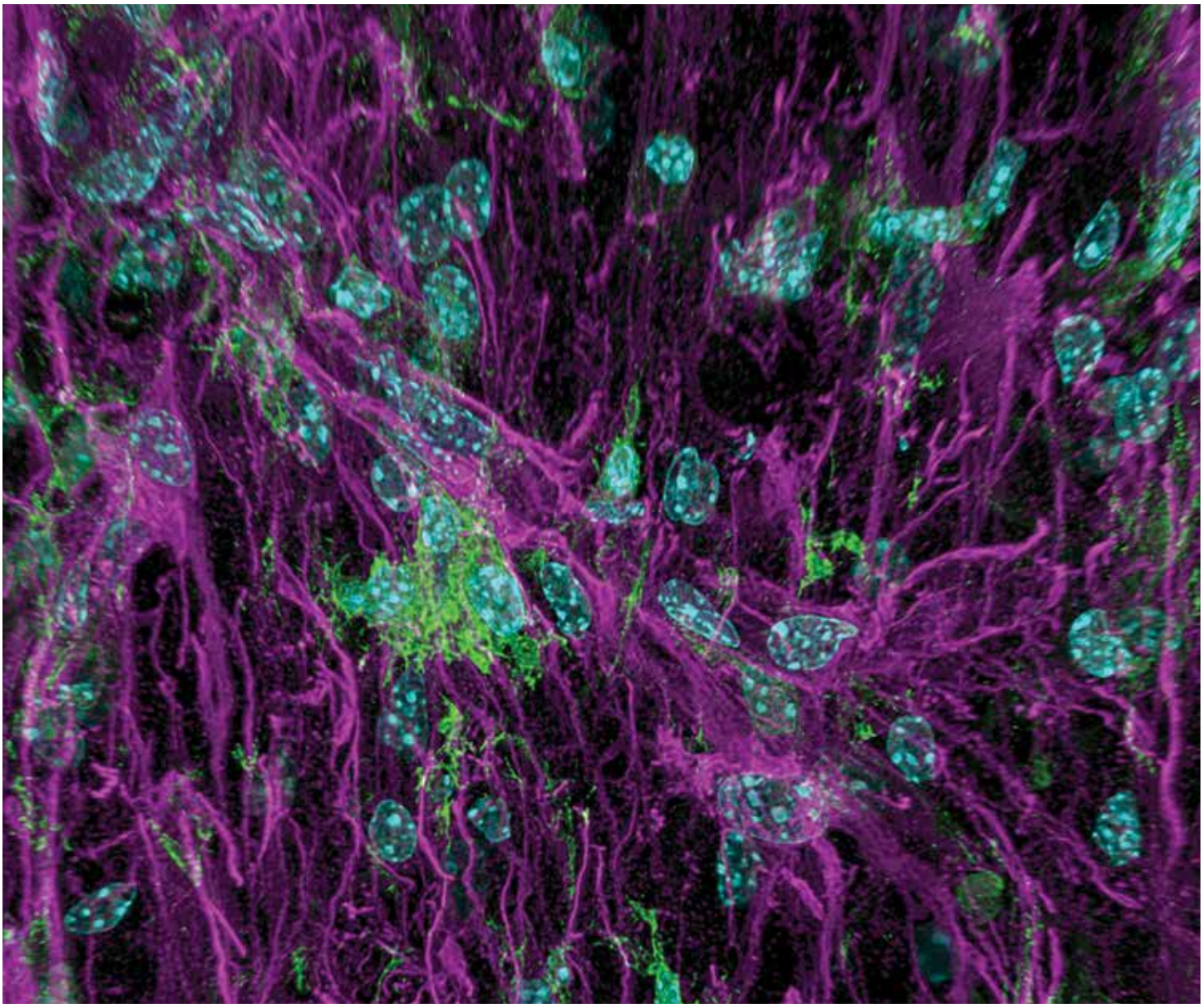

Neuroscience 2016

NEUROBIOLOGY OF DISEASE WORKSHOP

From Pediatric Encephalopathy to Alzheimer's: Linking Mitochondria to Neurological Diseases

Organizers: Heidi McBride, PhD, and Giovanni Manfredi, MD, PhD

SfN Society for Neuroscience

Neurobiology of Disease Workshop

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From Pediatric Encephalopathy to Alzheimer's: Linking Mitochondria to Neurological Diseases

Organized by: Heidi McBride, PhD, and Giovanni Manfredi, MD, PhD
Friday, November 11, 2016
8 a.m.–5 p.m.
Location: San Diego Convention Center | Room: 6A | San Diego, CA

TIME	TALK TITLE	SPEAKER
7:30–8 a.m.	**Check-in**	
8–8:10 a.m.	Opening Remarks	Heidi McBride, PhD • McGill University Giovanni Manfredi, MD, PhD • Cornell University, Weill Cornell Medicine
8:10–8:40 a.m.	Patient presentation	Richard Haas, MD • University of California, San Diego School of Medicine
8:40–9:20 a.m.	Biochemical and genetic basis of mitochondrial encephalopathies	Carlos Moraes, PhD • University of Miami, Miller School of Medicine
9:20–10 a.m.	Mitochondrial plasticity and neurodegeneration	Luca Scorrano, MD, PhD • University of Padua
10–10:40 a.m.	Mitochondrial motility in ALS/Parkinson's disease	Erika Holzbaur, PhD University of Pennsylvania, Perelman School of Medicine
10:40–11 a.m.	**Morning Break**	
11–11:40 a.m.	Mitochondrial contact sites and function in Alzheimer's disease	Eric Schon, PhD • Columbia University Medical Center
11:40 a.m.–12:20 p.m.	Mitochondrial quality control in Parkinson's disease	Richard Youle, PhD • National Institute of Neurological Disorders and Stroke
12:20–1 p.m.	Summary, discussion, Q&A, and breakout discussion guide	
1–2 p.m.	**Lunch**	Rooms 6D & 6E

AFTERNOON BREAKOUT SESSIONS | PARTICIPANTS SELECT DISCUSSION GROUPS AT 2 P.M. AND 3:30 P.M.

TIME	BREAKOUT SESSIONS	SPEAKERS	ROOM
2–3:30 p.m.	Mitochondrial bioenergetics in neurological disease	Elizabeth Jonas, MD Carlos Moraes, PhD Giovanni Manfredi, MD, PhD	1A
	Mitochondrial plasticity and neuronal survival	Luca Scorrano, MD, PhD Stuart Lipton, MD, PhD	1B
	Mitochondrial motility and neurodegeneration	Erika Holzbaur, PhD Xinnin Wang, PhD	2
	Mitochondrial/ER contact site function and dysfunction in Alzheimer's and Parkinson's diseases	Serge Przedborski, MD, PhD Eric Schon, PhD	4
	Mitochondrial quality control and neurodegeneration	Leo Pallanck, PhD Richard Youle, PhD	5A
	Mitochondrial roles in immunity: links to neuroinflammation	Heidi McBride, PhD Phillip West, PhD	5B
3:30–5 p.m.	**Repeat sessions above. Select a second discussion group.**		
5– 6 p.m.	**Reception**		3

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Introduction

There are a host of molecular triggers that lead to neurological diseases, but one common, underlying feature is the dysfunction of mitochondria, leading to a loss of cellular energy and signaling organized cell death programs. A common interpretation of these findings is that mitochondrial dysfunction is an end-stage phenomenon, not playing a major role in disease pathogenesis. However, this misconception has been recently challenged, as the field has developed a more precise understanding of the pathways that regulate mitochondrial behavior, function, and turnover. Whether or not these organelles are responsible for the initiation of disease, it is now clear that the rapid and dynamic response to cellular stress, the ability to reprogram metabolic pathways, and the ability to shape-shift and rapidly change their localization will together define the fate of diseased cells. The challenge for the neuroscience community is to define the role of mitochondrial metabolic and physical plasticity in neurons and glia and the deleterious consequences that arise when these fundamental processes go astray. This workshop brings together leaders in the fundamental studies of mitochondrial biology and disease to provide insight into the newest areas of research, with the broader aim of positioning these new discoveries within the context of neurological disease.

One of the greatest challenges in studying mitochondria is the vast array of cellular functions they fulfill. The five invited speakers will focus on distinct aspects of mitochondrial biology, and the topics will expand further during the breakout sessions. The lectures will provide a broad base for understanding mitochondrial biology: from their bacterial origin as metabolic organelles to exciting new concepts on how they behave within the cell.

In the first lecture, Carlos Moraes will focus on primary mitochondrial diseases caused by mutations of mitochondrial DNA (mtDNA) or nuclear DNA encoding mitochondrial proteins, providing insights into the complexity in their phenotypic presentation.

Later on, Luca Scorrano will explain the exciting world of mitochondrial dynamics, taking us through the molecular mechanisms and physiology of mitochondrial fusion, division, and cristae remodeling. The mitochondrial network is highly dynamic, yet the field is still learning the functional importance of these processes—particularly in complex cells like neurons.

Erika Holzbaur will address the critical role of mitochondrial motility along cytoskeletal tracks within neurons—an essential process that regulates mitochondrial distribution within axons and dendrites. Her work in this area has been transformative, providing exciting new insights into the molecular defects underlying neurodegenerative diseases like ALS.

A fascinating aspect of mitochondrial biology is the dynamic interaction with other organelles, such as endosomes, endoplasmic reticulum (ER), and peroxisomes. Interorganellar contact is emerging as a central feature of mitochondrial biology. Eric Schon will focus on the mitochondria–ER interactions and their physiological meaning, as these contacts are essential for calcium and lipid homeostasis. Dr. Schon has generated new evidence linking ER–mitochondrial contact sites with Alzheimer's disease.

Lastly, as a cellular furnace and iron forge, mtDNA, proteins, and lipids within the mitochondria are exposed to highly reactive oxygen and nitrogen species, providing strong evolutionary pressure for efficient quality control mechanisms. Richard Youle has transformed our understanding of mitochondrial quality control through his work on the molecular mechanisms that drive mitochondrial quality control and mitophagy—the targeted degradation of dysfunctional mitochondria. Dr. Youle

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discovered that two Parkinson's disease–related proteins, PINK1 and Parkin, are required to drive this process, providing a new understanding of the potential contribution of mitochondrial dysfunction to PD.

The goal of this workshop is for the speakers to engage the audience, leading to lively discussions within the afternoon breakout sessions. Additional discussion leaders, who are also leaders in the field, will widen the scope of each topic. The afternoon sessions will also include one new topic on the role of mitochondria in immune pathways, both adaptive and innate, as this is a very interesting emerging area of research that is certain to have a direct impact on neurological disease.

Overall, it is clear that mitochondria are much more than just the powerhouse of the cell, and their roles in neurological diseases continue to surprise us. We hope that this workshop will inspire the participants to think differently about mitochondria and to find new ways to explore their role in brain development, function, and disease.

Mitochondrial Encephalopathies

Carlos T. Moraes, PhD

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Miami, Florida

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Introduction

Mitochondria are essential organelles within the cell where most of the energy production occurs by the oxidative phosphorylation (OXPHOS) system. Components of the OXPHOS system are encoded by both the nuclear DNA (nDNA) and the mitochondrial DNA (mtDNA); therefore, mutations involving either genome can be deleterious to the cell. Postmitotic tissues, such as muscle and brain, are most sensitive to OXPHOS changes owing to their high energy requirements and nonproliferative status. The prevalence of OXPHOS diseases is estimated to be ⩾1 in 5,000 live births (Skladal et al., 2003).

In contrast to the large nuclear genome, which is organized into linear chromosomes, the human mitochondrial genome is a circular, double-stranded, supercoiled molecule comprising 16,569 bp and encoding for 37 genes (Fig. 1). MtDNA does not contain introns, and the majority of the genome is composed by coding regions, with only a small noncoding portion (1.1 kb), called the displacement loop (D-loop). The D-loop is essential for mtDNA replication and transcription because it contains the origin of H-strand replication (OH) and the promoter regions of the two strands (H-strand promoter [HSP] and L-strand promoter [LSP]). The two polycistronic RNAs transcribed from the two strands are processed to obtain 22 tRNAs molecules and 2 mitochondrial rRNA and are translated into 13 proteins (Chinnery and Hudson, 2013).

All the proteins encoded by the mtDNA are components of the four OXPHOS multi-subunit complexes. Complex II is the only exception, as all its components are encoded by the nDNA. The proteins involved in mtDNA transcription, translation, and replication as well as the other OXPHOS components, are all encoded by the nDNA, so mutations in these nuclear genes can also affect the stability of the mtDNA.

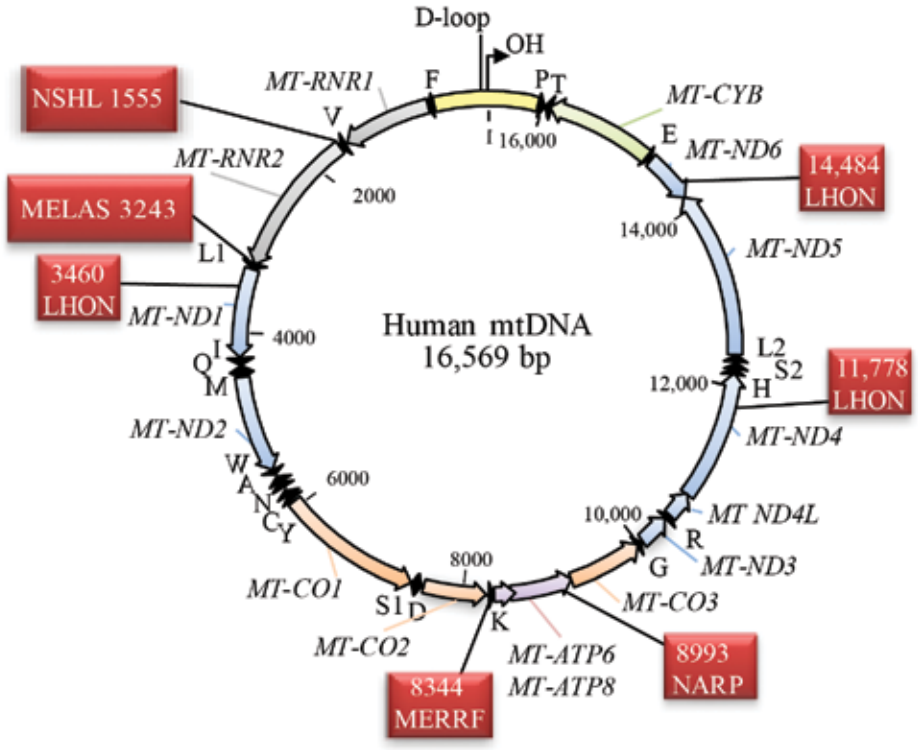

Figure 1. Schematic representation of human mitochondrial DNA. The illustration depicts the two mitochondrial rRNA genes, the 22 tRNA genes, the 13 genes coding for the subunits of Complexes I, III, IV, and V and the D-loop containing the H-strand origin of replication (OH). The red squares indicate the most prevalent mtDNA point mutations and the associated mitochondrial diseases. Light blue, subunits of Complex I of the mitochondrial electron transport chain; orange, subunits of cytochrome c oxidase (Complex IV); violet, subunits of the ATP synthase (Complex V); green, the *cytochrome b* gene, which is part of Complex III; black arrowheads, mitochondrial tRNA genes. MIDD, maternally inherited diabetes and deafness; NSHL, nonsyndromic hearing loss. Adapted with permission from Pinto M, Moraes CT (2014) Mitochondrial genome changes and neurodegenerative diseases, Biochim Biophys Acta 1842:1198–1207, their Fig. 1. Copyright 2014, Elsevier.

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Mitochondrial diseases are in general multi-symptomatic, with dysfunctions affecting different systems and tissues (e.g., loss of muscle coordination, muscle weakness, visual problems, hearing problems, learning disabilities, heart disease, liver disease, kidney disease, gastrointestinal disorders, respiratory disorders, neurological problems, diabetes, autonomic dysfunction, and dementia). However, the most affected tissues are the postmitotic ones, such as myocytes and neurons, which have high energy requirements.

Features of Pathogenic mtDNA Mutations

Multiple copies of mtDNA (~1000) exist in most cells, and these levels can vary depending on energy demands (Kelly et al., 2012). If all the mtDNA molecules present in a cell are identical (all wild-type or all carrying a mutation), this condition is known as "homoplasmy." When mtDNA with different sequences (pathogenic or not) are present in a single cell, the condition is known as "heteroplasmy." The latter is common for pathogenic mutations, as only a portion of the cellular mtDNA content is affected.

Heteroplasmy is a major factor that determines the clinical severity of mitochondrial diseases because mitochondrial function begins to be affected only when there is a relative high number of mutated mtDNA compared with wild type, usually >70–80% (Chinnery et al., 1997; White et al., 1999). This phenomenon is known as the "threshold effect" (Rossignol et al., 2003), and it can vary depending on the mutation, the cell type, the tissue, or even the affected individual.

Maternal inheritance is also a very important factor to take into consideration during the diagnosis of a mitochondrial disease because mtDNA transmission occurs only through the mother. MtDNA defects can be maternally inherited or sporadic. Point mutations are, in general, maternally inherited and heteroplasmic, with an estimated incidence of 1:5000 (Chinnery et al., 2012). They can affect mtDNA genes encoding proteins, tRNAs, or rRNAs. MtDNA rearrangements, like large-scale deletions, remove large portions of the mtDNA, leading to ablation of various genes, depending on the site and size of the deletion. They are consistently heteroplasmic and sporadic, and although their exact mechanism of formation is still controversial, it is believed that they can derive from errors in replication or inefficiency of the mtDNA repair system (Krishnan et al., 2008; Chen et al., 2011). The levels of these mitochondrial genomes may increase during life in view of the fact that deleted mtDNA molecules reportedly have a replicative advantage (Cortopassi et al., 1992; Diaz et al., 2002).

Mitochondrial Encephalopathies Caused by mtDNA Mutations

Patients with mtDNA large deletions commonly show one of three classic phenotypes: Pearson syndrome, chronic progressive external ophthalmoplegia (CPEO), and Kearns–Sayre syndrome (KSS). Patients with Pearson marrow–pancreas syndrome show a multi-symptomatic disease from birth and a 50% survival rate after 4 years of age. The main symptoms are sideroblastic anemia and exocrine pancreas dysfunction. Those who survive infancy are expected to develop KSS (McShane et al., 1991; Lee et al., 2007; Manea et al., 2009). CPEO is characterized by ptosis and ophthalmoplegia, and some patients also show proximal muscle weakness. Patients with CPEO can have brain, inner ear, and retinal disease in later stages of the disease, depending on the age of onset and the level of heteroplasmy (Aure et al., 2007). Isolated CPEO is commonly seen as a milder form of the disease, and clinical presentations can involve other muscles or symptoms and are sometimes referred to as "CPEO plus" (Moraes et al., 1989).

Although it is a multisystem disorder, CNS involvement is evident in KSS. The syndrome is defined by onset before 20 years of age, progressive external ophthalmoplegia (PEO), and pigmentary retinopathy. Moreover, patients may show cardiac conduction block (usually the cause of death in young adulthood), elevated CSF protein level, or cerebellar ataxia (Moraes et al., 1989). Other neurological problems may include proximal myopathy, exercise intolerance, ptosis, oropharyngeal and esophageal dysfunction, sensorineural hearing loss, and dementia.

Besides deletions, almost 600 pathogenic point mutations have been identified in the past 25 years, involving most of the mtDNA molecule (according to MITOMAP, 299 point mutations involving tRNA–rRNA and control regions and 274 involving OXPHOS proteins) (Lott et al., 2013). The most common mitochondrial encephalopathies caused by point mutations can be divided into clinical groups: Leber hereditary optic neuropathy (LHON); Leigh syndrome (LS); mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes (MELAS); myoclonic epilepsy with ragged red fibers (MERRF); and neuropathy, ataxia, retinitis pigmentosa (NARP).

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Approximately 95% of LHON cases show a mutation in one of three mtDNA genes: *G11778A*, *G3460A*, or *T14484C*, respectively encoding for ND4, ND1, and ND6: all subunits of Complex I of the electron transport chain (Fig. 1). The main characteristic of the disease is a painless, bilateral, subacute or acute visual failure, prevalently in young male adults, caused by the atrophy of the optic nerve. The neurodegeneration is limited to the retinal ganglion cell layer, and cell body and axonal degeneration, demyelination, and atrophy are observed from the optic nerves to the lateral geniculate bodies (Carelli et al., 2004).

LS can be caused by different mutations, both in mtDNA and in nDNA genes (see below). MtDNA mutations, which are commonly present in heteroplasmy, can occur in genes encoding for tRNAs as well as in genes encoding for Complex I, IV, or V of the OXPHOS system (Thorburn and Rahman, 1993; Santorelli et al., 1997; Kirby et al., 2003; Martin et al., 2005; Sarzi et al., 2007; Marin et al., 2013). The heterogenic symptoms include motor and intellectual developmental delay, bilateral brainstem disease, basal ganglia disease, elevated blood or CSF lactate levels, hypotonia, spasticity, chorea and other movement disorders, cerebellar ataxia, peripheral neuropathy, and respiratory failure secondary to brainstem dysfunction (Thorburn and Rahman, 1993).

Approximately 80% of MELAS cases are caused by a very common m.3243A>G mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene, although other mtDNA point mutations also have been associated with this phenotype (www.mitomap.org; Lott et al., 2013). As in the vast majority of the mitochondrial diseases, this is a multisystemic disorder, and its symptoms vary depending on heteroplasmy status and age of onset. Other than mitochondrial myopathy, encephalomyopathy, lactic acidosis, and strokelike symptoms, patients can show deafness, diabetes, migraines, gut immobility, and seizures (Debrosse and Parikh, 2012). Multiple strokes affect the patients, mainly in the cerebral cortex or in the subcortical white matter, causing multifocal necrosis with lesions that do not respect vascular territories and are often accompanied by profound neuronal cell loss, neuronal eosinophilia, astrogliosis, and spongiform degeneration (Tanji et al., 2001). A loss of Purkinje cells also takes place, causing cerebellar degeneration and a particularly prominent calcification in the basal ganglia.

In 90% of the cases of MERRF, the mutation responsible is the m.8344A>G transition in the $tRNA^{Lys}$ gene (Shoffner et al., 1990) (Fig. 1). Myoclonic epilepsy is the main symptom associated with this disease, together with the presence of clumps of diseased mitochondria accumulation in the subsarcolemmal region of the muscle fiber called "ragged red fibers." Other possible symptoms include ataxia, neuropathy, and cardiac abnormalities. Curiously, many patients with the A8344G mutation also show multiple lipomas in the back region (Larsson et al., 1995). The main neuropathological signs involve the olivocerebellar pathway, with severe neuron loss originating from the inferior olivary nucleus, Purkinje cells, and dentate nucleus.

Nonsyndromic hearing loss can be caused by mutations in mitochondrial tRNAs (Prezant et al., 1993) (Fig.1). NARP is associated mainly with the mutation T8993G (or T8993C) in the mtDNA encoding for the *MT-ATP6* gene (Fig. 1). This disease is characterized by sensory or sensorimotor axonal neuropathy, neurogenic muscle weakness, ataxia, cerebral or cerebellar atrophy, and retinitis pigmentosa. Other atypical neurological symptoms include seizures, learning problems, hearing loss, progressive external ophthalmoplegia, and anxiety. These same mutations, when present at high percentages, are also associated with LS (Lake et al., 2016).

Mitochondrial Diseases Caused by Mutations in nDNA Genes Affecting mtDNA Stability

MtDNA changes can be a consequence of mutations in nDNA-encoded genes involved in the maintenance of mtDNA integrity and mtDNA copy number. The most common mutations affect *POLG*, the gene encoding for the catalytic subunit of the mitochondrial DNA polymerase gamma, and the DNA helicase Twinkle (Spelbrink et al., 2001; Milenkovic et al., 2013). Both gene products are involved in mtDNA replication. Mutations in these genes provoke an accumulation of mtDNA point mutations, deletions, or depletion, eventually leading to different clinical manifestations.

More than 200 mutations in *POLG* associated with mitochondrial diseases have been identified, causing a plethora of heterogeneous disorders involving different tissues, time of onset, and severity. At least five major phenotypes can be distinguished: Alpers–Huttenlocher syndrome, childhood myocerebrohepatopathy spectrum, myoclonic epilepsy myopathy sensory ataxia, the ataxia neuropathy spectrum, and PEO with or without sensory ataxic neuropathy and dysarthria (Lamantea

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et al., 2002; Fratter et al., 2010; Brandon et al., 2013; Stumpf et al., 2013).

Recessive mutations in Twinkle protein cause severe, early-onset disorders that are also caused by defects in mtDNA maintenance, such as infantile-onset spinocerebellar ataxia and a hepatocerebral mtDNA depletion disorder characterized by severe epilepsy, migraine, and psychiatric symptoms (Fratter et al., 2010).

Several genes coding for proteins associated with nucleotide metabolism or transport to mitochondria have also been associated with mtDNA depletion and severe clinical phenotypes (Martí et al., 2003).

Mitochondrial Encephalopathies Caused by Nuclear DNA Defects

Defects in OXPHOS function can also be consequences of mutations in the nDNA, where most genes encoding for mitochondrial proteins are located. Although such defects can have different phenotypes, the most common one is LS. As mentioned earlier, LS is clinically heterogeneous, but onset occurs usually by 2 years of age. Patients present with developmental delays and regression, hypotonia, ataxia, dystonia, and ophthalmological abnormalities. The presentation can also be multisystemic; for example, cardiac, hepatic, gastrointestinal, and renal tubular dysfunction have been observed. Progression is often episodic and typically results in death by 3 years of age (Lake et al., 2016).

Neurological decline in LS patients is associated with the hallmark of the diagnosis: bilateral symmetric lesions within the brainstem and basal ganglia. Additional neuroradiological abnormalities such as white matter involvement and cerebral atrophy may also be observed. Thorburn and colleagues defined the criteria for diagnosing LS. They required that typical neuropathology or neuroradiology be accompanied by progressive neurodegeneration with (1) clinical evidence of brainstem and/or basal ganglia dysfunction; (2) intellectual and motor developmental delay; and (3) abnormal energy metabolism indicated by a severe defect in OXPHOS or pyruvate dehydrogenase complex (PDHc) activity, a molecular diagnosis in a gene related to mitochondrial energy generation, or elevated serum or CSF lactate (Lake et al., 2016).

Biochemical defects in each of the five OXPHOS complexes, and the electron carrier coenzyme Q_{10} (CoQ_{10}), have been observed in LS patients, although Complex V in LS has been associated only with mtDNA mutations. PDHc enables the generation of electron donors for OXPHOS; therefore, PDHc deficiency also represents a disorder of energy generation. Table 1 lists the genes that, when mutated, were found to cause LS.

Lessons from Mouse Models of Mitochondrial Diseases

Effective treatments for mitochondrial disorders are still unavailable, in part because of the poor understanding of the pathological mechanisms underlying these diseases. Therefore, during the past decade, several animal models were developed to improve our knowledge of the pathophysiology of mitochondrial disorders and to provide a platform

Table 1. List of nuclear genes causing Leigh syndrome when mutated

Pyruvate dehydrogenase	*PDHA1, PDHB, PDHX, DLAT, DLD, LIPT1, LIAS, TPK1, SLC19A3, SLC25A19*
Complex I	*NDUFV1, NDUFV2, NDUFS1, NDUFS2, NDUFS3, NDUFS4, NDUFS7, NDUFS8, NDUFA1, NDUFA2, NDUFA9, NDUFA10, NDUFA12, NDUFAF2, NDUFAF5, NDUFAF6, FOXRED1*
Complex II	*SDHA, SDHAF1*
Coenzyme Q_{10}	*PDSS2*
Complex III	*UQCRQ, BCS1L, TTC19*
Complex IV	*NDUFA4, SURF1, COX10, COX15, SCO2, PET100, LRPPRC, TACO1, ETHE1*
Mitochondrial DNA maintenance	*FBXL4, POLG, SUCLA2, SUCLG1*
Mitochondrial translation	*MTTI, MTTK, MTTL1, MTTV, MTTW, MTFMT, GTPBP3, TRMU, EARS2, FARS2, IARS2, NARS2, GFM1, GFM2, TSFM, C12orf65, PNPT1*
OXPHOS ± PDHc	*HIBCH, ECHS1, SERAC1, AIFM1*
Biotinidase	*BTD*

Adapted with permission from Lake NJ et al. (2016) Leigh syndrome: One disorder, more than 75 monogenic causes, Ann Neurol 79:193, their Table 1. Copyright 2015, American Neurological Association. Please refer to this publication for more details on the gene names and functions.

for testing therapeutic interventions. There are now several mouse models of OXPHOS defect, which were recently reviewed (Torraco et al., 2015).

We have created two neuron-specific mouse models of mitochondrial electron transport chain deficiencies involving defects in Complex III (CIII) or Complex IV (CIV). These conditional knockouts (cKOs) were created by *in vivo* ablation (CamKIIα-Cre) of the genes coding for the Rieske iron–sulfur protein (RISP) and COX10 (cyclo-oxygenase 10), respectively. RISP is one of the catalytic subunits of CIII, and COX10 is an assembly factor indispensable for the maturation of Cox1, one of the catalytic subunits of CIV. Although the rates of gene deletion, protein loss, and complex dysfunction were similar, the *RISP* cKO survived to 3.5 months of age, whereas the *COX10* cKO survived for 10–12 months. The *RISP* cKO had a sudden death, with minimal behavioral changes. In contrast, the *COX10* cKO showed a distinctive behavioral phenotype with onset at 4 months of age followed by a slower but progressive neurodegeneration. Curiously, the piriform and somatosensory cortices were more vulnerable to the CIII defect, whereas cingulate cortex (and to a less extent, piriform cortex) was affected preferentially by the CIV defect. In addition, the CIII model showed severe and early reactive oxygen species damage, a feature not observed until very late in the disease course of the CIV model (Diaz et al., 2012). These findings illustrate how specific respiratory chain defects have distinct molecular mechanisms, leading to distinct pathologies, akin to the clinical heterogeneity observed in patients with mitochondrial diseases.

Mouse models are also useful for evaluating therapies, although species-specific responses should always be kept in mind. We and others have shown that an increase in mitochondrial biogenesis in muscle has a strong protective effect in a mouse with a muscle CIV defect (Wenz et al., 2008; Viscomi et al., 2011). This correlation has been more difficult to obtain with CNS defects.

Recently, using a model of Complex I deficiency in the CNS (*Ndufs4*), the group of Vamsi Mootha found that chronic hypoxia markedly improved survival (Jain et al., 2016). This same model has also shown improved survival on administration of rapamycin (an mTOR pathway inhibitor), delaying the onset of neurological symptoms, reducing neuroinflammation, and preventing brain lesions. However, the mechanism is not yet understood (Johnson et al., 2013).

Acknowledgments

Parts of this paper were adapted with permission from previous publications. These include Pinto M, Moraes CT (2014) Mitochondrial genome changes and neurodegenerative diseases, Biochim Biophys Acta 1842:1198–1207, copyright 2014, Elsevier; and Diaz F et al. (2012) A defect in the mitochondrial complex III, but not complex IV, triggers early ROS-dependent damage in defined brain regions, Hum Mol Genet 21:5066–5077, copyright 2012, Oxford University Press.

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Mitochondrial Motility in Neurons

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Introduction

The active transport of organelles, proteins, and RNA along the extended axons of neurons has long fascinated scientists. The remarkable fact that the axon depends on the biosynthetic and degradative activities of the soma, located up to a meter away, highlights the importance of active transport. Genetic evidence confirms an essential role for active transport in the neuron, as defects in many of the proteins involved are sufficient to cause either neurodevelopmental or neurodegenerative disease.

Metabolic cell-labeling experiments in the 1960s demonstrated the rapid movement of newly synthesized proteins along the axon. Experiments with drugs that disrupt the cellular cytoskeleton demonstrated that microtubules are required for active transport along the axon (Kreutzberg, 1969). Pulse-chase labeling experiments led to the discovery of multiple phases of transport (Griffin et al., 1976). Organelles were observed to move outward from the cell body at "fast" speeds of ≤400 mm/d (~1 μm/s), while cytoskeletal proteins and some soluble proteins were observed to move via "slow" transport, at speeds of <8 mm/day (<0.1 μm/s). Outward-bound, anterograde (also known as orthograde) transport was most clearly defined by these metabolic labeling approaches. However, the retrograde transport of organelles from the distal axon back toward the cell body was also observed (Griffin et al., 1976). The development of live-cell imaging allowed the direct observation of organelle motility (Allen et al., 1982; Brady et al., 1982). These observations led to the discovery of the microtubule motor kinesin (Vale et al., 1985), now known as kinesin-1; cytoplasmic dynein was discovered soon after (Paschal et al., 1987). Breakthrough experiments using nerve ligation assays identified kinesin as a major motor for anterograde transport along the axon (Hirokawa et al., 1991) and dynein as the motor for retrograde transport (Hirokawa et al., 1990).

Since these initial discoveries, there has been considerable progress in understanding the mechanisms regulating the transport of organelles, including mitochondria, lysosomes, autophagosomes, and endosomes, as well as the transport mechanisms involved in neurotrophic and injury signaling. Together, these studies support a model in which the regulation of transport is cargo-specific. The complement of motors, adaptors, and scaffolding proteins bound to each cargo are organelle-specific, leading to distinct patterns of motility and localization along the axon. Although broad themes have emerged, the specific mechanisms regulating the transport of each organelle or protein complex may be unique. In addition, there is increasing evidence for the localized regulation of trafficking in key zones along the axon, such as the axon initial segment or the axon terminal.

In this chapter, modified from a broader review on axonal transport (Maday et al., 2014), we focus on the mechanisms regulating mitochondrial motility in neurons (Fig. 1).

Molecular Motors Drive Transport Along the Neuronal Cytoskeleton

The neuronal cytoskeleton

Microtubules, actin filaments, and intermediate filaments all contribute to the morphology and function of neurons, but axonal transport depends almost entirely on microtubules. Microtubules are polarized tubulin polymers with fast-growing plus ends and more stable minus ends, organized in a generally radial array in the soma with plus ends directed toward the cortex. In the axon, parallel microtubules form a unipolar array with plus ends oriented outward (Burton and Paige, 1981; Stepanova et al., 2003), whereas in dendrites, microtubule organization is more complex, with microtubules often organized in arrays with mixed polarity (Baas et al., 1988; Kwan et al., 2008; Kleele et al., 2014).

Microtubule-associated proteins, or MAPs, are bound along the length of axonal and dendritic microtubules. The canonical role for MAPs is to promote microtubule polymerization and stabilization; because of the high expression levels of MAPs in neurons, microtubules are generally more stable in these cells than in other cell types. MAPs may also function to regulate transport, as *in vitro* studies indicate they modulate the interaction of motors with the microtubule (Vershinin et al., 2007; Dixit et al., 2008).

Direct posttranslational modification of tubulin is widespread in neurons (Janke and Bulinski, 2011). Microtubule modifications directly modulate the activities of motor proteins (Sirajuddin et al., 2014), potentially contributing to the polarized trafficking of motors into axons (Jacobson et al., 2006; Konishi and Setou, 2009; Hammond et al., 2010). The nucleotide state of microtubules can also affect motor activity and contribute to polarized vesicle transport (Nakata et al., 2011).

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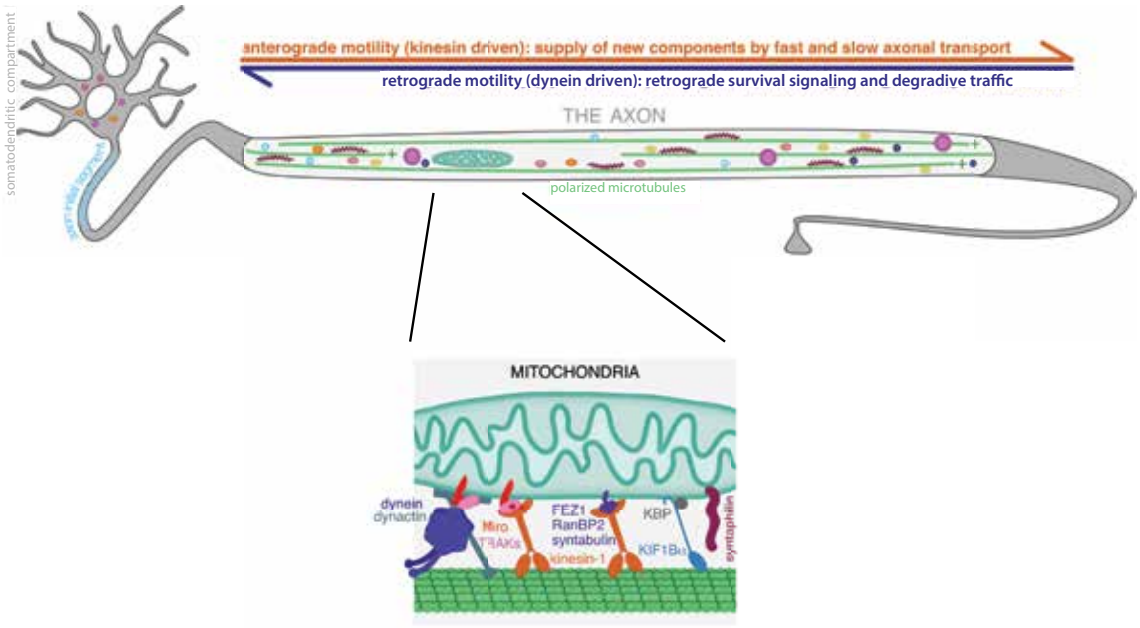

Figure 1. Molecular mechanisms of axonal transport. Microtubule motor proteins kinesin and dynein drive the movement of organelles including mitochondria, vesicles, RNA granules, and proteins along the axon. Kinesins drive anterograde transport outward from the soma, and dynein drives retrograde transport back from the distal axon. However, most cargos likely have both motor types bound simultaneously. Mitochondrial motility is regulated by a specific complement of molecular motors, scaffolding proteins, and adaptor proteins. These include kinesin-1, dynein and dynactin, Miro, TRAKs, FEZ1, RanBP2, syntabulin, KBP, KIF1Bα, and syntaphilin. Modified with permission from Maday S et al. (2014) Axonal transport: cargo-specific mechanisms of motility and regulation, Neuron 84: 294–295, their Fig. 1. Copyright 2014, Elsevier.

Kinesin and dynein motors drive axonal transport

The kinesin superfamily constitutes 45 genes in the human genome, 38 of which are expressed in brain (Miki et al., 2001). The neuronal motor proteome is more complex than that expressed in most other cell types, likely reflecting the enhanced importance of regulated and specific intracellular transport in neurons with their highly polarized morphology (Kuta et al., 2010; Silverman et al., 2010). A standardized nomenclature (Lawrence et al., 2004) groups kinesin genes into 14 subfamilies that share structural and functional similarities; motors from the kinesin-1, kinesin-2, and kinesin-3 families all contribute to axonal transport dynamics.

Members of the kinesin-1 family drive the transport of a wide range of cargos along the axon at velocities of ~0.5–1.0 μm/s, including vesicles, organelles, proteins, and RNA particles (Hirokawa et al., 2010) (Fig. 1). Active kinesin-1 motors are formed from a dimer of kinesin heavy chains (encoded by three mammalian genes: *KIF5A*, *KIF5B*, and *KIF5C*); a dimer of kinesin light chains is often but not always part of the complex (Sun et al., 2011) and contributes to the autoinhibitory mechanism of the motor.

Kinesin-2 and kinesin-3 motors are also critical for normal axonal transport. Kinesin-2 members can assemble into either homodimeric or heterotrimeric motors (Scholey, 2013), whereas kinesin-3 motors undergo cargo-mediated dimerization resulting in the formation of highly processive motors when bound to intracellular organelles (Soppina et al., 2014). Kinesin-2 motors drive the anterograde motility of fodrin-positive plasma membrane precursors (Takeda et al., 2000), N-cadherin and β-catenin (Teng et al., 2005), and choline acetyltransferase (Ray et al., 1999) and are associated with Rab7-positive late endosome–lysosome compartments in the neuron (Hendricks et al., 2010; Castle et al., 2014). Kinesin-3 motors drive the motility of synaptic vesicle precursors and dense core vesicles (Hall and Hedgecock, 1991; Okada et al., 1995; Lo et al., 2011).

Cytoplasmic dynein is the major motor driving retrograde transport. In contrast to the diversity

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of the kinesin superfamily, the motor subunit of cytoplasmic dynein is encoded by a single gene (reviewed in Roberts et al., 2013). Two dynein heavy chains dimerize by their N-terminal tail domains; additional intermediate chains, light intermediate chains, and light chains associate with the tails of the heavy chains to form a cargo-binding domain. Together, these proteins serve as the binding site for many of the proteins regulating dynein function in the cell. Whereas a single gene encodes the motor domain of cytoplasmic dynein, there is more diversity in the other subunits of the dynein complex—for example, there are two genes encoding dynein intermediate chains, one of which is neuron-specific (*DYNC1I1*), and two genes encoding dynein light intermediate chains (Kuta et al., 2010). There is evidence that these subunits can either coassemble (Zhang et al., 2013) or alternatively assemble into distinct complexes with specialized functions (Salata et al., 2001; Mitchell et al., 2012), which may allow for organelle-specific recruitment or regulation.

Most dynein functions in the cell require the dynein activator, dynactin. Dynactin is a highly conserved multiprotein complex (Schroer, 2004) essential for normal neuronal function (LaMonte et al., 2002; Moughamian and Holzbaur, 2012). The base of dynactin is formed from a 37-nm-long actin-like polymer. Projecting from this base is a dimer of the subunit $p150^{Glued}$ (Holzbaur et al., 1991), which binds directly to dynein intermediate chain (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995) and to microtubules via a cytoskeletal-associated protein glycine-rich (CAP-Gly) domain (Waterman-Storer et al., 1995). In neurons, the CAP-Gly domain of dynactin has a key role in the initiation of retrograde transport in the distal axon (Lloyd et al., 2012; Moughamian and Holzbaur, 2012).

The properties of kinesin and dynein motors have been explored *in vitro* at the single-molecule level. Kinesin-1 motors move in a highly processive manner toward the plus end of the microtubule, taking 8 nm steps in a straight path along a single protofilament. A single kinesin-1 motor has a stall force of 5–6 pN (Svoboda and Block, 1994), sufficient to move an organelle through the cytoplasm. Kinesin-2 motors have a stall force of similar magnitude (5 pN) but exhibit force-dependent detachment from the microtubule (Schroeder et al., 2012) that may affect interactions with other motors.

Studies with purified mammalian dynein indicate that dynein is a fast motor, with velocities of 0.5–1.0 μm/s. Unlike the highly processive unidirectional motility of kinesin-1, kinesin-2, and kinesin-3 motors, single mammalian dynein motors take frequent backsteps and sidesteps during movement along the microtubule (Mallik et al., 2005; Ross et al., 2006). However, either the coordinated activities of multiple dynein motors (Mallik et al., 2005) or the binding of activators such as BICD2 (McKenney et al., 2014; Schlager et al., 2014) convert dynein to a unidirectional and highly processive motor. Dynein is a much weaker motor than kinesin-1 or kinesin-2, with a stall force of ~1 pN (Mallik et al., 2004; Schroeder et al., 2010).

Although these observations might suggest that dynein is a less effective motor than kinesin, both the flexible nature of dynein and its ability to move backwards and sideways along a microtubule may allow the motor to function effectively in teams (Mallik et al., 2013) and to navigate around obstacles along its path (Dixit et al., 2008). In contrast, kinesin-1 motors are much less capable of effectively working in teams (Mallik et al., 2013) and are more likely than dynein to detach from the microtubule track when encountering obstacles (Vershinin et al., 2007; Dixit et al., 2008).

Opposing motors bind simultaneously to cargos along the axon

Many axonal cargos have multiple motor types bound simultaneously. For example, late endosomes/lysosomes copurify with kinesin-1, kinesin-2, and dynein motors (Hendricks et al., 2010). Even cargos that move processively in a single direction over long distances, such as autophagosomes, copurify with opposing dynein and kinesin motors (Maday et al., 2012). Quantitative analyses and live-cell trapping experiments suggest that 1–2 kinesins and 6–12 dyneins act together to move a single organelle along the microtubule (Hendricks et al., 2010, 2012; Rai et al., 2013).

Thus, it is essential to consider how multiple motors—and multiple types of motors—may interact either cooperatively or competitively to yield effective motility. Multiple models have been put forth (Gross, 2004, 2007; Welte, 2004; Muller et al., 2008; Fu and Holzbaur, 2014). The simplest model posits an unregulated tug-of-war between opposing kinesin and dynein motors. In a contrasting model, motors are coordinately regulated so that only a single motor type is active at any given time.

The autoinhibition of kinesin-1 is key to the regulated trafficking of many organelles. The binding of kinesin tail to the motor domain blocks motor

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function (Kaan et al., 2011); inhibition is relieved by specific binding partners such as the scaffolding proteins JIP1 (c-Jun N-terminal kinase–interacting protein 1) and JIP3 (Blasius et al., 2007; Sun et al., 2011; Fu and Holzbaur, 2013). In the mechanisms explored in detail to date, tight regulation of kinesin-1 activation by scaffolding proteins allows for sustained axonal transport of organelles in either the anterograde or retrograde directions. The regulation of other kinesin subfamilies is less well studied.

Regulation of dynein motors is also important to maintain axonal transport. Lis1 is a critical and conserved effector of dynein function. Structural studies indicate that Lis1 binds directly to the dynein motor domain and uncouples ATP hydrolysis from force production, leading to sustained attachment of the motor to the microtubule (Huang et al., 2012). Although induction of tight binding might be expected to block effective transport, instead it has been found that depletion of Lis1 inhibits the dynein-driven transport of late endosomes and lysosomes along the axon (Pandey and Smith, 2011; Moughamian et al., 2013) as well as mitochondrial motility within axons (Shao et al., 2013). Nde1 (also known as NudE) and Ndel1 (also known as NudE-like or NudEL) form a complex with Lis1 and are similarly required for normal axonal transport of at least some dynein cargos (Pandey and Smith, 2011; Shao et al., 2013).

The Bicaudal D homolog (BICD) proteins are also key dynein effectors. BICD1 and BICD2 recruit dynein–dynactin to Rab6-positive Golgi and cytoplasmic vesicles (Matanis et al., 2002) as well as mRNAs including FMRP (Fragile X mental retardation protein) (Bianco et al., 2010). BICD1 was also shown to control the trafficking of activated neurotrophin receptors to degradation routes in order to balance the neuronal response to neurotrophin stimulation (Terenzio et al., 2014).

Multiple additional mechanisms have been proposed to regulate motor activity on cargos moving along the axon. Rab GTPases have been shown to regulate motor recruitment to several cargos (Akhmanova and Hammer, 2010). Scaffolding proteins are also key: huntingtin is involved in the regulation of BDNF-positive vesicles (Gauthier et al., 2004) and autophagosomes (Wong and Holzbaur, 2014); JIP1 is involved in the regulation of APP (amyloid precursor protein)–positive vesicles; JIP3 regulates the injury-signaling pathway in mammalian cells and lysosomal motility in zebrafish (Drerup and Nechiporuk, 2013); and the Miro–TRAK (mitochondrial Rho GTPase–trafficking protein, kinesin-binding) complex regulates motors bound to mitochondria (Macaskill et al., 2009a; Wang and Schwarz, 2009). Finally, there is evidence implicating upstream kinases in the regulation of transport including Cdk5, JNK (c-Jun N-terminal kinase), and p38 MAPK (mitogen-activated protein kinase) (Horiuchi et al., 2007; Pandey and Smith, 2011; Fu and Holzbaur, 2013; Morfini et al., 2013; Klinman and Holzbaur, 2015), but the mechanisms involved have not yet been fully elucidated.

Both common themes and cargo-specific mechanisms operate in the axonal transport of diverse axonal cargos

Live-cell and *in vivo* imaging of fluorescently tagged organelles moving along axons has revealed a surprising diversity in the movement of specific populations, indicating that the regulation of the motors that drive transport likely occurs primarily at the level of the organelle rather than reflecting an overall regulatory environment within the axon. Although the observed patterns of motility are diverse, some common themes are emerging:

1. Motors remain stably associated with a cargo during transport along the axon, even when they are inactive.

2. Only a small complement of motors is necessary to effectively move even large (>1 μm) organelles along the microtubule. These motors function in groups that usually include opposing motor activities.

3. Motors are regulated by mechanisms that may include Rab-specific recruitment, upstream regulation by kinases and phosphatases, and scaffolding proteins that control motor activity.

4. Mutations in motors, their adaptors, or their regulators can lead to neurodegeneration or neuronal cell death, consistent with an essential role for axonal transport in maintaining neuronal homeostasis.

Despite these common themes, accumulating evidence suggests that the motility of each cargo actively transported along the axon is regulated by a distinct mechanism. Here, we focus specifically on the mechanisms regulating mitochondrial motility in neurons.

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Mitochondrial Transport

Localized regions within the neuron, such as growth cones and synapses, experience significant energy demands. This requirement for ATP cannot be sustained by diffusion from the cell soma and must be handled locally within the neuron. Mitochondria, the organelles responsible for ATP production and intracellular calcium buffering, are actively shuttled and positioned within the neuron to meet the localized needs of the cell (Fig. 1). Thus, mitochondrial motility facilitates a dynamic response to balance environmental demands. In axons of hippocampal neurons grown *in vitro*, ~20–30% of mitochondria are motile, moving equally in both anterograde and retrograde directions; the remaining ~70–80% are stationary (Hollenbeck and Saxton, 2005). *In vivo*, axonal mitochondria are ~10% motile and exhibit a greater bias in flux in the anterograde direction than seen in neurons in culture; ~70% are anterograde and ~30% are retrograde (Pilling et al., 2006; Misgeld et al., 2007).

Mitochondrial transport is regulated by neuronal activity (Sajic et al., 2013). Elevated intracellular calcium levels resulting from enhanced synaptic activity arrest mitochondrial motility in a highly localized fashion, since mitochondria as little as 15 μm away from the stimulation site remain motile (Li et al., 2004; Macaskill et al., 2009a; Wang and Schwarz, 2009). Passing mitochondria become immobilized in areas of locally high Ca^{2+} at active synapses where demands for energy and calcium buffering are high. The distribution of mitochondria at synapses in turn affects synaptic transmission and strength. Stable positioning of mitochondria at presynaptic boutons maintains a steady release of synaptic vesicles (SVs), resulting in steady amplitudes of EPSCs (Sun et al., 2013).

Mitochondrial distribution is also coupled to the balance between mitochondrial fission and fusion. Mutations in the mitochondrial fission protein DRP1 (dynamin-related protein-1) result in the accumulation of mitochondria in the soma of both *Drosophila* motor neurons (Verstreken et al., 2005) and cultured hippocampal neurons (Li et al., 2004). The resulting decrease in mitochondrial density at presynaptic terminals of the neuromuscular junction impairs SV release, a defect rescued with exogenous ATP (Verstreken et al., 2005).

The calcium-dependent arrest of mitochondrial motility is mediated by Miro (Fransson et al., 2003; Guo et al., 2005). Miro has two Ca^{2+} binding EF-hand domains and two GTPase domains, and binds the kinesin-1 adaptors TRAK1 and TRAK2, also known as Milton in *Drosophila* (Fransson et al., 2006; Macaskill et al., 2009b). Ca^{2+} binding to Miro induces mitochondrial arrest; however, controversy still surrounds the mechanism. One model proposes that high levels of calcium promote binding of Miro1 to the motor domain of kinesin-1, thereby sterically inhibiting access to the microtubule (Wang and Schwarz, 2009). A second model posits that elevated calcium levels cause the dissociation of kinesin-1 from mitochondria and the Miro–TRAK complex (Macaskill et al., 2009a). Differences between axonal versus dendritic modes of regulation may underlie some of these observations. Syntaphilin is enriched on stationary mitochondria in the axon, and knockout mice show enhanced axonal mitochondrial motility, with no effect observed on the motility of dendritic mitochondria (Kang et al., 2008). Calcium promotes binding of syntaphilin to both microtubules and kinesin-1, thereby decreasing the ATPase rate of kinesin-1 and acting as a brake on motility (Chen and Sheng, 2013), but only in the axon. Thus, the differing models may reflect cell-compartment-specific regulatory mechanisms for mitochondrial movement.

In addition to the Miro–TRAK complex, syntabulin (Cai et al., 2005), FEZ1 (fasciculation and elongation protein ζ-1) (Fujita et al., 2007; Ikuta et al., 2007), and RanBP2 (Ran-binding protein 2) (Cho et al., 2007; Patil et al., 2013) have all been shown to recruit kinesin-1 to mitochondria to regulate mitochondrial motility. Whether these proteins can interact with the Miro1 complex or act independently remains to be established. However, in the absence of kinesin-1, a small population of mitochondria is still motile (Pilling et al., 2006), indicating that other kinesins also drive mitochondrial motility. There is evidence that both KIF1Bα (kinesin superfamily protein 1B alpha) (Nangaku et al., 1994) and KLP6 (kinesin-like protein 6) (Tanaka et al., 2011) contribute to the intracellular transport of mitochondria.

The role of dynein in mitochondrial trafficking is less well studied. Mutations in kinesin-1 and the Ca^{2+}-dependent inactivation of kinesin-1 arrest mitochondrial motion in both anterograde as well as retrograde directions (Pilling et al., 2006; Macaskill et al., 2009a; Wang and Schwarz, 2009; Chen and Sheng, 2013), suggesting that the activity of oppositely directed motors is coordinated (Pilling et al., 2006). The TRAK proteins interact with the dynein–dynactin complex and may modulate

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this coordination (van Spronsen et al., 2013). Loss of Miro affects both anterograde and retrograde transport (Russo et al., 2009), also consistent with an integrated regulatory mechanism.

The Energy Requirements of Axonal Transport

Axonal transport is an energetically costly process as molecular motors hydrolyze ATP to carry out the work of stepping along microtubules. The conventional kinesin-1 motor consumes one molecule of ATP for every 8 nm step taken (Hackney, 1994). Measurements to date indicate a typical vesicle has one to two kinesins bound and exerting force at any one time (Soppina et al., 2009; Hendricks et al., 2010, 2012; Encalada et al., 2011; Rai et al., 2013). Taking the example of an average axon in the rat cortex, 40 mm in length, a single vesicle traversing this axon in the anterograde direction would require ~5×10^6 ATP molecules to do so (assuming no tug-of-war or switch events occur, which can be frequent *in vivo*) (Soppina et al., 2009; Hendricks et al., 2010). In the 1-m-long axons of human motor neurons, the minimum ATP consumed per anterograde transport event reaches ~1.25×10^8 ATP molecules.

Unlike the consistent unidirectional stepping of kinesin-1 motors, the step size of single cytoplasmic dynein motors purified from mammalian brain ranges from 8 to 32 nm in length and can include backsteps (Mallik et al., 2004; Ross et al., 2006). However, recent *in vitro* and *in vivo* measurements show that dynein acts in teams of 6–12 motors per vesicle to produce persistent retrograde motility, and under these conditions, motor teams show a step size of 8 nm (Soppina et al., 2009; Hendricks et al., 2010; Rai et al., 2013). Thus, a single vesicle traversing a human motor neuron from neuromuscular junction back to the soma would require a minimum of ~7.5×10^8 ATP molecules.

Strikingly, however, the amount of ATP hydrolyzed during axonal transport is relatively inconsequential compared with the amount of ATP consumed by those same neurons to fire action potentials and maintain resting potentials. A single action potential propagated along a 40-mm-long axon would require ~1×10^8 ATP molecules, and thus, axonal transport likely amounts to a fraction of the 25% of energy allocated to the housekeeping budget of the gray matter (Harris and Attwell, 2012).

One mechanism proposed to specifically address the energy demands of axonal transport is based on the finding that glycolytic enzymes are bound to the surface of vesicles moving along the axon and can serve as an independent source of ATP for the motors driving transport of these vesicles (Zala et al., 2013). The identification of an energy source independent of mitochondria that can power vesicular transport is intriguing and may allow cargos to transit any gaps in ATP gradients between unevenly dispersed mitochondria along the axon (Macaskill and Kittler, 2010; Zala et al., 2013). However, it remains unclear whether onboard energy production by glycolysis is required for axonal transport *in vivo*, as the energetic lives of glia and neurons are intimately linked (Saab et al., 2013). Glia supply neurons with lactate under conditions of glucose shortage, bypassing glycolysis in the axon. Indeed, myelinated axons can survive for extended periods with only lactate, whereas fast axonal transport would be predicted to stop under these conditions if solely dependent on glycolysis. Further, there are several forms of axonal transport that do not involve vesicular membranes, including slow axonal transport and the movement of RNA granules. Without an onboard ATP supplier, these transport processes would experience regions of slow to no motility in the hypothesized low-ATP regions. Alternatively, diffusion may be sufficient to maintain consistent levels of ATP along the axon. In either case, an onboard mechanism of glycolysis might become more relevant in situations of fast action potential firing—a high-energy task that increases local ATP demands, potentially restricting the ATP available for housekeeping tasks.

Common Themes and Outstanding Questions

The compartmentalized nature of neurons requires active mechanisms of transport to distribute organelles to localized regions of demand. The differing patterns of motility observed for distinct organelles may reflect underlying functional differences. For example, mitochondrial motility facilitates distribution to sites of need, where these organelles become tethered to supply local needs for energy production and calcium buffering. Similarly, the bidirectional movement of mRNA granules may effectively distribute these particles to sites of local synthesis. Other organelles, such as signaling endosomes, must relay information across the extended distance of the axon and thus undergo long journeys with highly processive, unidirectional motility to efficiently move from distal axon to cell soma. And degradative organelles such as autophagosomes must efficiently clear damaged organelles and aggregated proteins, recycling components back to the cell body for reuse.

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Many major outstanding questions remain unanswered. How is organelle movement in the axon choreographed? How is the complement of motors associated with each organelle regulated? Further work is also required to uncover regional-specific differences in organelle transport within the neuron. Advances in imaging technology will continue to facilitate the study of these pathways and provide insights into the alteration of these pathways in stress and disease. A growing number of human diseases, both neurodevelopmental and neurodegenerative, are caused by mutations in the axonal transport machinery. Further, axonal transport is misregulated in many of the major neurodegenerative diseases affecting human populations, including ALS and Alzheimer's, Huntington's, and Parkinson's diseases (Millecamps and Julien, 2013). Thus, continued research into the molecular mechanisms involved in axonal transport and its regulation should provide new insights pointing toward the development of novel therapeutic approaches in future.

Acknowledgment

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Alzheimer's Disease: Mitochondrial Contact Sites and Function

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NOTES

Introduction

Beginning in grade school, and continuing on through high school and college, the approach to teaching and understanding the structure of the eukaryotic cell has been dominated by what one might call the "pigeonhole" view. That is, the cell is presented not as a holistic entity but rather as an object containing a set of independent subcellular elements—e.g., the nucleus, the Golgi body, the endoplasmic reticulum, peroxisomes, endosomes, lysosomes, and mitochondria—each with its own unique place within the cell and each with its own function. This view is so entrenched that we have even anthropomorphized many of these functions: the nucleus is the cell's "information center," the mitochondrion is the "powerhouse of the cell," the lysosome is the cell's "garbage disposal and recycling center," and so forth.

The reality, of course, is much more complicated. Indeed, each subcellular compartment has its own role to play, but to work properly both in space and in time, the individual function of each organelle has to be coordinated with that of every other organelle. Moreover, our description of organelles with individual functions obscures the fact that organelles can have multiple overlapping and/or complementary functions. For example, cholesterol biosynthesis requires the interplay of at least five organelles (the plasma membrane [PM], endoplasmic reticulum [ER], Golgi, mitochondria, and nucleus), whereas calcium trafficking requires at least three (PM, ER, and mitochondria).

Nowhere is this interdependence seen more clearly than in the multiple functions of the ER, which makes physical connections with the nucleus (as the nuclear envelope), peroxisomes (in the "pre-peroxisomal" compartment), the Golgi body (at ER exit sites), the plasma membrane (at plasma membrane–associated membranes), and lipid droplets, among other locations (Lynes and Simmen, 2011; English and Voeltz, 2013). One other important ER connection point, and one that will inform the rest of this review, is the association of ER with mitochondria, at mitochondria-associated ER membranes (MAMs). The role of the MAM as a discrete yet highly dynamic entity in its own right, and its intimate association with neurodegenerative disease, have been revealed only in the past decade or so. We will focus here on the relationship of MAM to the pathogenesis of Alzheimer's disease (AD).

Mitochondria-Associated ER Membranes

MAM is a subdomain of the ER that communicates with mitochondria, both biochemically and physically (Rusinol et al., 1994; Csordas et al., 2006; Hayashi et al., 2009; Raturi and Simmen, 2013). It is a distinct biochemical/biophysical entity within the overall ER network: as opposed to "free ER," "MAM ER" is a lipid-raft-like domain rich in cholesterol and sphingomyelin (Hayashi and Fujimoto, 2010; Area-Gomez et al., 2012) and is enriched in a number of specific proteins with specific functions. Among these are proteins involved in calcium homeostasis, e.g., IP3 (inositol 1,4,5-triphosphate) receptors (Mendes et al., 2005; Szabadkai et al., 2006); in cholesterol metabolism, e.g., acyl-CoA:cholesterol acyltransferase 1 (ACAT1) (Rusinol et al., 1994); in phospholipid metabolism, e.g., phosphatidylserine synthase (Vance et al., 1997; Stone and Vance, 2000); in lipid transfer between mitochondria and ER, e.g., fatty acid transfer protein 4 (Jia et al., 2007); and in the maintenance of mitochondrial morphology, e.g., dynamin-related protein 1 (Friedman et al., 2011). In addition, other proteins stabilize and regulate the apposition of ER to mitochondria at an interorganellar distance of ~10–30 nm (Csordas et al., 2006). These proteins include phosphofurin acidic cluster sorting protein 2 (Simmen et al., 2005) and mitofusin 2 (de Brito and Scorrano, 2008), although their exact "tethering" mechanism is unknown.

Alzheimer's Disease and the Amyloid Cascade Hypothesis

AD is the most common neurodegenerative dementia of aging (Querfurth and LaFerla, 2010). It is defined by its distinct neuropathology, namely, the accumulation in the brain of extracellular plaques composed predominantly of β-amyloid (Aβ) and of intracellular tangles consisting of hyperphosphorylated forms of the microtubule-associated protein tau (Querfurth and LaFerla, 2010).

Familial AD (FAD) affects ~1% of all AD patients. It is inherited as an autosomal-dominant trait and is caused by mutations in genes specifying one of three proteins: presenilin-1 (PS1), presenilin-2 (PS2), and the amyloid precursor protein (APP). The presenilins are aspartyl proteases that form the enzymatically active core of the γ-secretase complex that, together with β-secretase 1 (BACE1), processes APP to produce Aβ. Cleavage of the ~700-aa APP

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by BACE1 produces a 99-aa C-terminal fragment (C99), which is then cleaved by γ-secretase to produce an "APP intracellular domain (~50 aa)" and a set of Aβ fragments that average ~40 aa in the normal situation and ~42 aa in AD. In sporadic AD (SAD), which comprises ~99% of cases, the mechanistic connection to Aβ formation and to its deposition in plaques is less clear. Although this form of AD is sporadic, there are genetic risk factors that predispose an individual to the disease. Foremost among them is the ε4 allele of apolipoprotein E (ApoE4), which is a component of lipoproteins associated with intercellular cholesterol trafficking (Huang, 2010; Holtzman et al., 2012). The reason for the elevated risk conferred by ApoE4 as compared with the more common ApoE3 allele is unknown.

The pathogenetic mechanisms underlying AD are also unknown. However, the fact that mutations in PS1 and PS2 (which process APP) and in APP itself (a substrate of the γ-secretase complex that contains those presenilins) in FAD became the basis of the most dominant and commonly accepted hypothesis to explain AD pathogenicity, namely, the "amyloid cascade" (Hardy and Higgins, 1992). In brief, the hypothesis proposes that the disease arises when, as noted above, APP is cleaved aberrantly by γ-secretase to produce a range of Aβ fragments that average not ~40 aa in length, but ~42 aa, with a concomitant increase in the ratio of $A\beta_{42}$:$A\beta_{40}$. Whereas "normal" $A\beta_{40}$ is essentially soluble, "AD-associated" $A\beta_{42}$ is fibrillogenic and accumulates in the plaques. This extraneuritic amyloid is toxic and promotes tau hyperphosphorylation, leading to the neurofibrillary tangles; both the plaques and the tangles promote cell death, thereby causing the disease (Hardy and Higgins, 1992; Selkoe, 2011).

The amyloid cascade hypothesis is attractive not only because it helps explain findings from many different approaches to the disease but also because it explains why mutations in both APP and the presenilins cause FAD. However, besides the problem of trying to explain plaque formation in SAD, the amyloid cascade hypothesis does not address other features of AD that have received less attention in the field (Schon et al., 2010, 2013). These include altered metabolism of fatty acid (Fraser et al., 2010), glucose (Hoyer et al., 1988; Liu et al., 2009), cholesterol (Stefani and Liguri, 2009), and phospholipid (Pettegrew et al., 2001); perturbed calcium homeostasis (Bezprozvanny and Mattson, 2008); and mitochondrial dysfunction (Wang et al., 2009). Notably, these "other" features of AD are the very ones that are in large part regulated by proteins enriched in the MAM.

The MAM Hypothesis

Based on the striking coincidence between the symptomatology of AD and the functions associated with MAM, we hypothesized that there might be a cause-and-effect relationship between the two. In fact, we and others found that the presenilins (Area-Gomez et al., 2009; Newman et al., 2014)—and γ-secretase activity itself (Area-Gomez et al., 2009; Schreiner et al., 2015)—are located mainly at the MAM. Moreover, the demonstration that MAM is an intracellular lipid raft (Hayashi and Fujimoto, 2010; Area-Gomez et al., 2012) is consistent with the finding that the lipid rafts in which PS1 and γ-secretase activity reside (Vetrivel et al., 2004) are located not only at the cell surface (Marambaud et al., 2002; Area-Gomez et al., 2009) but also inside the cell, at the MAM.

Moreover, MAM function is also altered in AD. For example, it is well known that calcium homeostasis, which is essentially a MAM-mediated process (Hayashi et al., 2009; Csordas et al., 2010; Patergnani et al., 2011), is altered in patients with AD (Peterson and Goldman, 1986; Sims et al., 1987; Gibson et al., 1997; Mattson, 2010; Supnet and Bezprozvanny, 2010; Liang et al., 2015) and in *PSEN1*-mutant mice (Sun et al., 2014). Similarly, mitochondrial dynamics (e.g., organellar fission, fusion, and localization) and bioenergetics have been shown to be altered in AD (Peterson and Goldman, 1986; Gibson and Huang, 2004; Stokin et al., 2005; Wang et al., 2008; Ferrer, 2009; Riemer and Kins, 2013).

Other aspects of MAM function have received less attention, but these, too, are perturbed in AD. These include altered phospholipid profiles (Pettegrew et al., 2001) and altered cholesterol metabolism (Stefani and Liguri, 2009), both of which are increased significantly in PS-mutant cells and in fibroblasts from AD patients (Area-Gomez et al., 2012). Altered phospholipid profiles were measured by the trafficking of phosphatidylserine from the ER to mitochondria via the MAM (Voelker, 2005), and altered cholesterol metabolism was measured by the conversion of free cholesterol to cholesteryl esters by the MAM-localized enzyme ACAT1 (Rusinol et al., 1994). The finding of increased ACAT1 activity in AD cells (Area-Gomez et al., 2012) is particularly noteworthy because ACAT1 activity has been shown to be required for the production of Aβ (Puglielli et al., 2001, 2004), albeit via a currently unknown mechanism.

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Notably, upregulated MAM-mediated phospholipid and cholesterol metabolism was observed not only in FAD cells containing presenilin mutations but also in fibroblasts from FAD patients with mutations in APP, and even more strikingly, in cells from SAD patients in which the *presenilin* and *APP* genes are presumably normal (Area-Gomez et al., 2012). These data imply that increased ER–mitochondrial communication and upregulated MAM function may be present in essentially all AD patients, and that perturbed MAM behavior likely occurs before plaque and tangle formation.

The centrality of altered MAM behavior to AD pathogenesis was recently underscored by studies of the effects of ApoE4 on MAM function (Tambini et al., 2016). As noted above, the ε4 allele of *ApoE* is the single most important genetic risk factor for developing SAD (Holtzman et al., 2012). Strikingly, cells treated with lipoproteins containing ApoE4 upregulated MAM function to a significantly greater degree than did those containing ApoE3 (Tambini et al., 2016). Notably, these effects were apparently the result of the role of ApoE as a component of lipoproteins, not as the free, unlipidated protein (Tambini et al., 2016); these findings imply that the deleterious effects of ApoE4 on MAM function derived from its function in lipoprotein-mediated cholesterol trafficking and metabolism. Thus, ApoE4's contribution to disease risk in AD may, in fact, be caused by its effects on MAM function via perturbed cholesterol homeostasis. In support of this view, we note that genetic variants in a number of cholesterol metabolism–related genes (Wollmer, 2010), such as *ABCA7* (Steinberg et al., 2015), which is required for cholesterol and phospholipid mobilization (Abe-Dohmae et al., 2004), predispose an individual to developing AD.

Conclusion and Implications

Taken together, these observations support a view of AD pathogenesis that differs from that offered by the amyloid cascade hypothesis. Rather than being the result of cellular toxicity due to the accumulation of plaques and tangles, we hypothesize that the development and progression of the disease result from increased ER–mitochondrial communication. This increase, in turn, affects numerous cellular functions, both directly (e.g., via increased ACAT1 activity and cholesterol ester synthesis) and indirectly (e.g., via increased lipid droplet formation). The increased ER–mitochondrial apposition and increased MAM function are consistent with the features described earlier: aberrant phospholipid profiles; the perturbed cholesterol homeostasis; the changes in mitochondrial function, morphology, and distribution; the increased calcium trafficking between the two organelles; and the elevated ratio of $A\beta_{42}$:$A\beta_{40}$. Thus, we propose that the functional cause of AD is increased ER–mitochondrial communication and upregulated MAM function. However, the biochemical cause of this ER–mitochondrial hyperconnectivity, and how APP processing plays a role in this process, remain to be elucidated.

Acknowledgments

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Role of Mitochondrial Quality Control in Parkinsonism

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Introduction

Mutations in genes encoding PINK1 and Parkin cause early-onset familial Parkinson's disease (PD). Consistent with genetic studies in *Drosophila* indicating that PINK1 functions upstream of Parkin in the same pathway (Clark et al., 2006; Park et al., 2006; Poole et al., 2008), biochemical and cell biology studies have shown that PINK1 recruits Parkin to damaged mitochondria, where Parkin can induce selective autophagy of damaged mitochondria (mitophagy) (Narendra et al., 2008, 2010; Geisler et al., 2010; Vives-Bauza et al., 2010). PINK1 is a kinase that phosphorylates Parkin and triggers Parkin, an E3 ubiquitin ligase, to associate with mitochondria and ubiquitinate scores of substrates on the surface of mitochondria (Sarraf et al., 2013). These substrates, linked with both K48 and K63 ubiquitin chains, recruit adaptors such as p62, NBR1, Optineurin, and NDP52 to mitochondria that are key for initiating autophagosome recognition of damaged mitochondria (Wong and Holzbaur, 2014; Lazarou et al., 2015). Chemical uncouplers (Narendra et al., 2008), mitochondrial DNA (mtDNA) mutations (Suen et al., 2010), or even unfolded proteins (Jin and Youle, 2013) can induce Parkin translocation and mitophagy.

In Vivo Support for the Mitochondrial Quality Control Hypothesis

The hypothesis which holds that insufficient mitophagy may cause disease phenotypes in patients with mutations in PINK1 and Parkin was initially based on results in cultured cells (Narendra et al., 2008). However, this model has been recently supported by *in vivo* results in *Drosophila* (Vincow et al., 2013) and murine models (Fig. 1) (Pickrell et al., 2015).

We have found that Parkin protects dopaminergic neurons in mice from damage caused by mtDNA mutations. Polymerase gamma (POLG) is responsible for the replication of mtDNA. *Mutator* mice express a proofreading-deficient POLG that causes a progressive accumulation of mtDNA mutations throughout the body (Trifunovic et al., 2004; Kujoth et al., 2005). This leads to a premature aging phenotype characterized by early hair loss, anemia, osteoporosis, sarcopenia, cardiomyopathy, and decreased lifespan resulting from the decline of mitochondrial function (Trifunovic et al., 2004; Kujoth et al., 2005). *Mutator* mice have a loss of oxidative phosphorylation activity in the CNS, but do not display signs of neurodegeneration (Kujoth et al., 2005; Ross et al., 2010; Dai et al., 2013). We crossed this aging mouse model to a *Parkin* knockout (KO) mouse background. Although the *Parkin* KO mouse has a minor phenotype and no loss of dopaminergic neurons, we found a substantial loss of dopaminergic neurons in *Mutator* mice in the absence of endogenous Parkin (Fig. 1). *Mutator* mice had no loss of TH+ neurons at 52 weeks of age, whereas the *Mutator Parkin KO* mice showed a decrease of ~40% of TH+ neurons in both substantia nigra (SN) and ventral tegmental area (VTA) regions.

We performed the pole test, which is sensitive and specifically indicative of nigrostriatal dysfunctions (Matsuura et al., 1997), on 52-week-old *Mutator* and *Mutator Parkin* KO mice. *Mutator Parkin* KO mice latency times before descending the pole were significant longer than those of either *Mutator, Parkin* KO, or control (wild-type [WT]) mice, suggesting that the loss of TH+ axons and neurons at this age (Fig. 1C) caused a behavioral deficit in motor coordination. Levodopa (L-DOPA) treatment was able to completely reverse this climbing defect of the

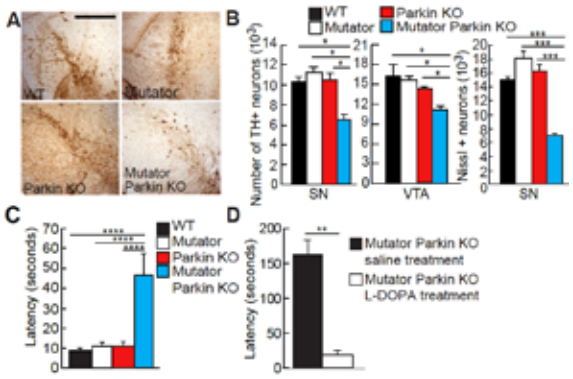

Figure 1. Dopaminergic neurons degenerate and cause a movement disorder after the loss of endogenous Parkin in a mouse model of mitochondrial dysfunction. ***A,*** Representative images of the SN detected in midbrain sections after staining with anti-TH antibodies. Scale bar, 500 µm. ***B,*** The estimated number of TH^+ neurons in the SN and VTA, and $Nissl^+$ neurons in the SN in 48- to 52-week-old mice. *n* = 4/group. ***C,*** Latency time (s) recorded for 48- to 52-week-old mice performing the pole test. *n* = 6–8/group. ***D,*** Latency time (s) recorded for 56-week-old *Mutator Parkin* KO mice performing the pole test. Each mouse was scored for a baseline performance for comparison after L-DOPA treatment. *n* = 4/group. Reprinted with permission from Pickrell AM, et al. Endogenous Parkin preserves dopaminergic substantia nigral neurons following mitochondrial DNA mutagenic stress. Neuron 87:373; their Figs. 1*A, B, G,* and *H*. Copyright 2015, Elsevier.

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Mutator Parkin KO mice, indicating that the motor impairment resulted from the loss of dopaminergic neurons (Fig. 1*D*). These results show that in whole-body *Parkin* KO mice, whole-body mtDNA mutation accumulation causes a specific defect in the midbrain dopaminergic cells. They also show that endogenous Parkin protects neurons *in vivo* from a mitochondria-specific deficit induced by the mutant mtDNA polymerase.

Implications for Therapy in Humans

Genetic studies have shown that increasing the expression of Parkin can rescue mice from 6-OHDA (Vercammen et al., 2006; Yasuda et al., 2011) and MPTP-induced dopaminergic neuron loss (Yasuda et al., 2011) and rescue *Drosophila* from aging and associated proteotoxicity (Pimenta de Castro et al., 2012; Rana et al., 2013). Consistent with its role in stress mitigation, *Parkin* gene expression is upregulated by the unfolded protein response transcriptional factor, ATF4 (Bouman et al., 2011). Thus, increasing PINK1 or Parkin expression or activity in humans might foster mitochondrial quality control and have therapeutic benefits for patients with mtDNA mutation diseases, PD, or other diseases linked to mitochondrial dysfunction.

Animal studies corroborate cell-culture models suggesting that one key role that PINK1 and Parkin normally play is to mediate mitochondrial quality control, and that individuals with mutations in PINK1 and Parkin may develop parkinsonism because of a deficit in this pathway. Moreover, drugging this pathway to augment mitochondrial quality control may eventually help patients with inherited mtDNA mutations, metabolic disorders such as type 2 diabetes, and possibly forms of PD. Understanding the molecular deficits leading to inherited forms of PD also could yield insights into the etiology of sporadic PD.

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