in 3% BSA/PBS for 30 min at RT, and re-rinsed. Coverslips were rinsed and stored in PBS at 4ºC until visualization. All rinses were with PBS and performed in quadruplicate for 3 min each.

Immunoblotting

Immunoblotting for mfn-1 was performed using rabbit anti-mfn-1 antibody (provided by Dr. Ansgar Santel) and goat anti-rabbit IgG coupled to horseradish peroxidase as the secondary antibody. Normalization was performed using mouse anti-β-actin (Sigma, St. Louis, MO) and goat anti-mouse IgG coupled to horseradish peroxidase. Immunoblotting for drp-1/dlp-1 was performed using mouse anti-dlp-1 IgG (BD Bioisciences, San Jose, CA) and goat anti-mouse IgG coupled to horseradish peroxidase as the secondary antibody. Normalization was performed using rabbit anti-β-actin (Biolegend, San Diego, CA) and goat anti-rabbit IgG coupled to horseradish peroxidase. All horseradish peroxidase-coupled antibodies were purchased from Pierce Biotechnology (Rockford, IL).

Cells were scraped from 6-well plates after 5 or 14 DIV, collected in PBS and centrifuged at 14,000 rpm for 5 minutes. Pellets were washed twice with PBS and lysed for 45 min in buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 % Triton X-100, 5 mM EGTA, 20 μM Leupeptin, 1 mM AEBSF, 1 mM $NaVO_3$, 10 mM NaF, and 1 tablet of protease inhibitor. Protein was collected from the supernatant after a 5 min spin at 14,000 rpm and concentration was determined using a micro-protein assay using the BCA protein assay kit according to manufacture's instruction (Pierce Biotechnology). 20 μg protein were loaded with 6x SDS sample loading buffer (60 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM 2-mercaptoethanol, 20% glycerol, and 2% SDS) and size-fractionated by electrophoresis on 4-15 % Tris-HCl Ready Gels (Bio-Rad Labs, Hercules, CA) at 20-30 mA for 1 hr. Protein was

transferred to a nitrocellulose membrane at 80 V for 2 hr at 4°C. The membrane was blocked with 5% dry milk for 1 hr at RT, rinsed in 1% Tween/PBS (PBS-T), incubated in primary antibody diluted 1:500-1:1000 in PBS-T overnight at 4°C, rinsed in PBS-T, incubated in secondary antibody diluted 1:1000 in PBS-T for 1 hr at RT, and rinsed in PBS-T. Chemiluminescence was performed using the SuperSignal West Dura Extended Duration Substrate Kit (Pierce Biotechnology).

Imaging

Cells were perfused with HEPES-buffered salt solution (HBSS) adjusted to pH 7.4 with NaOH and composed of (in mM) NaCl 137, KCl 5, $NaHCO_3$ 10, Hepes 20, glucose 5.5, KH_2PO_4 0.6, Na_2HPO_4 0.6, $CaCl_2$ 1.4 and $MgSO_4$ 0.9. $CaCl_2$ was omitted from the buffer for experiments measuring $[Ca^{2+}]_i$ after FCCP treatment. HBSS was warmed to 37°C and flowed at a rate of 5 ml/min throughout the duration of each experiment. Imaging was performed at a rate of 1 frame/6 seconds except where noted, at a magnification of 40x using Compix Inc. imaging systems and SimplePCI software (Compix Inc., Cranberry PA). The excitation wavelengths used were 495 nm for eYFP and rh123, 340/380 nm for mag-fura-2AM and fura-2AM, and 540 nm for Alexa 546 goat anti-mouse polyclonal antibody.

Analysis of mitochondrial movement, length, occupancy and number

Using SimplePCI, 1-3 circular regions of interest (ROIs) of 1.81 μm diameter were evenly spaced along the processes of each neuron. Mitochondrial movement was measured as the average number of mitochondria entering a ROI over time, as detected by an increase in fluorescence of at least 20 units. SimplePCI functions were employed to determine average mitochondrial length, occupancy and number in a 255 x 255 pixel subfield that excluded

proximal processes clearly containing more than one mitochondrion in their cross-section. Briefly, user-defined thresholds for pixel intensity and object size were used to identify the total number of measurable mitochondria in a frame. Length was calculated from skeletonized objects, which consisted of a 1 pixel-wide line running the length of each mitochondrial object. All neuronal processes in the field were then converted into line objects, their total length was calculated, and mitochondrial occupancy was computed as total mitochondrial length/process length. A paired t-test was used for statistical analysis.

Measurement of $[Ca^{2+}]_i$ and mitochondrial membrane potential

Spontaneous intracellular Ca^{2+} transients were identified as transient changes in fura-2AM ratios that were synchronous across neurons in the recording field. Cells were loaded with 5 μM fura-2AM/HBSS (Molecular Probes) for 15-20 min at 37°C. Neurons were imaged at excitation wavelengths of 340/380 nm at a rate of 1 frame/3 sec before and during a 3 min perfusion of 200 nM TTX (Sigma, St. Louis, MO). Using SimplePCI, $[Ca^{2+}]_i$ was represented as the fura-2 ratio of cell bodies after subtraction of background ratio.

Intracellular Ca^{2+} changes after glutamate or FCCP treatment was measured as previously described (Brocard et al., 2001). Briefly, neurons were loaded with 5 μM mag-fura-2AM (Molecular Probes) in 5 mg BSA/ml HBSS for 10 minutes at 37°C. Neurons were imaged at excitation wavelengths of 340/380 nm during a 5 minute treatment with 30 μM glutamate/1 μM glycine or 750 nM FCCP followed by a 10 minute washout. The area under the mag-fura-2 ratio curve (AUC) after subtraction of background fluorescence was calculated for the 0-6.5 minute time point after each drug was applied. $Baseline_{AUC}$ was extrapolated from the AUC during the 1 minute immediately preceding drug treatment. A paired t-test was used for statistical analysis.

Mitochondrial membrane potential ($\Delta\psi_m$) was measured as the increase from baseline rhodamine 123 (rh123) fluorescence after dissipation of $\Delta\psi_m$ by FCCP. Briefly, neurons were loaded with 5 μM rh123 (Molecular Probes) in HBSS for 10 minutes at 37°C and rinsed thoroughly until baseline rh123 fluorescence stabilized. Cells were imaged during a 3 minute treatment with 750 nM FCCP. Mean background fluorescence was subtracted from the mean fluorescence of each non-saturating cell body, fluorescence values were normalized to baseline fluorescence taken 30 seconds before FCCP treatment, and $\Delta\psi_m$ was calculated as the difference between normalized maximum fluorescence after FCCP and baseline fluorescence. A paired t-test was used for statistical analysis.

2.4 RESULTS

Functional synapses are rare in young cortical neurons

In vitro synaptic development of cortical neurons was visualized by immunostaining for the pre-synaptic vesicle protein, synaptophysin, and the post-synaptic scaffolding protein, PSD-95 in neuronal cultures of different developmental stages. Representative images at 2, 5-6, 8 and 12 days *in vitro* (DIV) are shown in Figure 1. Synaptophysin expression was detectable at earlier time points than PSD-95 expression. In general, neurons from 2-6 DIV had considerably less immunostaining of pre- and post-synaptic components compared to 8 DIV neurons and mature 12 DIV neurons. This is consistent with a previous report of the development of synaptic contacts in neurons in culture (Weiss et al., 1986). Therefore, we performed experiments at 5 DIV when expression of synaptic components is minimal, and at 14 DIV when neurons contain many synapses.

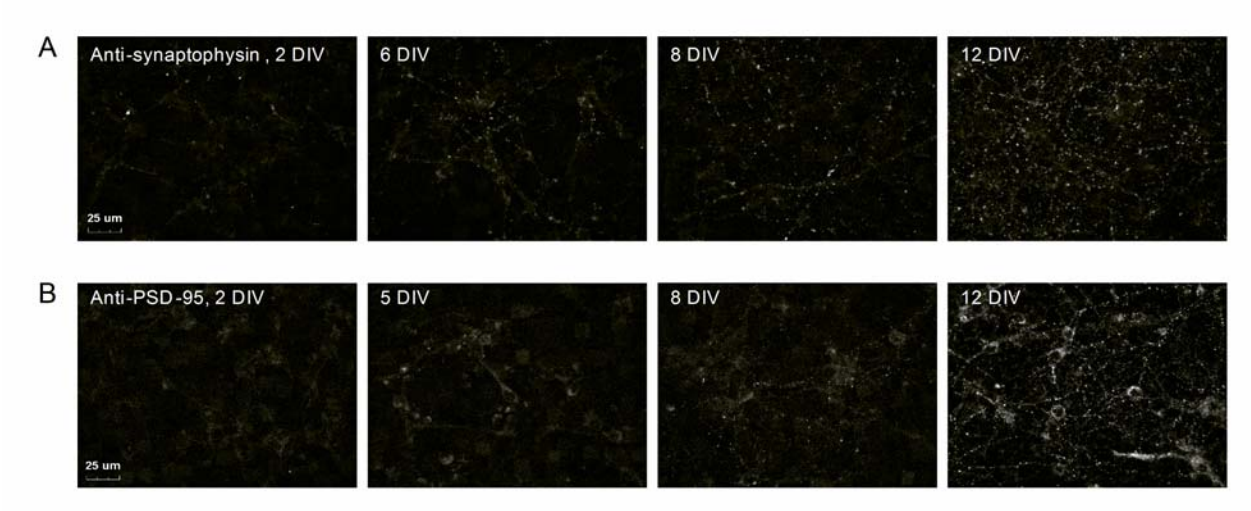

Figure 2.1 Synaptic structures were uncommon in young neurons but abundant in mature neurons.
Immunostaining for synaptophysin (A) and PSD-95 (B) of neurons aged 2 through 12 DIV demonstrated the time course of expression of pre- and post-synaptic components, respectively. Synaptophysin expression was rare at 2 DIV and gradually increased through 12 DIV. PSD-95 expression was not observed until after 5 DIV and was pronounced by 12 DIV. Images are representative of 2-3 separate cultures.

Five DIV neurons had negligible intracellular Ca^{2+} influx after treatment with 30 μM glutamate/1 μM glycine, as measured by the fluorescent $[Ca^{2+}]_i$ indicator mag-fura-2 (Figure 2A). On the other hand, 14 DIV neurons had a nearly 5-fold greater response to glutamate ($p < 0.01$; Figure 2A, B). Furthermore, our examination of spontaneous $[Ca^{2+}]_i$ transients representative of spontaneous synaptic activity in cortical neurons demonstrated prominent synchronous fluxes at 14 DIV which were completely absent at 5 DIV (Figure 2C, D). We confirmed that these $[Ca^{2+}]_i$ transients were mediated by synaptic activity by perfusing neurons with 200 nM tetrodotoxin (TTX), a blocker of voltage-sensitive Na^+ channels in excitable membranes. Transients in 14 DIV neurons were abolished after approximately 30 seconds and the pattern of $[Ca^{2+}]_i$ fluxes reverted to that seen in 5 DIV neurons. Therefore, we conclude that our cortical neurons are functionally synaptically immature at 5 DIV and mature at 14 DIV.

Throughout the remainder of the text, we will refer to 5 DIV neurons as "young/immature" and 14 DIV neurons as "old/mature".

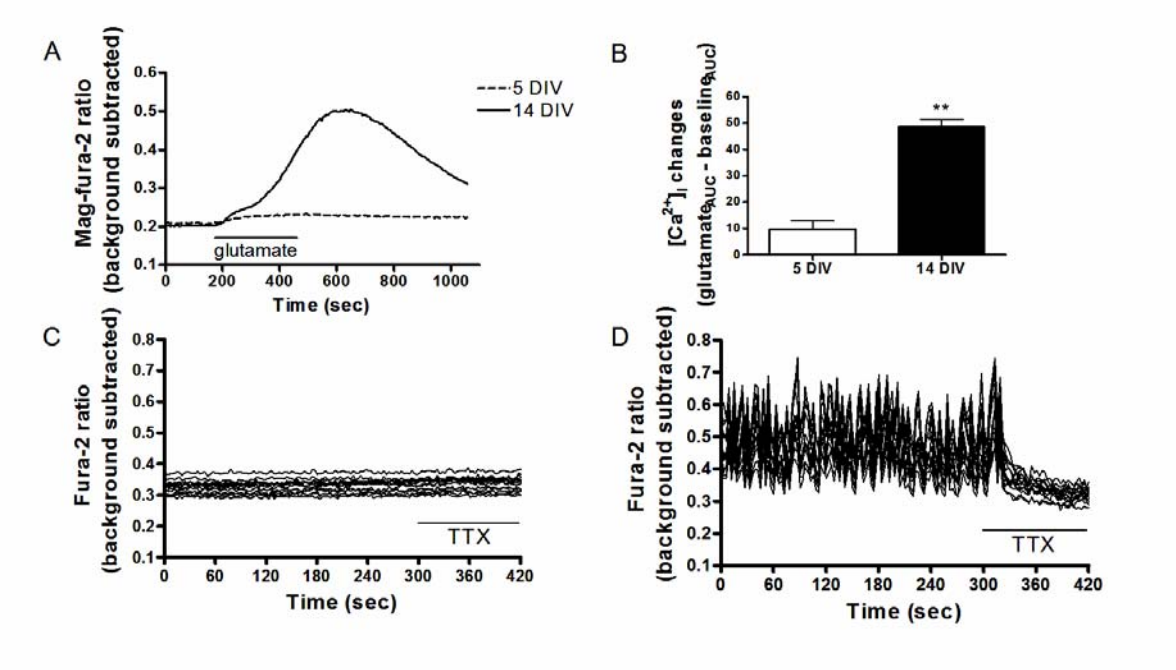

Figure 2.2 Functional synaptic activity was absent in 5 DIV cortical neurons but apparent in 14 DIV neurons.

(**A**) Intracellular Ca^{2+} influx after a 5 min perfusion with 30 μM glutamate/1 μM glycine was minimal in 5 DIV neurons and robust in 14 DIV neurons. Traces are mean mag-fura-2 ratios after subtraction of background fluorescence and represent 3 coverslips each from 3 separate cultures. (**B**) $[Ca^{2+}]_i$ was depicted as the area under mag-fura-2 ratio curves (AUC) for 6.5 min after treatment with 30 μM glutamate/1 μM glycine with baseline AUC subtracted. $[Ca^{2+}]_i$ influx was significantly greater in 14 DIV neurons ($p < 0.01$). Values are shown as mean ± SE of 3 coverslips each from 3 separate cultures. (**C, D**) Spontaneous calcium fluxes measured by fura-2 were indicative of basal synaptic activity and were inhibited by 200 nM TTX. Such fluxes were absent in immature, 5 DIV neurons but prominent and synchronous in mature, 14 DIV neurons. Traces represent 13-14 cells from a single coverslip. Experiments were repeated 2-3 times each on 3 separate cultures.

Mitochondrial movement, morphology and distribution vary with neuronal maturity

We next investigated the impact of the development of synaptic connections on mitochondrial movement, morphology and distribution. Interestingly, transfection of cortical neurons with the mitochondrially-targeted mito-eYFP plasmid revealed that mitochondria have markedly different

movement patterns and appearances depending on the maturation state of the cells. Figure 3A illustrates the typical appearance of mitochondria in 5 and 14 DIV transfected neurons. Mitochondrial movement was measured over time by recording the number of mitochondria moving into 1.81 μm diameter regions of interest (ROIs) that were evenly spaced along neuronal processes and that were initially unoccupied by mitochondria. It is important to note that our measurement of mitochondrial movement can be influenced by both the number of moving mitochondria and the velocity with which they move. Significantly fewer mitochondria were found to enter any given ROI per minute in synaptically mature cells compared to mitochondria in immature cells (0.32 ± 0.072 vs 0.49 ± 0.083 $p < 0.05$; Figure 3B). We therefore conclude that mitochondria become less motile with neuronal maturity.

The fractional occupancy of neuronal processes by mitochondria (Figure 3C) was increased in 14 DIV neurons (Figure 3C). That is, in a given 255 x 255 pixel subfield, the fraction of total neuronal process length that was occupied by mitochondria was $24.8 \pm 2.4\%$ in 14 DIV neurons compared to $16.8 \pm 1.5\%$ in 5 DIV neurons ($p < 0.05$). Interestingly, the number of mitochondria per μm of neuronal process did not differ with neuronal age (Figure 3D). The main contributor to increased fractional occupancy of neuronal processes by mitochondria was therefore mitochondrial length, which was significantly greater in 14 DIV neurons, measuring 2.44 ± 0.12 μm versus 2.01 ± 0.14 μm in 5 DIV neurons ($p < 0.05$; Figure 3E). Recent studies suggest that mitochondrial morphology is controlled by the balance of effects of proteins that promote fission or fusion of mitochondria (Rube and van der Bliek, 2004). However, in this case the difference in morphology was not mirrored by the elevated expression of a mitochondrial fusion protein or reduced expression of a fission protein in older neurons. Mature neurons had about one fourth the expression of the fusion protein, mfn-1, and a

nearly 2-fold increase in expression of the fission protein, drp-1, compared to immature neurons ($p < 0.05$; Figure 4A-C). Thus, although the expression of these proteins suggests that fission should be enhanced in mature relative to immature neurons, the data show that neurons are actually longer, consistent with fusogenic activity.

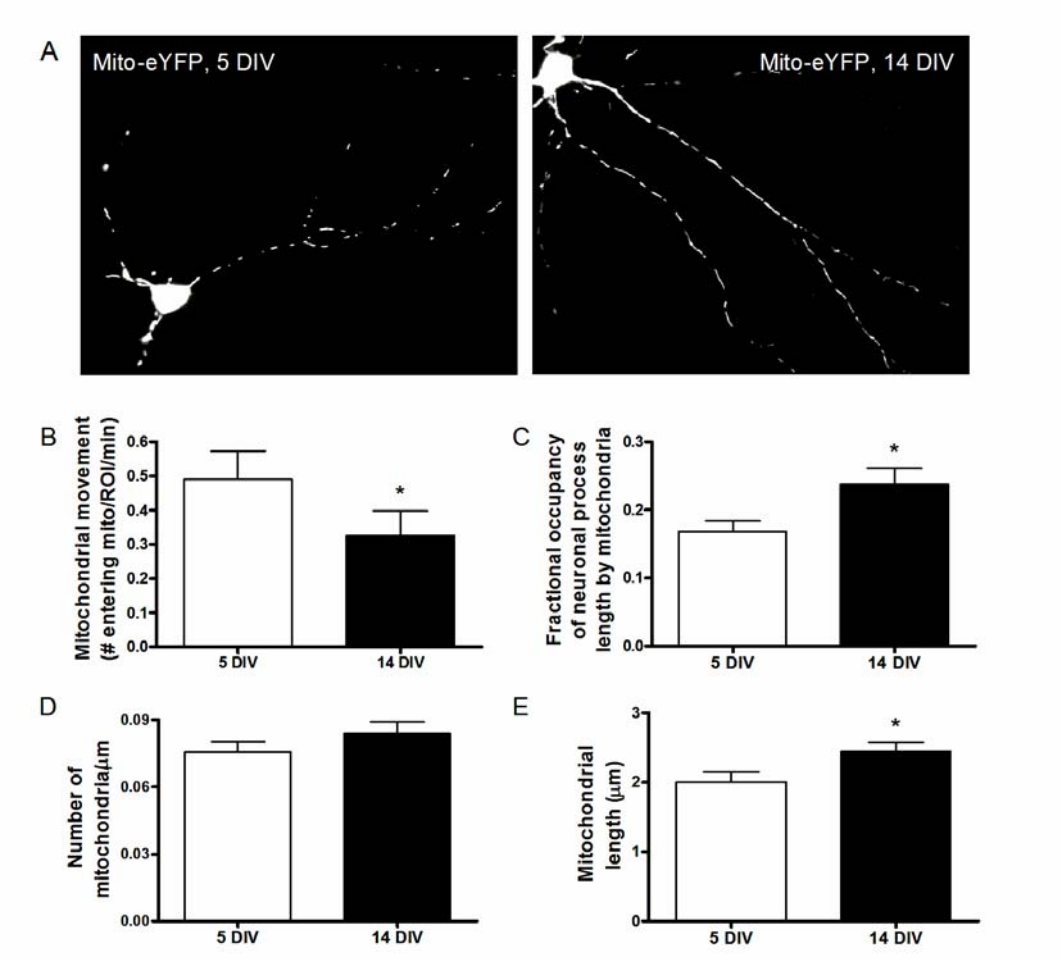

Figure 2.3 Mitochondrial movement, morphology and distribution differ between synaptically immature and mature neurons.

(**A**) Transfection of cortical neurons with mito-eYFP revealed a different mitochondrial morphology and distribution in 5 DIV neurons compared to 14 DIV neurons. Images are representative of 4-5 separate cultures. (**B**) The mean number of mitochondria entering a given region of interest (ROI) on a neuronal process per minute was significantly lower in 14 DIV neurons compared to 5 DIV neurons. (**C**) Mitochondria in mature neurons were significantly longer than in immature neurons. (**D**) Mitochondrial occupancy of neuronal processes was significantly greater in mature neurons. This was measured as the total length of all mitochondria divided by the length of all neuronal

processes in a given field. (**E**) The number of mitochondria/µm of neuronal process did not differ in neurons of different age. Values are shown as mean ± SE from at least 3 coverslips each from 4-5 separate cultures. P-values < 0.05 were considered significant.

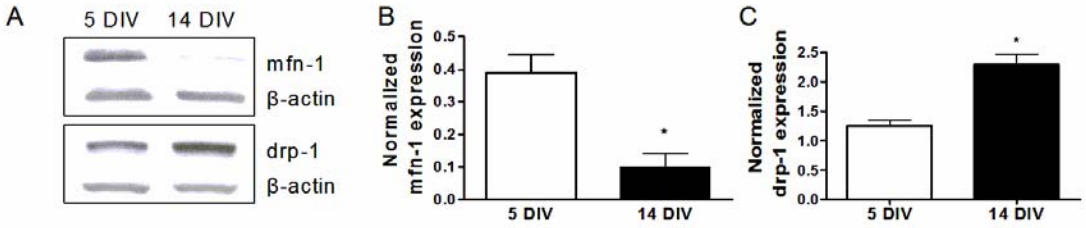

Figure 2.4 The expression levels of mitochondrial morphology-regulating proteins differed between immature and mature neurons.

(**A**) Immunoblot for the mitochondrial fusion protein, mfn-1 and the fission protein, drp-1, at 5 DIV and 14 DIV. (**B**) Mfn-1 expression was significantly lower in 14 DIV neurons. (**C**) Drp-1 expression was significantly greater in 14 DIV neurons compared to immature neurons. Data represent 4-5 separate cultures (mean+/- SE, with p-values < 0.05 considered significant).

Mitochondria in immature and mature cortical neurons have similar membrane potential and Ca^{2+} sequestration properties

We next investigated whether the neuronal age-dependent differences in mitochondrial movement, morphology and distribution seen in Figure 3 could be caused by an inherent dissimilarity in mitochondrial function. Mitochondrial membrane potential ($\Delta\psi_m$) was measured using the potentiometric dye, rhodamine 123 (rh123), which is sequestered and then quenched by mitochondria in a $\Delta\psi_m$-dependent manner. Upon dissipation of $\Delta\psi_m$ with a 3 min perfusion of 750 nM FCCP, mitochondria release rh123 into the cytosol, which is detected by an increase in rh123 fluorescence. Therefore, $\Delta\psi_m$ is represented by the difference in baseline fluorescence and maximum fluorescence after FCCP treatment. Figure 5A-B demonstrates that mitochondria in 5

DIV neurons indeed maintain a membrane potential that is equivalent to that seen in 14 DIV neurons ($p < 0.05$).

In agreement with our findings of similar $\Delta\psi_m$ in younger and older neurons, we also observed that mitochondria sequestered comparable concentrations of Ca^{2+} when neurons were in the basal state. Using mag-fura-2, we measured Ca^{2+} release from mitochondria into the cytosol after dissipation of $\Delta\psi_m$ with a 5 min perfusion of 750 nM FCCP (Figure 5C). $[Ca^{2+}]_{mito}$, represented by calculating the area under the mag-fura-2 ratio curve, was almost identical between 5 DIV and 14 DIV neurons (Figure 5D). Therefore, we conclude that mitochondria in synaptically immature neurons are functionally capable of maintaining $\Delta\psi_m$ and sequestering intracellular Ca^{2+} in a manner that is largely comparable to mitochondria in mature neurons.

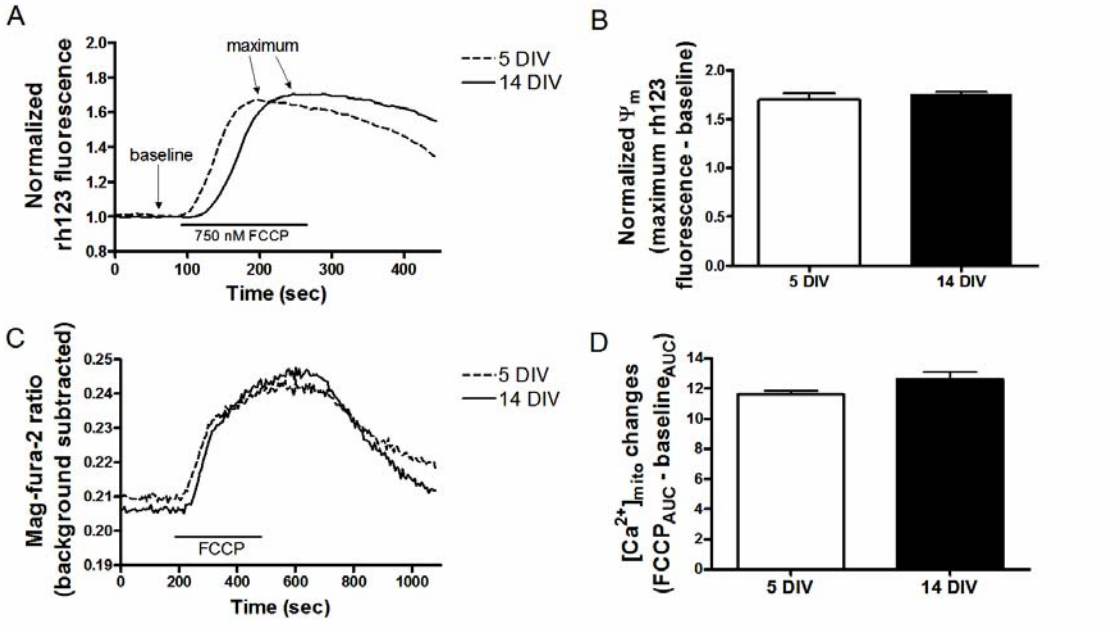

Figure 2.5 Mitochondrial function is broadly similar between synaptically immature and mature neurons.
(**A**) Mitochondria in immature neurons maintain a membrane potential that is dissipated upon a 3 min perfusion with 750 nM FCCP, as measured by change in rh123 fluorescence. (**B**) $\Delta\psi_m$ was similar in 5 DIV and 14 DIV neurons. This was measured as the difference between maximum normalized rh123 fluorescence after FCCP treatment and baseline fluorescence taken 30 sec before FCCP. (**C**) Mitochondria in immature neurons basally sequester similar

amounts of intracellular Ca^{2+} compared to mature neurons, as determined by the mag-fura-2 ratio curve after a 5 min perfusion with 750 nM FCCP. (**D**) There was no difference in $FCCP_{AUC}$ calculated as the area under the mag-fura-2 ratio curve (AUC) for 6.5 min after the start of FCCP treatment with $baseline_{AUC}$ subtracted. Mean traces represent 3 coverslips each from 3-4 separate cultures; corresponding chart values are shown as mean ± SE calculated from the same experiments.

2.5 DISCUSSION

Our results show that mitochondria in synaptically immature cortical neurons are more mobile, are shorter, and occupy a smaller percentage of total neuronal process length compared to synaptically mature neurons. However, the number of mitochondria per neuronal process length was similar between the two age groups. These findings demonstrate that mitochondrial morphology and trafficking vary as a function of synaptic maturity. Several developmental issues that might affect mitochondrial movement, morphology and distribution should be considered: (i) mitochondria in young neurons are functionally different or immature, and this difference is manifest as altered morphology and increased movement, (ii) the intracellular environment in young developing cells may have different energetic demands compared to mature cells, especially with regard to synaptogenesis and neurite outgrowth, and (iii) the maintenance and modulation of synaptic communication between mature neurons may impart special demands on mitochondrial movement and morphology.

We demonstrated that mitochondria in 5 DIV and 14 DIV neurons were capable of maintaining equivalent $\Delta\psi_m$ (Figure 5B). This is a new finding in cortical neurons isolated from the same source but aged for different time periods *in vitro*. Figure 5D shows that mitochondria in immature and mature cells also basally sequester comparable amounts of intracellular Ca^{2+}, possibly because cells of both ages are similar with regard to resting intracellular Ca^{2+}

homeostasis and mitochondrial calcium cycling. We previously showed that mitochondria in mature neurons stop moving when $\Delta\psi_m$ is dissipated and adopt a shorter, rounder morphology when the cell is exposed to large Ca^{2+} loads after glutamate treatment, especially in the cytosolic compartment (Rintoul et al., 2003b). In contrast to those findings in injured neurons, we now demonstrate that mitochondrial movement in young, healthy neurons is increased, but not because they have larger $\Delta\psi_m$. Furthermore, mitochondria in young neurons are shorter, but in a manner that is independent of $[Ca^{2+}]_{cyto}$ large enough to increase mitochondrial Ca^{2+} uptake.

The different cellular processes and energetic demands of 5 DIV neurons compared to 14 DIV neurons may impose important requirements on mitochondrial movement, morphology and distribution. Developing neurons must not only actively synthesize the bulk of cellular proteins and organelles, but also elongate axons and dendrites and form new synapses. Synaptogenesis is rapidly dynamic and energy-demanding, involving the contact of advancing axon growth cones and motile dendritic filopodia, recruitment of synaptic vesicle and receptor components over the course of 1-2 hours, and functional and morphological differentiation at pre- and post-synaptic sites (reviewed by Cohen-Cory, 2002). It is possible that the enhanced mitochondrial mobility we observed in developing neurons exists to provide adequate ATP to multiple sites of need at any given time. Increased mitochondrial movement may be assisted by shorter mitochondrial length, so that less energy is required to drive molecular motors for organelle transport. It is also possible that specific docking sites for mitochondria develop subsequent to the formation of relatively stable synaptic contacts in these cultures. Although no specific docking mechanisms have been identified for mitochondria in neurons, the presence of such a mechanism has been inferred by the studies of Chada and Hollenbeck (2004) who found that NGF decreased mitochondrial motility in axons. In this way, the decreased movement of mitochondria in mature

neurons could be the consequence of a decrease in the need for motility as the energy demands change, and also an increase in the impact of docking signals.

Interestingly, our measurements of mitochondrial morphology did not correlate with the expression levels of mitochondrial fusion and fission proteins, mfn-1 and drp-1, respectively (Figures 3D, 4A-C). Given that the number of mitochondria per μm of neuronal process is similar at 5 and 14 DIV (Figure 3C), it is logical to consider that the longer mitochondrial length in mature neurons is the result of either increased mitochondrial biogenesis with fusion to already existing mitochondria, or elongation of initial mitochondria over time (Bakeeva et al., 1981; Chen et al., 2003). The low level of mfn-1 expression we observed at 14 DIV supports the latter. Furthermore, the heightened expression of drp-1 in mature neurons might allow division and distribution of healthy mitochondria to meet the changing energetic demands of local environments distal to the cell body, and to compensate for the removal of unhealthy mitochondria (Smirnova et al., 1998; Pitts et al., 1999; Smirnova et al., 2001; Priault et al., 2005). In contrast, mitochondrial fission may be less important in young neurons where neuronal processes do not extend as far from the large mitochondrial pool in the cell body and mitochondrial turnover may be less frequent. One important caveat to our experiments, however, is that mitochondrial morphology was measured exclusively in neuronal processes, whereas immunoblotting for protein expression was performed on whole cells. It is possible that the population of mitochondria in the soma may have different characteristics than those in the processes, although the extent to which somatic vs. distal mitochondria contribute to the immunoblotting results remains unclear. Differential expression patterns of proteins that regulate mitochondrial morphology other than mfn-1 and drp-1 may also account for the observed differences in morphology, as could post-translational processes that affect the activity

of fission and fusion proteins (Fekkes et al., 2000; Mozdy et al., 2000; Tieu and Nunnari, 2000; Bach et al., 2003; Varadi et al., 2004).

After the organellar and synaptic pool are established in mature cells, mitochondrial homeostasis may become relatively more static for long-term support of energy-demanding processes. An obvious site of high demand for mitochondria in neuronal processes is the synapse. In mature neurons, mitochondria closely associate with synapses in electron micrographs and even tether to vesicle release sites (Kageyama and Wong-Riley, 1982; Rowland et al., 2000). Synaptic transmission requires mitochondrial ATP generation and control of local Ca^{2+} concentrations for neurotransmitter exocytosis, vesicle recruitment, activation of ion conductances, signaling at ionotropic glutamatergic synapses and synaptic plasticity (Bindokas et al., 1998; Zucker, 1999; David and Barrett, 2000; Zenisek and Matthews, 2000; Calupca et al., 2001; Billups and Forsythe, 2002; David and Barrett, 2003; Yang et al., 2003). The reduction in mitochondrial movement we observed in mature neurons suggests that the demands for mitochondria are more stably located throughout neuronal processes than in immature neurons, as would be expected from mature synapses. Mitochondrial morphology may also be elongated to better serve multiple synapses and to optimize mitochondrial function. This may be a particularly advantageous morphology since there is minimal requirement for increased energy expenditure for motility.

In conclusion, our study provides the first comparison of mitochondrial movement, morphology and distribution between synaptically immature and mature cortical neurons. We suggest that the dynamic energetic requirements of developing neurons, especially with regard to synaptogenesis, are met by highly motile mitochondria that adopt a shortened morphology to optimize motility. As neurons age and establish mature synapses, mitochondria move less and

elongate to better serve multiple synaptic sites simultaneously over longer time periods. Mitochondria in 5 DIV and 14 DIV neurons had identical $\Delta\psi_m$ and sequestered similar amounts of intracellular Ca^{2+}. Therefore, we propose that mitochondrial movement and morphology are governed by cellular demands in healthy neurons and that the regulating mechanisms are independent of $\Delta\psi_m$ of $[Ca^{2+}]_{mito}$, which have been implicated in injury models.

3.0 MITOCHONDRIAL TRAFFICKING TO SYNAPSES IN CULTURED PRIMARY CORTICAL NEURONS

3.1 ABSTRACT

Functional synapses require mitochondria to supply ATP and regulate local $[Ca^{2+}]_i$ for neurotransmission. Mitochondria are thought to be transported to specific cellular regions of increased need such as synapses. Yet little is known about how this occurs, including the spatiotemporal distribution of mitochondria relative to pre- and postsynaptic sites, whether mitochondria are dynamically recruited to synapses, and how synaptic activity affects these trafficking patterns. We studied primary cortical neurons in culture because they form synaptic connections and show spontaneous synaptic activity under normal conditions. Moreover, mitochondria must be transported over extensive distances to reach synapses on distal processes. Neurons were cotransfected with mitochondrially-targeted CFP and eYFP-tagged presynaptic label, synaptophysin, or postsynaptic label, PSD-95. Fluorescence microscopy revealed longer dendritic mitochondria that occupied a greater fraction of neuronal process length than axonal mitochondria. Mitochondria localized significantly to pre- and postsynaptic sites; but while this localization was preserved during synaptic inactivity, it was elevated in dendrites and reduced in axons during overactivity. Mitochondrial movement and recruitment to synapses also differed between axons and dendrites under basal conditions and when synaptic activity was altered. Additionally, we show that movement of dendritic mitochondria can be selectively impaired by

neuronal insults. We conclude that mitochondrial trafficking to synapses is dynamic in neurons and is modulated by changes in synaptic activity. Furthermore, mitochondrial morphology and distribution may be optimized differentially to best serve the synaptic distributions in axons and dendrites. Lastly, selective cessation of mitochondrial movement in dendrites suggests early postsynaptic dysfunction in neuronal injury and degeneration.

3.2 INTRODUCTION

Mitochondria are vital to the function of cells not only because they are the main source of energy but also because they regulate intracellular Ca^{2+} homeostasis. Since mitochondria are thought to be synthesized in the perinuclear region, they must be trafficked appropriately to meet demands throughout the cell (Wong-Riley, 1989; Miller, 1992; Davis and Clayton, 1996). This has particular relevance to neurons where processes can extend well beyond the cell body. Mitochondrial movement is dynamic, with individual organelles exhibiting various velocities and motility patterns in axons and dendrites (Overly et al., 1996; Ligon and Steward, 2000a). Microtubules and actin filaments serve as cytoskeletal substrates on which kinesins and dyneins transport the organelles, likely through interaction with accessory proteins such as Milton and syntabulin (Hirokawa et al., 1990; Nangaku et al., 1994; Morris and Hollenbeck, 1995; Tanaka et al., 1998; Ligon and Steward, 2000b; Stowers et al., 2002; Cai et al., 2005). Interestingly, mitochondrial morphology is also dynamic and can be regulated, for example through fusion and fission (reviewed by Rube and van der Bliek, 2004). An elongated morphology may confer bioenergetic advantages to ATP generation and dispersal (reviewed by Skulachev, 2001). Additionally, it is speculated that Ca^{2+} may be buffered over a greater surface area by longer

mitochondria, whereas shorter mitochondria may be transported more efficiently by molecular motors.

While mitochondria are thought to be trafficked to specific cellular regions such as those with high metabolic demand or elevated $[Ca^{2+}]_i$, few targets for mitochondrial recruitment have been unambiguously identified (Morris and Hollenbeck, 1993; Yi et al., 2004). An obvious site of high demand for mitochondria in neuronal processes is the synapse. An analysis of mature neurons by electron microscopy demonstrated that mitochondria closely associate with synapses and tether to vesicle release sites (Kageyama and Wong-Riley, 1982; Rowland et al., 2000). Synaptic transmission requires mitochondrial ATP generation and control of local Ca^{2+} concentrations for neurotransmitter exocytosis, vesicle recruitment, activation of ion conductances, signaling at metabotropic receptors, potentiation of neurotransmitter release and synaptic plasticity (Bindokas et al., 1998; Zucker, 1999; David and Barrett, 2000; Zenisek and Matthews, 2000; Calupca et al., 2001; Billups and Forsythe, 2002; Vanden Berghe et al., 2002; David and Barrett, 2003; Kann et al., 2003; Yang et al., 2003). However, recent studies in mutant *Drosophila* neurons with perinuclearly clustered mitochondria also suggest that direct mitochondrial localization to presynaptic terminals may be less critical for neurotransmission processes under physiological conditions than for Ca^{2+} buffering and mobilization of reserve pool vesicles during tetanic stimulation (Guo et al., 2005; Verstreken et al., 2005). The question then arises if and how dynamic mitochondrial trafficking patterns and regulation of morphology mediate appropriate synaptic support, both presynaptically and postsynaptically. Some evidence exists for mitochondrial recruitment to dendritic spines during morphogenesis in hippocampal neurons after repetitive depolarizing stimulation (Li et al., 2004). However, the normal

trafficking patterns of mitochondria to synapses in spontaneously active neurons remains to be elucidated.

Since mitochondria are important for supporting synaptic transmission, it is likely that impairment of mitochondrial transport could result in synaptic dysfunction in addition to inadequate distribution of ATP and sequestration of $[Ca^{2+}]_i$. This is particularly critical in injured neurons because many insults are known to stop mitochondrial movement (Rintoul et al., 2003; Malaiyandi et al., 2005; Reynolds and Santos, 2005). The work of Li et al. (2004) and Guo et al. (2005) suggests that different mechanisms account for mitochondrial distribution in axons and dendrites. For example, formation of dendritic spines but not of synaptic boutons may require local recruitment of mitochondria. Therefore, if mitochondrial movement in axons and dendrites has different susceptibilities to neuronal insults, the result could be selective dysfunction of pre- or postsynaptic neurotransmission. In this study, we show not only the differential trafficking patterns of mitochondria to presynaptic terminals and postsynaptic densities, but also reveal dynamic modulation of mitochondrial trafficking, morphology and distribution that accompany changes in synaptic activity. Additionally, we provide evidence for heightened vulnerability of dendritic mitochondria to movement cessation after neurotoxic glutamate and zinc exposure, suggestive of dysfunction in dendrites prior to axons.

3.3 EXPERIMENTAL PROCEDURES

Materials

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Cell culture

All animals used for this study were maintained in accordance with the University of Pittsburgh *Guidelines for the Care and Use of Animals*. Primary neurons were dissociated for culture from the cortices of embryonic day 17 Sprague Dawley rat pups and grown under serum-free conditions as previously described (Malaiyandi et al., 2005).

Transfection of cortical cultures

The mito-CFP construct was obtained from Clonetech (Mountain View, CA) and targets cytochrome c oxidase subunit VIII. The PSD-95-eYFP and synaptophysin-eYFP plasmids were provided by Dr. Ann Marie Craig. The mito-eYFP construct obtained from Dr. Roger Tsien (Llopis et al., 1998) employs a cytochrome c oxidase subunit IV mitochondrial localization sequence. Plasmids were purified and amplified using the Qiagen Plasmid Maxi Kit (Valencia, CA) according to manufacturer's instructions. Cotransfections were performed on neurons after 11 days *in vitro* (DIV), using 1 ug each of mito-CFP and PSD-95-eYFP or synaptophysin-eYFP and 2.5 µl Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) in an added volume of 250 µl DMEM. Transfections with mito-eYFP were performed with 1 ug plasmid and otherwise identical conditions. Media was completely replaced with conditioned media 6-8 hours after transfection and neurons were imaged 3-4 days after transfection.

Imaging

Cells were perfused with HEPES-buffered salt solution (HBSS) adjusted to pH 7.4 with NaOH and composed of (in mM) NaCl 137, KCl 5, $NaHCO_3$ 10, Hepes 20, glucose 5.5, KH_2PO_4 0.6, Na_2HPO_4 0.6, $CaCl_2$ 1.4 and $MgSO_4$ 0.9. HBSS was warmed to 37°C and flowed at a rate of 5 ml/min throughout the duration of each experiment. For fluorescence recording, we used a

BX61WI Olympus Optical (Tokyo, Japan) microscope, a CCD camera (Hamamatsu, Shizouka, Japan) and a Lambda-LS xenon arc lamp light source with a Lambda 10-2 optical filter changer (Sutter, Novato, CA). Imaging was performed at a rate of 1 frame/10 seconds at a magnification of 60x except where noted, using Compix Inc. imaging systems and SimplePCI software (Compix Inc., Cranberry PA). Dual wavelength fluorescence imaging was performed using the following excitation wavelengths: 440/495 nm for mito-CFP and eYFP-tagged synaptic components, and 340/380 nm for fura-2AM. Single wavelength fluorescence imaging of mito-eYFP was performed at a 495 nm excitation wavelength.

Measurement and pharmacologic modulation of spontaneous $[Ca^{2+}]_i$ fluxes

Spontaneous $[Ca^{2+}]_i$ transients were represented by fluxes in fura-2AM ratios. Cells were loaded with 5 μM fura-2AM/HBSS (Molecular Probes, Eugene, OR) for 15-20 min at 37°C. Neurons were imaged at a rate of 1 frame/3 sec at 40x magnification. $[Ca^{2+}]_i$ was represented as the fura-2 ratio of cell bodies after subtraction of background fluorescence using SimplePCI. Drug treatments were 1 hr pre-incubation with 200 nM TTX prepared from a 1 mM stock dissolved in distilled water, 24 hr pre-incubation with 1 μM TTX or 30 min pre-treatment with 250 nM veratridine prepared from a 1 mM stock dissolved in ethanol. Experiments were performed during the 30 mins following each pre-treatment with continuous perfusion of 200 nM TTX or 250 nM veratridine.

Analysis of mitochondrial length, occupancy and number

SimplePCI functions were employed to determine average mitochondrial length, occupancy and number in a subfield that excluded proximal processes clearly containing more than one mitochondrion in their cross-section. User-defined thresholds for pixel intensity and object size

were used to identify the total number of measurable mitochondria in a frame. Length was calculated from skeletonized objects, which consisted of a 1 pixel-wide line running the length of each mitochondrial object. All neuronal processes in the field were then converted into line objects, their total length was calculated, and mitochondrial occupancy was computed as total mitochondrial length/process length. A student's t-test was used for comparisons between axonal and dendritic controls. A one-way ANOVA with Dunnett's Multiple Comparison Test to control was used to analyze data in axons or dendrites after pharmacologic treatments.

Analysis of synapse number, mitochondrial localization and movement parameters relative to synaptic sites

Synapses were designated using only the eYFP image so that the observer was blinded to the location of mitochondria. Clusters of eYFP labeled PSD-95 and synaptophysin proteins were identified as stable synaptic components if they were approximately 1 μm in diameter and did not display net lateral movement > 1 μm during the course of each 30 min experiment. Therefore, small or mobile fluorescent clusters were excluded from our analysis. The total number of synaptophysin or PSD-95 clusters identified in this manner was divided by the total neuronal process length in a given subfield to yield the number of synapses/μm. A student's t-test was used for comparisons between axonal and dendritic controls. A one-way ANOVA with Dunnett's Multiple Comparison Test to control was used to analyze data in axons or dendrites after pharmacologic treatments.

Using SimplePCI, rectangular regions of interest (ROIs) with a width of 1.1 μm were placed around each identified synaptic component cluster using the eYFP image. As controls, ROIs were placed along the same neuronal processes in locations that were void of eYFP-labeled clusters during the 30 min experiment. Mitochondrial parameters were then analyzed from

imaging files containing the ROIs and only the CFP image so that the observer was blinded to the synaptic contents of each ROI. The mitochondrial localization and movement parameters that were measured are shown in Table-1. Mitochondria occupying at least half of the width of an ROI were considered to be localized in that ROI. A mitochondrion was considered to occupy an ROI for as long as the mitochondrial edge did not completely move out of the ROI. The number of mitochondria moving past each ROI was counted over the entire course of each movie and those that localized in the ROI according to the definition above were considered to stop during their trajectory. The length of time that these moving mitochondria stopped in ROIs was also measured. A student's paired t-test was used for comparisons between synaptic and nonsynaptic sites in the same cells. A one-way ANOVA with Dunnett's Multiple Comparison Test to control was used to analyze data in axons or dendrites after pharmacologic treatments.

Measurement of drug-induced changes in mitochondrial movement and morphology

For experiments with glutamate treatment, axons were identified in cortical neurons by selective expression of synaptophysin-eYFP and dendrites were identified by selective expression of PSD-95-eYFP. Proximal and distal axon segments were imaged 3-4 and 10-12 imaging fields away from the cell soma, which corresponded to 200-500 μm and 1-1.4 mm, respectively. Cells were perfused for 10 min with HBSS, followed by 10 min with 30 μM glutmate/1 μM glycine, followed by a 10 min HBSS wash. Mitochondrial length was measured as described above using frames corresponding to the start of HBSS perfusion, the end of glutamate treatment, and the end of HBSS wash. Mitochondrial roundness was computed from the same frames as length using a Simple PCI function as 4π*area/√perimeter. Overall mitochondrial movement was measured during the first 190 sec of HBSS perfusion, the last 190 sec of glutamate treatment, and the last 190 sec of HBSS wash as previously described (Rintoul et al., 2003). Briefly, a custom Visual

Basic macro was used to quantify movement as *average event count/average number of mitochondrial pixels*, where *event count* is the number of corresponding pixels that vary by at least 20 fluorescence units between 2 consecutive frames, and the *number of mitochondrial pixels* is the number of pixels per frame that are 20 fluorescence units above background fluorescence. The value of 20 units was chosen because a masking function in SimplePCI software effectively identified mitochondria as pixels 20 units above background fluorescence.

For experiments involving FCCP, oligomycin, zinc, rotenone and 4-bromo-A23187 treatment, neurons were transfected with mito-eYFP and axons and dendrites were imaged at a rate of 1 frame/6 sec. Movement was analyzed using the macro described above during the following 2 min time periods: before drug treatment, at the end of drug perfusion, and at the end of a 15-20 min HBSS wash. The treatment conditions were either 5 min perfusion with 750 nM FCCP, 10 min perfusion with 10 μM oligomycin, 5 min perfusion with 2 μM rotenone, 5 min perfusion with 1 μM 4-bromo-A23187, or 10 min perfusion with 3 μM $ZnCl_2$/20 μM Na-pyrithione. $ZnCl_2$ treatment was followed by a 5 min perfusion with 25 μM zinc chelator N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN). $CaCl_2$ was omitted from HBSS in experiments involving FCCP and $ZnCl_2$. For each cell, mitochondrial movement during treatment and after washout was normalized to movement prior to treatment. A student's paired t-test was used for statistical analysis of movement recovery.

3.4 RESULTS

Mitochondria are visualized concurrently with pre- and postsynaptic labels

Mitochondria were imaged simultaneously with pre- or postsynaptic sites by cotransfecting primary cortical neurons with a mitochondrially-targeted cyan fluorescent protein (mito-CFP) and either the presynaptic vesicle protein, synaptophysin, or the postsynaptic density protein, PSD-95 tagged to an enhanced yellow fluorescent protein (eYFP). A single neuron transfected with mito-CFP, synaptophysin-eYFP and PSD-95-mRFP is shown in Fig. 1A. Fig. 1B and C show labeled mitochondria with PSD-95 clusters in a dendrite and with synaptophysin clusters in axon branches, respectively. Axonal and dendritic differences in synaptic density and mitochondrial morphology and distribution were readily observed. Localization of mitochondria to a subset of pre- and postsynaptic sites was also seen. These mitochondrial morphology and distribution patterns were quantified and are discussed below.

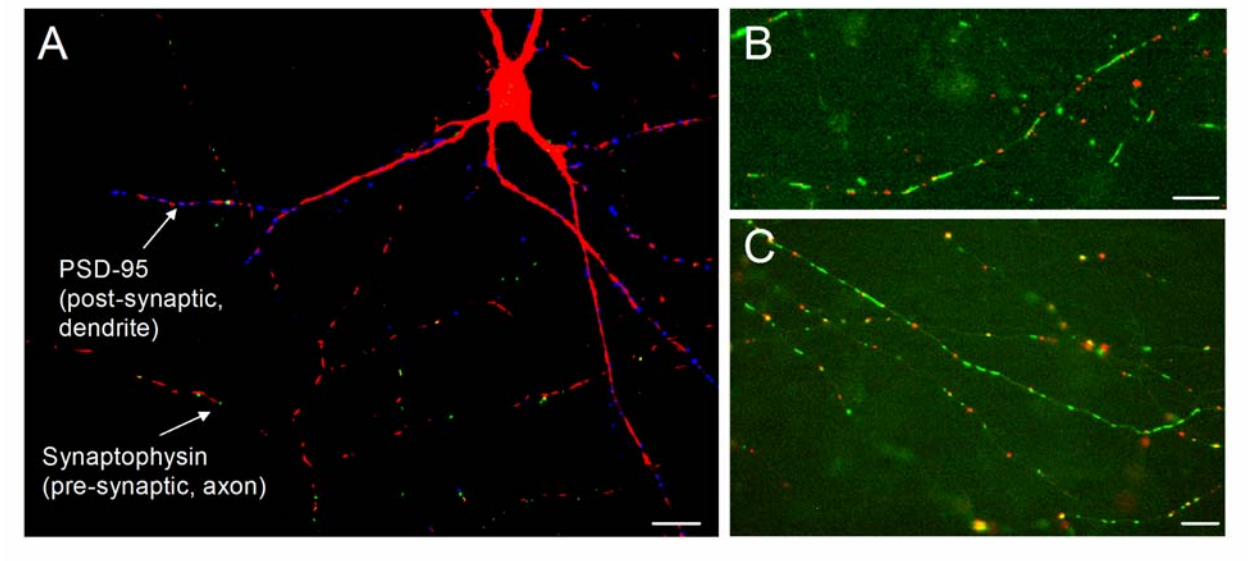

Figure 3.1 Mitochondrial localization and trafficking were visualized relative to pre- and postsynaptic sites by cotransfecting neurons with fluorescent proteins.

(**A**) Primary cortical neuron transfected with mito-CFP (red), presynaptic label synaptophysin-eYFP (green) and postsynaptic label PSD-95-mRFP (blue). (**B**) Dendritic segment from a transfected neuron showing mito-CFP labeled mitochondria (green) and PSD-95-eYFP clusters (red). (**C**) Axonal branches from a transfected neuron showing mito-CFP labeled mitochondria (green) and synaptophysin-eYFP clusters (red). Images are representative of 3-5 cultures. Scale bar, 10 µm.

Time-lapse fluorescence imaging permitted us to measure multiple parameters of mitochondrial movement patterns. Imaging movies demonstrated the variability and complexity of mitochondrial trafficking to pre- and postsynaptic sites. Specifically, we observed mitochondria that (1) localized to synapses for long or short time periods, (2) paused at synaptic sites as they traveled unidirectionally along a neuronal process, (3) shuttled bidirectionally between multiple neighboring synapses, (4) moved to and briefly resided at a synapse before dividing and leaving one fragment at the synapse while the remaining fragment moved elsewhere and (5) elongated to bridge nearby synapses. To study the more accessible characteristics of mitochondrial trafficking to synapses, we measured the parameters listed in Table 1. Three populations of mitochondria with specific movement patterns emerged from our data, as diagramed in Fig. 2A. These included relatively immobile mitochondria that remained stationary for long time periods (generally > 15 min), and mobile mitochondria that paused at specific locations for shorter (< 20 sec) or longer (mean 1.5-2 min, data not shown) time periods. We distinguished sites where mitochondria halted by whether they contained a synaptic component. The trafficking patterns of these mitochondrial populations are discussed in detail below.

Parameter	Method of measurement
Mitochondrial localization frequency	Fraction of ROIs that contain mitochondria
Mitochondrial residence time	Fraction of total movie length (~ 30 min) that the mitochondria initially found in an ROI remained stationary
# passing mitochondria/min	Calculated from the number of mitochondria moving past each ROI during the entire movie
Fraction of passing mitochondria that stop	Fraction of mitochondria moving past an ROI that localized in that ROI for at least 1 imaging frame

Table 1 Parameters of mitochondrial movement measured.

Regions of interest (ROIs) were placed around synaptophysin and PSD-95 clusters and measurements were performed in a blinded manner using mitochondrial images. As a comparison with mitochondrial localization and trafficking to nonsynaptic sites, measurements were also made at ROIs placed throughout neuronal processes where synaptophysin and PSD-95 clusters were absent.

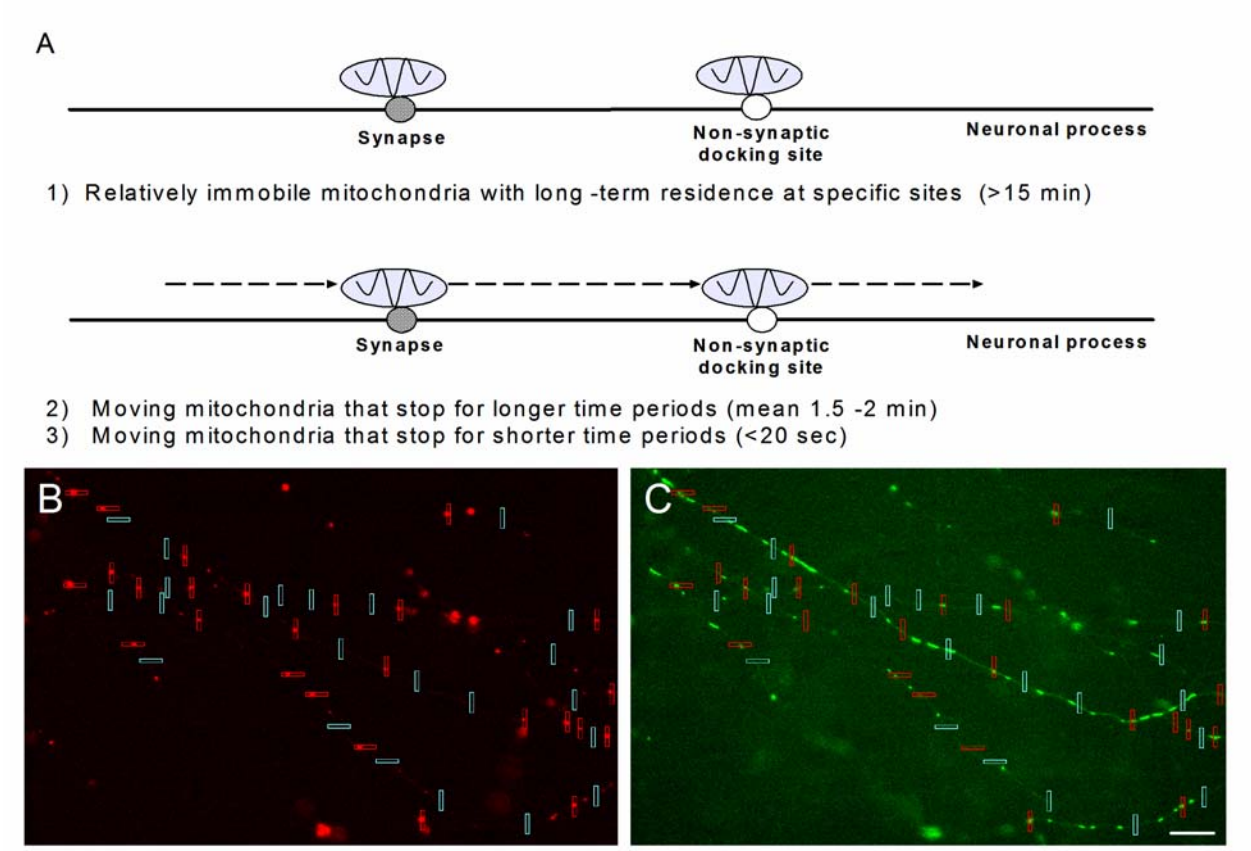

Figure 3.2 Populations of mitochondria with different movement patterns were observed and their trafficking patterns were analyzed in a blinded manner.

(**A**) Mitochondria that were relatively immobile tended to remain stationary at synaptic and nonsynaptic sites for > 15 min. Relatively mobile mitochondria exhibited saltatory movement, making stops at synaptic and nonsynaptic sites for shorter or longer time periods. (**B**) Representative axonal image prepared for blinded data analysis shows regions of interest (ROIs) placed around synaptophysin clusters (red boxes) and ROIs placed at nonsynaptic locations (blue boxes). (**C**) Corresponding mitochondrial image with ROIs, shown here before box colors were made uniform, was used to measure the localization and trafficking parameters listed in Table 1. Scale bar, 10 μm.

Since mitochondria localized to synapses are thought to be functional in providing ATP or regulating local $[Ca^{2+}]_i$, we predicted that synaptic overactivity would induce mitochondrial recruitment to synapses and synaptic quiescence would release localized mitochondria to perform other duties. The spontaneous synaptic firing of neurons in culture was overactivated by veratridine, an inhibitor of Na^+ channel inactivation. As a counterpart, we silenced synaptic activity with tetrodotoxin (TTX), a blocker of voltage-sensitive Na^+ channels in excitable

membranes. Synaptic activity was monitored by the fluorescent $[Ca^{2+}]_i$ indicator, fura-2AM, as the spontaneous and synchronous $[Ca^{2+}]_i$ transients that cultured neurons exhibit when firing. As shown in Fig. 3, veratridine caused intense, high-magnitude $[Ca^{2+}]_i$ spiking activity while TTX treatment completely obliterated $[Ca^{2+}]_i$ transients. We confirmed that these activity patterns were maintained throughout the duration of our drug treatments, and drug was continuously perfused on cells during each imaging experiment.

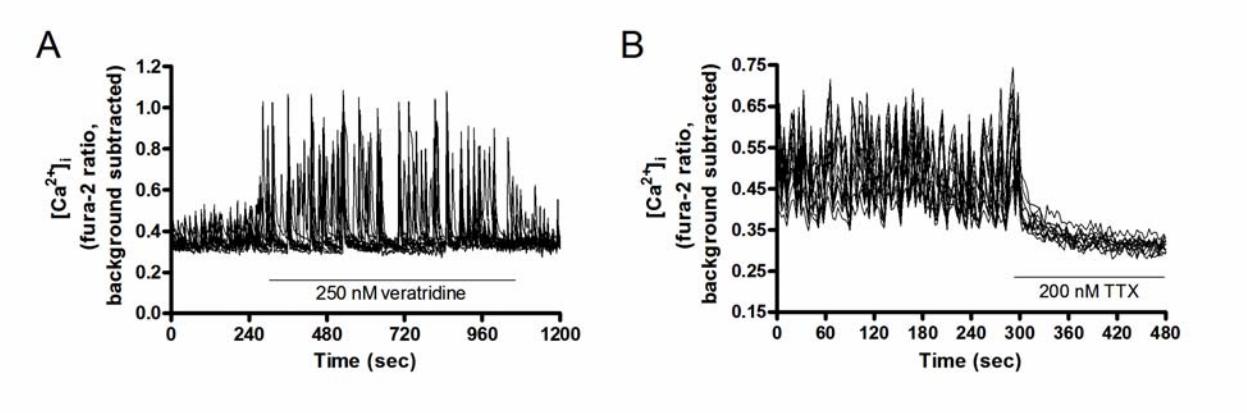

Figure 3.3 Spontaneous synaptic activity of cultured neurons was pharmacologically modulated to determine the effects on mitochondrial trafficking.

(**A**) Spontaneous $[Ca^{2+}]_i$ fluxes measured by fura-2AM were indicative of basal synaptic activity and were amplified in magnitude by 250 nM veratridine. (**B**) $[Ca^{2+}]_i$ spiking was inhibited by 200 nM TTX. Traces represent 11-13 cells from a single coverslip. Experiments were repeated 2-3 times each on 3 separate cultures.

Mitochondrial morphology and distribution differ in axons and dendrites and are differentially modulated by changes in synaptic activity

The morphology of mitochondria and their distribution relative to synaptic sites was clearly variable between axons and dendrites (Fig. 1). First, mitochondria were more fully distributed through dendrites, occupying a large fraction of neuronal process length. In contrast, mitochondria were visibly shorter and more sparsely distributed throughout axons. The

distribution of presynaptic sites and postsynaptic densities also differed; axons were far less densely populated by synaptophysin clusters than were dendrites populated by PSD-95 clusters.

In accordance with these observations, we found that mitochondria occupied 26.3 ± 0.02% of any given dendritic segment, which was significantly greater than the 17.4 ± 0.02% mitochondrial occupation of any given axonal segment ($p < 0.01$; Fig. 4A). This increase in mitochondrial occupancy of dendritic process length was attributed to a remarkable increase in mitochondrial length, 2.2 ± 0.1 μm in dendrites compared to 1.4 ± 0.1 μm in axons ($p < 0.0001$; Fig. 4B). However, the number of discrete mitochondria per μm of neuronal process was equivalent in axons and dendrites (Fig. 4C). Interestingly, the increased mitochondrial length and occupancy in dendrites correlated with a 2-fold increase in density of PSD-95 clusters on dendrites compared to synaptophysin clusters on axons ($p < 0.01$; Fig. 4D). Therefore, mitochondria seem to be more fully distributed through processes that have high synaptic density.

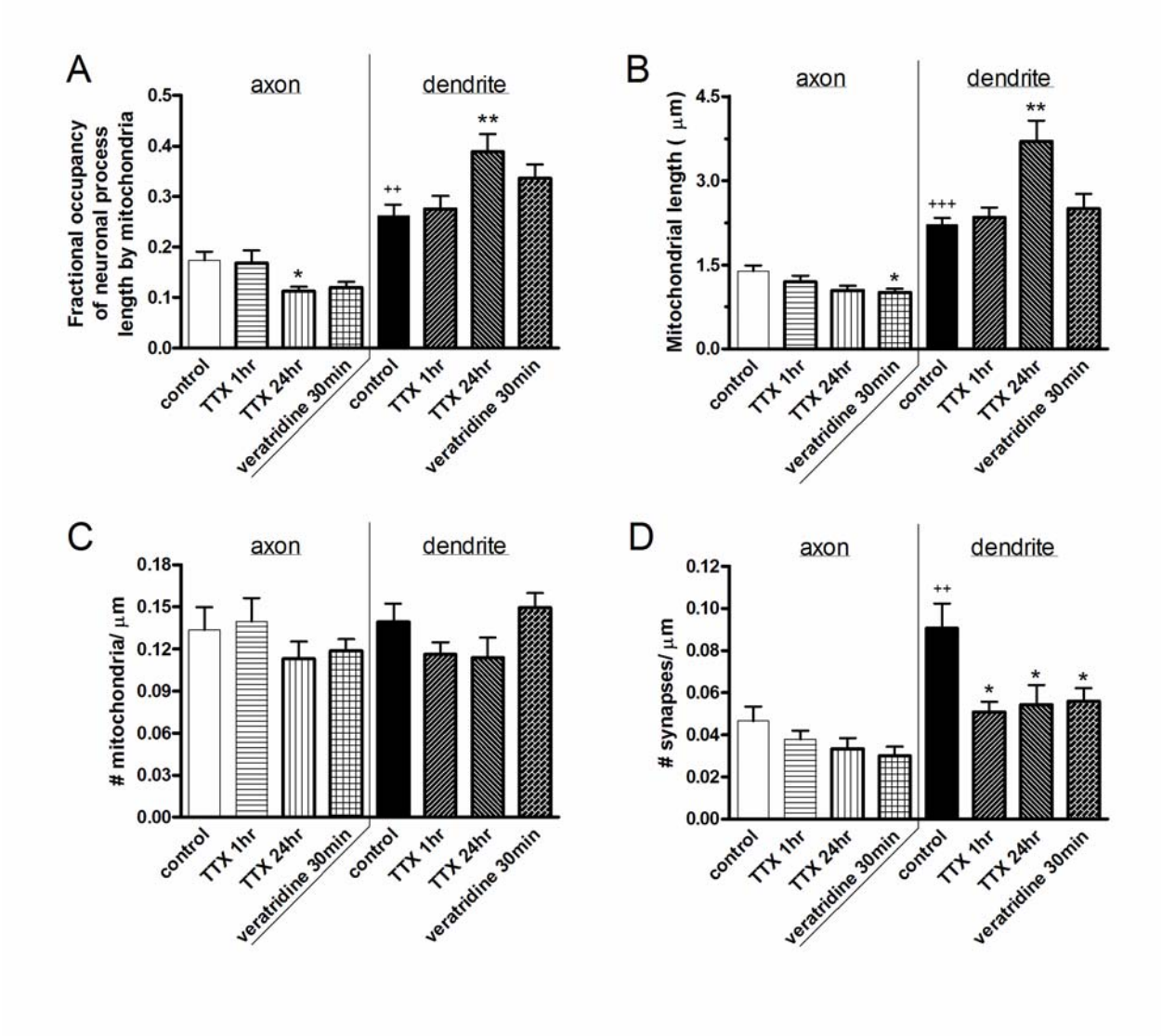

Figure 3.4 Mitochondrial distribution and morphology differed between axons and dendrites and was differentially modulated by changes in synaptic activity.

(**A**) Mitochondrial occupancy of axons was significantly lower than that of dendrites. This was measured as the total length of all mitochondria divided by the length of all neuronal processes in a given field. Treatment with 1 μM TTX for 24 hr or 250 nM veratridine for 30 min reduced mitochondrial occupancy of axons but increased occupancy of dendrites. (**B**) Mitochondrial length was significantly shorter in axons than in dendrites. Treatment with TTX for 24 hr increased mitochondrial length in dendrites. Veratridine also reduced mitochondrial length in axons. (**C**) Number of mitochondria per μm of neuronal process was similar between axons and dendrites and was not affected by changes in synaptic activity. (**D**) Significantly more stationary clusters of PSD-95 populated dendrites compared to synaptophysin clusters on axons. TTX and veratridine treatments reduced the number of stationary PSD-95 clusters. Values are shown as mean ± SE from 2-3 coverslips each from 4-5 separate cultures. $p < 0.05$ was considered significant, where + represents comparisons between axons and dendrites of untreated cells, and * represents comparisons between untreated cells and pharmacologically treated cells.

When synaptic activity was modulated, mitochondrial morphology and distribution surprisingly changed in opposite directions on axons compared to dendrites. Fig. 4A demonstrates that 24 hr TTX treatment reduced mitochondrial occupancy of axons from 17.4 ± 0.02% to 11.2 ± 0.01% ($p < 0.05$) but increased mitochondrial occupancy of dendrites from 26.2 ± 0.02% to 38.8 ± 0.03% ($p < 0.01$). The change in mitochondrial occupancy in dendrites was coincident with a significant increase in mitochondrial length but no significant change in the number of mitochondria in neuronal processes (Fig. 4B, C). Veratridine treatment resulted in a significantly shorter mitochondrial length and a trend toward reduced mitochondrial occupancy in axons but increased mitochondrial occupancy in dendrites. It is important to note that all pharmacologic manipulations of synaptic activity reduced the number of stationary PSD-95 clusters per μm of dendritic processes ($p < 0.05$; Fig. 4D). However, treatments never altered the total number of labeled clusters (data not shown); thus, the effects seen in Fig. 4D are a result of increased cluster mobility. For our measurements, mobility was considered to be net movement greater than 1 μm over the course of 30 mins. In summary, these results demonstrate the ability of mitochondrial morphology to be dynamically modulated by global changes in synaptic activity. Furthermore, axonal and dendritic mitochondria responded differently to single pharmacologic manipulations, suggesting that distinct mechanisms operate within these neuronal compartments to alter mitochondrial morphology and distribution.

Mitochondria localize to synaptic sites on axons and dendrites for extended time periods

We observed mitochondria localized to subsets of pre- and postsynaptic sites for varying time periods. To measure mitochondrial localization frequency at synaptic sites, we placed regions of interest (ROIs) of width 1.1 μm around synaptophysin and PSD-95 clusters as well as throughout neuronal processes where such clusters were absent (Fig. 2B). The presence of mitochondria in

ROIs and the length of time those mitochondria remained stationary were then blindly scored (Fig. 2C). During a 30 minute span of imaging, we found that the mitochondria that were localized in ROIs tended to reside there for at least 15 min and therefore likely represented a generally stationary pool of mitochondria (Fig. 2A).

Our results demonstrate that mitochondria localized significantly to pre- and postsynaptic sites. The percentage of synaptophysin clusters that contained mitochondria was 36.1 ± 0.06% compared to the 14.3 ± 0.03% chance of finding mitochondria at locations on axons that did not contain synaptophysin clusters ($p < 0.05$; Fig. 5A). Furthermore, mitochondria that localized to labeled presynaptic terminals remained stationary for 77.0 ± 0.09% of the movie duration, significantly more than the 47.4 ± 0.07% that mitochondria resided at nonsynaptic sites ($p < 0.05$; Fig. 5B). Interestingly, veratridine treatment caused mitochondria to (i) localize to synaptic sites and nonsynaptic sites at similar frequencies and (ii) remain stationary at synaptic sites for similar time periods as at nonsynaptic sites (Fig. 5A, B).

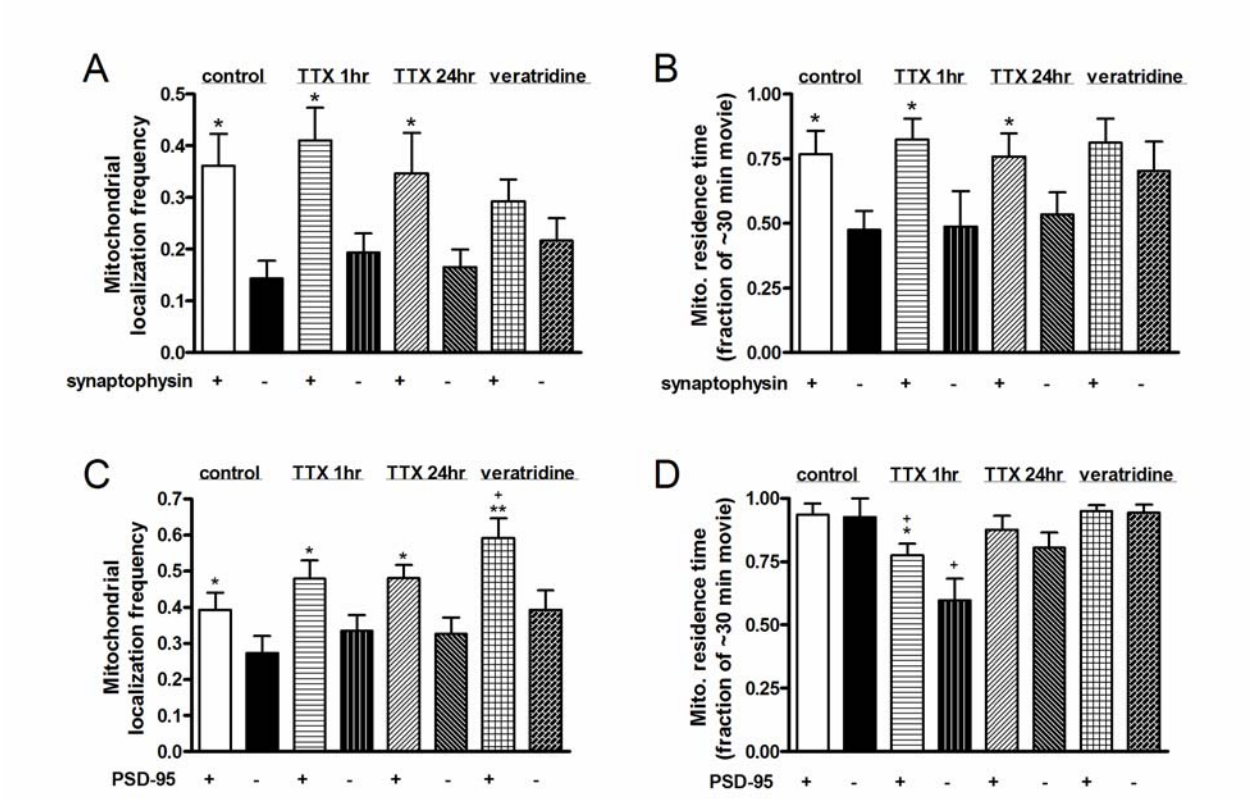

Figure 3.5 Mitochondria localized significantly to pre- and postsynaptic sites and their distribution relative to synapses changed in response to altered synaptic activity.

(**A**) Mitochondrial localization frequency to presynaptic sites was measured as the fraction of synaptophysin clusters that contained mitochondria compared to the fraction of randomly selected sites void of synaptophysin clusters that contained mitochondria. Mitochondria localized preferentially to synaptophysin clusters in untreated and TTX-treated cells but not in veratridine-treated cells. (**B**) Residence times of the mitochondria localized to synaptic and nonsynaptic sites in *A* were compared. Residence time was calculated as the length of time that the colocalized mitochondria remained stationary divided by the duration time of the imaging movie. Mitochondria resided significantly longer at synaptophysin clusters than nonsynaptic sites in untreated and TTX-treated cells but not veratridine-treated cells. (**C**) Mitochondrial localization frequency at PSD-95 clusters was measured as in *A*. Mitochondria localized preferentially to PSD-95 clusters and veratridine treatment increased this localization frequency relative to untreated cells. (**D**) Residence time of mitochondria at PSD-95 clusters was measured as described in *B*. Treatment with 200 nM TTX for 1 hr reduced mitochondrial residence times in dendrites, yet mitochondria resided at PSD-95 clusters longer than at nonsynaptic sites. Values are shown as mean ± SE from 2-3 coverslips each from 3-5 separate cultures. $p < 0.05$ was considered significant, where * represents comparisons between synaptic sites and nonsynaptic sites in the same cells, and + represents comparisons between untreated cells and pharmacologically treated cells.

Mitochondria also localized to postsynaptic sites on dendrites, but exhibited different patterns of distribution than on axons. Mitochondria colocalized to 39.3 ± 0.05% of PSD-95 clusters compared to 27.4 ± 0.05% at sites that did not contain postsynaptic densities ($p < 0.05$; Fig. 5C). The increased frequency of finding mitochondria at nonsynaptic sites on dendrites compared to axons can be attributed to the higher fractional occupancy of dendrites by mitochondria (Fig. 4A). We found mitochondria were relatively stationary in dendrites, and as a result, they resided at both synaptic and nonsynaptic sites for nearly the entire duration of our 30 min imaging experiments (Fig. 5D). However, results from TTX treatment show that after 1 hr, mitochondria remained stationary for shorter time periods, and actually resided at sites containing PSD-95 clusters significantly longer than at sites void of clusters ($p < 0.05$; Fig. 5D). On the other hand, veratridine treatment caused mitochondria to associate with 59.2 ± 0.06% of PSD-95 clusters compared to 39.3 ± 0.05% of clusters in control ($p < 0.05$; Fig. 5C). These results indicate that mitochondria associate significantly with a subset of pre- and postsynaptic sites and selectively reside at presynaptic terminals for extended time periods. Furthermore, changes in synaptic activity can modulate mitochondrial distribution to synaptic sites differently in axons and dendrites.

Mitochondrial movement is greater in axons than dendrites and increases during synaptic inactivity

We measured mitochondrial movement as the number of mitochondria that move past ROIs placed along neuronal processes over the course of 30 min. It is important to note that our measurement of mitochondrial movement can be influenced by both the number of moving mitochondria and the velocity with which they move. Mitochondrial movement past synaptic sites was identical to nonsynaptic sites (data not shown). This suggests that we were measuring

a population of highly mobile mitochondria that made brief stops lasting well under 30 minutes. We therefore distinguish this population of mitochondria from the generally immobile population described above and in Fig. 5.

Remarkably more mitochondrial movement was observed in axons compared to dendrites: 0.13 ± 0.02 vs 0.03± 0.008 mitochondria moved past a given point per minute in axons and dendrites, respectively ($p < 0.001$; Fig. 6A). Synaptic silence by TTX for 1 hr caused mitochondrial movement to increase dramatically in all neuronal processes. The number of mitochondria passing a given point increased to 0.21 ± 0.02 ($p < 0.05$) in axons and to 0.09 ± 0.01 ($p < 0.01$) in dendrites. Movement remained somewhat elevated in dendrites after 24 hr of TTX treatment, but completely returned to control values in axons. On the other hand, veratridine treatment had no significant effects on movement in either axons or dendrites.

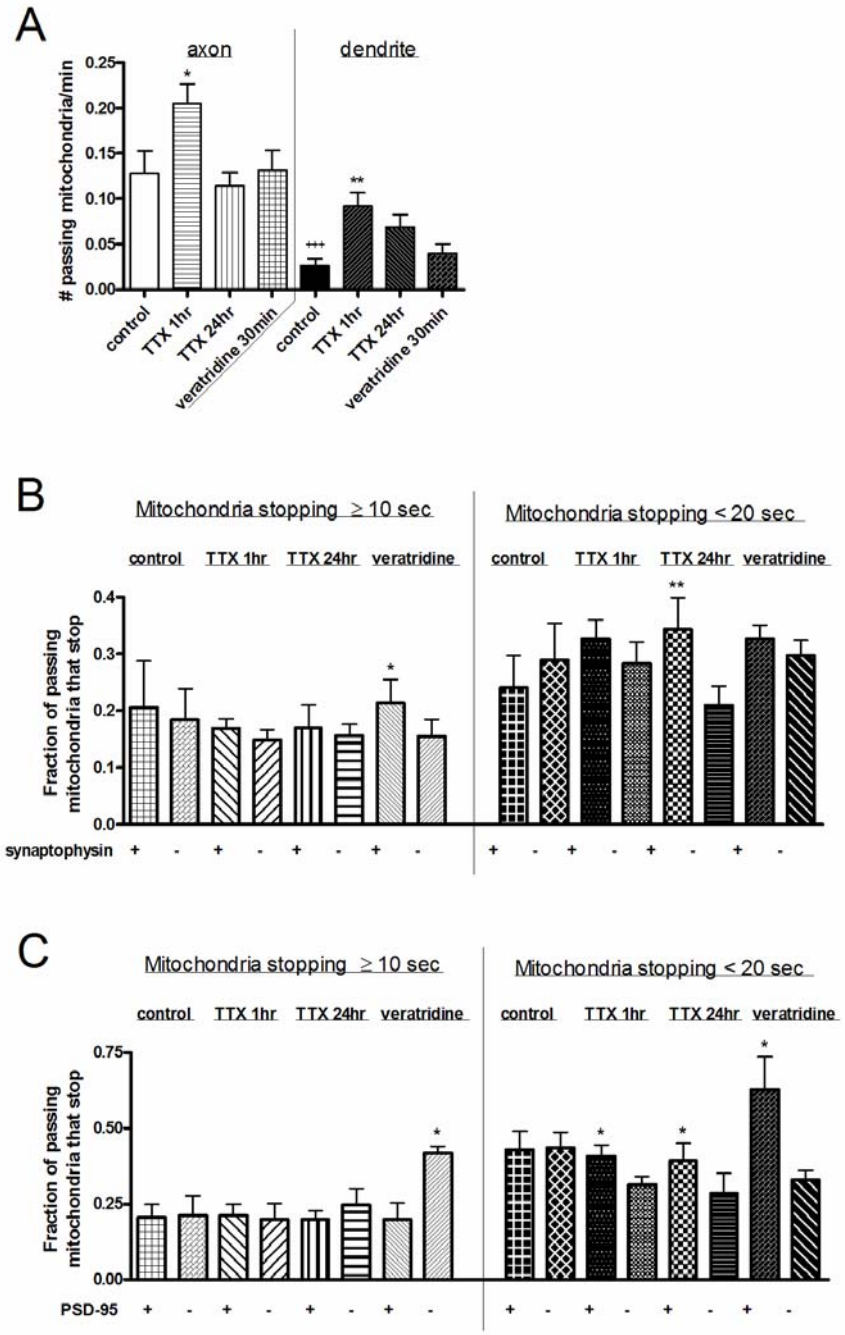

Figure 3.6 Mitochondrial movement patterns differed between axons and dendrites and was altered in response to changes in synaptic activity.

(**A**) Mitochondrial movement was greater in axons than dendrites and was increased in all processes by 1 hr treatment with 200 nM TTX. Movement was measured as the number of mitochondria passing a given point on a neuronal process over time. (**B**) The fraction of passing mitochondria measured in *A* that paused at synaptophysin

clusters on axons was compared to the fraction that paused at nonsynaptic sites. Mitochondria that stopped ≥ 10 sec (average 1.5 min) showed a preference toward synaptophysin clusters after 30 min treatment with 250 nM veratridine. Mitochondria that stopped for < 20 sec showed a preference toward synaptophysin clusters after 24 hr treatment with 1 μM TTX. (**C**) The fraction of passing mitochondria measured in *A* that paused at PSD-95 clusters on dendrites was compared to the fraction that paused at nonsynaptic sites. Mitochondria that stopped ≥ 10 sec (average 2 min) showed a preference toward PSD-95 clusters after veratridine treatment. Mitochondria that stopped for < 20 sec showed a preference toward nonsynaptic sites after TTX and veratridine treatments. Values are shown as mean ± SE from 2-3 coverslips each from 3-5 separate cultures. $p < 0.05$ was considered significant. In *A*, + represents comparisons between axons and dendrites of untreated cells, and * represents comparisons between untreated cells and pharmacologically treated cells. In *B* and *C*, * represents comparisons between synaptic sites and nonsynaptic sites in the same cells.

Saltatory movement of mitochondria is modulated by changes in synaptic activity

Overall mitochondrial movement in neuronal processes can be largely attributed to a mobile subset of mitochondria that is distinct from a stationary subset of mitochondria. Mobile mitochondria have been reported to account for 5-20% or 35-45% of the total mitochondrial pool in cultured hippocampal neurons (Overly et al., 1996; Ligon and Steward, 2000a). These mitochondria exhibit saltatory movement, pausing briefly along their trajectory and also changing direction mid-course. As diagramed in Fig. 2A, some mitochondria, most often those that travel with high velocity, paused for increments less than 20 sec; others paused for longer time periods, on average 1.5 min in axons and 2 min in dendrites (data not shown). Therefore, we separated these two groups of mitochondria as those that stopped for < 20 sec and those that stopped for ≥ 10 sec. Some obligatory overlap in these groups exists due to limitations of time-lapse image acquisition, but no mitochondria were counted in both groups.

Our measurements of mitochondria that stopped at synaptic sites and at sites that did not contain synaptophysin or PSD-95 clusters indicate that such movement patterns change in response to altered synaptic activity (Fig. 2B, C). In the basal state, moving mitochondria were

equally likely to stop at a given location regardless of whether a synapse was present (Fig. 6B, C). However, among mitochondria that paused for < 20 sec, a greater fraction stopped at labeled presynaptic terminals after 24 hr TTX treatment ($p < 0.01$), and a greater fraction stopped at PSD-95 clusters after 1 hr and 24 hr TTX compared to sites that did not contain synaptic components ($p < 0.05$). The same pattern of selectivity toward pausing at synaptic sites was found after veratridine treatment among mitochondria that stopped for ≥ 10 sec on axons and < 20 sec on dendrites ($p < 0.05$). There was also a notable increase in the fraction of mitochondria that stopped for ≥ 10 sec at nonsynaptic sites relative to postsynaptic sites on dendrites after veratridine treatment ($p < 0.05$). Therefore, saltatory movement to both synaptic and nonsynaptic sites was altered by changes in synaptic activity.

Axonal and dendritic mitochondria exhibit different susceptibilities to drug-induced cessation of movement and morphological remodeling

Our data demonstrate that axonal and dendritic mitochondria have different patterns of movement, morphology and distribution under control conditions and when synaptic activity is altered (Fig. 4-6). We next sought to determine whether drugs that trigger mitochondrial or neuronal injury impair mitochondrial movement or cause morphologic remodeling of mitochondria selectively in one compartment over the other. Mitochondrial movement is thought to be crucial for neuronal health and mitochondrial morphology is thought to impart some functional property to the organelles. Therefore, if mitochondria in certain neuronal processes are more vulnerable to cessation of movement or morphological remodeling during injury, then those processes may also be more likely to degenerate. This could provide a mechanism for selective axonal or dendritic dysfunction and degeneration in neuronal injury and disease.

We and others previously showed that mitochondria in injured and apoptotic neurons and other cell types stop moving and adopt a punctate, fragmented morphology rather than the traditional thin, elongated shape (Bossy-Wetzel et al., 2003; Rintoul et al., 2003; Vanden Berghe et al., 2004). The remodeling of mitochondrial morphology can be directly visualized during an acute excitotoxic glutamate exposure and is thought to be mediated by Ca^{2+} influx into the cell (Rintoul et al., 2003). To further that investigation, we examined the ability of glutamate to stop movement and cause rounding of mitochondria in dendrites, proximal axon segments and distal axon segments. Remodeling of mitochondrial morphology was observed in dendrites (Fig. 7A, B), but not in axonal segments at least 200 μm from the cell body (Fig. 7C, D). Quantification revealed that glutamate reduced the length of dendritic mitochondria to approximately one-fourth the initial length and well below the length of axonal mitochondria (Fig. 7E). Additionally, only dendritic mitochondria rounded after glutamate treatment (Fig. 7F).

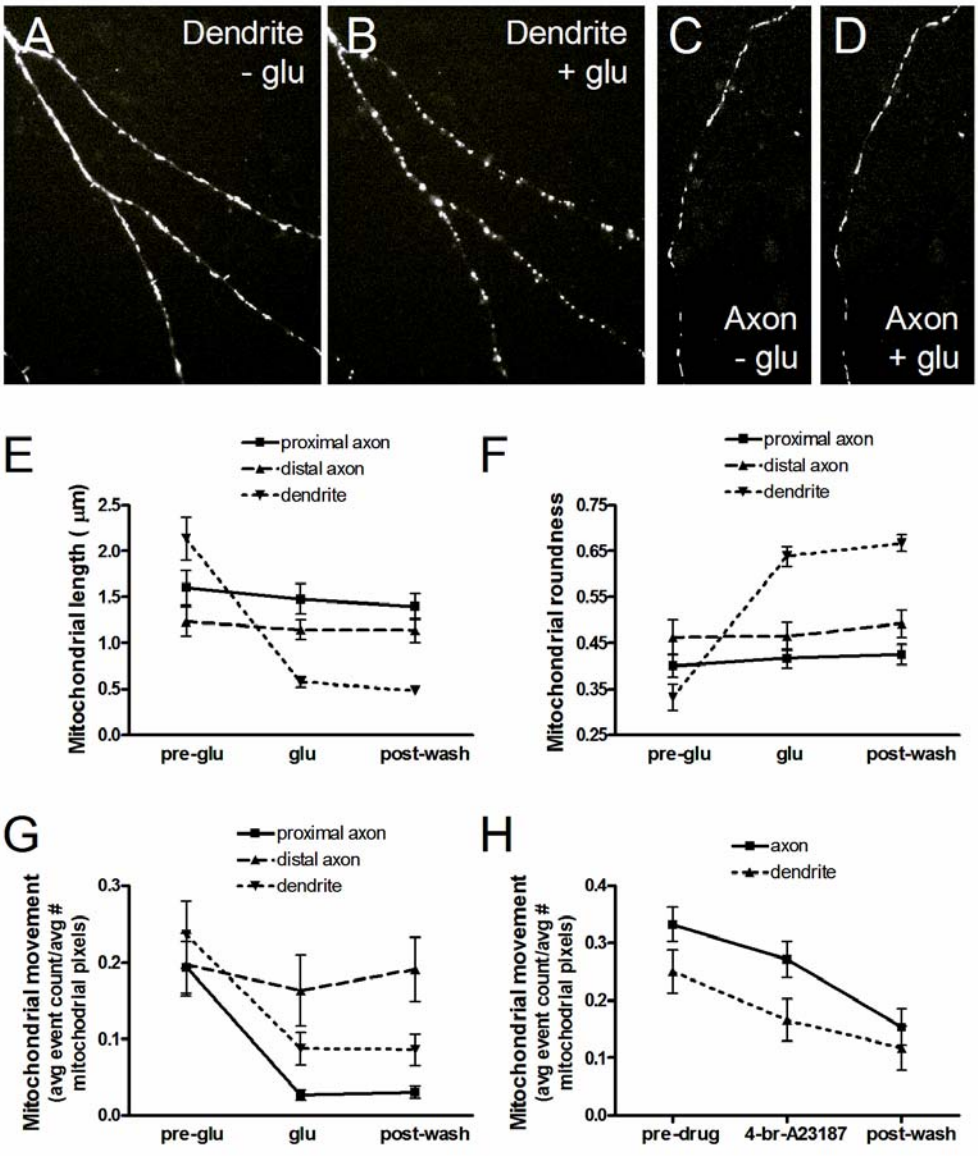

Figure 3.7 Acute treatment with excitotoxic glutamate concentrations caused remodeling of mitochondrial morphology solely in dendrites and cessation of mitochondrial movement in dendrites and proximal axon segments.

(**A, B**) Dendritic mitochondria displayed morphological remodeling and are shown before and after a 10 min treatment with 30 µM glutamate/1 µM glycine. (**C, D**) Axonal mitochondria did not demonstrate morphological remodeling and are shown before and after glutamate treatment. (**E**) Mitochondrial length shortened in dendrites but not axons after glutamate treatment. No recovery was observed after a 10 min wash. (**F**) Mitochondria rounded selectively in dendrites after glutamate treatment with no recovery after a 10 min wash. (**G**) Mitochondrial movement in dendrites and proximal axon segments decreased significantly after glutamate treatment, but was

unaffected in distal axon segments. (H) Mitochondrial movement was reduced in all neuronal processes when $[Ca^{2+}]_i$ was uniformly increased by a 5 min treatment with 1 μM 4-Br-A23187, a Ca^{2+}-ionophore. Note that the difference in mitochondrial morphology precludes the absolute values for axonal and dendritic movement to be compared by our measurement technique in *G* and *H*. Also, movement values should not be compared between *G* and *H* because images were acquired at different rates. Values are shown as mean ± SE from 2-3 coverslips each from 3-4 separate cultures. $p < 0.05$ was considered significant.

Analysis of mitochondrial movement after glutamate treatment revealed an interesting susceptibility of mitochondria in dendrites and proximal axon segments to cessation, while mitochondria in distal axons were unaffected (Fig. 7G). We tested the hypothesis that the spatial differences in cessation of movement were due to $[Ca^{2+}]_i$ gradients after glutamate treatment, which were expected to be elevated near glutamate receptors on dendrites and the cell body, and decline progressively from the cell body into the axonal compartment as $[Ca^{2+}]_i$ is buffered. Perfusion of cells with the Ca^{2+} ionophore, 4-Br-A23187, stopped mitochondrial movement in all neuronal processes (Fig. 7H). At higher concentrations of ambient Ca^{2+}, 4-Br-A23187 also caused rounding of mitochondria in all cellular compartments (data not shown). Therefore, short-term excitotoxic glutamate treatment causes mitochondria to stop moving before rounding and these effects are most likely caused by the distribution of elevated $[Ca^{2+}]_i$, which is particularly elevated in the dendritic compartment where glutamate receptors are located.

We next tested whether other drugs that impair mitochondrial function or are related to neurotoxicity also stopped mitochondrial movement selectively in dendrites. Unlike glutamate treatment, which causes elevated $[Ca^{2+}]_i$ largely in the dendritic compartment, the drugs we tested here presumably gain equivalent entry into axons and dendrites. We examined the effects of acute (1) depolarization of mitochondrial membrane potential (ψ_m) by FCCP, (2) inhibition of mitochondrial ATP synthase by oligomycin, (3) elevation of intracellular zinc to neurotoxic concentrations followed by chelation with TPEN, and (4) inhibition of electron transport chain

(ETC) complex I by rotenone, which is implicated in Parkinson's disease (Betarbet et al., 2000). Unlike glutamate, these four drug treatments reduced mitochondrial movement to a similar degree between axons and dendrites. However, we found an interesting difference in acute recovery of mitochondrial movement after washout of drug (Fig. 8). Only axonal mitochondria regained movement after FCCP ($p < 0.01$) and zinc/TPEN treatment ($p < 0.05$). It is important to note that mitochondrial movement in axons did not recover after zinc treatment unless TPEN was applied (data not shown). Movement also did not recover in axons or dendrites after oligomycin or rotenone, both of which are nearly irreversible inhibitors. In summary, mitochondrial movement in dendrites was shown to be selectively impaired or unable to acutely recover from glutamate, FCCP and zinc/TPEN treatment. This has important implications for abnormal dispersion of ATP and sequestration of Ca^{2+} by mobile mitochondria in dendrites following neuronal insults.

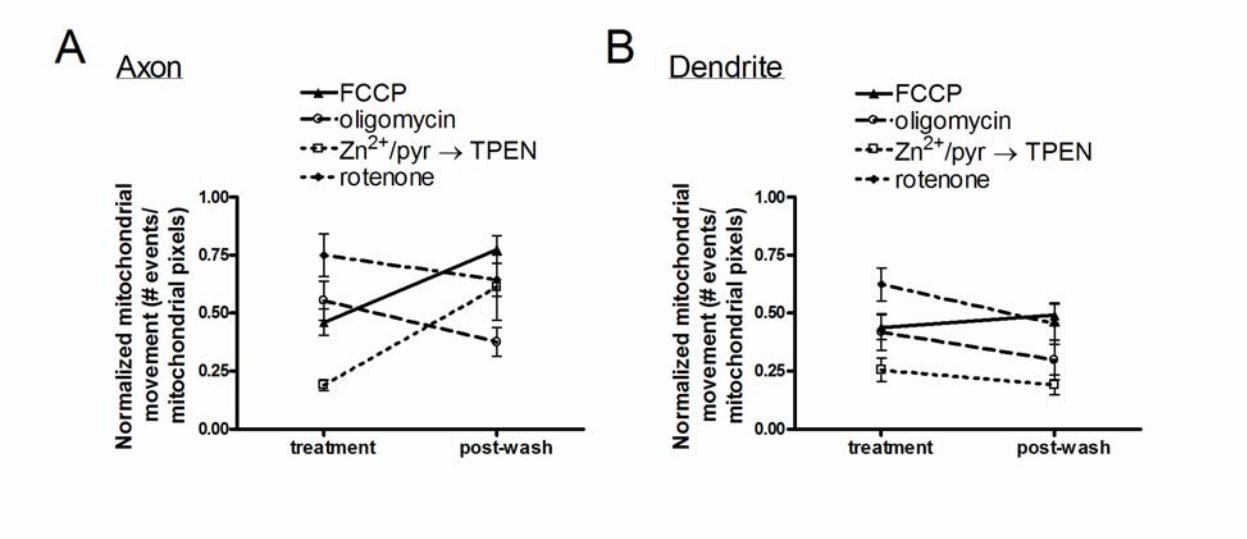

Figure 3.8 Axonal and dendritic mitochondrial movement were equally susceptible to cessation after FCCP, oligomycin, rotenone and zinc exposures, but only axonal mitochondria acutely recovered movement after washout of FCCP and chelation of zinc.

(**A**) Mitochondrial movement in axons significantly recovered 15 min after depolarization with 5 min 750 nM FCCP treatment, and 20 min after exposure to 10 min 3 μM $ZnCl_2$/20 μM Na-pyrithione treatment followed by chelation

with 5 min 25 μM TPEN. Movement did not recover after 10 min treatment with 10 μM oligomycin or 5 min treatment with 2 μM rotenone. (**B**) Mitochondrial movement in dendrites did not acutely recover after treatment with FCCP, oligomycin or zinc/pyrithione followed by TPEN. Movement continued to decrease significantly in dendrites 20 min after rotenone was washed out. Values were normalized to mitochondrial movement before treatment and are shown as mean ± SE from 2-6 coverslips each from 2-4 separate cultures. $p < 0.05$ was considered significant.

3.5 DISCUSSION

Our results demonstrate that mitochondrial morphology, distribution and movement are different in axons and dendrites, yet mitochondria in both compartments localize to and remain stationary at a subset of synaptic sites. Furthermore, we reveal that these dynamic properties of mitochondria change under conditions of synaptic silence and overactivity. Many studies examining synaptic mitochondria employ electron microscopy and are limited to static images (Rowland et al., 2000; Sakata and Jones, 2003; Briones et al., 2005). More recently, synaptic function was investigated in mutant neurons that exhibited abnormal distribution and transport of mitochondria (Guo et al., 2005; Verstreken et al., 2005). However, it is difficult to account for the dynamics of normal mitochondrial movement and morphology in these models. Li et al. (2004) recently demonstrated recruitment of mitochondria to developing and morphogenic dendritic spines. We now provide new insight into the normal trafficking of mitochondria to both pre- and postsynaptic sites, thereby advancing our understanding of how the mitochondrial pool is allotted to meet dynamic demands throughout the cell.

Axonal and dendritic mitochondria differ in morphology, distribution and movement

We show that the number of mitochondria per μm of neuronal process is the same in axons and dendrites of primary cortical neurons, but mitochondria occupy a smaller fraction of axonal

process length due to a shorter morphology (Fig. 4A-C). Furthermore, we correlate this with a smaller number of presynaptic terminals per μm on axons than postsynaptic densities on dendrites (Fig. 4D). This compartmental difference in mitochondrial morphology may be functionally relevant in that longer mitochondria could more efficiently distribute energy to multiple, closely apposed sites of high metabolic demand on dendrites (reviewed by Skulachev, 2001). This explanation coincides with evidence that mitochondria in hippocampal neuron dendrites are more metabolically active than in axons, as measured by the mitochondrial membrane potential sensitive dye JC-1 (Overly et al., 1996). Popov et al. (2005) recently used 3D ultrastructural reconstructions to describe long filamentous networks of mitochondria in dendrites and discrete mitochondrial bodies in axons of hippocampal slices from adult ground squirrels and rats. As discussed in that paper, differences in experimental procedures may explain why such extensive mitochondrial networks have not been seen by others; however, we certainly observed similar patterns of mitochondrial distribution and morphology in cortical neurons.

Unlike postsynaptic densities on dendrites, presynaptic terminals are not only farther spaced from each other on axons, but are also distributed across a much longer and more extensively branched distance. Presynaptic terminals require mitochondria at least for sequestering Ca^{2+}, powering the plasma membrane Ca^{2+}-ATPase and releasing Ca^{2+} for post-tetanic potentiation (Tang and Zucker, 1997; Zenisek and Matthews, 2000; Calupca et al., 2001; Medler and Gleason, 2002). Mitochondrial ATP generation may also prove important for the dynamic actin rearrangements associated with synaptic vesicle cycling and synaptic plasticity (reviewed by Dillon and Goda, 2005). Since mitochondria were found to occupy only 17.4 ± 0.02% of axonal length and associate with only 36.1 ± 0.06% of labeled presynaptic terminals at

any given time, it is likely that mitochondrial transport plays a more important role in delivering mitochondria throughout axons than dendrites (Fig. 4A, 5A). This conjecture is supported by a greater than 4-fold increase in the number of mitochondria found to pass a given point on axons compared to dendrites (Fig. 6A). Our finding could be the result of both a larger number of motile mitochondria and a greater distance traveled by mitochondria in axons, as was concluded in hippocampal neurons by Overly et al. (1996) but not by Ligon and Steward (2000) who found similar movement profiles in axons and dendrites. Furthermore, it is possible that a shorter mitochondrial morphology is maintained in axons so that molecular motors can more efficiently transport the organelles.

Manipulation of spontaneous synaptic activity of cultured neurons demonstrated that mitochondrial morphology and distribution can be differentially altered in axons and dendrites by a single drug treatment. Specifically, we observed that after 24 hr TTX treatment, the fractional occupancy of axons by mitochondria decreased, yet that of dendrites increased (Fig. 4B). Unlike Li et al. (2004), who found a similar effect in hippocampal dendrites after 20 min TTX treatment, we did not see an acute difference after 1 hr TTX. Mitochondria may elongate only following prolonged TTX treatment in an attempt to bridge stationary postsynaptic densities that became fewer and farther apart (Fig. 4B, D). The reduced number of stationary PSD-95 clusters we observed was due to increased cluster mobility since the total number of clusters was not changed by drug treatments (Fig. 4D and data not shown). Since our criteria for cluster motility was necessarily strict to permit data to be analyzed blindly, the cluster movements we observed may represent changes in the motility, restructuring and turnover of spines, all of which are known to be modulated by synaptic activity (Kirov and Harris, 1999; Okabe et al., 1999; Konur and Yuste, 2004). Additionally, PSD-95 proteins themselves exhibit a degree of

dynamics (Rasse et al., 2005). In summary, we provide the first evidence for the distinct compartmental regulation of mitochondrial morphology and distribution in axons and dendrites, both in the basal state and during altered synaptic activity.

Mitochondrial localization and trafficking to pre- and postsynaptic sites

Presynaptic terminals have been described as containing abundant mitochondria (Kageyama and Wong-Riley, 1982; Nguyen et al., 1997; Popov et al., 2005). It was therefore surprising that when measuring within the 1 μm surrounding synaptic sites, we found mitochondria at only 36.1 ± 0.06% of presynaptic labels at any given time (Fig. 5A). This is similar to an electron microscopy study finding only 41% of synaptic boutons in CA3→CA1 axons in rat hippocampal slices (Shepherd and Harris, 1998). Given the ability of mitochondria to rapidly move through processes, it is likely that transient metabolic demands are met by moving mitochondria or maybe by nearby but not specifically localized mitochondria. On the other hand, synaptic sites requiring longer-term support, perhaps for post-tetanic potentiation and mobilization of reserve pool vesicles, may act as more stable "docking sites" for mitochondria (Tang and Zucker, 1997; Verstreken et al., 2005). Indeed, we found that mitochondria that localized to synaptic sites remained stationary for the majority of our 30 min imaging experiments (Fig. 5B). Furthermore, our examination of developing neurons that had not yet formed functional synapses revealed significantly more mitochondrial movement, presumably due to the absence of synaptic "docking sites" (submitted manuscript). In general, our findings that not all presynaptic terminals have associated mitochondra agree with recent reports of normal neurotransmission during physiological levels of stimulation in mutant *Drosophila* axons with perinuclearly distributed mitochondria (Guo et al., 2005; Verstreken et al., 2005). However, the importance of mitochondria at presynaptic terminals, especially during overstimulation, suggests that

mitochondria would be recruited to presynaptic sites during veratridine treatment. Yet we did not observe any redistribution of mitochondria to synaptophysin clusters other than a modest increase in the fraction of moving mitochondria that paused at synaptic sites relative to nonsynaptic sites on axons (Fig. 5A, 5B, 6B). In fact, the redistribution trend of relatively stationary mitochondria was more toward nonsynaptic sites on axons, possibly to power ion channels for membrane repolarization (Fig. 5A, B). A possible explanation is that the normal mitochondrial distribution still allowed adequate Ca^{2+} buffering, as $[Ca^{2+}]_i$ transients continued to show full recovery (Fig. 3A).

The distribution of mitochondria in dendrites relative to postsynaptic densities and spines is not clearly defined. Unlike presynaptic terminals, mitochondria in hippocampal neurons are not usually found in dendritic spines, although they have been seen penetrating the bases of thorny excrescences (Popov et al., 2005). Our results show 39.3 ± 0.05% of labeled PSD-95 clusters were associated with mitochondria at any given time, which was significantly higher than the distribution of mitochondria at nonsynaptic sites (Fig. 5C). Stimulation with veratridine caused significantly more PSD-95 clusters to be associated with mitochondria and more moving mitochondria to pause at postsynaptic densities for less than 20 sec (Fig. 5C, 6C). This suggests mitochondrial proximity is important for supporting processes at excitatory postsynaptic sites, such as local $[Ca^{2+}]_i$ regulation in conjunction with smooth ER (Pivovarova et al., 2002). Additionally, local ATP supply may power synapse-associated polyribosome complexes and clathrin-dependent endocytic machinery at postsynaptic sites (Steward and Levy, 1982; Racz et al., 2004). Unfortunately we could not precisely resolve spine morphology at PSD-95 clusters; thus, it is possible that subtypes of spines, such as those with shorter necks or fewer spine Ca^{2+} pumps, are more dependent on mitochondrial uptake of Ca^{2+} that diffuses into the dendrite

(Pivovarova et al., 1999; Majewska et al., 2000; Pivovarova et al., 2002). Some PSD-95 clusters that recruited mitochondria after stimulation could also represent morphogenic spines (Li et al., 2004). Alternatively, mitochondria may be recruited to provide ATP for ubiquitination and proteasome-dependent degradation of PSD-95, which has been shown to occur after NMDA treatment of hippocampal neurons in the time frame of our experiments (Colledge et al., 2003).

The dramatic increase in mitochondrial movement in both axons and dendrites after 1 hr TTX treatment suggests a transition of mitochondria from the stationary state to the mobile state, as well as an increase in mobility of already moving mitochondria (Fig. 6A). In support of this, we observed that dendritic but not axonal mitochondria remained stationary for significantly shorter time periods after 1 hr TTX (Fig. 5B, D). Therefore, moving mitochondria likely have increased velocity in axons and dendrites; additionally more mitochondria probably become mobile in dendrites. Our results are in agreement with the effects of TTX in hippocampal dendrites, but not in myenteric nerve fibers where TTX was shown to reduce the fraction of moving mitochondria and have no effect on velocity (Li et al., 2004; Vanden Berghe et al., 2004).

Interestingly, we found that despite the remarkable increase in mitochondrial movement during TTX treatment, there were negligible or modest changes in the fraction of passing mitochondria that stopped at synaptic and nonsynaptic sites (Fig. 6B, C). In other words, mitochondria increased overall mobility but without targeted destinations. Furthermore, the fraction of synaptic sites associated with mitochondria was unaffected by TTX up to 24 hr after treatment (Fig. 5A, C). Therefore, we conclude that inactive synapses still serve as targets for mitochondrial localization. This suggests several possibilities: (i) structural rather than activity-dependent mechanisms may cause mitochondria to stop at synaptic sites, (ii) pre- and

postsynaptic compensation mechanisms may require mitochondrial support, as evidenced by a moderate preference for moving mitochondria to stop at synaptic sites during TTX treatment (Fig. 6B, C).

Dendrite-specific impairments of mitochondrial movement in injury

Our results that movement of dendritic mitochondria were more susceptible than axonal mitochondria to impairment by neurotoxic concentrations of glutamate and zinc, as well as by depolarization of ψ_m, suggest that inadequate mitochondrial movement may be a more important pathogenic process in dendrites than axons. We previously showed that acute exposure to excitotoxic glutamate concentrations caused mitochondria to stop moving and round in dendrites due to elevated intracellular Ca^{2+} influx through NMDA receptors that disrupts cytosolic structure (Rintoul et al., 2003). We now confirm that this is a regional effect related to the distribution of glutamate receptors on dendrites since axonal mitochondria exhibited the same response only when $[Ca^{2+}]_i$ was elevated with a Ca^{2+}-ionophore (Fig. 7). Furthermore, our observation that mitochondria in proximal but not distal axons also stopped moving after glutamate treatment suggests that $[Ca^{2+}]_i$ is relatively well buffered spatially and that movement cessation precedes morphological remodeling. However, an important implication is that despite preserved mitochondrial movement in the majority of the axon, impaired trafficking to and from the cell body could have consequences on the distribution of mitochondria and clearance of damaged organelles by retrograde transport (Hollenbeck, 1993).

Interestingly, whereas dissipation of ψ_m is also known to stop mitochondrial movement, we now demonstrate that only axonal mitochondria recover movement within 15 min of FCCP washout (Fig. 8) (Rintoul et al., 2003; Vanden Berghe et al., 2004). The question then arises if this recovery is due to a faster rate of mitochondrial repolarization in axons, perhaps because the

electrochemical gradient can be better re-established in mitochondria of smaller volume. However, we have observed mitochondria in the cell body fully repolarize 8-10 min after identical FCCP treatments, well before dendritic mitochondria recover movement (data not shown). A closer examination of ψ_m in axons and dendrites could resolve this issue. Alternatively, subtle cytoskeletal rearrangements in dendrites may be responsible for delayed recovery of mitochondrial movement. In contrast to the reversible effects of FCCP, mitochondrial movement in neither axons nor dendrites recovered from inhibition of ATP synthase and ETC complex I by oligomycin and rotenone, respectively. These data confirm that mitochondrial ATP production is important for mitochondrial movement throughout all neuronal processes.

Lastly, we show that zinc chelation caused an interesting selective recovery of movement by axonal mitochondria after treatment with neurotoxic concentrations of zinc that do not cause significant ψ_m depolarization (Fig. 8) (Malaiyandi et al., 2005). In these experiments, zinc was applied concurrently with the zinc ionophore, pyrithione, so axons and dendrites received the same zinc exposure. Chelation of zinc in all processes was then effected with TPEN. We previously reported that the Zn^{2+}/TPEN treatment conditions used here cause irreversible, PI-3 kinase-dependent cessation of mitochondrial movement in undistinguished neuronal processes and over 75% cell death in cultured primary cortical neurons (Malaiyandi et al., 2005). We now advance those findings by showing that zinc chelation actually leads to significant acute recovery of mitochondrial movement selectively in axons, which does not recover without chelation. Therefore, it seems that the TPEN-independent PI3-kinase signaling cascade that is activated by acute zinc exposure occurs either only in dendrites or at a much slower rate in axons. Regardless, this data strongly suggests that our previous finding of significant neurotoxicity after

acute Zn^{2+}/TPEN treatment is related to a dendritic pathology in which impaired mitochondrial movement is an early event.

Not only are mitochondria crucial for supporting cellular functions, but mitochondrial trafficking is also important for transporting damaged mitochondria to cellular locations where they can be repaired or degraded (Hollenbeck, 1993; De Vos et al., 2000; Tolkovsky et al., 2002; Miller and Sheetz, 2004). Therefore, selective impairment of mitochondrial movement in dendrites may cause abnormal distribution of ATP and regulation of $[Ca^{2+}]_i$ in these processes, in addition to production of harmful reactive oxygen species from damaged mitochondria that are not properly removed. In light of the findings in this study that mitochondrial morphology, localization and trafficking to synapses are dynamic in the basal state and when synaptic activity is altered, we predict that impaired mitochondrial movement will also lead to aberrant support of synaptic transmission. This may become particularly important if moving mitochondria support presynaptic compensation in response to the reduced postsynaptic reception of signal, which could then lead to overstimulation if and when dendritic mitochondrial movement recovers. In summary, we provide the first evidence for selective dendritic alterations in mitochondrial morphology and movement after neurotoxic glutamate and zinc treatments that may prove crucial to our understanding of cell death in excitotoxicity and ischemia.

4.0 MUTANT HUNTINGTIN AGGREGATES IMPAIR MITOCHONDRIAL MOVEMENT AND TRAFFICKING IN CORTICAL NEURONS

4.1 ABSTRACT

Huntington's disease (HD) is a neurodegenerative disorder caused by a polyglutamine repeat in the huntingtin gene (Htt). Mitochondrial defects and protein aggregates are characteristic of affected neurons. Recent studies suggest that these aggregates impair cellular transport mechanisms by interacting with cytoskeletal components and molecular motors. Here, we investigated whether mutant Htt alters mitochondrial trafficking and morphology in primary cortical neurons. We demonstrate that full-length mutant Htt was more effective than N-terminal mutant Htt in blocking mitochondrial movement, an effect that correlated with its heightened expression in the cytosolic compartment. Aggregates impaired the passage of mitochondria along neuronal processes, causing mitochondria to accumulate adjacent to aggregates and become immobilized. Furthermore, mitochondrial trafficking was reduced specifically at sites of aggregates while remaining unaltered in regions lacking aggregates. We conclude that in cortical neurons, an early event in HD pathophysiology is the aberrant mobility and trafficking of mitochondria caused by cytosolic Htt aggregates.

4.2 INTRODUCTION

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by uncontrolled movement, dementia, emotional disturbance and premature death. HD is caused by an expanded CAG repeat in the first exon of the huntingtin (Htt) gene. However, it is not fully understood how the expanded polyglutamine repeat makes the Htt protein neurotoxic. It is evident that fragments of Htt containing the mutant polyglutamine tract aggregate in various subcellular compartments within transfected cells and neurons of transgenic mice and HD patients (Cooper et al., 1998; Hazeki et al., 1999; Kuemmerle et al., 1999; Meade et al., 2002). Cell death may result from disruption of transcription factors or by sequestration of other important proteins into nuclear Htt aggregates (Perez et al., 1998; Chai et al., 1999; Steffan et al., 2000; Nucifora et al., 2001; Mitsui et al., 2002). Interestingly, Htt aggregate-like structures are also found surrounding mitochondrial membranes in neurons of transgenic mice (Li et al., 2001; Panov et al., 2002). Mitochondrial involvement in HD is further implicated by the observation that animals and humans exposed to the mitochondrial toxin 3-nitropropionic acid exhibit neuropathological outcomes similar to HD patients (Ludolph et al., 1991; Sipione and Cattaneo, 2001; Rubinsztein, 2002). Furthermore, lymphoblast mitochondria from HD patients have lower membrane potentials and depolarize in response to smaller calcium loads than controls (Panov et al., 2002). Defects in mitochondrial calcium homeostasis caused by mutant Htt may trigger neuronal injury through enhanced cytochrome c release, caspase activation and increased vulnerability to excitotoxicity (Jana et al., 2001; Zeron et al., 2001; Panov et al., 2002).

Mitochondria are vital to the function of cells not only because they are the main source of energy but also because they regulate intracellular Ca^{2+} homeostasis. Therefore, mitochondria must be trafficked appropriately to meet energy demands throughout the cell. This has particular

relevance to neurons where axons and dendrites can extend well beyond the cell body. In addition to supporting cellular functions, mitochondrial trafficking may be important for transporting damaged mitochondria to cellular locations where they can be repaired or degraded (Hollenbeck, 1993; De Vos et al., 2000; Tolkovsky et al., 2002; Miller and Sheetz, 2004). Impairment of mitochondrial transport could then result in the persistence of injured mitochondria in the cell. This would have severe consequences, as damaged mitochondria promote apoptosis by producing reactive oxygen species and releasing cytochrome c (Lee and Wei, 2000). Our laboratory and others have demonstrated that mitochondrial movement is inhibited by agents implicated in neurological diseases, including glutamate, rotenone and zinc (Rintoul et al., 2003b; Vanden Berghe et al., 2004; Malaiyandi et al., 2005). Therefore, defects in mitochondrial transport could have important implications for a wide range of neurodegenerative diseases, as cellular energy and Ca^{2+} homeostasis may be further compromised if energy-generating and Ca^{2+}-buffering mitochondria are not appropriately distributed.

A mechanism for mitochondrial dysfunction in HD that has only been described by one group is the impact of mutant Htt on mitochondrial transport (Trushina et al., 2004). Electron microscopy has revealed mutant Htt aggregates to be so large as to occupy almost the entire axonal cross-sectional diameter (Li et al., 2003). Indeed, aggregates in axons and dendrites associate with mitochondria, block protein transport in neurites, and lead to neuritic degeneration (Li et al., 2001). While vesicular transport defects are emerging as an important pathologic contributor in HD, mitochondrial transport is subject to different modes of regulation, and therefore may exhibit different susceptibilities to mutant Htt (Li et al., 2001; Gunawardena et al., 2003; Szebenyi et al., 2003; Lee et al., 2004). In fact, recent work demonstrated global

disruptions of vesicular transport and mitochondrial motility in mouse striatal neurons (Trushina et al., 2004). We previously utilized primary cortical neurons for in depth analyses of mitochondrial mobility, morphology and the impact of various insults on these properties (Rintoul et al., 2003b; Malaiyandi et al., 2005). Although the cortex is the second most afflicted region in HD, different cell vulnerabilities probably account for there being only one-third to one-half as much loss in cross-sectional area compared to the neostriatum (de la Monte et al., 1988; Mann et al., 1993). We therefore performed detailed examinations of mitochondrial dynamics and function in primary cortical neurons expressing N-terminal and full-length mutant Htt and revealed selective defects that are likely to contribute to neuronal cell demise.

4.3 EXPERIMENTAL PROCEDURES

Materials

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

DNA constructs

The mito-eYFP construct obtained from Dr. Roger Tsien (University of California, San Diego) was produced by insertion of the gene for eYFP into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA) and employs the targeting sequence of cytochrome c oxidase subunit IV (Llopis et al., 1998). The mitochondrially-targeted mRFP construct was generated by cloning in-frame with a cytochrome c oxidase subunit VIII targeting sequence (Invitrogen). The truncated Htt constructs (provided by Dr. Christopher Ross, Johns Hopkins University) were composed of the NH_2-terminal 63 amino acids of huntingtin with a normal (Htt-N63-19Q) or

expanded (Htt-N63-99Q) glutamine repeat inserted into the pcDNA3.1-Myc-His vector (Jiang et al., 2003). The full-length Htt constructs (provided by Dr. Michael Hayden, University of British Columbia) contained 15 or 138 glutamine repeats inserted in exon 1 of the Htt gene and were cloned as described (Wellington et al., 2000). The GFP-tagged Htt constructs (provided by Dr. Steven Finkbeiner) encoded the first exon of human Htt containing 25 or 103 glutamine repeats (Kazantsev et al., 1999). The P-LUC plasmid consisted of the luciferase gene placed in a mammalian expression vector under the control of a CMV promoter (Gossen and Bujard, 1992). Plasmid constructs were purified and amplified using the Qiagen Plasmid Maxi Kit (Valencia, CA) according to manufacturer's instructions.

Cell culture

All animals used for this study were maintained in accordance with the University of Pittsburgh *Guidelines for the Care and Use of Animals*. Primary neurons were isolated for culture from embryonic day 17 Sprague Dawley rat pups and grown in a 37˚C incubator containing 5% CO_2. Cortices were removed, trypsinized at 37˚C for 30 minutes, and plated on poly-D-lysine-coated 31 mm glass coverslips. Cells were plated in medium containing Dulbecco's Modified Essential Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Five hours after plating, plating medium was completely removed and replaced with N2-NB media (Neurobasal media with 0.5% penicillin/streptomycin and 1% N2 supplement). Four days after plating, approximately one-third of the media was removed and replaced with an equal volume of fresh N2-NB media. Eight and 11 days after plating, approximately one-third of the media was removed and replaced with B27-NB media (Neurobasal media with 0.5 % penicillin/streptomycin and 2% B27 Supplement Minus AO). All cell culture reagents were purchased from Invitrogen.

Cell transfection

All transfections were performed on neurons after 11-15 days in culture, with no further media changes. By this time point, neurons exhibit physiologic maturity and are responsive to excitotoxicity. Modified calcium phosphate transfections with 2 μg eYFP plasmid or cotransfections with 2 μg each of eYFP and Htt-N63-19Q, Htt-N63-99Q, Htt-25Q or Htt-103Q plasmids were performed according to Xia et al. (Xia et al., 1996), resulting in efficiencies of 1-2%. Neurons were then returned to the incubator until time of use. Alternatively, for experiments with mito-mRFP and GFP-tagged Htt plasmids, transfections were performed using 1 μg of each plasmid per 1.25 μl Lipofectamine 2000 reagent (Invitrogen) in an added volume of 250 μl DMEM. Media was completely replaced with conditioned media 24 hr after transfection and cells were incubated until time of use.

Imaging

Cells were perfused with HEPES-buffered salt solution (HBSS) adjusted to pH 7.4 with NaOH and composed of (in mM) NaCl 137, KCl 5, $NaHCO_3$ 10, HEPES 20, glucose 5.5, KH_2PO_4 0.6, Na_2HPO_4 0.6, $CaCl_2$ 1.4 and $MgSO_4$ 0.9. HBSS was warmed to 37°C and flowed at a rate of 5 ml/min throughout the duration of each experiment. Imaging was performed at a rate of 1 frame/6 seconds using Compix Inc. imaging systems and SimplePCI software (Compix Inc., Cranberry, PA). The excitation wavelengths used were 495 nm for eYFP and GFP, 560 nm for mRFP, 340/380 nm alternatively for mag-fura-2, 540 nm for Alexa 546 goat anti-mouse polyclonal antibody, and 340 nm for Hoechst dye.

Immunocytochemistry and quantification of Htt aggregates

Coverslips containing transfected neurons were removed from culture plates and rinsed once with Dulbecco's PBS (Invitrogen) for 1-3 min. Cells were then fixed for 10 min at RT with a freshly made solution of 4% paraformaldehyde and 4% sucrose in PBS composed of 90 mM dibasic sodium phosphate and 10 mM monobasic sodium phosphate, adjusted to pH 7.4 with NaOH. Coverslips were rinsed, permeabilized for 30 sec with ice-cold acetone and re-rinsed. Incubation with mouse monoclonal anti-HA antibody diluted 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-Htt aa 2146-2541-antibody (Chemicon, Temecula, CA) diluted 1:500 in 1% bovine serum albumin (BSA)/PBS was performed for 1 hr at RT. Cells were then rinsed, incubated with Alexa 546 goat anti-mouse polyclonal antibody (Molecular Probes, Eugene, OR) diluted 1:500 in 1% BSA/PBS for 30 min at RT, and re-rinsed. DNA was stained by incubation of cells in 1.5 μM Hoechst 33258 dye for 10 min. Coverslips were rinsed and stored in PBS at 4ºC until visualization. All rinses were with PBS and performed in quadruplicate for 3 min each. The time-course of Htt aggregation was scored by a blinded observer. Transfected cells were first identified under the eYFP fluorescence wavelength; the wavelength was then changed to 540 nm to determine the presence, number and localization of stained aggregates.

Analysis of mitochondrial movement

Cells transfected with the non-GFP-tagged Htt constructs were imaged at a magnification of 40x for 2 min 2-4 days after transfection, and mitochondrial movement was determined as we previously described (Rintoul et al., 2003b). Briefly, a custom Visual Basic macro was used to quantify movement as *average event count/average number of mitochondrial pixels*, where *event count* is the number of corresponding pixels that vary by at least 20 fluorescence units between 2 consecutive frames, and the *number of mitochondrial pixels* is the number of pixels per frame

that are 20 fluorescence units above background fluorescence. The value of 20 units was chosen because a masking function in SimplePCI software effectively identified mitochondria as pixels 20 units above background fluorescence. A student's t-test was used for statistical comparison.

For experiments with the GFP-tagged Htt constructs, dual-wavelength fluorescence imaging of mitochondria and Htt was performed for 4.5 minutes at a magnification of 60x. Using SimplePCI, circular regions of interest (ROIs) of 1.81 μm diameter were evenly spaced along the processes of each neuron. These ROIs either contained an Htt aggregate or did not, for a controlled comparison. The GFP-Htt images were then removed from the mito-mRFP images so that the presence of aggregates in the ROIs was unknown. An observer who was also blinded to the transfection conditions then scored whether a mitochondrion was present in the ROI, whether that mitochondrion displayed net movement, and the number of mitochondria outside of the ROI that moved into and out of the region during the course of imaging. A one-way ANOVA with Dunnett's Multiple Comparison Test to GFP-Htt-25Q was used for statistical analysis.

Glutamate-induced excitotoxicity, mitochondrial remodeling and mitochondrial Ca^{2+}-buffering capacity

Excitotoxic cell death was measured by cotransfecting neurons with p-LUC, a luciferase – expressing plasmid, along with the Htt constructs in a 1:1 ratio as described above. Approximately 72 hr after transfection, coverslips were rinsed twice with HBSS, treated with a solution of 30 μM glutamate/1 μM glycine for 10-15 min, and rinsed 3 times with HBSS, all at 37°C. This treatment resulted in approximately 35-50% decrease in luminescence compared to controls. Control cells were subject to the same procedures except replacing the glutamate treatment with a 10-15 min incubation in HBSS. Twenty-four hr after glutamate treatment, cells

were scraped off coverslips, collected in PBS and the Gene-Lux (Perkin Elmer, Boston, MA) luciferase assay was performed according to manufacturer's instructions. Samples were measured on a Victor 2 luminometer. A student's t-test was used for statistical comparison.

The effects of intense glutamate stimulation on mitochondrial morphology were observed as we previously described (Rintoul et al., 2003b). Cells were imaged 3-4 days after transfection during a 5 min treatment of 30 μM glutamate/1 μM glycine. SimplePCI functions were employed to determine the average mitochondrial length and roundness for each imaging frame. Briefly, user-defined pixel intensity and object size thresholds identified measurable mitochondria in a frame. Length was calculated from skeletonized objects, which consisted of a 1 pixel-wide line running the length of each object. Roundness was calculated by the formula 4π*area/√perimeter.

Mitochondrial Ca^{2+} buffering capacity after glutamate treatment was measured as we previously described (Brocard et al., 2001). Briefly, neurons were loaded with 5 μM mag-fura-2AM (Molecular Probes, Eugene, OR) in 5 mg BSA/ml HBSS for 10 min at 37°C. Neurons were imaged at excitation wavelengths of 340/380 nm during the treatment sequence of 5 min with 30 μM glutamate/1 μM glycine, 10 min washout, 5 min with 750 nM FCCP in Ca^{2+}-free HBSS and a 5 min washout. The area under the mag-fura-2 ratio curve after subtraction of background fluorescence was calculated during the 6.5 min after glutamate ($glutamate_{AUC}$) or FCCP ($FCCP_{AUC}$) was applied. Thus, the mitochondrial Ca^{2+} buffering capacity was represented by $FCCP_{AUC}/glutamate_{AUC}$. A student's t-test was used for statistical comparison.

4.4 RESULTS

Huntingtin expression and aggregation

Immunostaining of primary neurons cotransfected with mitochondrially-targeted eYFP (mito-eYFP) and N-terminal mutant Htt revealed various forms of Htt expression. Pilot data indicate that >95% of cotransfected neurons express both plasmids. As shown in Fig. 1, N-terminal mutant Htt was found in "small" (diameter < 1.6 μm), "large" (1.6 μm ≤ diameter ≤ 3.3 μm) and "giant" (diameter > 3.3 μm) nuclear aggregates as well as diffusely throughout the nucleus. Interestingly, some Htt aggregates were also colocalized with mitochondria in neuronal processes (Fig. 1). Fig. 2 demonstrates that full-length Htt was robustly expressed in the cytosol where we predicted interactions with mitochondria would occur, although there was no clear visual evidence of cytosolic aggregates.

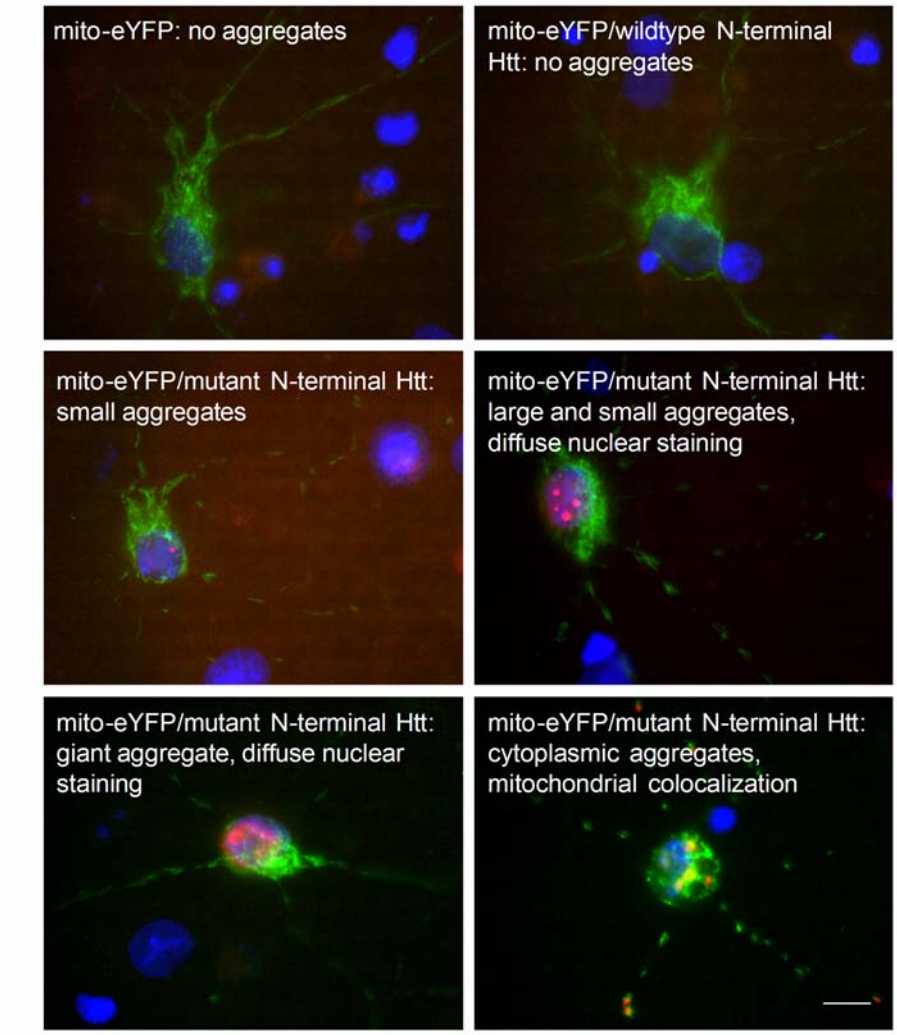

Figure 4.1 Htt-N63-99Q transfected in primary cortical neurons forms intracellular aggregates of variable size, often in nuclei.

Htt (red) expression was identified by immunostaining and nuclei (blue) were labeled with Hoechst dye. An aggregate was considered "small" if the diameter < 1.6 μm, "large" if 1.6 μm < diameter < 3.3 μm, and "giant" if the diameter > 3.3 μm. Diffuse expression of mutant Htt throughout the nucleus was also observed. Neurons were simultaneously transfected with mito-eYFP, which revealed colocalization of cytosolic aggregates with mitochondria (green). Neurons are shown 2-5 days after transfection and are representative of 3-5 cultures. Scale bar, 10 μm.

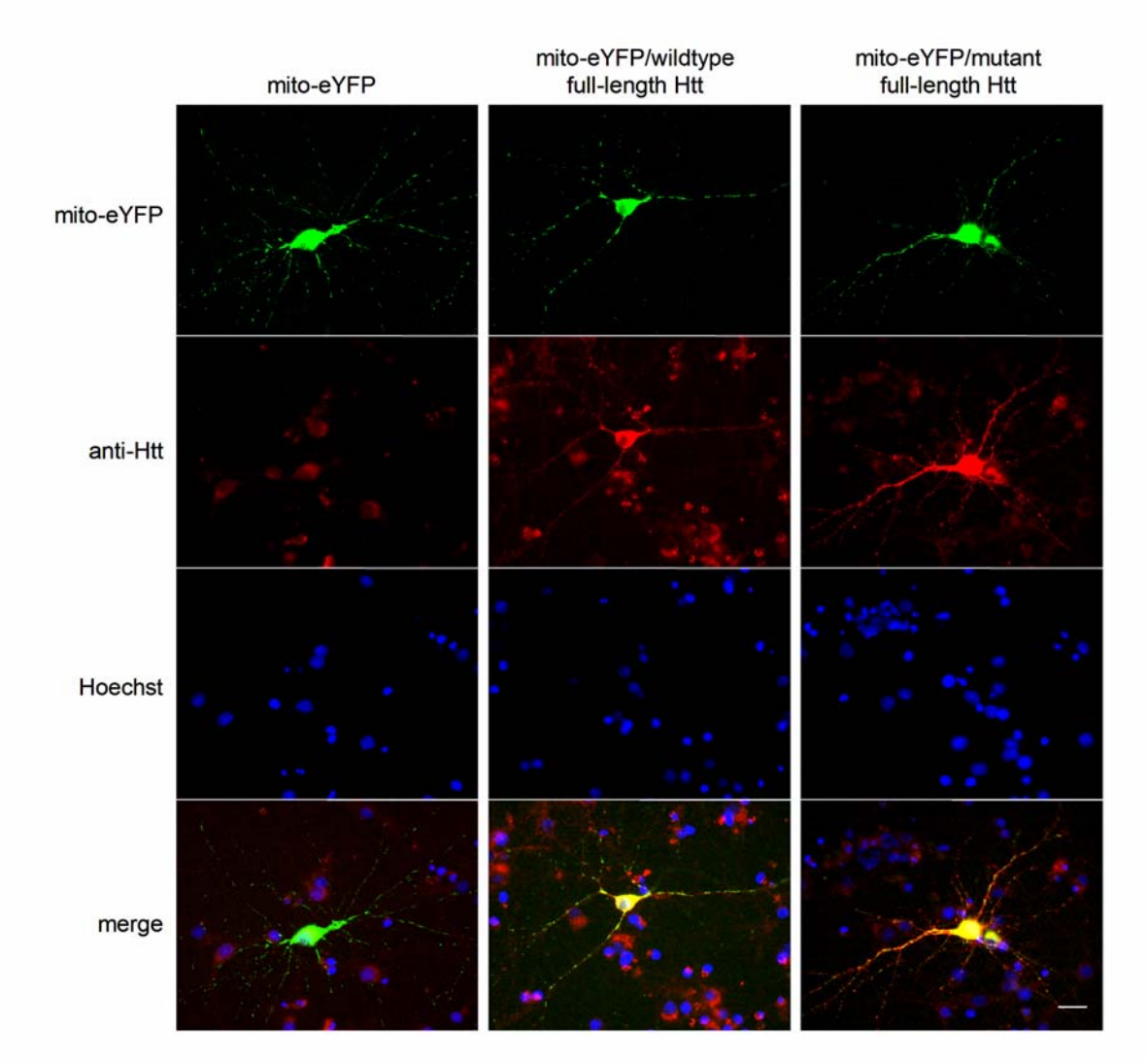

Figure 4.2 Full-length Htt-103Q transfected in primary cortical neurons was expressed diffusely in the cytosol with no clear evidence of aggregation.

Htt (red) was identified by immunostaining, mitochondria (green) were identified by cotransfection with mito-eYFP and nuclei (blue) were stained with Hoechst 33258. Neurons are shown 3 days after transfection. Scale bar, 20 μm.

Aggregation of N-terminal mutant Htt was found to be a time-dependent process beginning in the cytosol and progressing to the nuclear compartment. The percentage of cells with nuclear aggregates increased to 70.0 ± 0.09% over the course of 4 days after transfection with N-terminal mutant Htt (Fig. 3A). On the other hand, cytosolic aggregates were generally maintained in less than 10% of cells regardless of time. Interestingly, the number of nuclear

aggregates per cell did not increase over time. An initial peak of 5.6 ± 1.1 aggregates per cell were seen on the second day after transfection, which declined to 2.3 ± 0.7 aggregates on day 3 and decreased slowly thereafter (Fig. 3B). This decline in the of number aggregates after an early surge may result from proteasomal degradation (Martin-Aparicio et al., 2001). There were no remarkable changes in aggregate sizes from the third through fifth day after transfection, suggesting that aggregates were stably established in transfected cells by the third day.

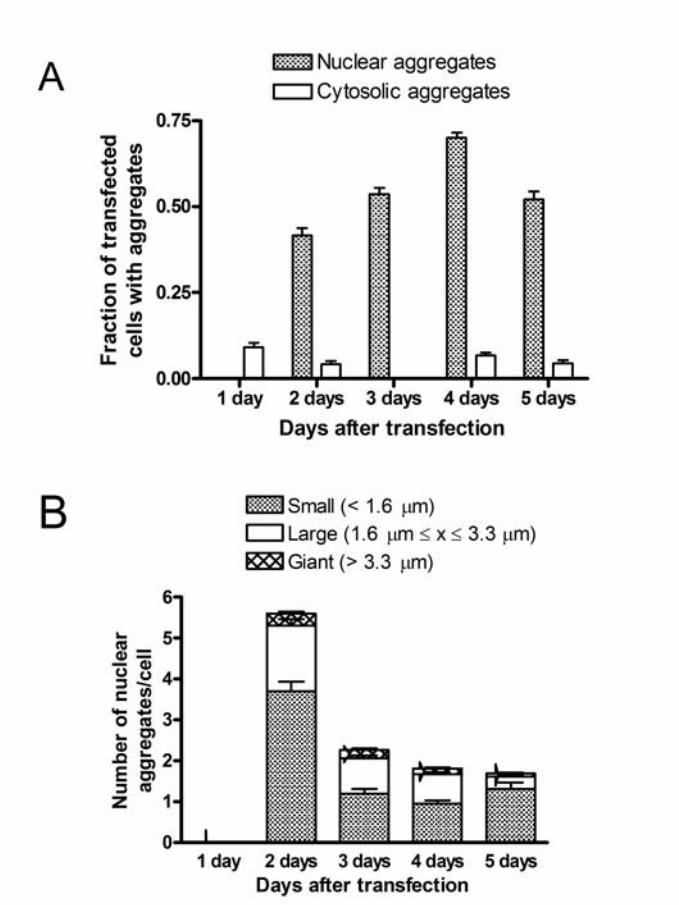

Figure 4.3 Mutant N-terminal Htt formed aggregates in transfected neurons.

(**A**) Aggregation was a progressive process with a greater fraction of cells expressing nuclear aggregates over time. Cytosolic aggregates were observed in a small fraction of cells. Values are represented as mean ± SEM from 2-3 coverslips each from 3 separate cultures. (**B**) The number of nuclear aggregates declined on the third day after transfection and was stable thereafter. The size distribution of aggregates was also relatively constant after the third day. Values are represented as mean ± SE taken from aggregate-positive cells observed in 3A.

Global mitochondrial movement and morphology

Mitochondria were fluorescently labeled by cotransfecting neurons with eYFP fused to the cytochrome c oxidase subunit IV mitochondrial localization sequence (mito-eYFP). Global mitochondrial movement was measured as the net movement of mitochondrial pixels over time in a field of neuronal processes. We did not differentiate between axons and dendrites or the nature of Htt expression in measured cells. Analysis of normalized global mitochondrial movement revealed a modest 17.4% decrease in movement in neurons transfected with full-length but not N-terminal mutant Htt ($p < 0.05$; Fig. 4). Presumably, this results from heightened expression of full-length mutant Htt in the cytosolic compartment (Fig. 1-3) where interactions with mitochondria, molecular motors and cytoskeletal components would be maximal. In comparison, N-terminal mutant Htt fragments seem to be largely sequestered into the nucleus where they cannot interact with mitochondria. The observation that global mitochondrial movement continues to occur rather effectively suggests that at this stage of mutant Htt expression, mechanisms of mitochondrial trafficking are largely able to bypass the impediments that are posed. However, given the likely importance of mitochondrial trafficking in sustaining cellular and mitochondrial health, it is possible that even moderate impairments in mitochondrial movement can cause pathogenic defects over the long course of HD.

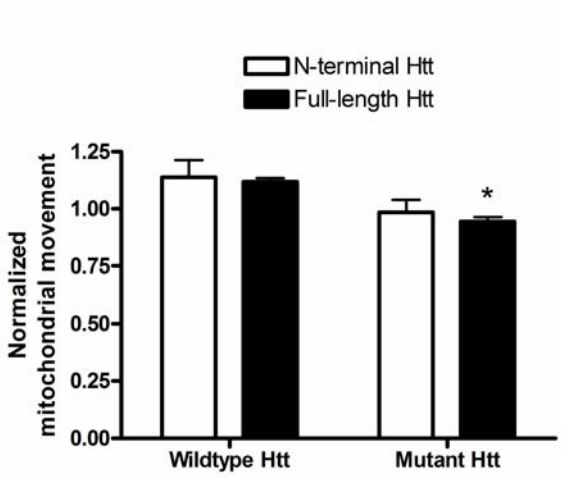

Figure 4.4 Global mitochondrial movement was modestly but significantly impaired in neuronal processes transfected with mito-eYFP and full-length mutant Htt but not N-terminal mutant Htt.

Values are represented as mean ± SE event count/mitochondrial pixels taken from at least 3 coverslips from 3-4 cultures and are normalized to neurons from respective cultures transfected with only the mito-eYFP plasmid.

We also examined whether mutant Htt expression caused aberrant mitochondrial morphology. We and others previously showed that mitochondria in injured and apoptotic neurons and other cell types adopt a punctate, fragmented morphology rather than the traditional thin, elongated shape (Bossy-Wetzel et al., 2003; Rintoul et al., 2003b; Vanden Berghe et al., 2004). This remodeling of mitochondrial morphology can be directly visualized during an acute excitotoxic glutamate stimulus and is thought to be mediated by influx of Ca^{2+} into the cell (Rintoul et al., 2003b). This paradigm was important to test because increased excitotoxic susceptibility, as seen in HD, could manifest as altered mitochondrial remodeling in response to glutamate. However, Fig. 5A-C revealed no differences in initial mitochondrial length, roundness or distribution in neurons expressing N-terminal or full-length mutant Htt, nor in start-time or rate of remodeling during a 5 minute perfusion with 30 μM glutamate/1 μM glycine. The final remodeled mitochondrial morphology was also identical between mutant Htt-transfected cells and controls. Therefore, we conclude that full-length mutant Htt reduces global

mitochondrial movement, but not mitochondrial morphology or the gross mitochondrial remodeling response to an excitotoxic glutamate stimulus.

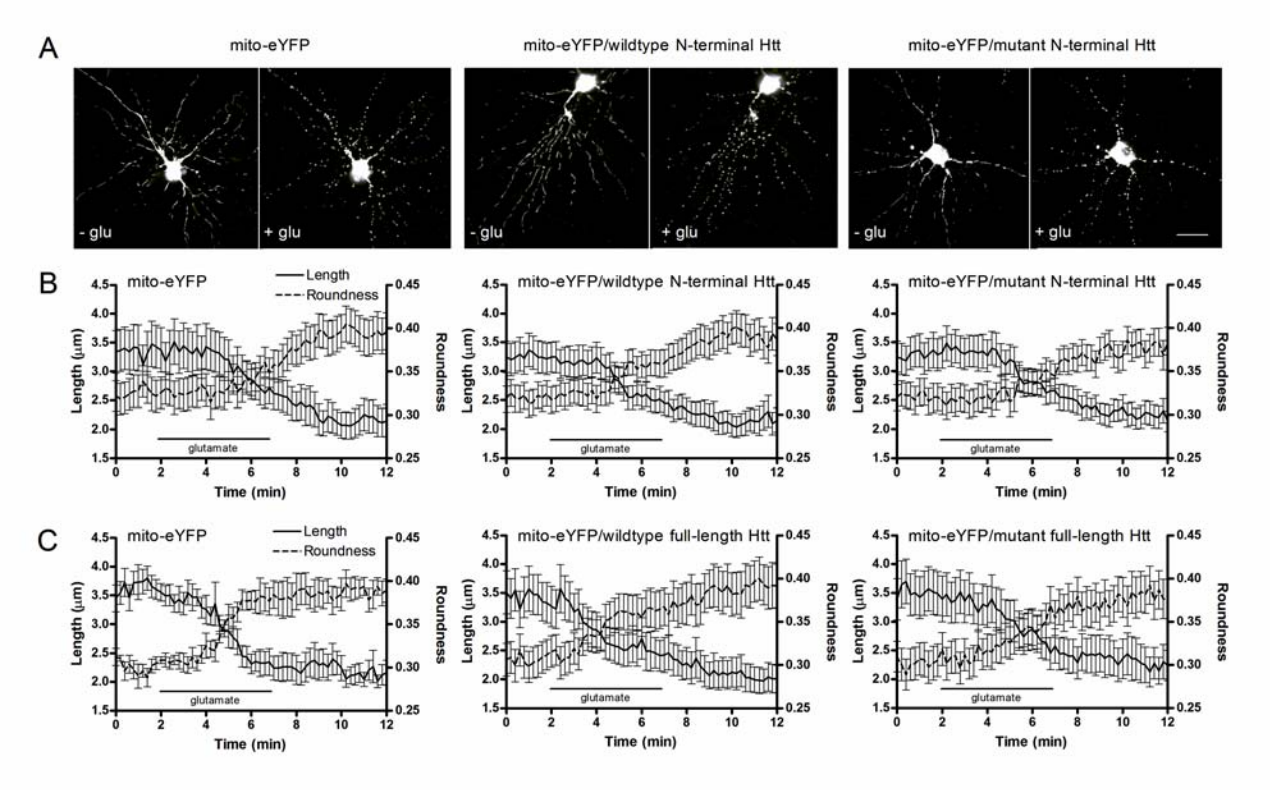

Figure 4.5 N-terminal or full-length mutant Htt does not alter mitochondrial morphology or glutamate-induced remodeling in transfected neurons.

(**A**) Mitochondrial remodeling after a 5 min perfusion of neurons with 30 μM glutamate/1 μM glycine is observed as a shortening and rounding of mitochondrial morphology. Scale bar, 20 μm. (**B**) Quantification of mitochondrial length and roundness during glutamate-induced remodeling revealed no differences in initial or terminal morphology or rate of remodeling in neurons transfected with N-terminal mutant Htt. (**C**) Transfection of neurons with full-length mutant Htt also revealed no differences in mitochondrial morphology or remodeling. Values are represented as mean ± SE from at least 3 coverslips each from 3-4 separate cultures.

Excitotoxicity and Ca^{2+}-buffering capacity

The mitochondrial remodeling response we observed is an immediate and robust effect presumably caused by elevated intracellular Ca^{2+} entry through NMDA receptor activation (Rintoul et al., 2003b). We next tested whether neurons expressing N-terminal and full-length mutant Htt were more vulnerable to delayed glutamate excitotoxicity due specifically to altered

mitochondrial Ca^{2+}-buffering capabilities. The fraction of cells susceptible to glutamate excitotoxicity was determined by cotransfecting neurons with a luciferase-expressing plasmid and assaying for luminescence 24 hr after treatment with 30 μM glutamate/1 μM glycine. As shown in Fig. 6, expression of either full length or N-terminal mutant Htt did not render neurons more susceptible to excitotoxicity. In fact, N-terminal mutant Htt expression exhibited a trend towards neuroprotection against glutamate excitotoxicity.

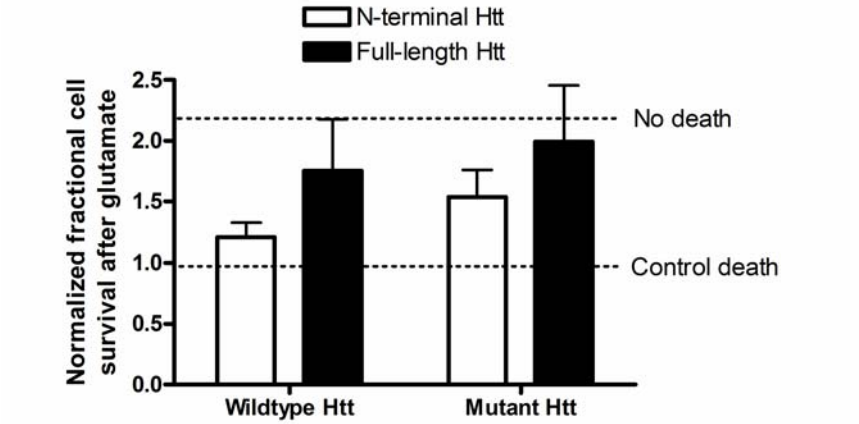

Figure 4.6 Impact of mutant Htt on excitotoxic injury.

Mutant Htt did not reduce fractional cell survival 24 hr after treatment with 30 μM glutamate/1 μM glycine, but the trend was in the opposite direction, particularly with N-terminal mutant Htt. Cell survival was measured by cotransfecting neurons with the luciferase-expressing plasmid, p-LUC, and assaying for luminescence from an entire coverslip. Dead cells lose luciferase expression; thus, fractional cell survival is calculated as the luminescence from glutamate-treated cells divided by luminescence from buffer-treated cells. The dotted line labeled "no death" represents mean luminescence of p-LUC-transfected neurons treated with buffer alone; the dotted line labeled "control death" represents mean (luminescence of p-LUC-transfected neurons treated with glutamate)/(luminescence from p-LUC-transfected neurons treated with buffer alone). Values are shown as mean ± SE luminescence units of glutamate-treated cells divided by that of untreated cells from 3 coverslips each from 3-6 separate cultures, and are normalized to values obtained from neurons from respective cultures transfected with only the p-LUC plasmid (dotted line labeled "control death").

Using a paradigm we previously described (Brocard et al., 2001), we then examined whether intracellular Ca^{2+}-influx or mitochondrial Ca^{2+}-uptake after glutatmate treatment were altered. $[Ca^{2+}]_c$ was monitored using mag-fura-2AM (Fig. 7A); the area under the 340/380 nm ratio curve (AUC) after glutamate treatment represented Ca^{2+} entry into the cell and the AUC after application of the uncoupler FCCP represented mitochondrial uptake of intracellular Ca^{2+} (Fig. 7B). Thus, the mitochondrial contribution to removal of elevated cytosolic Ca^{2+} was calculated as $FCCP_{AUC}/glutamate_{AUC}$ (Fig. 7C). In accordance with the pattern seen in Fig. 6, we found a significant 17.9 ± 4.8% reduction in normalized $[Ca^{2+}]_c$ changes after glutamate treatment in neurons transfected with N-terminal mutant Htt compared to controls ($p < 0.05$). Despite this observation, we did not find any overall effect on the mitochondrial ability to maintain Ca^{2+} homeostasis following an excitotoxic glutamate exposure as reflected by the accumulated mitochondrial calcium load. In summary, we conclude that neurons expressing mutant Htt generally resemble control neurons in their mitochondrial and cellular responses to excitotoxic glutamate treatment.

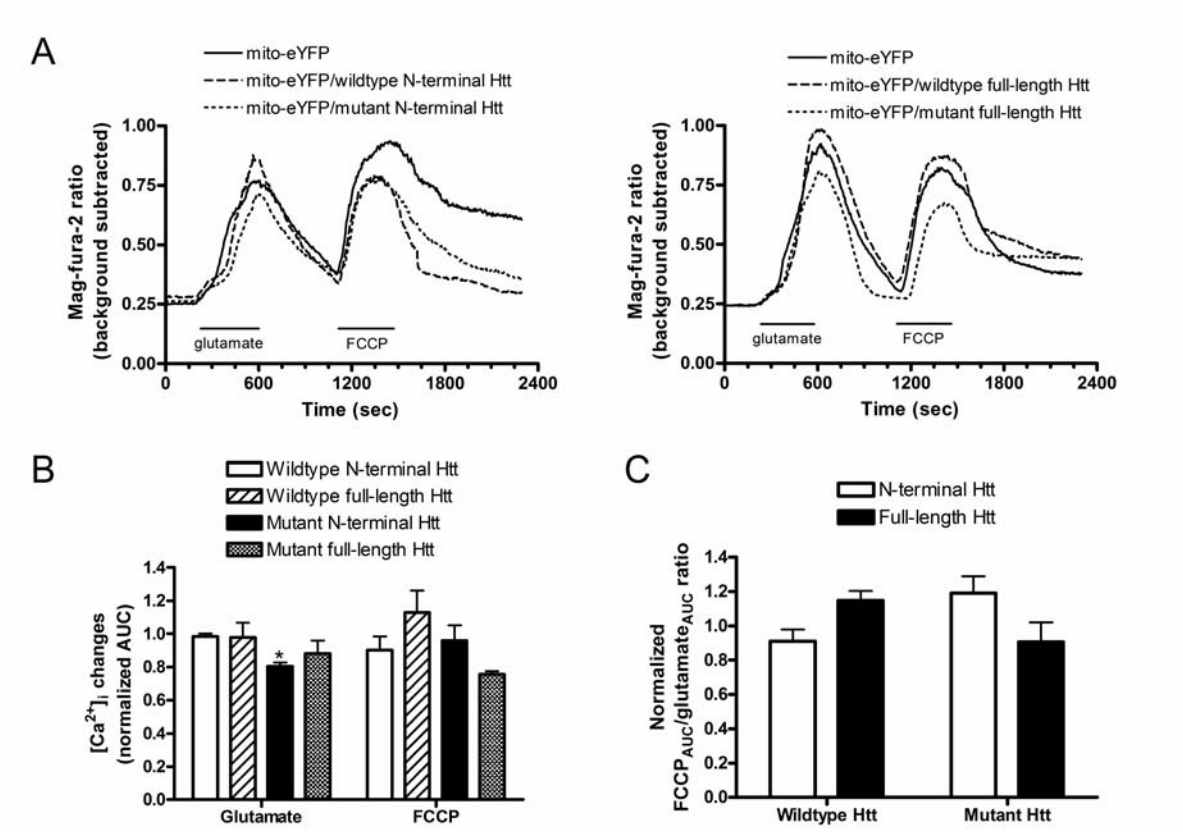

Figure 4.7 Neurons expressing mutant Htt demonstrated relatively normal calcium handling in response to an excitotoxic treatment of 30 µM glutamate/1 µM glycine.

(**A**) Intracellular Ca^{2+} influx after glutamate/glycine treatment and mitochondrial Ca^{2+} release after 750 nM FCCP treatment are shown in neurons transfected with either N-terminal (left panel) of full-length (right panel) mutant Htt. Traces are mean mag-fura-2 ratios after background subtraction and represent at least 3 coverslips each from 3 separate cultures. (**B**) Areas under mag-fura-2 curves (AUC) were calculated for 6.5 min after the start of glutamate/glycine and FCCP treatments. $Glutamate_{AUC}$ was significantly smaller in neurons transfected with N-terminal mutant Htt than controls ($p < 0.05$). (**C**) The mitochondrial contribution to buffering Ca^{2+} after glutamate/glycine treatment was calculated as $FCCP_{AUC}/glutamate_{AUC}$. Values in panels B and C are shown as mean ± SE from at least 3 coverslips each from 3 separate cultures and are normalized to neurons from respective cultures transfected with only the mito-eYFP plasmid.

Localized inhibition of mitochondrial movement by Htt aggregates

Various mechanisms have been proposed by which mutant Htt is able to impair axonal transport. These include the binding of cytoskeletal components and the titration of molecular motors,

perhaps through interactions with wildtype Htt (Gunawardena et al., 2003; Lee et al., 2004; Trushina et al., 2004). We therefore examined whether the slight reduction in global mitochondrial movement we observed in neurons expressing full-length mutant Htt (Fig. 4) was the result of (*i*) general slowing of all mitochondrial transport, as might be expected if molecular motors or cytoskeletal components were titrated out of operational pools, or (*ii*) mutant Htt aggregates acting as localized blockades of mitochondrial trafficking in neuronal processes. Neurons were cotransfected with GFP-tagged mutant Htt_{exon1}-103Q or wildtype Htt_{exon1}-25Q and a mitochondrially-targeted monomeric RFP (mito-mRFP). These experiments allowed us to directly measure the movement of mitochondria relative to cytosolic Htt aggregates. Wildtype Htt_{exon1} was expressed diffusely throughout the cytosol while mutant Htt_{exon1} was either diffusely expressed or aggregated (Fig. 8A-E).

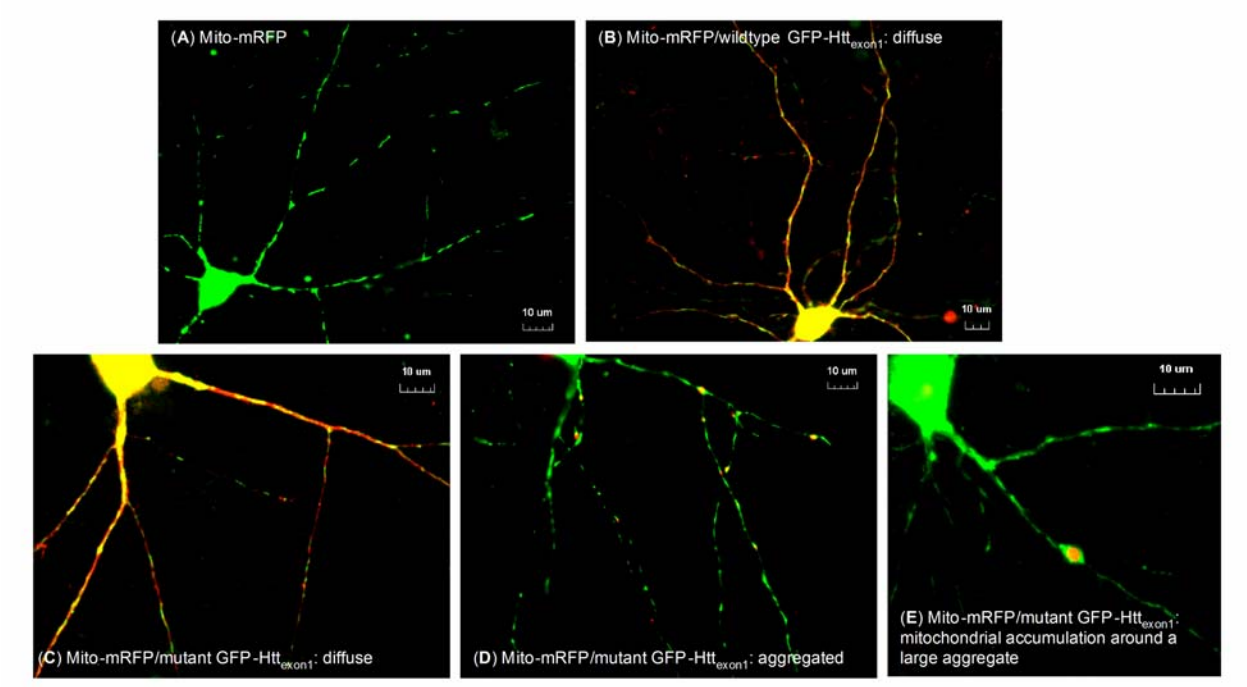

Figure 4.8 GFP-tagged mutant Htt formed cytosolic aggregates colocalized with mitochondria.

(**A**) Mitochondria were labeled by cotransfecting neurons with mito-mRFP (green). (**B**) Wildtype GFP-Htt_{exon1}-25Q (red) was expressed cytosolically. (**C, D**) Mutant GFP-Htt_{exon1}-103Q (red) was expressed either diffusely throughout

the cytosol or as aggregates colocalized with mito-mRFP-labeled mitochondria (green) as well as. (**E**) Large accumulations of mitochondria (green) could be seen surrounding aggregates (red) in neurons transfected with mutant GFP-Htt_{exon1}. Neurons are shown 3 days after transfection and are representative of at least 3 coverslips each from 5 separate cultures. 10 μm scale bars are shown.

We first examined whether mitochondria colocalized with Htt aggregates by assessing the normal characteristics of mitochondria distribution in processes. The probability that a mitochondrion would normally occupy any given point on a neuronal process was calculated by dividing the length of all mitochondria by the length of all neuronal processes in a field. Mitochondria were found to occupy 27.2 ± 3.7% of the length of the processes. If one assumes that this distribution is essentially random then for any given point on a process mitochondria should be found 27% of the time. However, we found that a remarkable 99.1 ± 0.9% of sites where aggregates were present also contained mitochondria ($p < 0.0005$; Fig. 9A), which suggests that the presence of aggregates changes the distribution of mitochondria in processes. This may be the consequence of close if not direct physical contact between aggregates and mitochondria that could impair movement of mitochondria, especially since aggregates were immobile. In addition, our results revealed that mitochondria which were colocalized with aggregates were less likely to be mobile than controls ($p < 0.05$; Fig. 9B) and also that aggregates significantly limited the passage of non-colocalized mitochondria ($p < 0.05$; Fig. 9C). Intriguingly, we also observed a significant decrease in the number of mitochondria entering regions of neuronal processes occupied by aggregates ($p < 0.05$; Fig. 9D). It is important to note that these effects on movement and trafficking were observed only at sites where aggregates were present. That is, in cells expressing aggregates, mitochondria that were not colocalized with aggregates moved in the same manner as controls. In cells expressing mutant Htt diffusely throughout the cytosol, mitochondria also moved similar to controls. Therefore, we conclude

that mutant Htt aggregates are immobile obstacles in cortical neuronal processes that locally impair mitochondrial trafficking and cause gradual accumulation of mitochondria out of the cellular pool and into the peri-aggregate region (Fig. 8E). However, mitochondria outside of the immediate vicinity of an aggregate or in cells expressing mutant Htt diffusely seemed unaffected.

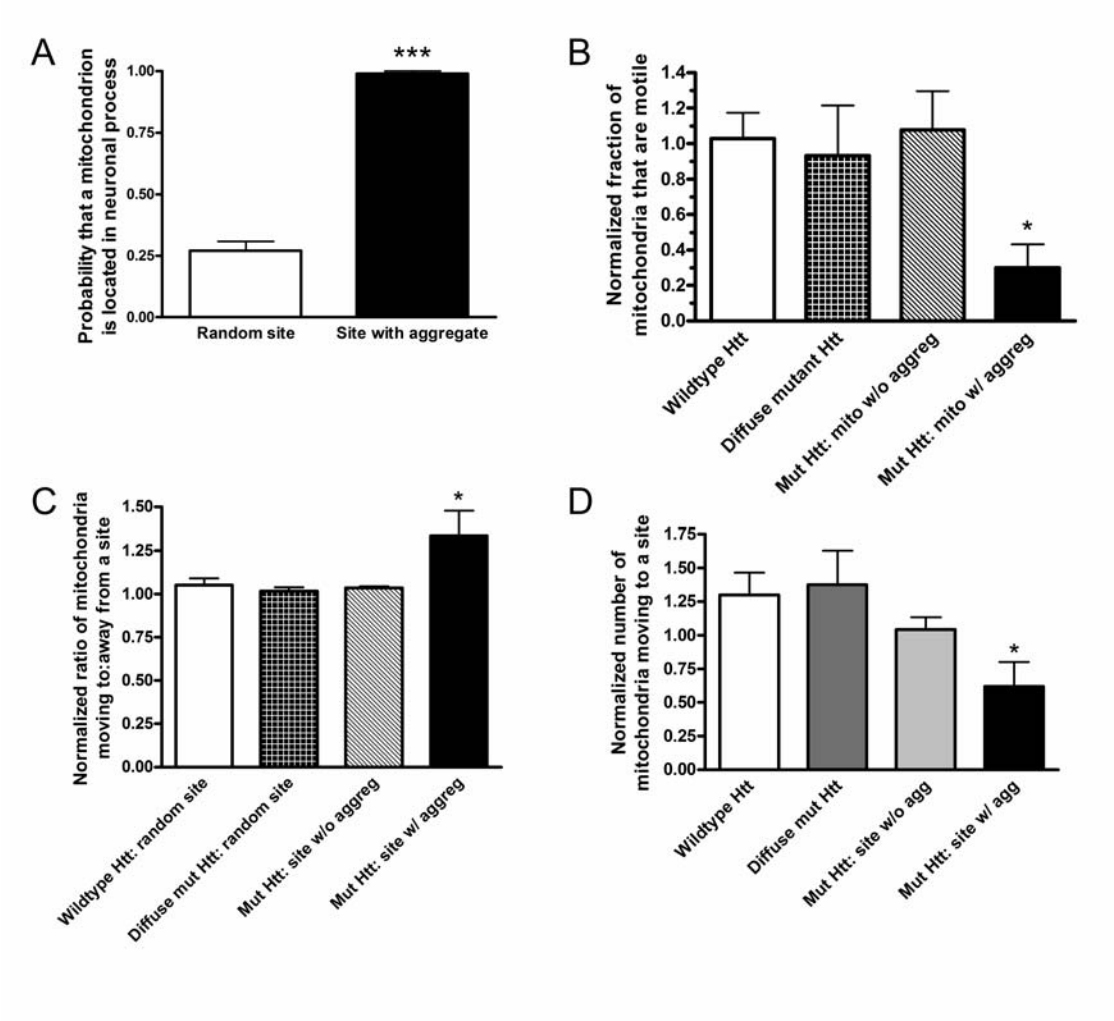

Figure 4.9 Mitochondria colocalized with and were immobilized by mutant Htt aggregates.

Mitochondrial movement was specifically impaired at the sites of aggregates and fewer mitochondria were trafficked to these sites. (**A)** The probability that a mitochondrion was located at the site of an aggregate was significantly higher than the chance of finding a mitochondrion at any randomly selected site, as determined by the average fraction of neuronal process length occupied by mitochondria ($p < 0.0005$). Values are shown as mean ± SEM from at least 2 coverslips from 4 cultures. (**B**) A significantly smaller fraction of mitochondria that colocalized with aggregates were motile compared to mitochondria not colocalized with aggregates in the same

cells, mitochondria in cells expressing diffuse mutant Htt, and mitochondria in controls ($p < 0.05$). (**C**) The ratio of mitochondria moving to:away from the site of an aggregate was significantly increased compared to sites where aggregates were absent in the same cells, and compared to randomly selected sites in cells expressing diffuse mutant Htt and in control cells ($p < 0.05$). (**D**) The number of mitochondria moving to site of an aggregate was significantly reduced compared to sites where aggregates were absent in the same cells, and compared to randomly selected sites in cells expressing diffuse mutant Htt and in control cells ($p < 0.05$). Values are shown as mean ± SE from at least 3 coverslips each from 5 separate cultures and are normalized to respective cultures transfected with only the mito-mRFP plasmid.

4.5 DISCUSSION

Our results show that Htt aggregates act as physical roadblocks for mitochondrial transport in cortical neurons with the consequence that healthy, motile mitochondria gradually accumulate around aggregates and are also less frequently trafficked toward aggregates. Our findings shed new light on the sequence of events in HD pathophysiology and the possible cell selectivity for mitochondrial trafficking defects. Specifically, we conclude that abnormal mitochondrial trafficking in cortical neurons manifests as small subpopulations of mitochondria becoming trapped and immobilized by mutant Htt aggregates, while the vast majority of mitochondria that do not contact aggregates have normal movement patterns, regardless of whether aggregates are present in the processes or if mutant Htt is expressed throughout the cytosol (Fig. 9B-D). We propose that this impairment in mitochondrial movement is an early pathogenic event, occurring before mitochondrial and cellular dysfunction in cortical neurons. Blockage of mitochondrial transport by aggregates could gradually titrate healthy mitochondria out of the operating pool and lead to neurodegeneration.

Trushina et al. (2004) recently concluded that in mouse striatal neurons, an early step in HD pathology is aberrant mitochondrial motility caused by sequestration of wildtype Htt and

trafficking proteins by mutant Htt. To gain further insights beyond collective mitochondrial populations, we examined movement of mitochondria that were (*i*) specifically colocalized with cytoplasmic aggregates, (*ii*) upstream or downstream of but not colocalized with aggregates, and (*iii*) in cells expressing mutant Htt diffusely throughout the cytosol. We were thus able to carefully dissect the role of aggregates as physical roadblocks for mitochondria separately from the effects of aggregated or non-aggregated mutant Htt interactions with cytoskeletal and motor proteins that others have found to broadly affect protein and vesicle transport in rat striatal neurons, *Drosophila* larval neurons and axoplasm isolated from squid giant axons (Li et al., 2001; Gunawardena et al., 2003; Szebenyi et al., 2003; Lee et al., 2004). That is, if aggregates in cortical neurons also sequestered transport proteins to the extent described by others, or if diffusely expressed mutant Htt disrupted wildtype Htt interactions with transport molecules, then we would expect mitochondrial movement to be reduced accordingly in conditions (*ii*) and (*iii*) above. However, in neither condition did mitochondria have a smaller probability of moving or did fewer mitochondria move past a given point on a neuronal process over time (Fig. 9B-C).

Physical blockage of mitochondria by aggregates may be one of the first signs of abnormal trafficking in cortical neurons, with substantial global disruption of mitochondrial movement developing at a delayed pace relative to prior reports in striatal neurons, if at all. In fact, there is the distinct possibility that local disruption of cytoskeletal components by aggregates may have caused our observations of decreased mitochondrial trafficking specifically to sites of aggregation (Fig. 9D) (Trushina et al., 2003). It is also possible that mitochondria trapped in the vicinity of aggregates stopped moving because they became damaged and depolarized or that local ATP depletion occurred in these regions, as would be expected from the bioenergetic defects associated with HD (Schon and Manfredi, 2003). This is supported by our

previous findings that mitochondrial motility depends on ψ_m and mitochondrial ATP synthesis (Rintoul et al., 2003b). It remains to be learned whether such trafficking defects radiate out from locations of aggregates to encompass entire neuronal processes over time. If this does prove to be the case, then it is likely that the global mitochondrial trafficking defects described by Trushina et al. (2004) in striatal neurons may eventually develop in cortical neurons. Another possibility is that cellular signals may locally protect the mitochondrial pool from damaging regions in the cell by limiting trafficking to those locations. Regardless of whether mitochondrial trafficking to aggregates is purposely or inadvertently reduced, a likely result is that neuronal processes will degenerate at sites of aggregates because mitochondria are not shuttled to these regions and those organelles that do enter are trapped and deteriorate. Local regions of degeneration could very well expand and cause broad mitochondrial and cellular dysfunctions over time as a result of the loss of function in distal processes.

Indeed, we show that mitochondrial movement was blocked by aggregates before neurons displayed heightened glutamate excitotoxicity or any overall alterations in mitochondrial Ca^{2+}-handling or morphology (Fig. 4-7). Our results therefore confirm that aberrant mitochondrial transport is an early event in the pathogenesis of HD associated with the formation of Htt aggregates in neuronal processes, even though the trafficking defects manifest differently in cortical and striatal neurons (Trushina et al., 2004). Long term consequences of impaired mitochondrial movement by aggregates and a gradual decline in functional mitochondria from the cellular pool could be neurodegeneration and cell death mediated by the combination of insufficient ATP for cellular processes, dysregulation of local $[Ca^{2+}]_i$, mitochondrial dysfunction, release of reactive oxygen species and induction of apoptosis. Our proposed mechanism accounts for the mitochondrial defects and oxidative damage observed in HD, and additionally

provides information into the role of cytosolic aggregates in impairing cellular transport (Browne et al., 1997; Panov et al., 2002; Panov et al., 2003; Choo et al., 2004; Ruan et al., 2004; Saulle et al., 2004). Furthermore, a longer time course for development of global mitochondrial trafficking defects may account for the improved health of cortical neurons which are less susceptible to glutamate excitotoxicity than striatal neurons, possibly due to reduced calcium-induced mitochondrial permeability transition (Greene et al., 1998; Brustovetsky et al., 2003; Snider et al., 2003). The surprising lack of glutamate excitotoxicity we observed in Htt-transfected cells could also be accounted for by mitochondrial depolarization at lower $[Ca^{2+}]_i$, thus preventing uptake of pathological amounts of Ca^{2+} and inorganic phosphate that would activate mitochondrial permeability transition and cell death (Panov et al., 2003; Panov et al., 2005).

Several recent studies on mutant Htt focused on impaired vesicular transport, and suggest aggregation in small-diameter axons and synaptic terminals may lead to neuronal and synaptic defects (Gunawardena et al., 2003; Li et al., 2003; Lee et al., 2004; Gunawardena and Goldstein, 2005). Our examination of spontaneous intracellular calcium fluxes due to synaptic activity did not reveal any differences, supporting the idea that impaired mitochondrial movement is an earlier pathogenic event than synaptic dysfunction (data not shown). Interestingly, there also appeared to be no selectivity for aggregation in neuronal processes of smaller diameter or any net movement of aggregates as these reports suggest. In fact, large aggregates that were prominently surrounded by mitochondrial accumulations were more often found proximal to the cell body (Fig. 8E). This is probably because the most mitochondrial traffic occurs in the large-diameter processes to and from the cell soma where mitochondrial density is high. Thus, mitochondria traveling anterogradely and retrogradely rapidly build up around such aggregates, further

enlarging the "roadblock" and impairing transport of smaller particles such as vesicles. We propose that the large size of mitochondria renders these organelles the most vulnerable to impaired transport by mutant Htt aggregates, and aggregates may have the most severe consequences when they form in the proximal axon. It is important to note that mitochondrial movement may also be unaffected by separate but concurrent trafficking disruptions mediated by wildtype Htt and its associated proteins. For example, Gaulthier et al. demonstrated reduced transport velocity of BDNF vesicles but not of mitochondria in neuronal cell lines derived from mutant Htt knock-in mice (Gauthier et al., 2004). Our data agrees with their findings, as we found normal movement in mitochondria that were not associated with mutant Htt aggregates.

We conclude from our results comparing the effects of N-terminal and full-length mutant Htt that the context of the polyglutamine repeat influences its pathologic function, as others have (Hackam et al., 1998; Yu et al., 2003). We found a significant difference in global mitochondrial movement only in full-length mutant Htt-transfected neurons (Fig. 4). Our GFP-tagged Htt experiments reinforce the importance of cytosolic aggregates as the pathologic entity (Fig. 9). This agrees with findings that neuropil aggregates associated with structures resembling mitochondria are more commonly found in striatal neurons and lead to selective neurodegeneration (Li et al., 2001). We therefore suspect that N-terminal Htt was largely localized to the nucleus whereas full-length Htt formed more cytosolic aggregates in transfected neurons (Cooper et al., 1998; Xia et al., 2003; Cornett et al., 2005). In other words, the context of the polyglutamine tract in the Htt gene might be influential in its provision of different proteolytic cleavage sites that optimize cytosolic rather than nuclear aggregate formation. Furthermore, it is likely that the earlier onset and enhanced formation of aggregates that occurs with increased polyglutamine length leads to more rapid and substantial impairment of

mitochondrial transport, thus worsening disease severity (Scherzinger et al., 1999; Chen et al., 2001; Chen et al., 2002).

In conclusion, our study provides evidence for impairment of mitochondrial transport in cortical neurons by the physical blockage of neuronal processes by mutant Htt aggregates, as well as reduced trafficking of motile mitochondria to aggregates. We suggest that this is one of the earliest steps in the molecular pathogenesis of HD, as the titration of mitochondria out of operational pools and into accumulations localized around aggregates is likely to be a gradual process. Furthermore, global mitochondrial trafficking defects may develop slower in cortical neurons than in striatal neurons, resulting in selective neurodegeneration. Since mitochondria are numerous and exhibit dynamic motility, it is likely that considerable time is required before unaffected mitochondria fail to compensate for the reduction in number of functional mitochondria, the growing impediments in neuronal processes, and the localized sites of disrupted mitochondrial delivery. We predict that cellular and mitochondrial dysfunctions manifest slowly, just as HD symptoms and neurodegeneration occur over the course of many years.

5.0 DISCUSSION

5.1 OVERVIEW

Mitochondrial trafficking in neurons is a fundamental property with vast implications for normal and abnormal cellular and mitochondrial function. The explosive interest in the field over the three years during which this dissertation was completed is a clear indication of the growing appreciation of its importance. However, mitochondrial trafficking and morphology are extraordinarily complex in neurons, and as such, their study is riddled with difficulty. Furthermore, because mitochondrial movement is so intimately tied with the functional status of cells and of mitochondria themselves, results obtained from experimental manipulations, whether intended to be physiological or injurious, can be a challenge to interpret.

This discussion is organized into two major parts. The first reviews the complexities of studying mitochondrial trafficking, describes approaches that could be adopted for the future (5.2), and evaluates what can be learned from recent examinations of mitochondrial trafficking to synapses (5.3). The second part concentrates on impaired mitochondrial movement as a cause of neuronal dysfunction, with an emphasis on the pathologic implications (5.4) and possible mechanisms (5.5) of heterogeneous movement abnormalities in neurons. The discussion closes with a look into what the future will uncover about mitochondrial trafficking and how that knowledge can be harnessed to develop pharmacologic therapies for neuronal injury and disease.

5.2 ADOPTING A REDUCTIONIST APPROACH TO THE INVESTIGATION OF MITOCHONDRIAL TRAFFICKING

5.2.1 Too many mitochondria moving for too many reasons

Mitochondrial movement in neurons is extremely diverse with velocity, direction changes and pauses during movement varying among individual organelles, different neuronal processes, and different cells. There is also likely to be tremendous variability in the regulatory influences on movement between individual mitochondria such that (i) they may be targeted to different cues in cells, (ii) these cues may have various distributions in different neuronal processes, (iii) the characteristics of motor and adaptor proteins that mediate movement may differ between organelles and may be subject to different functional modifications, and (iv) cytoskeletal arrangements and interactions with microtubule-associated proteins can vary between neuronal processes and among different regions of a single process (discussed in Chapter 1.4). For scientists, the diversity of mitochondrial movement is both a gift and a curse. On the one hand, it is fascinating because there are multiple levels of complexity to explore. But ultimately, the complexity makes it nearly impossible to study any single variable in a controlled manner. There are several oversimplified schemes that can be adopted for the study of mitochondrial movement. One approach starts with mitochondria and tries to identify trafficking cues by observing where mitochondria stop and go. Another method starts with a cellular signal and determines how mitochondria move relative to that signal. Ultimately, the results from one approach are tested by the other approach to confirm the identity and effects of a physiological signal on normal mitochondrial trafficking.

5.2.2 Subdividing the mitochondrial pool for analysis

5.2.2.1 *Nonspecific mitochondrial measurements*

There are two theoretically straightforward approaches to studying trafficking of the mitochondrial pool – either in totality or in partiality. The first involves measuring movement of all mitochondria in entire cells. This is not so much of an issue in cell lines with relatively indiscriminate distributions of cellular contents, but polarized neurons provide a different challenge. Sampling from the whole mitochondrial pool usually has little or no regard for the anatomic regions or activities of neuronal processes being examined, and sometimes even with processes from more than one cell at a time being imaged. Most experiments are done in this manner, as it is simpler, yields data more rapidly, and in many cases, pharmacologic treatments seem to affect mitochondrial movement quite uniformly in cells (Rintoul et al., 2003b; Vanden Berghe et al., 2004; Yi et al., 2004; Verstreken et al., 2005). Yet true differences in mitochondrial movement in various cellular regions after drug application can easily be overlooked when those regions comprise a small fraction, if any, of the imaging field. For example, we uncovered a dendrite-specific rounding of mitochondria after acute glutamate exposure and a preservation of mitochondrial movement in distal axons that were previously concealed by the all-inclusive imaging approach (Chapter 3).

5.2.2.2 *Measuring regional subpopulations of mitochondria*

The next step toward achieving specificity of measurements is distinguishing between anatomic regions of neurons, such as axons and dendrites or proximal and distal segments of processes. The axon-dendrite comparison has been made in previous reports characterizing mitochondrial movement patterns in hippocampal neurons (Overly et al., 1996; Ligon and Steward, 2000a). A

differentiation between proximal and distal dendrites, where microtubule polarities are known to differ, was performed as well (Overly et al., 1996). Additionally, mitochondrial movement was compared in proximal and distal axons to determine the effect of growth cone proximity on trafficking (Morris and Hollenbeck, 1993). A portion of this dissertation work was also devoted to the study of differential regulation of mitochondrial morphology and movement relative to synapses on axons compared to dendrites (Chapter 3). These studies have been important in describing mitochondrial movement activities on a smaller spatial scale, but still suffer from lack of specificity because mitochondria with varying movement patterns almost always occupy a given region. In terms of studying the mitochondrial pool, the finest detail can be achieved by tracking the movement of an individual mitochondrion.

5.2.2.3 *Studying trafficking of an individual mitochondrion*

An understanding of how the variable movement patterns of single mitochondria contribute to the diversity observed in the whole mitochondrial pool can be derived from observing the trafficking of individual mitochondria. This can best be achieved by time-lapse imaging of neurons transfected with a photoactivatable fluorescent protein targeted to mitochondria (Rintoul et al., 2003a). Focal photoactivation would result in the labeling of a discrete group of mitochondria which could be analyzed individually once they moved away from each other. This method would be a marked improvement over current techniques which employ fluorescent lipophilic cationic dyes or transfection of neurons with fluorescent plasmids containing mitochondrial targeting sequences. Commonly used dyes for labeling mitochondria in live cells are rhodamine 123 and MitoTracker variants (Overly et al., 1996; Ligon and Steward, 2000a; Chada and Hollenbeck, 2003; Vanden Berghe et al., 2004; Cai et al., 2005). With the exception of MitoTracker Green, the sequestration of these dyes into mitochondria relies on the negative

mitochondrial membrane potential. Therefore, de-energized mitochondria which may possess certain characteristics that influence movement patterns such as respiratory damage or increased metabolic activity, could be excluded from labeling. Perhaps more importantly, these dyes have the ability to uncouple and inhibit respiration and depolarize ψ_m depending on cell type and dye concentration (Nicholls and Ward, 2000; Buckman et al., 2001). Compromised mitochondrial function can clearly alter mitochondrial movement (Chapter 1.9). Technically, loading cells with fluorescent dyes also poses a problem because the mitochondria of all cells on a coverslip become labeled, making it nearly impossible to distinguish mitochondria in one overlapping process from another.

A fairly effective solution to the overlabeling problem, and the method we used in the dissertation work, is to transfect neurons with a mitochondrially targeted fluorescent protein (Li et al., 2004; Guo et al., 2005; Verstreken et al., 2005). Transfection efficiencies of primary neurons are low, typically 1-5%, which permits individual neurons to be imaged independently. An additional advantage is that localization of eYFP to the mitochondrial matrix using a cytochrome c oxidase subunit IV targeting signal does not appear to compromise cell or mitochondrial function (Llopis et al., 1998 and our own observations). However, even when only one cell is considered, the abundance of mitochondria in a single process, combined with their dynamic movements, fusions and fissions, can significantly complicate the ability to track a single organelle. This difficulty is exacerbated as mitochondria move past each other, often pausing where other mitochondria are located and sometimes fusing with those mitochondria or undergoing fission. The identity of the departing mitochondrion can therefore be obfuscated. The ability to photoactivate only a subset of mitochondria provides an additional level of sophistication that would allow more detailed and explicit tracking of individual mitochondrion.

Observing the movement patterns of an individual mitochondrion would reveal basic trafficking properties that are still undefined. A fundamental question is if mitochondria with different motility properties serve different trafficking functions, or if all mitochondria are subject to the same movement and recruitment cues in cells. For instance, if a particular site on a neuronal process requires ATP, does it indiscriminately recruit passing mitochondria until the demand is met, or does it recruit faster or slower moving mitochondria depending on the expected duration on demand? By the same token, are mitochondria that travel with high speed more likely to serve sites with transient demands, or is their observed speed a result of being targeted to a distant destination where they will become stationary? The idea of a destination also raises the question of whether individual mitochondria are targeted to specific sites, or if they just continuously circulate along neuronal processes and respond to recruitment signals as they are encountered. Observations by Chada and Hollenbeck (2004) over a 10 minute interval where many but not all mitochondria moving past an NGF-coated bead stopped, and almost half of these mitochondria continued to move away from the bead after pausing, suggest that (i) almost all moving mitochondria can respond to a strong recruitment stimulus, (ii) mitochondrial residence time at a single recruitment signal varies between individual organelles, and (iii) some mitochondria may not veer from their intended trajectory or may not recognize certain recruitment signals.

Separately tracking the movement trajectories of individual mitochondria could very well reveal that organelles with certain velocities also exhibit different trafficking patterns. Mitochondria with different speeds and movement patterns have been anecdotally described, but it is unclear whether distinct populations of moving mitochondria really exist. For example, an oversimplified scheme may categorize mitochondria as (i) relatively stationary, (ii) slow movers

or (iii) fast movers. Frequency distributions of mitochondrial velocities indicate that velocities exist on a continuum up to approximately 1 μm/sec, and are found in more discrete distributions at velocities greater than 1 μm/sec (Morris and Hollenbeck, 1995; Ligon and Steward, 2000a, b). This agrees with the thought that structural properties of individual motor proteins dictate that protein's power stroke, and there must be a finite number of motor protein isoforms associated with mitochondria (Trinczek et al., 1999). Alternatively, a combination of differentially regulated isoforms may transport a single organelle, thus allowing for marked velocity changes during its trajectory. Collecting movement data from single mitochondria could easily reveal if this is the case. Furthermore, correlating velocity with the frequency with which individual mitochondria stop, the time duration for which they stop, and whether stopping points are distributed at similar intervals for different organelles would provide invaluable information on whether mitochondria tend to be equally responsive to cellular cues or if different mitochondria respond to different cues, perhaps because of different mobility characteristics.

5.2.3 Studying cellular targets and modulators of mitochondrial movement

5.2.3.1 *Measuring mitochondrial trafficking to a single cue*

An alternative to deriving cellular cues from mitochondrial movement patterns is to select some cellular signal and study mitochondrial trafficking in response to changes in that signal. This was accomplished quite elegantly by Chada and Hollenbeck (2004), who plotted incoming and outgoing mitochondrial movements in the vicinity of an NGF-coated bead placed next to an axon. In contrast to control beads which mitochondria moved past with relatively continuous motion, more mitochondria moved toward NGF beads and tended to remain stationary after arrival. This resulted in a net accumulation of mitochondria specifically around the NGF beads,

which was not observed with endosomes, lysosomes or vesicles. As a valuable counterpart, the authors demonstrated that elevated NGF must be applied focally to recruit mitochondria because addition of high NGF concentrations to the culture medium dissipated the effects of NGF beads (Chada and Hollenbeck, 2003). It should be noted that the researchers did not explore the possibility that whole-cell application of NGF prevented recruitment of mitochondria to NGF beads simply by stopping general mitochondrial movement. In summary, their work provides an example of a single chemical target to which mitochondria are recruited and when the target is effectively diffused, mitochondrial recruitment ceases.

The experimental system used in the NGF studies offers several conveniences to the investigation of mitochondrial movement (Chada and Hollenbeck, 2003, 2004). First, large focal concentrations of NGF are not distributed throughout neuronal processes, but are instead found specifically at growth cones of developing axons where NGF promotes elongation and sprouting of collateral branches (Letourneau, 1978; Diamond et al., 1992; Gallo and Letourneau, 1998). This allowed the investigators to largely avoid the effects of confounding mitochondrial trafficking to high physiological NGF concentrations by placing experimental beads at regions of axons at least 100 μm from the growth cone. It should be noted however, that while the effects of exogenous and endogenous NGF sources could be isolated quite effectively, the measurement of mitochondrial movement to NGF beads is probably somewhat confounded by the signals that govern where mitochondria would normally move. This was addressed by the use of control heat-denatured NGF beads; thus the increased mitochondrial trafficking to NGF beads compared to control beads convincingly represented recruitment of mitochondria away from their normal trajectories. Another advantage of the Chada and Hollenbeck system is that they used young, developing neurons that were still elongating their axons. Synapses, which have dynamic

influences on mitochondrial movement, are probably not structurally assembled at this time. Our own work confirms that mitochondrial movement is significantly greater in synaptically immature neurons (Chapter 2). Therefore, a large contribution to confounding mitochondrial "docking" signals along axons was avoided.

Obviously, the advantages of the system used to study mitochondrial trafficking to focal NGF stimulation cannot be easily translated to broader examinations of mitochondrial movement to cues such as ATP and $[Ca^{2+}]_i$ gradients. In these cases, there are many endogenous sources that are distributed throughout axons and dendrites. Furthermore, synaptically mature neurons are likely to provide more physiologically relevant information. Neurons are post-mitotic and therefore almost their entire lifespan is spent in the mature state. Synaptic transmission is the major specialized function of neurons. Ideally, an understanding of how mitochondrial trafficking to synapses operates in healthy mature neurons will help us deduce how disruptions of trafficking could lead to synaptic dysfunction in aging, neuronal injury and neurodegeneration. Approaches that have been used to examine mitochondrial trafficking to synapses and to $[Ca^{2+}]_i$ gradients, including our own, are discussed in the following sections.

5.2.3.2 *Studying mitochondrial movement responses to global pharmacologic treatments*

The adoption of a reductionist approach to studying mitochondrial trafficking is also relevant to pharmacologic manipulations. Although describing normal mitochondrial movement patterns is highly informative, it is rarely enough to answer mechanistic questions about movement regulation. Studying the response of mitochondrial movement to pharmacologic alterations in cellular ion concentrations, neuronal activity and mitochondrial activity can elucidate some aspects of movement regulation. Almost every study to date has applied drugs universally to cells on a coverlip through pre-incubation and/or perfusion. This is more physiologically

relevant in some cases than in others. For example, in a model of ischemic excitotoxicity during which glutamate release is known to increase profoundly and cause elevated Ca^{2+} entry into cells, global perfusion of neurons with glutamate seems appropriate (Rintoul et al., 2003b). During ischemia, physiologic glutamate release from cells occurs at presynaptic terminals with glutamate-containing vesicles as well as by reverse transport of glutamate through glutamate transporters (Nishizawa, 2001). Presumably, drug perfusion might activate some glutamatergic receptors that would not normally encounter physiologically released glutamate, but this is probably a minor concern given the substantially overactivated glutamatergic signaling that occurs during excitotoxicity.

In another situation where researchers attempted to conclude that locations of physiological $[Ca^{2+}]_i$ spikes recruit mitochondria for Ca^{2+} uptake, vasopressin was applied systemically to mobilize Ca^{2+} from ER/SR stores in the H9c2 cardiac cell line (Yi et al., 2004). This resulted in increased $[Ca^{2+}]_{cyto}$ that was roughly simultaneous with reduced mitochondrial motility. However, the authors' conclusion that mitochondria stop specifically at ER/SR where $[Ca^{2+}]_i$ might be highest was weakened by their findings that (1) cessation of mitochondrial movement occurred throughout entire cells, (2) no colocalization was shown between ER and mitochondria and (3) mitochondrial Ca^{2+} uptake was not even demonstrated except in cells where microtubules were disrupted by nocodazole. Furthermore, no difference in $[Ca^{2+}]_i$ was seen between vasopressin-treated control cells and cells where mitochondria were depolarized with extremely high concentrations of FCCP/oligomycin. This argues against the idea that mitochondria were needed to uptake the Ca^{2+} concentrations released in their experiments. On the other hand, their $[Ca^{2+}]_{cyto}$ measurements, which were performed with the fluorescent Ca^{2+}-indicator fura-2, did not have the spatial resolution to detect microdomains of more elevated

$[Ca^{2+}]_i$ that would be expected near ER. Since most mitochondria already stopped moving when $[Ca^{2+}]_{cyto}$ reached 500 nM, recruitment to $[Ca^{2+}]_i$ microdomains in their experiments is unlikely and actually suggests impaired movement might be an inadvertent rather than intentional consequence of elevated $[Ca^{2+}]_{cyto}$. Regardless of the flaws in this study, $[Ca^{2+}]_i$ microdomains are still highly regarded as potential targets for mitochondrial trafficking.

Previous examinations of intact mitochondrial function in the form of ψ_m or ATP synthesis as a requirement for normal mitochondrial movement have also employed global drug perfusion. Use of uncouplers such as FCCP indicates that ψ_m depolarization inhibits mitochondrial function (Rintoul et al., 2003b; Vanden Berghe et al., 2004). However, by applying the drug to entire coverslips, certain confounding factors are exacerbated. For example, uncoupling oxidative phosphorylation alone dissipates ψ_m and consequently removes the driving force for protons into the mitochondrial matrix through ATP synthase. Instead, protons flow in the opposite direction and cellular ATP can be rapidly depleted (Nicholls and Ward, 2000). Thus, mitochondrial movement could be impaired largely due to lack of mitochondrially-produced ATP rather than just ψ_m depolarization. Indeed, perfusion with the ATP synthase inhibitor oligomycin, which also results in ~5 mV ψ_m hyperpolarization, has been shown to stop mitochondrial movement (Chapter 3) (Scott and Nicholls, 1980). Yet this is also not a straightforward conclusion because ATP is still provided through the glycolytic pathway and molecular motors are known to operate at very low ATP concentrations (Hollenbeck, 1996). Another problem with FCCP is that it is a protonophore which is not necessarily selective for mitochondrial membranes and it can also disrupt ion homeostasis either directly or as a result of effects on mitochondrial ψ_m and ATP synthesis. Plasma membrane depolarization, elevated $[Na^+]_i$ and cytoplasmic acidification were observed in isolated nerve terminals with nM

concentrations of FCCP (Tretter et al., 1998). Importantly, intracellular Ca^{2+} homeostasis can be influenced by altered Na^{+} homeostasis since the ions are often exchanged in unison. In carotid body cells, FCCP also blocked K^{+} conductances and caused voltage-gated Ca^{2+} entry due to plasma membrane depolarization (Buckler and Vaughan-Jones, 1998). Although mitochondrial sequestration of Ca^{2+} is relatively minor in the basal state, we have demonstrated a small increase in $[Ca^{2+}]_i$ after FCCP treatment (Chapter 2). This can further complicate interpretation of the effects of drugs that dissipate ψ_m and inhibit ATP synthase because elevated cytosolic Ca^{2+} is known to impair mitochondrial movement (Rintoul et al., 2003b; Yi et al., 2004). Furthermore, the mitochondrial rounding seen after excitotoxic elevations of $[Ca^{2+}]_i$ can still be observed, albeit to a lesser degree, with FCCP treatment alone (unpublished observations). Therefore, while investigations of mitochondrial function yield some interesting interpretations about movement regulation, the global perfusion of drugs produces multiple physiological changes in whole cells that can also influence movement indirectly.

A third tactic used to understand the regulation of mitochondrial movement was by global pharmacologic modulation of synaptic activity. Our analysis of mitochondrial localization and trafficking to pre- and postsynaptic sites relied on long- and short-term treatments of neurons with modulators of Na^{+} channel activity to achieve synaptic silence and overactivity (Chapter 3). This is a reasonable approach to gaining a global view of mitochondrial trafficking in entire cells when all synaptic sites are presumed to be behaving similarly. The case is simpler when considering synaptic silence by TTX treatment because action potential propagation is blocked, thus activation of postsynaptic receptors do not result in signal transmission down axons and presynaptic terminals do not depolarize for neurotransmitter release. Cell-to-cell communication ceases as evidenced by lack of synchronous $[Ca^{2+}]_i$

transients in cultures. Our global approach to silencing synaptic activity actually was ideal for revealing an overall increase in nonspecific mitochondrial movement that was corroborated by different qualitative measurements by Li et al. (2004). This finding would clearly be obfuscated if fewer mitochondria or smaller regions of neuronal processes were studied. Interestingly, this mitochondrial movement response existed concurrent with the synaptic remodeling that is known to occur with prolonged inactivity, although that was not explicitly shown in these experiments (Kirov and Harris, 1999; Murthy et al., 2001).

Unfortunately, the examination of mitochondrial movement during global synaptic overactivity is much more complicated. We perfused cells with an inhibitor of Na^{+} channel inactivation which produced robust, recoverable Ca^{2+} transients as neurons fired spontaneously (Chapter 3). Presumably, this causes larger or prolonged neurotransmitter release that leads to postsynaptic overactivation. Postsynaptically, we looked at mitochondrial trafficking to PSD-95 clusters, a marker for glutamatergic synapses containing NMDA receptors. However, we could not identify locations of non-PSD-95-labeled postsynaptic sites which surely contribute an important population on dendrites. These include at least GABAergic and cholinergic synapses which have been shown to mediate spontaneous synaptic firing in our cultures (data not shown). Therefore, the possibility exists that many "nonsynaptic" sites that were used as a control comparison to demonstrate specificity for PSD-95-targeted trafficking actually contained unlabeled postsynaptic sites. This is a background signal that probably could not be avoided with global drug application even if all true postsynaptic sites could be visualized because the high synaptic density on dendrites prevents one active postsynaptic site from being effectively resolved from its neighbor. The complexity is enhanced when activity-dependent synaptic

remodeling is considered, which can be a highly variable process in different synapses (Okabe et al., 1999; Konur and Yuste, 2004).

Alternate approaches to overactivating synaptic firing with agents such as agonists of excitatory receptors or antagonists of inhibitory receptors would also be experimentally complicated. Perfusion of excitatory stimuli or agonists can result in sustained receptor stimulation, thus eliminating the physiological transient $[Ca^{2+}]_i$ spiking to be studied. Membrane depolarization by perfusion with high KCl, such as that used by Li et al. (2004) also seemed excessively nonphysiological, as it resulted in large sustained Ca^{2+} influx that slowly recovered, following which spontaneous $[Ca^{2+}]_i$ were substantially dampened (data not shown). The use of bicuculline, an antagonist of inhibitory $GABA_A$ receptors, was quite successful in increasing the amplitude of $[Ca^{2+}]_i$ transients while maintaining the typical spiking pattern. However, we also uncovered a degree of heterogeneity in our cultured neurons such that a small population of neurons actually became synaptically silent during bicuculline treatment, perhaps because they received excessive inhibition by non-GABA neurotransmitters. Concurrent calcium imaging would have to be performed during mitochondrial trafficking experiments to confirm that we had not selected an anomalous cell. This would introduce another variable in an already complicated system in addition to bordering on practical unfeasibility. Therefore, while our method of measuring mitochondrial trafficking to synapses during synaptic overactivation with veratridine was not void of experimental pitfalls, we considered it the most physiological approach to using global drug treatment.

5.2.3.3 *Local pharmacologic manipulations as a future approach for studying mitochondrial movement*

The investigation of targets for mitochondrial trafficking, how the functional status of mitochondria affects motility, and the functional role that mitochondria play at specific neuronal locations can all greatly benefit from shifting pharmacologic modulations from a macro-scale to a micro-scale. For example, instead of perfusing drugs over entire coverslips, a micro-perfusion system can be utilized to apply drugs locally over a small segment of neuronal process or over a small population of mitochondria. With regard to the studies described above, local application of (1) a Ca^{2+}-mobilizing drug, Ca^{2+} ionophore or an NMDA receptor agonist could reveal mitochondrial recruitment to regions of elevated $[Ca^{2+}]_i$ requiring mitochondrial buffering, (2) an uncoupler could successfully depolarize a small subset of mitochondria without resulting in substantial loss of ATP synthesis or disrupted ion homeostasis, and (3) global TTX treatment combined with local application of a postsynaptic receptor agonist or depolarizing agent could selectively activate synapses on a very small and measurable segment of neuronal process.

Measuring mitochondrial trafficking under conditions of locally controlled synaptic activity actually opens a wealth of opportunity for the study of the mitochondrial support required at different types of synapses with different activities and plasticities, especially when combined with electrophysiological techniques. Furthermore, the function of mitochondria at these different synaptic sites as Ca^{2+}-buffers or ATP synthesizers can be explored in detail with the combined use of locally applied modulators of ψ_m and mitochondrial ATP synthesis as well as fluorescent calcium- and ψ_m-indicators. Some highly informative experiments include (*i*) measuring mitochondrial trafficking to excitatory or inhibitory synapses containing different receptor types under various stimulation protocols, (*ii*) determining how *local* mitochondrial

ATP depletion and/or impairment of mitochondrial Ca^{2+}-uptake influence synaptic firing during physiological and long term stimulations, (*iii*) investigating whether local inhibition of mitochondrial function causes recruitment of normally functioning mitochondria and (*iv*) studying whether mitochondria are recruited when synapses are stimulated to undergo short or long-term plasticity and what Ca^{2+}-buffering and ATP generating roles they play once recruited. In summary, the ease of pharmacologic manipulation of whole neurons has contributed over a short time period a great deal to our understanding of mitochondrial movement and the influences thereupon. However, the yield of such methodology is rapidly diminishing because there is a real difficulty in achieving specificity of results when drugs can have a host of undesired effects on whole cells that alter movement, and the diversity in mitochondrial populations and physiological responses to changes in whole neurons can complicate data interpretation. The future of the mitochondrial movement field will benefit substantially from more sophisticated, local manipulations and mitochondrial measurements.

5.3 AN EVALUATION OF STUDIES OF MITOCHONDRIAL TRANSPORT TO SYNAPSES

5.3.1 Using genetic mutants to study mitochondrial movement

The complexity of studying mitochondrial movement in a controlled manner in wildtype neurons has led to the recent use of genetic mutants with disrupted mitochondrial distribution as a tool to derive the functions of normal mitochondrial movement (Stowers et al., 2002; Li et al., 2004; Guo et al., 2005; Verstreken et al., 2005). To emphasize the relevance of the dissertation work, these reports are all related to proper mitochondrial localization for synaptic function. These

studies contribute some of the most mechanistic details to our understanding of mitochondrial distribution and synaptic function. However, an important caveat is that any genetic alteration that grossly affects mitochondrial transport or morphology is also bound to have complex effects on ATP distribution and Ca^{2+} homeostasis that are not only restricted to synaptic sites. Additionally, these studies did not usually emphasize the possibility of altered mitochondrial function when normal movement and morphology are disrupted.

5.3.2 Significance of mitochondrial transport to presynaptic terminals for synaptic function

5.3.2.1 *Milton mediates mitochondrial transport and distribution in Drosophila neurons*

Stowers et al. (2002) were the first to describe a genetic mutant with disrupted axonal transport of mitochondria and defects in synaptic transmission. They employed a genetic screen of *Drosophila* for mutants with defective photoreceptor function. Flies were further analyzed by electroretinogram to identify those with intact phototransduction but disrupted synaptic transmission to second-order cells. This method identified Milton as a novel protein that when homozygously mutated, caused disrupted transmission of a light-evoked response that was similar to that seen when synaptotagmin, a protein mediating neurotransmitter release, was mutated. The investigators then showed that endogenous Milton and transfected Milton colocalized with mitochondria in peripheral nerves of *Drosophila* larvae and in HEK293T cells, respectively, and that Milton coimmunoprecipitates with kinesin heavy chain (KHC). Furthermore, Milton immunostaining was present in synaptic regions of adult brains, and mitochondria in Milton mutants and null larvae were absent in photoreceptor terminals and instead located entirely within cell bodies. The overall conclusions were that Milton, through

some association with KHC, mediates axonal transport of mitochondria to synapses, and that the lack of mitochondria at synaptic terminals prevents normal neurotransmission by photoreceptors.

The Milton study is important as an identifier of a novel protein that is likely to mediate mitochondrial transport. However, their suggestion that Milton is responsible for synapse-specific mitochondrial trafficking is much less convincing. This conclusion was largely based on finding heavy immunostaining of Milton in brain regions containing high densities of synaptic terminals and axons. The high metabolic activity and Ca^{2+} fluxes of these regions may simply require more mitochondria so that Milton staining indicates overall mitochondrial distribution rather than selective transport to synapses over other locations. This is supported by their data of somatically accumulated mitochondria in Milton mutants, rather than a phenotype of normal mitochondrial distribution in axons but absent in synaptic terminals. Another concern is the health of the Milton larval mutants used, which were sluggish, did not progress past the second larval instar and died 3-5 days after hatching. Mitochondria are known to redistribute perinuclearly in HeLa cells treated with hydrogen peroxide as well as in heat-shocked or TNF-α-treated fibroblasts (De Vos et al., 2000; Lyamzaev et al., 2004). Additionally, mitochondria can localize around the nucleus when cells are treated with uncouplers and inhibitors of respiration (Lyamzaev et al., 2004). Even if cell health was not compromised enough in Milton mutants to cause the abnormal mitochondrial distribution, defects in mitochondrial function were certainly possible and could have accounted for the observed phenotype. No examination of mitochondrial function was performed. In spite of the questionable health of the mutants, photoreceptor axons seemed structurally normal and capable of phototransduction. However, terminals were populated by about half the normal amount of synaptic vesicles. It is really quite surprising then that electroretinogram transients associated with synaptic transmission were

completely absent unless mitochondrial function or some other mechanism associated with neurotransmitter release was also severely compromised. Neither of these possibilities were examined by the authors, but more recent studies have characterized synaptic dysfunctions in different mutants in much greater detail (Guo et al., 2005; Verstreken et al., 2005).

5.3.2.2 *Drosophila dmiro mutants lack presynaptic mitochondria and exhibit abnormal synaptic transmission with prolonged stimulation*

The study by Guo et al. (2005) provides an interesting contrast to the Stowers work whereby disrupted mitochondrial distribution to *Drosophila* neuromuscular junctions (NMJs) was achieved by mutating the mitochondrial Rho-GTPase dMiro. The Miro family proteins are found on the outer mitochondrial membrane and may link cellular signaling events with mitochondrial morphology dynamics (Frederick et al., 2004). Genetic screens identified the *dmiro* mutants as having defective activity-dependent synaptic transmission. Like the lethal Milton mutants, homozygous *dmiro* mutants displayed abnormal locomotion, paralysis and death in the larval to early-pupal stage. Mitochondria were scarce in NMJ axons and presynaptic terminals and were instead arranged in rows in neuronal cell bodies. Additionally, there were an increased number of synaptic boutons but they were abnormally small and clustered and lacked typical microtubule loops. Although some vesicular transport impairment was found in mutant axons, NMJs contained the normal number of synaptic vesicles and synapses. Interestingly, reintroducing dMiro presynaptically in mutants largely restored normal synaptic bouton structure, microtubule loops and vesicular transport but mitochondrial distribution was abnormally restored with accumulations in the most distal bouton of each terminal branch. Prolonged or high frequency NMJ stimulation evoked reduced excitatory junctional potentials (EJPs) that could not be attributed to defective vesicle recycling since miniature EJP frequency

actually increased after stimulation. Lastly, $[Ca^{2+}]_i$ was elevated in resting mutant boutons and reached higher amplitude during stimulation than control boutons. Therefore, the authors concluded that dMiro specifically controls anterograde mitochondrial transport and that mitochondrial localization to presynaptic terminals is required for adequate neurotransmitter release and mitochondrial Ca^{2+}-buffering at NMJs during prolonged stimulation.

While the dMiro study adds a level of sophistication to the Milton study, the defects in their *Drosophila* mutants are more complicated to interpret. It is difficult to reconcile that dMiro specifically mediates anterograde mitochondrial transport when so many other defects are seen with synaptic and microtubule structure as well as vesicle transport. The likelihood that inappropriate mitochondrial distribution alone could account for these other phenotypes does not agree with their findings of (1) just a modest 10-45 nM difference in resting $[Ca^{2+}]_i$ in boutons, which is probably smaller in the remaining cytosol, suggesting that $[Ca^{2+}]_i$ homeostasis is not grossly disrupted by somatically clustered mitochondria, (2) normal mitochondrial ultrastructure and ψ_m, which indicates mitochondrial ATP synthesis is intact, and (3) microtubule defects in *dmiro* mutant muscles where clustered mitochondria remained distributed throughout the tissue so insufficient ATP diffusion could be excluded as a cause. It should be noted, however, that ψ_m was concluded from only visual observation of the presence of polarized mitochondria using the fluorescent dye, JC-1, which accumulates in mitochondria and exhibits different fluorescence properties in a ψ_m-dependent manner. Although mutants resembled controls, few polarized mitochondria were seen in either case and no quantification of ψ_m was performed.

While presynaptic expression of dMiro in mutant NMJs restored many synaptic defects and redistributed mitochondria to synaptic terminals, the functional consequence of these effects were not explored. Demonstration that mitochondria in "rescued" synaptic terminals uptake

Ca^{2+} during stimulation, which they showed in controls, and that normal $[Ca^{2+}]_i$ at boutons and EJPs are restored during prolonged stimulation would have supported their case. Alternatively, the rescued synaptic function could be attributed to some restoration of synaptic structure, although they had already excluded a problem with the physical ability of vesicles to release neurotransmitter and recycle. Another possibility is that restored vesicle density at terminals could account for a rescue of activity. Impaired vesicle transport in *dmiro* mutants may have caused subtle changes in synaptic vesicle content at boutons that were not detected by their measurements. Proper redistribution of vesicles to synaptic terminals could further complicate the attribution of a rescued phenotype to restored mitochondrial transport since vesicles are also thought to contribute to Ca^{2+} regulation at synapses (Israel et al., 1980; Parducz and Dunant, 1993). In summary, this investigation provides a great deal of insight into an evolving picture of the extent that direct mitochondrial localization to presynaptic terminals is required for appropriate function. It no longer seems that general synaptic function is strictly dependent on local supply of ATP and Ca^{2+} sequestration properties by mitochondria. Since reduced EJPs and elevated bouton $[Ca^{2+}]$ were only observed during prolonged stimulation, mitochondria may actually play a role in supporting synapses with heightened activity.

5.3.2.3 *Drp1 mutant synaptic terminals have reduced mitochondria and abnormal neurotransmission; mitochondrial ATP facilitates mobilization of the synaptic vesicle reserve pool*

Verstreken et al. (2005) used another genetic mutant to study the importance of proper mitochondrial distribution for mobilization of reserve pool vesicles in *Drosophila* NMJs. The animals they used were mutant for an ortholog of the mitochondrial fission protein, drp1. Mitochondria in mutant motor neurons had normal ψ_m but showed increased density in cell

bodies whereas mitochondria in axons displayed an elongated morphology. Synaptic structure was not disrupted except for a reduction in the number and hence volume of mitochondria in boutons. Like the *dmiro* mutants, *drp1* mutant NMJs exhibited reduced EJP amplitude during prolonged stimulation as well as increased $[Ca^{2+}]_i$ at rest and after prolonged stimulation. Bathing motor nerves in ATP resulted in a significant but not full recovery of EJP amplitude; it was concluded from this finding that ATP depletion in mutants impairs regaining fusion-competent vesicles. This is an interesting interpretation given that (1) ~70% of mutant synaptic boutons still contained mitochondria, (2) elongated mitochondria were still distributed throughout axons, and (3) mitochondrial function was presumably normal. Furthermore, processes at synaptic terminals necessary for neuronal signaling such as recycling of second messengers and neurotransmitters consume under 10% of total energy usage for neuronal signaling (Laughlin, 2001). The suggestion then seems to be that a ~75% reduction in mitochondrial volume in boutons causes a severe reduction in ATP supply that cannot be met by diffusion of ATP from the abundant mitochondria in the axon shaft but can be partially met by diffusion of exogenously supplied ATP. It might be considered that more synaptic dysfunctions would be observed if ATP depletion were such an issue. Yet Ca^{2+} clearance from terminals, which depends on ATP-powered pumps and direct mitochondrial uptake, was normal during stimulation periods under 30 seconds and vesicle endocytosis and exocytosis were fully operational even after prolonged stimulation. One other caveat to their findings of rescued EJP potentials after nerves were bathed in ATP is that extracellular ATP can also cause synaptic excitation by activating purinergic receptors. In fact, exogenous ATP application at concentrations 1-2 orders lower than that used by Verstreken et al. has been shown to promote

excitatory postsynaptic currents and $[Ca^{2+}]_i$ increases in rat hippocampal neurons, vagus neurons and cerebellar Purkinje neurons (Illes and Alexandre Ribeiro, 2004).

The authors then used the membrane probe FM1-43 to demonstrate an interesting difference in vesicle pool mobilization in *drp1* mutants that they again attributed to insufficient mitochondrial ATP supply. FM1-43 dye is nonfluorescent in aqueous solution but becomes fluorescent when it inserts into cell membranes. It is a valuable tool for labeling actively firing synaptic terminals because recycling vesicles uptake the dye and become highly fluorescent. *Drp1* mutants exhibited normal endocytosis and exocytosis of vesicles into the recycling pool, which is mobilized and continuously recycled during moderate physiological stimulation (Rizzoli and Betz, 2005). However, when the stimulation protocol was modified to label both the recycling and reserve vesicle pool, which is seldom recruited during physiological activity, less dye was taken up by mutant synapses and less dye was retained after the recycling pool was unloaded. This was said to indicate a defect in reserve pool vesicle cycling so they were not loaded during the protocol. Yet it is also possible that the elevated bouton $[Ca^{2+}]$ in mutants facilitated the release of reserve pool vesicles during stimulation of the recycling pool. The recycling vesicle pool loading and unloading profiles in mutants were almost identically recapitulated in controls treated with either the electron transport chain inhibitor antimycin or ATP synthase inhibitor oligomycin. Lastly, more retained dye was evident in oligomycin treated terminals compared to control when high stimulation was provided to unload the reserve pool. While the conclusion was made that mitochondrial ATP synthesis was required for reserve pool vesicle loading and/or mobilization, these drugs were applied to entire larval preparations rather than just at the boutons being measured. Therefore some disagreement exists between their similar findings in neurons with global block of ATP synthesis compared to *drp1* mutants where

mitochondria are theoretically capable of normal ATP synthesis but a problem might exist in locally distributing that ATP to boutons. It should be noted that glycolytic ATP synthesis is unaccounted for, although this is thought to contribute under 5% of cellular ATP in the brain (Erecinska and Silver, 1989). Treatment of *drp1* neurons with ATP restored the vesicle loading/unloading profile, but this may also be an effect on ATP-dependent restoration of $[Ca^{2+}]_i$ homeostasis in terminals which might affect vesicle release. Their attempt to rule out mitochondrial Ca^{2+} sequestration as an influence using TPP^+ was not adequate because TPP^+ inhibits mitochondrial efflux rather than uptake by blocking the mitochondrial Na^+/Ca^{2+} exchanger (Karadjov et al., 1986). In fact, there are no known permeable blockers specifically of mitochondrial Ca^{2+} uptake. Finally, their results suggest that the drp1 blockage of reserve pool mobilization is mediated by inhibition of myosin light chain kinase (MLCK) which normally activates MLC for binding with actin. Myosin mediates vesicle transport on actin filaments, but it may also mediate mitochondrial docking on actin (Chada and Hollenbeck, 2004). Maldistribution of mitochondria at synaptic terminals in their control neurons treated with MLCK inhibitors was not examined and could alter their data interpretations.

In summary, Verstreken et al. offer a mechanistic study of unprecedented detail for the mitochondrial ATP dependence of vesicle pool dynamics in *Drosophila* NMJs. However, the lack of characterization of mitochondrial function coupled with the global application of drugs that significantly affect function of all mitochondria results in ambiguities about (1) the role of ATP supply in general to power vesicle cycling vs. local ATP supply by mitochondria at presynaptic terminals and (2) the role of altered $[Ca^{2+}]_i$ homeostasis in terminals that can be affected by both local mitochondrial sequestration and ATP production and have important implications for neurotransmitter release especially during potentiating stimuli (Tang and

Zucker, 1997). This study would benefit greatly from adapting pharmacologic applications to a more minute scale, as discussed in Chapter 5.2.3, which might also remove the need to perform studies in an inadequately characterized genetic mutant.

5.3.3 Mitochondrial transport to morphogenic dendritic spines

Finally, an investigation by Li et al. (2004) of mitochondrial localization at dendritic spines provides an interesting postsynaptic comparison to the previous presynaptic studies that also highlights the compartmental differences in mitochondrial movement and morphology that we describe in Chapter 3. Using hippocampal neurons, the authors conclude that mitochondria localize to spines during their development and their morphogenesis after stimulation in a manner dependent on drp1-mediated mitochondrial fission. First, mitochondrial association with dendritic protrusions was shown to decline as hippocampal neurons aged *in vitro*. Although it is logical that mitochondria are needed to support recruitment of synaptic components and assembly of postsynaptic specializations during synaptogenesis, it is somewhat surprising that mitochondria are not retained locally to mediate Ca^{2+} signaling at excitatory synapses. If developing spines act as mitochondrial "docking" signals, then their finding also contrasts with our observations of increased rather than decreased mitochondrial movement in synaptically immature neurons. However, the authors only report an 8-12% association between developing spines and mitochondria that could be overlooked in our study. Additionally, the neurons we used, which were cortical rather than hippocampal, may not have contained enough sites of synaptogenesis to exert a noticeable effect on mitochondrial movement. After intense stimulation of cultured neurons and hippocampal slices, formation of new dendritic protrusions were seen, the association between mitochondria and protrusions increased, mitochondria

occupied a smaller fraction of the dendrite shaft and mitochondria moved at slower velocities. This was suggested to be due to fission of mitochondria and redistribution out of the dendrite shaft into protrusions. However, it should be noted that the stimulation protocol used – 3 min perfusions with 90 mM KCl at 10 min intervals repeated in quadruplicate – causes substantial Ca^{2+} influx into cells that recovers slowly. In fact, we tested this very protocol on our cortical neurons and found that recovery of spontaneous synaptic activity was markedly reduced after each KCl pulse and completely lost by the end of the protocol. Elevated $[Ca^{2+}]_i$ is an important consideration because it can cause mitochondria to stop moving and shorten as Li et al. described. They confirm that blocking Ca^{2+} entry through L-type Ca^{2+} channels or NMDA receptors prevents the reduction in movement. Therefore, the cessation of mitochondrial movement could very well be a broad consequence of elevated $[Ca^{2+}]_i$ with little relation to localization into new spines.

To test the role drp1-mediated mitochondrial fission in redistributing mitochondria into dendritic protrusions after stimulation, the investigators transfected neurons and slices with drp1 and a dominant negative mutant, drp1-K38A. Strangely, mitochondrial morphology was longer in drp1-transfected dendrites where fission was shown to predominate. Likewise, mitochondrial morphology was shorter and even punctate in drp1-K38A-expressing dendrites where fusion was more frequent than fission. Compared to controls, more spines were seen in drp1-transfected cells and more PSD-95 puncta formed after KCl treatment. Fewer spines were seen and no increase in PSD-95 density was observed after KCl treatment in drp-K38A-transfected cells. The interpretation that modifying mitochondrial morphology independent of some regulatory signal in the cell can cause mitochondria to adopt a new function, such as the formation of new spines, suggests morphology regulation might be an initial signaling step rather than an effecter.

This is somewhat difficult to reconcile with the identification of regulatory proteins for mitochondrial morphology and the ability to alter morphology with different stimuli as described in Chapters 1.5 and 1.6. Furthermore, it seems to shift the prevailing theory about mitochondrial trafficking and morphology from (A) mitochondria being recruited to signals originating at morphogenic spines or other sites of mitochondrial demand to (B) smaller or divided mitochondria providing a novel function in neurons such as inducing spine formation irregardless of the physiological state of the neuron. This seemingly counterintuitive conclusion suggests that divided mitochondria would have increased metabolic activity, which has not been reported thus far. Another possibility is that the increased number of mitochondria generated by fission may now be freely recruited to targeting factors that could not be served by the normal number of mitochondria.

The authors argue that drp1 is required for activity-dependent regulation of mitochondrial movement since drp1-K38A transfected cells did not demonstrate any changes in movement with TTX or KCl treatment that were seen in controls. However, if mitochondrial movement is related to recruitment into spines which might explain why TTX increases movement in controls, then movement would be expected to be elevated in drp1-K38A-transfected cells because they have fewer spines and do not demonstrate KCl-induced increase in PSD-95 density. Alternatively, drp1-K38A may be toxic to cells. This is supported by their results in transfected neurons because (1) mitochondria were punctate and resembled that which is observed in injured cells, although no measure of mitochondrial function was made, (2) mitochondrial movement was reduced as is the case in injured cells, although there was a large inconsistency between separate measurements in drp1-K38A transfected cells, (3) spine number was markedly reduced perhaps due to degeneration, (4) PSD-95 density was not upregulated after KCl treatment

perhaps because neurons were unhealthy and did not confer the appropriate ion changes and signaling events necessary for postsynaptic remodeling, and (5) activity-dependent alterations in fusion and fission events was absent, even though mitochondrial fusion mechanisms should still be intact and were shown to predominate after TTX treatment in control and even drp1-expressing cells.

Although this study uses the much simpler experimental system of cultured cells and slices rather than genetic mutants, it is perhaps the least clear story. The mitochondrial morphology of transfected cells contradicts the known function of drp1 as a fission protein and the data are too incohesive to implicate drp1-mediated fission as a regulator of mitochondrial morphology and distribution dynamics for spine morphogenesis. What is most convincing though, is that mitochondria can be observed extending into dendritic protrusions over the course of a few hours after stimulation. This implicates morphogenic spines as a new target for mitochondrial trafficking. The fact that only a small fraction of protrusions seemed to recruit mitochondria, approximately 3% before and 12% after stimulation, further suggests that mitochondria are dynamically trafficked to subsets of synapses with specific activity characteristics. The dissertation work examined mitochondrial trafficking with high temporal resolution while the work of Li et al. achieved high spatial resolution, and both studies are in agreement. The next logical step is to identify distinguishing functional qualities of synaptic sites that recruit mitochondria. Perhaps it is also these very qualities that serve as cues for mitochondrial targeting.

5.4 IMPAIRMENT OF MITOCHONDRIAL MOVEMENT IN INJURY AND DISEASE

Mitochondrial trafficking is emerging as a more complex process than previously appreciated. On the one hand, neuronal development seems to proceed even when anterograde movement of mitochondria is hindered, and presynaptic function is even intact during short stimulations. Although the requirements for mitochondrial localization and recruitment to synapses may be dictated by other qualities of individual synaptic firing, mitochondrial distribution throughout axons and dendrites seems important for neuronal health. After all, defective mitochondrial movement likely contributes to the early death of mutant *Drosophila* larvae where mitochondria are clustered near the cell body. In this section, we discuss how acquired impairments in mitochondrial movement can be related to neuronal injury and disease. Although this has been implicated in a few studies, a clear cause and effect has yet to be demonstrated. We address mitochondrial trafficking impairments in both acute, severe insults as well as in the development of localized synaptic dysfunctions and neuritic degeneration relevant to chronic neurodegeneration.

5.4.1 Neuritic degeneration

Central neurons are highly susceptible to injury. They are postmitotic and therefore cannot reproduce after injury like cells of many other tissues. Unlike peripheral neurons, central neurons also have a highly limited capacity for axonal regeneration. This is because brain extracellular matrix lacks proteins such as laminin and fibronectin that promote axon growth, and mature neurons typically stop synthesizing intracellular proteins, such as GAP-43, that mediate axon growth during development. Neuronal injury can also be extensively destructive because

cells that synaptically connect to or receive synaptic connections from injured neurons often degenerate (Kandel et al., 1991).

Axonal degeneration may be a more substantial process than dendritic degeneration and it has certainly been studied more extensively. Axons are much longer than dendrites and distal regions are therefore more removed from protein synthetic machinery near the cell soma and proximal dendrites that could facilitate reaction to injury. Also, the highly branched nature of axons might make segments downstream from the site of injury more vulnerable to deterioration. Wallerian degeneration is activated after severe traumatic injury such as axotomy and is characterized by swelling of the axon stumps due to continued axonal transport, rapid loss of synaptic transmission in the distal terminals, cytoskeletal breakdown and degradation of the distal axonal segment. Glial cells assist in removing cellular debris and synaptic contacts between the injured cell and postsynaptic neurons (Griffin et al., 1995). Elevated Ca^{2+} entry also occurs in the damaged axon and can disrupt cell function extensively as described in Section 1.7. Chromatolysis occurs in some cells, which involves restructuring of the nucleus and ER for increased RNA and protein synthesis likely related to repairing the severed axon (Kandel et al., 1991).

Selective elimination of neuronal processes also occurs during less severe neuronal injury, disease and aging. This can have a protective role by removing sources of dysfunctional synaptic transmission. "Dying back degeneration" is a term used to describe initial degeneration of distal axon segments that proceeds back toward the cell body and results in axon fragmentation resembling Wallerian degeneration (Cavanagh, 1964). The mechanisms of neuritic degeneration are not well understood, but probably involve some sequence of NAD metabolism, microtubule and neurofilament breakdown, and activation of the ubiquitin-

proteasome system perhaps for degradation of neuroprotective proteins (Zhai et al., 2003; Araki et al., 2004). Neuritic degeneration has been shown to be independent of caspase-dependent cell death such that caspase inhibition and Bcl-2 overexpression can prevent apoptosis but not axon degeneration. However, delaying axonal degeneration in a genetic mouse model of spinal muscular atrophy actually improved symptoms and increased lifespan (Luo and O'Leary, 2005). Gradual degeneration of neuronal processes therefore seems to be an important contributor to neurodegenerative symptoms before overt neuron loss.

Since trafficking of mitochondria through axons and dendrites is important for appropriate ATP distribution and Ca^{2+}-buffering, notably for neurotransmission, impaired mitochondrial movement would likely cause synaptic dysfunctions, ATP loss, altered $[Ca^{2+}]_i$ homeostasis and production of ROS. Some or all of these pathological findings characterize HD, PD, AD, ALS, spinobulbar muscular atrophy and ischemic injury. Furthermore, our dissertation work and other studies confirm that mitochondrial transport is defective in these conditions and suggest that it may be a pathologic contributor common to many neurodegenerative processes.

5.4.2 Extent and distribution of stopped mitochondria as a determinant of pathology

The dissertation work contributes an exciting perspective for future studies whereby mitochondrial movement may actually be impaired with regional selectivity in neurons under different injury conditions (Chapter 3). Not only is the spatial distribution of impaired mitochondrial transport important, but the temporal span and intensity of the insult also contribute to the degree of impaired movement. Therefore, pathophysiological events, neuritic degeneration and clinical manifestations may have remarkably different manifestations depending on whether mitochondrial transport is affected throughout neurons, in axons,

dendrites, or in localized regions of certain neuronal processes, and whether movement recovers. For example, when mitochondrial movement is slowly and progressively impaired, degeneration will probably start in distal neuronal processes where transport from the cell body poses the greatest challenge. When mitochondrial movement halts suddenly, neuritic degeneration might be expected to proceed rapidly to cell death, unless mitochondrial movement recovers before irreversible downstream signaling cascades are activated. The discussion that follows speculates on the degenerative processes involved in various neuronal injuries as the characterization of mitochondrial movement impairments causing clear pathological consequences remains largely undefined.

5.4.2.1 *Whole-cell impairments in mitochondrial movement*

Our findings indicate that pathological conditions that cause ψ_m depolarization, inhibition of mitochondrial ATP synthesis, and elevated $[Ca^{2+}]_i$ throughout entire neurons will also impair mitochondrial movement uniformly in axons and dendrites during the insult (Chapter 3). As discussed in Chapters 1.7 and 1.8, these three attributes are highly intertwined. Mitochondrial Ca^{2+}-uptake can depolarize ψ_m which would reduce the drive for ATP synthesis. Depolarization of ψ_m can also result from electron transport chain defects, which would limit ATP synthesis and mitochondrial Ca^{2+} uptake. Elevated cytosolic $[Ca^{2+}]$ may also mediate morphologic remodeling of mitochondria, the physiological consequence of which is yet to be determined. The effects of impaired mitochondrial ATP synthesis on powering active Ca^{2+} clearance and maintaining ion gradients that can affect oxidative phosphorylation depend on the compensatory ability of glycolytic ATP production and probably become more important over long time periods.

Impairment of mitochondrial movement in a cell by mechanisms that involve dysfunctions of any given mitochondrion is probably most relevant to genetic diseases of

mitochondrial proteins, but could also include chronic neurodegenerative diseases. First, let us consider diseases caused by mtDNA mutations. Mitochondria contain multiple copies of mtDNA which can be nonidentical, so respiratory chain malfunction and expression of a diseased phenotype depends on adequate accumulation of mtDNA mutations within both mitochondria and cells, the severity of the mutation, the replicative advantage of the mutation over the normal allele, and the cell's vulnerability to the mutation (Graff et al., 2002). Even though mutated mtDNA may initially be contained in a subpopulation of mitochondria, sharing of mtDNA varies between organelles and can contribute to distribution of mtDNA mutations throughout the entire mitochondrial population. As a few examples, mitochondrial damage that impairs movement could result from respiratory defects due to mutations in mtDNA encoding mitochondrial tRNA causing mitochondrial encephalopathy lactic acidosis and stroke-like episodes (MELAS) and myoclonus epilepsy and ragged red fibres syndrome (MERFF) and mtDNA mutations encoding complex I genes leading to Leber's hereditary optic neuropathy (LHON).

Defective mitochondrial proteins can also be acquired from mutations of nuclear-encoded genes. The incorporation into mitochondria of abnormal proteins transported from the cytoplasm is probably nonspecific and heterogeneous between individual organelles. Diseases arising from mutated nuclear-encoded mitochondrial genes include Leigh syndrome caused by a complex II defect and encephaolomyopathy resulting from a complex I gene mutation (Orth and Schapira, 2001; Graff et al., 2002). Other diseases caused by genetic mutations in nuclear genes encoding mitochondrial proteins unrelated to oxidative phosphorylation, including Friedreich's ataxia, hereditary spastic paraplegia and Wilson's disease involve pathogenic processes such as disrupted metal homeostasis and oxidative stress that are not as easily attributable to the entire

mitochondrial population. As we have shown, zinc impairs mitochondrial movement differently in axons and dendrites, and a compartmental distinction for the cessation of movement by oxidative stressors has not yet been made.

The pathogeneses of chronic neurodegenerative diseases are also highly complex. However, common features of AD, ALS and HD are ψ_m depolarization, respiratory defects and abnormal $[Ca^{2+}]_i$ homeostasis (Chapter 1.8). It is reasonable to predict that prolonged disease courses, coupled with ongoing disruptions such as oxidative stress and mtDNA damage, may predispose the vast mitochondrial population to injury. Our findings of indiscriminately impaired mitochondrial movement with rotenone, the complex I inhibitor implicated in PD, provides a disease correlate where oxidative stress is thought to be the primary effecter of neuronal death, although our laboratory has also found that chronic subtoxic rotenone treatment depolarizes ψ_m (unpublished data). It should be noted that global mitochondrial trafficking defects in chronic disease can also be caused by factors other than mitochondrial dysfunctions, such as interactions between diseased proteins and cytoskeletal or motor protein constituents (Trushina et al., 2004). Additionally, concurrent local mitochondrial trafficking defects can be contributed by insoluble protein inclusions that form with or without compartmental specificity in AD, ALS and HD as discussed later in this chapter.

Chronic mild mitochondrial dysfunction can gradually slow mitochondrial transport and lead to synaptic and cellular dysfunctions at an even slower pace corresponding to the protracted course of neurodegenerative disease. This is supported by findings of only modest disruption in vesicle cycling at synaptic terminals when mitochondrial ATP synthesis is blocked, with the greatest defects in mobilization of the reserve pool which is not recruited at physiological stimulation levels anyway (Verstreken et al., 2005). Furthermore, if mitochondrial movement

stops “in place” as our experiments show, then mitochondria that presumably still retain some functional capacity would continue to be well distributed in neuronal processes and support cell demands statically. The overall consequence of persistent and progressive mitochondrial movement impairment can be considered nonspecific dysfunction at pre- and postsynaptic terminals and degeneration of axons and dendrites. This would likely progress in a dying back degenerative pattern initiating in distal neuronal process segments, especially axons, that are less likely to receive ATP from the large mitochondrial population in the cell body. Similarly nonspecific degeneration of pre- and postsynaptic neurons contacting the diseased cells could then follow, probably with postsynaptic neurons degenerating first in correspondence with the earlier axonal degeneration of the injured neurons. In summary, impaired mitochondrial trafficking can be an early initiator of synaptic dysfunction and neuritic degeneration that may take years to cause substantial neuron loss and clinical symptoms in progressive diseases.

5.4.2.2 *Axon- or dendrite-specific impairments in mitochondrial movement*

One of the most important contributions of this dissertation work is the discovery that mitochondrial movement can be impaired differently in axons and dendrites after neuronal insults relevant to acute injuries such as ischemia and trauma. Movement impairment could be characterized as either cessation during the insult or the irrecoverable persistence of that cessation after drug is washed away. Both situations were demonstrated selectively in dendritic mitochondria – the former after glutamate treatment and the latter after zinc/chelation treatment. Additionally, acute recovery of mitochondrial movement after uncoupling oxidative phosphorylation was only observed in axons.

These are interesting findings because studies on neuritic degeneration often focus on axons rather than dendrites. In fact, it might be presumed that dendrites are less vulnerable to

dysfunction and degeneration because even their most distal ends are relatively close in proximity to the cell body from where proteins mediating repair can be more rapidly recruited. Additionally, mitochondria are more fully distributed through dendrites, so it might be predicted that ATP can be spatially dispersed over a reasonable area even as mitochondrial movement and function become compromised. The most significant consequence of impaired mitochondrial trafficking in dendrites might therefore be the dynamic recruitment of mobile mitochondria into excitatory spines. This may be especially relevant as remodeling of postsynaptic structures might be relevant during intense release of neuromodulators of excitatory synapses, including glutamate and zinc, into the extracellular space during ischemic or epileptic events (Benveniste et al., 1984; Lobner and Lipton, 1990; Bresink et al., 1996; Paoletti et al., 2000). Postsynaptic dysfunction might therefore be an early consequence of selectively impaired mitochondrial movement or slow recovery of movement in dendrites during excitotoxic injury involving glutamate and zinc.

The idea that irreversible cessation of mitochondrial movement in dendrites leads to degeneration and cell death is supported by the finding that under our experimental conditions, chelation of zinc after a neurotoxic exposure selectively restores axonal mitochondrial movement but does not provide any protection against neurotoxicity (Chapter 3 and Malaiyandi et al., 2005). If impaired dendritic mitochondrial movement and subsequent dendritic degeneration significantly mediate zinc-induced toxicity, then findings in other experiments demonstrating chelation-mediated protection from cell death may have been performed during the permissive exposure durations or concentrations where dendritic mitochondrial movement could still be reversed (Canzoniero et al., 2003). Further support for cessation of mitochondrial movement and general disruption in dendrites comes from our studies with glutamate. After an intense, acute

glutamate exposure, mitochondria stop moving in all dendrites with recovery over hours and in proximal axons with recovery over minutes while mitochondria in distal axons are spared. Yet 30-50% neurons proceed to die by delayed excitotoxicity. Prolonged cessation of movement and mitochondrial dysfunction in dendrites may exacerbate recovery of cytoskeletal disruptions, $[Ca^{2+}]_i$ homeostasis and synaptic function. Indeed, loss of dendritic spines after severe ischemia and recovery of spine structure after reperfusion may be mediated in part by effects on mitochondrial movement (Zhang et al., 2005).

While our results strongly implicate regional selectivity of impaired mitochondrial movement as a potential cause of dendritic degeneration during acute neuronal injury, the same reasoning may be applied to axonal degeneration in AD. One aspect of AD pathophysiology begins with overexpression of tau, a microtubule-associated protein (MAP) found in axons, which blocks motor protein binding sites on microtubules and thus reduces axonal transport of mitochondria, vesicles and endoplasmic reticulum (Ebneth et al., 1998). Tau hyperphosphorylation is thought to be a protective measure taken by the cell to dislodge the protein from microtubules. However, hyperphosphorylated tau forms neurofibrillary tangles in axons that can further act as physical roadblocks for transport. Lastly, the dislodgement of tau exposes microtubules to severing proteins that can ultimately result in axonal degeneration (Baas and Qiang, 2005). Therefore, mitochondrial movement can be selectively impaired in axons or dendrites, contributing to degeneration of the affected processes.

5.4.2.3 *Discrete sites of impaired mitochondrial movement*

Mitochondrial transport can be inhibited by roadblocks of insoluble protein accumulations in neuronal processes. This mechanism is implicated in several neurodegenerative diseases with protein abnormalities and probably occurs concurrently with the gradual decline in trafficking of

damaged mitochondrial described in the previous sections. Discrete sites of movement impairment represent a different pathology where the mitochondrial pool as a whole probably retains normal function, but gradual titration of organelles out of the pool reduces the availability of recruitable mitochondria and the efficiency with which ATP can be dispersed and $[Ca^{2+}]_i$ can be buffered. Since mitochondrial movement is so dynamic and can probably compensate for reduced mitochondrial number quite effectively, synaptic and neuritic degeneration likely develops over a very long time period. Synaptic dysfunction may actually be one of the first manifestations since we and others have shown mitochondrial trafficking to synaptic sites over minute to hour timeframes (Chapter 3 and Li et al., 2004).

Cytosolic aggregates and transport defects are part of the pathology of multiple diseases, although it is not always clear if the aggregates are acting as physical roadblocks or if they impair trafficking more broadly by interactions with transport-related proteins and chaperones. Furthermore, the conclusion that transport is inhibited often relies on fixed images revealing particle accumulations rather than dynamic particle tracking that would provide qualitative and quantitative information about the defect. With relation to HD, we specifically found mitochondria to accumulate around and become immobilized by mutant Htt aggregates (Chapter 4). In AD, neurofibrillary tangles and altered APP expression are associated with axonal transport defects (Ebneth et al., 1998; Torroja et al., 1999; Gunawardena and Goldstein, 2001). Formation of neurofilament "torpedoes" and a corresponding reduction of organelles were found in Purkinje cell axons of a patient with the polyglutamine disease spinocerebellar ataxia type 6 (Yang et al., 2000). In spinobulbar musclar atrophy, androgen receptors containing a mutant polyglutamine repeat aggregate in neurites and colocalize with mitochondria (Piccioni et al., 2002). As a final example, increased mitochondria, especially with a rounded morphology, were

observed in axon hillocks and initial axonal segments of anterior horn cells in human and mouse models for ALS (Sasaki and Iwata, 1996; Sasaki et al., 2005). This is thought to be caused by impeded axonal transport of mitochondria by neurofilament accumulations near the cell soma, ultimately causing axonal degeneration (Collard et al., 1995).

It is less straightforward to predict how neuronal processes will degenerate in diseases where insoluble proteins aggregates form indiscriminately in a cell. One possibility is that blockage and accumulation of mitochondria by aggregates in distal processes will be more severe because those regions receive less total mitochondrial traffic that could help compensate for reduced mitochondrial numbers. Alternatively, it can be predicted that mitochondrial movement would be most significantly impaired where aggregates are largest and where titration of organelles is facilitated by high mitochondrial traffic. The most mitochondrial traffic converges on proximal branches of axons and dendrites and the larger caliber of these segments probably also permits larger aggregates to form. It is in these regions where perpetuation of mitochondrial accumulation around aggregates and increasing aggregate size is probably maximal. Severe blockages in proximal segments of axons and dendrites could facilitate degeneration of all downstream branches. It is important to note that cytosolic aggregates often block other transported proteins and organelles as well, so titration of synaptic vesicles, receptors, chaperones and proteasomes can also contribute to cellular dysfunction and degeneration.

5.5 MECHANISMS FOR REGIONAL DIFFERENCES IN MOVEMENT IMPAIRMENTS

5.5.1 Source of insult

One potential mechanism to account for mitochondrial movement to preferentially stop in one cellular region over another is that the insult is targeted to or distributed over a specific region. We showed that mitochondrial movement in axons and dendrites is equally susceptible to impairment by elevated $[Ca^{2+}]_i$, but intense Ca^{2+} entry is rarely distributed over entire neurons under pathological conditions. Instead, the source of stimulus for Ca^{2+} entry must be considered, as well as the locations of Ca^{2+} entry points in the affected cell. Elevated glutamate release has been demonstrated during ischemic events and traumatic brain injury (Nishizawa, 2001). Activation of ionotropic glutamate receptors on dendrites mediates the profound and neuroxtoxic Ca^{2+}-influx into cells. Therefore, it can be expected that cessation of mitochondrial movement will be most severe in the dendritic compartment, with axonal involvement dependent on the ability for neurons to spatially buffer the elevated Ca^{2+}. Indeed, our results confirmed this regionally selective effect on mitochondrial movement after acute treatment with an excitotoxic glutamate stimulus. NMDA receptors are also known to mediate elevated $[Ca^{2+}]_i$ in hippocampal neurons during seizures, acquired epilepsy and excitotoxic susceptibility during alcohol withdrawal (Nagy, 2004; Raza et al., 2004). Selective impairments in mitochondrial movement may therefore play a pathophysiological role in dendritic degeneration observed in injuries involving Ca^{2+} influx through glutamate receptors (Lin et al., 1997).

Another example of a localized source of pathologic Ca^{2+} influx is that which occurs during axon shearing and stretch injury after head trauma. The pathophysiologic process in these

cases involves axonal deformation triggering Na^+ influx through voltage-gated Na^+ channels, followed by inward flow of Ca^{2+} through the Na^+/Ca^{2+} exchanger and voltage-gated Ca^{2+} channels. Influx of Na^+ and Ca^{2+} may be potentiated by Ca^{2+}-mediated proteolysis of the Na^+ channel α subunit that prevents its inactivation (Wolf et al., 2001; Iwata et al., 2004). Altered $[Ca^{2+}]_i$ homeostasis and prolonged elevations in $[Ca^{2+}]_i$ result in secondary damage to axons from downstream signaling cascades and mitochondrial failure (Weber et al., 1999). Mitochondrial movement can be predicted to stop at the site of axonal injury and may involve expanding regions of elevated $[Ca^{2+}]_i$. Clearance of $[Ca^{2+}]_i$ may be further compromised by the inability of healthy mitochondria to transport to the damaged axon segment. Therefore, impaired mitochondrial movement in axons may be an important contributor to axonal degeneration after traumatic injury.

A third example of source-specific impairments in mitochondrial movement is that which is produced by insoluble protein aggregates in neuronal processes. The underlying pathologic processes governing aggregate formation would dictate any selectivity for axons or dendrites and a proximal or distal distribution. For example, the axonal distribution of tau means neurofibrillary tangles form preferentially in axons and block axonal transport in AD. At least in our investigation of mutant Htt aggregates, we found no preference for axonal or dendritic aggregate formation nor in the vulnerability of mitochondrial transport in any specific region to blockage. The degree of impairment may therefore rely on qualities such as aggregate size, time required for aggregate formation, and amount of mitochondrial traffic in the vicinity of the aggregate. In summary, while all mitochondria may exhibit the same physiological vulnerability to movement cessation by certain insults, the physiology of injury and disease can stop mitochondrial movement and cause neuritic degeneration with regional selectivity.

5.5.2 Influences of axonal and dendritic physiology

Axons and dendrites differ vastly with regard to anatomy and function, so compartmental differences in protein distributions, signaling mechanisms and cytoskeletal arrangements could cause mitochondrial movement to stop or recover in different manners. Studies of *C. elegans* and mammalian motor proteins suggest that different kinesins can display selectively for axons or dendrites (Setou et al., 2004). This allows cargos such as synaptic vesicles or neurotransmitter receptors to be transported to the appropriate neuronal processes. If the same targeting rules apply to mitochondrial cargos, then molecular motor isoforms in axons and dendrites probably differ and their differential regulations could very well affect movement cessation and subsequent recovery. Any compartmental differences in cytoskeletal properties that mediate mitochondrial movement and docking may also affect how rapidly mitochondria stop moving or recover after cytoskeleton-modifying insults like glutamate. Examples of cytoskeletal properties to consider are microtubule assembly and disassembly processes, volume of cytoskeletal substrates, activities of microtubule-severing proteins, and phosphorylation states of microtubule-associated proteins that are themselves often distributed preferentially in axons or dendrites (Baas et al., 2005; Baas and Qiang, 2005). Movement recovery may also be delayed in dendrites if the relatively longer mitochondria require reformation of longer microtubule tracks for transport.

With regard to our studies with zinc, either separate signaling pathways or faster activation of the same pathway may account for zinc's ability to cause irreversible cessation of mitochondrial movement in dendrites but chelation-dependent recovery of movement in axons during a single exposure. Zinc-mediated inhibition of mitochondrial movement and toxicity depends on the concentration and duration of zinc treatment. Impaired movement after less

severe zinc exposure can be reversed by chelation in axons and dendrites, but increased exposure activates a PI3-kinase mediated signaling cascade of movement cessation that can no longer be reversed by chelation in dendrites (Malaiyandi et al., 2005). This may be due to (1) improved Zn^{2+}-buffering in axons by greater availability of Zn^{2+}-binding proteins such as metallothioneins which can protect against zinc toxicity, (2) enhanced efflux of toxic cytoplasmic Zn^{2+} levels into the extracellular space or into cellular compartments by metal transporters and (3) the presence of high capacity zinc storage vesicles in presynaptic terminals whose release onto postsynaptic neurons might promote excitotoxicity (Palmiter et al., 1996; Aschner et al., 1997; Cole et al., 1999; Frederickson et al., 2004; Liuzzi and Cousins, 2004). These possibilities suggest that the $[Zn^{2+}]_i$ needed to activate downstream pathways mediating irreversible movement inhibition can eventually develop in axons once Zn^{2+}-clearance mechanisms are saturated. An alternate hypothesis is that zinc signaling actually differs in axons and dendrites so that either PI 3-kinase-mediated inhibition of movement is never activated or downstream effecters are effectively neutralized perhaps by increased phosphatase activity in axons. Interestingly, movement cessation in dendrites upon PI 3-kinase activation may also be mediated by phosphorylation of neurofilaments which has been shown to increase mitochondrial binding and immobilization (Wagner et al., 2003).

5.5.3 Functional differences in individual mitochondria

The mitochondrial population is diverse at least with regard to trafficking and morphology, so it makes sense that functional variation also exists between individual organelles. First of all, mitochondrial lifespan is limited so there will inevitably be a subset of unhealthy mitochondria in a cell, even if they are being transported for removal (Miller and Sheetz, 2004). Among healthy

mitochondria, functional heterogeneity in ψ_m may result from different numbers of oxidative phosphorylation enzymes or ion pumps that maintain appropriate gradients across mitochondrial membranes. Functional heterogeneity of mitochondria could very well influence how the movement of a given organelle will be affected by injurious stimuli. Since ψ_m-depolarization reduces movement, mitochondria with inherently lower ψ_m might reach the threshold for movement cessation more rapidly when faced with depolarizing insults including elevated $[Ca^{2+}]_i$. Similarly, mitochondria that can regenerate ψ_m faster could also recover movement earlier after an insult is removed.

Variation in the capacities of individual organelles to safely uptake Ca^{2+} before frank ψ_m depolarization and activation of membrane permeability transition is another important consideration. In order to sequester large amounts of cytosolic Ca^{2+} without dangerously increasing matrix free $[Ca^{2+}]$, the mitochondrial matrix contains inorganic phosphates (P_i) that precipitate out Ca^{2+} as well as various Ca^{2+} binding proteins (David, 1999). Thus, mitochondrial heterogeneity in Ca^{2+} uptake can be achieved by differences in ψ_m, numbers of Ca^{2+} uniporter, and concentrations of P_i and other Ca^{2+} buffering proteins in the matrix. Indeed, we have seen that individual mitochondria isolated from rat brain exhibit heterogeneity in ψ_m depolarization in response to Ca^{2+}, although the mechanism for this has yet to be determined (Vergun et al., 2003). Individual mitochondrial Ca^{2+} uptake properties can modulate movement by their influences on ψ_m and $[Ca^{2+}]_{cyto}$.

Mitochondrial morphology also has important implications for movement because it can impact the volume of mitochondrial compartments, the ion concentrations within them, and consequently the ability of enzymes to maintain ψ_m and efficiently generate ATP. Given the dynamic fission and fusion events and the interchange of membranes, enzymes and mtDNA that

occur between mitochondria, it is doubtful that functional homogeneity can be conserved between all mitochondria. However, it is interesting to consider that if mitochondria of similar morphology are functionally similar, then this could contribute to a general difference in mitochondrial movement response in axons and dendrites since the axonal population of mitochondria are stereotypically shorter than that of dendrites. Additionally, mitochondria are exposed to different local demands for $[Ca^{2+}]_i$ uptake in cells that could impact the energetic state of individual mitochondria (Park et al., 2001). A last possibility unrelated to mitochondrial function per se, is that mitochondrial motor isoforms which are known to be differentially modulated by phosphorylation and protein interactions, may be variably distributed on individual organelles (Sheetz, 1999; Reilein et al., 2001). This may account for different motility responses between different mitochondria, and may also explain the axonal and dendritic difference if each compartment employs separate motor isoforms.

It is important to note that while the end result of many of our pharmacologic manipulations is homogenous cessation of mitochondrial movement in the affected compartment, individual organelles with increased duration of retained motility can certainly be identified. Even more striking is the heterogeneity of movement recovery after drug washout. Sometimes a sole moving mitochondrion can be observed in a given field; other times a small population of mitochondria regain motility. Unquantified observations suggest that the mitochondria that regain movement earliest tend to travel with high velocity. It is interesting to speculate on whether some functional properties of fast-moving mitochondria predisposes them to rapid movement recovery, or if certain mitochondrial recruitment sites develop after drug treatment to which the small number of mitochondria that have regained motility are rapidly targeted after washout. It should be noted that we often applied relatively sizeable

concentrations of drugs to attain robust drug actions. The organellar heterogeneity in movement cessation is accentuated when concentrations are significantly reduced, as we have observed with glutamate and rotenone (unpublished observations and Reynolds and Santos, 2005).

5.6 OVERALL ASSESSMENT AND FUTURE DIRECTIONS

The discussion thus far has reviewed why mitochondrial trafficking is challenging to quantitatively and mechanistically study, what the dissertation work and past studies have revealed about mitochondrial trafficking patterns and the regulation thereof, and the limitations of those studies. We proposed that reducing the scale of pharmacologic and imaging techniques should be adopted in future experiments to gain unprecedented resolution of trafficking mechanisms among individual mitochondria. By combining fluorescence imaging, electrophysiology and local pharmacologic manipulations of individual synapses, we will be able to uncover the dynamics and functional purposes of mitochondrial localization and recruitment for supporting synapses during physiological synaptic activity, short and long term plasticity. Local irreversible inhibition of ATP synthesis by mitochondria normally localized at a synapse could reveal not only what neurotransmission properties depend on local ATP supply but also whether compensatory recruitment of intact mitochondria occurs. This would provide crucial information on the significance of mitochondrial trafficking in normal neurotransmission related to learning and memory, as well as how synaptic transmission and plasticity can fail if mitochondrial movement is impaired. Elucidating these physiological and pathophysiological properties would be relevant to any neurodegenerative disease characterized by synaptic dysfunction and especially to AD where memory loss is an early and devastating clinical feature.

Studies often focus on where and why mitochondria are transported to specific locations, but much is still unknown about the mediators of the physical trafficking of mitochondria. This includes the individual motive properties of mitochondria-transporting motor protein isoforms, the identity of scaffolding proteins that associate motor proteins with mitochondria, and how mitochondrial motility patterns can be modulated by regulatory modifications of scaffolding proteins, motors and other accessory proteins. More detailed analysis will reveal whether certain mitochondrial populations, for example those in axons or dendrites or those with shorter or longer morphology, tend to associate with certain scaffolds and motors that dictate motile capabilities. In terms of the saltatory properties of mitochondrial movement, chemical and physical cues that recruit mitochondria need to be clarified, as do signals, mediators and regulatory events required for physical docking of mitochondria on the cytoskeleton. Understanding how mitochondrial trafficking is executed and how those executioners can be regulated will provide insight into how dysregulation during injury alters mitochondrial movement. Just as importantly, it will provide specific targets for pharmacologic manipulations developed to restore impaired mitochondrial movement.

We then discussed the relationship between impaired mitochondrial movement and neuronal injury and disease. This is a logical relationship but one that is difficult to elucidate because of concurrent and ongoing pathophysiological events in acute and chronic neurological conditions. It is necessary to determine whether impaired mitochondrial movement is a cause of further pathology or if it is an effect of more significant effecters of injury. Our examination of mutant Htt aggregates offers an example of a subtle trafficking defect that precedes other mitochondrial, synaptic or cellular disruptions. However, the story is rarely so simple. Cessation of mitochondrial movement is highly sensitive to many stimuli present in disease

states such as elevated $[Ca^{2+}]_i$, oxidative stress, inhibition of electron transport and uncoupling of oxidative phosphorylation. In most cases mitochondrial movement can therefore be predicted to stop as a result of some other pathophysiological effecter. The important questions that need to be addressed in the future then, are at what point in the injury process mitochondrial trafficking is compromised, what the functional consequences of stopped movement are, and the time required for irreversible cellular damage and overt phenotypic expression. Understanding these qualities will allow researchers to decide what pharmacologic interventions should be targeted to preserving mitochondrial movement and when those interventions should be performed.

While a great deal is understood about mitochondrial physiology and cell survival and death mechanisms in neurons, our grasp of mitochondrial trafficking is only beginning to emerge. Despite all the complexities, the last decade has been especially fruitful in producing detailed descriptions of movement patterns, identifying potential targets and functional roles for mitochondrial trafficking, and implicating disrupted mitochondrial transport in injurious and degenerative conditions in neurons. Efficient progress is largely contributed by the growing appreciation for the importance of mitochondrial movement and the interdisciplinary enthusiasm in studying mitochondrial function in neurons, trafficking mechanisms, and mitochondrial dysfunction in disease. Increasingly sophisticated experimental techniques can be harnessed to finally study mitochondrial trafficking at the microscopic level needed to make specific mechanistic conclusions. Ultimately, it is from our understanding of how normal mitochondrial trafficking supports cellular function that we can derive how abnormal trafficking will cause cellular dysfunction and neuritic degeneration in injury and disease.

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