Mitochondrial encephalomyopathies are common and devastating multisystem genetic disorders characterized by neuromuscular dysfunction and tissue degeneration. Point mutations in the human mitochondrial ATP6 gene are known to cause several related mitochondrial disorders: NARP (neuropathy, ataxia, and retinitis pigmentosa), MILS (maternally inherited Leigh’s syndrome), and FBSN (familial bilateral striatal necrosis). We identified a pathogenic mutation in the Drosophila ATP6 gene that causes progressive, adult-onset neuromuscular dysfunction and myodegeneration. Our results demonstrate ultrastructural defects in the mitochondrial innermembrane, neural dysfunction, and a marked reduction in mitochondrial ATP synthase activity associated with this mutation. This Drosophila mutant recapitulates key features of the human neuromuscular disorders enabling detailed in vivo studies of these enigmatic diseases.

Key words: ATP6; ANT; ATP synthase; neurodegeneration; muscle degeneration; aging; mitochondria

Introduction

Mitochondrial impairment results in a host of devastating conditions known generally as respiratory chain diseases (DiMauro and Schon, 2003). Furthermore, mitochondrial dysfunction has been conclusively implicated in nearly every chronic human neurodegenerative disease, including mitochondrial encephalomyopathies, amyotrophic lateral sclerosis, and Huntington’s, Alzheimer’s, and Parkinson’s diseases (Orth and Schapira, 2001; Schapira and Cock, 2003). Primary mutations in several distinct mitochondrial genes, as well as nuclear genes that encode proteins localized to the mitochondria, are known to cause a variety of related and devastating encephalomyopathies with complex clinical features, including neurological and muscular dysfunction that is often complicated by renal, endocrine, cardiac, and hepatic involvement (for review, see Schapira and Cock, 1999; Hart et al., 2002; DiMauro and Schon, 2003). There are over 150 mutations causing these related diseases and range from point mutations to large deletions. Because there are numerous mutagenic targets, these mitochondrial disorders have an extremely high prevalence similar to that of muscular dystrophy, ~10–15 cases per 100,000 individuals (DiMauro and Schon, 2003).

Point mutations in the human mitochondrial ATP6 gene are known to cause neuropathy, ataxia, and retinitis pigmentosa (NARP), maternally inherited Leigh’s syndrome (MILS) and familial bilateral striatal necrosis (FBSN) diseases. Also, disease is associated with a common 5kb mitochondrial genome deletion of ATP6 (Table 1). The ATP6 protein is an essential component of the mitochondrial F1F0-ATP synthase (Complex V) that functions as a hydrogen ion channel serving to couple ion transport with rotary ATP catalysis (Mitchell and Moyle, 1967; Walker, 1995; Boyer, 1997; Stock et al., 2000). Bioenergetic impairment and oxidative damage resulting from respiratory chain dysfunction are two prominent hypotheses for the pathogenesis of human mitochondrial encephalopathies. However, mechanistic details of pathogenesis associated with chronic mitochondrial impairment remain unclear, and as a result, therapeutic approaches remain limited.

Autosomal dominant progressive external ophthalmoplegia (adPEO) results from mutation of three distinct nuclear loci: 4q34–35, 10q24 (Suomalainen et al., 1995), and 15q22–26 (Van Goethem et al., 2001). The 4q PEO locus was recently identified as the ANT1 gene, wherein four different mutation sites segregating in six PEO families were discovered (Kaukonen et al., 2000; Napoli et al., 2001; Komaki et al., 2002). Patients with PEO are predisposed to secondary mitochondrial mutations that appear to underlie, or at least contribute to, the pathophysiological basis for their degenerative disease (Servidei et al., 1991; Suomalainen et al., 1997). The basis for the mutator phenomenon associated with PEO disease is not understood.
We discovered a mitochondrial ATP6 mutation as a materi-
nally inherited component in our Drosophila sesB1 (ANT1) mu-
tant stock. To our knowledge this is the first pathogenic mito-
chondrial mutation isolated in an intact genetic animal system. 
Our studies of the Drosophila ATP6 and sesB1(ANT1) mutations 
revealed common phenotypes, including conditional paralysis, 
shortened lifespan, and degeneration of the neuromuscular sys-
tem. The observed locomotor impairment, myodegeneration, 
nerval dysfunction, and severe shortening of lifespan observed in 
the fly recapitulate key features of MILD disease in humans. Ul-
trastructural analysis of ATP6 revealed a novel mitochondrial 
morphological defect associated with ATP synthase impairment. 
ATP synthase activity was absent in ATP6 mutants. Surprisingly, 
respiration rates were unaltered in these mutants. These data 
demonstrate that impaired ATP synthase function associated 
with ATP6 dysfunction can cause severe encephalomyopathy in 
the absence of a defect in respiration rate in vivo.

Materials and Methods

Drosophila stocks and culture. Standard cornmeal molasses fly media was 
used, unless otherwise noted. sesB1 and DSR (Wolbachia-infected posi-
tive control) flies were obtained from Michael Ashburner (University of 
Cambridge, Cambridge, UK) and Michael Clark (University of Chicago, 
Chicago, IL), respectively. All other stocks were obtained from the 
Bloomington stock center. Canton S was our wild-type (WT) strain. 
Tetracycline treatment of flies involved maintaining cultures on standard 
Drosophila stocks and culture.

Materials and Methods

Table 1. Human diseases associated directly with ATP6 mutation

<table>
<thead>
<tr>
<th>AA</th>
<th>Mutation</th>
<th>Disease</th>
<th>Notes and key references</th>
</tr>
</thead>
<tbody>
<tr>
<td>S99P</td>
<td>T8821C</td>
<td>Oligospermia</td>
<td>Holyoke et al., 1999</td>
</tr>
<tr>
<td>W109R</td>
<td>T8951C</td>
<td>FBSM</td>
<td>De Meirel et al., 1995</td>
</tr>
<tr>
<td>L56R</td>
<td>T8993G</td>
<td>NARP/MLS/HCN</td>
<td>Holt et al., 1990; Fryer et al., 1994; Pastores et al., 1994; White et al., 1999; Marin-Garcia et al., 2001</td>
</tr>
<tr>
<td>L56P</td>
<td>T8993C</td>
<td>NARP/MLS</td>
<td>de Vries et al., 1993; Santorelli et al., 1994; White et al., 1999; Sciacco et al., 2003</td>
</tr>
<tr>
<td>I92T</td>
<td>T9101C</td>
<td>LHON</td>
<td>Not a highly conserved residue or dramatic amino acid change (Lamminen et al., 1995)</td>
</tr>
<tr>
<td>L217P</td>
<td>T9176C</td>
<td>FBSM/MLS</td>
<td>Thyagarajan et al., 1995; Campos et al., 1997</td>
</tr>
<tr>
<td>L217R</td>
<td>T9176G</td>
<td>MILS</td>
<td>Carrozzi et al., 2001</td>
</tr>
<tr>
<td>N/A</td>
<td>Deletion</td>
<td>PEO</td>
<td>Commonly removes ATP6 (Moraes et al., 1989; Schon et al., 1989; Zeviani et al., 1989; Mita et al., 1990)</td>
</tr>
<tr>
<td>N/A</td>
<td>Deletion</td>
<td>KSS</td>
<td>Commonly removes ATP6 (Moraes et al., 1989; Schon et al., 1989; Mita et al., 1990)</td>
</tr>
<tr>
<td>N/A</td>
<td>Deletion</td>
<td>Myopathy</td>
<td>Commonly removes ATP6 (Holt et al., 1989)</td>
</tr>
</tbody>
</table>

ATP6 is a single disease defined by either neuropathy, ataxia, retinitis pigmentosa or neurogenic muscle weakness, ataxia, retinitis pigmentosa. HCM, Hypertrophic cardiomyopathy; KSS, Kearns–Sayre syndrome; LHON, Leber hereditary optic neuropathy.
of the final models was increased by 20% to compensate for radiation-induced thinning of the plastic section (Deng et al., 1999).

**ATP synthase assays.** The micromolar quantity of ATP produced per microgram of protein was determined using a modified version of a previously described assay (Manfredi, 2001). Mitochondria were isolated from ~2 g of adult flies. Flies were homogenized with a glass Dounce homogenizer, three times with plunger A, in 50 ml of mitochondrial isolation buffer (320 mM sucrose, 1 mM EDTA K-salt, 10 mM Tris, pH 7.4). The homogenate was centrifuged at 1500 × g for 10 min at 4°C. The supernatant was homogenized, three times with plunger B, and centrifuged at 17,000 × g for 10 min at 4°C. The mitochondrial pellet was resuspended in ATP synthesis buffer A (150 mM KCl, 25 mM Tris-HCl, 2 mM EDTA, 0.1% BSA, 10 mM KH2PO4, 0.1M MgCl2, pH 7.4). Each sample is split into two tubes, and each tube contains the following concentrations of reagents (in μM): 0.15 di(adenosine)pentaphosphate, 0.1 ADP, 1 malate, and 1 pyruvate. Oligomycin at 10 μg/ml was added to only one of the two tubes. An ATP standard curve was generated (0.0, 0.01, 0.1, 1.0, and 10.0 μM). ATP synthase measurements were taken in a black 96-well plate after the addition of buffer B (0.8 mM Luciferin, 20 μg/ml Luciferase, 0.5 mM Tris-acetate, pH 7.75), and the luminescence was immediately recorded using a Victor2 plate reader (PerkinElmer, Wellesley, MA). The light emission was integrated for 1 s every 30 s for a total of 3 min. The data from this experiment were normalized to the total protein in the sample. The homogenate was brought to a final concentration of 0.1 M NaOH and sonicated for 10 s (setting 5 Fisher sonic dismembrator model 100; Fisher Scientific, Houston, TX). The concentration of protein was determined using a colorimetric protein assay reagent (Biorad, Hercules, CA) and a BSA protein standard curve. There was no significant difference in ATP synthase activity between untreated ATP6I and oligomycin-treated wild type, demonstrating that the ATP6 protein encoded by the mutant is not simply oligomycin insensitive.

**Population respiration rates.** We directly measured respiration of ~5 g of flies (~5000 flies) as the emergence of CO2 in a fixed volume respirometer. The respirometer was a sealed acrylic 3.570 L box containing a dual wavelength infrared- absorbance analyzer to monitor CO2 concentration between 0 and 10,000 ppm (model 2820; Bacharach, New Kensington, PA) and 30 glass shell vials each containing 8 ml of yeast-free food and ~150 flies. Cheesecloth was used to confine flies to within the vials while allowing free air exchange. Flies aged 2–10 d were placed in the respirometer, which was purged with 60% N2, 40% O2 (20 min) to remove CO2 and establish a standard initial O2 concentration. The intake and exhaust ports were sealed, and the CO2 levels were recorded every 2 min for 5 h. We performed this experiment, sans flies, to determine the integrity of the container and identify any other sources of CO2. No detectable CO2 was produced in all experiments without flies over the course of the experiment. The vials were removed immediately after the experiment, and respiration data are presented as CO2 emergence per unit mass (parts per million per gram of fly) as a function of time. The effective respirometer chamber volume (total volume less volume of vials, food, meter, and flies) is ~2.760 L. Experiments were performed alternating wild-type and age-matched mutant flies (n = 4).

**Individual metabolic measurements.** Flies eclosed at room temperature and were placed in a 25°C incubator with a 12/12 h photoperiod for at least 48 h to acclimate to standard metabolic conditions. Resting metabolic rates were measured on flies that were 5–6 d postemergent using methods described previously (Van Voorhies et al., 2003, 2004). Briefly, respiration was measured as the CO2 produced by individual flies maintained in a 2.2 mL glass sealed chamber flushed with CO2-free, water-saturated (100% relative humidity) air. Gas samples were removed from the chamber with a Hamilton SampleLock syringe (Hamilton, Reno, NV) and directly injected into a 150 μl/min (±1%) standard temperature pressure dry, CO2-free carrier air stream. Flow of the carrier air stream was controlled with a mass flow meter (Sierra Instruments, Monterey, CA) and was scrubbed of water vapor with a magnesium perchlorate filter before entering into a Li-Cor 6251 carbon dioxide gas analyzer (Li-Cor, Lincoln, NE). The Li-Cor 6251 analyzer has a sensitivity of <0.1 ppm and an accuracy of <1 ppm. The amount of CO2 produced by each fly was calculated using DATACAN software (Sable Systems International, Henderson, NV). Each fly examined was briefly frozen at ~80°C, and its mass was determined using a Sartorius (Goettingen, Germany) M2P microbalance. The CO2 gas analysis system was zeroed daily against CO2 free air and calibrated against a 51 ppm certified gas standard (Air Products, Long Beach, CA.). Previous studies have established that CO2 production provides a sensitive and accurate proxy for oxygen consumption in Drosophila melanogaster (Van Voorhies et al., 2004). Results expressed in microwatts/ fly are as follows: 19.6 ± 0.7 ATP6 females, 20.8 ± 0.9 wild-type females, 15.7 ± 0.4 ATP6I-positive control females, 15.1 ± 0.5 ATP6I males, 13.3 ± 0.5 wild-type males, and 10.2 ± 0.2 ATP6I-positive control males (SE provided). Our studies revealed severely reduced respiration in ATP6I flies that served as a positive control for these studies. ATP6I flies are heterozygous for ATP6, the Drosophila gene encoding the Na+/K+ ATPase α subunit (Palladino et al., 2003).

**Results**

**Maternally inherited mutation causes conditional paralysis.** Mutations affecting ANTI in Drosophila were originally identified as a stress-sensitive strain of animals (sesB) that are conditionally paralytic in response to mechanical stress (Homyk and Sheppard, 1977). It was later discovered that sesB is an adult viable hypomorphic allele of the gene encoding ANTI and that null alleles are lethal (Zhang et al., 1999). Previous studies have shown that conditional paralytic mutants in Drosophila are enriched for those that cause neurodegeneration (Palladino et al., 2002). During the course of studying sesB(ANTI), a separate maternally inherited genetic component was discovered that enhances the conditional paralysis of sesB(ANTI) and itself causes conditional paralysis. This maternal component was genetically isolated and named for the maternally derived phenotype MATBS (maternal isolate, bang sensitive; see Materials and Methods for details). MATBS animals transmit conditional paralysis and reduced lifespan (with 100% penetrance) to every strain tested but only when maternal inheritance is maintained. The maternal effect is stable; MATBS strains have been maintained for numerous generations without phenotypic erosion.

Previous studies have shown that a pathogenic variant of the bacterium Wolbachia, a naturally occurring insect symbiont, could infect Drosophila and cause a shortening of lifespan (Min and Benzer, 1997b). To test whether a pathogenic variant of Wolbachia could be the source of the MATBS maternally inherited phenotypes, Wolbachia-specific primers and an established PCR detection protocol were used as an assay for the presence of these bacteria (Min and Benzer, 1997b). Wolbachia were detected in a positive control strain known to harbor the bacteria but were not present in our mutant strain (data not shown). Furthermore, tetracycline treatment for two generations has been shown to completely rid flies of microbial parasites, including Wolbachia, and this treatment did not modify the phenotypes associated with MATBS (Min and Benzer, 1997b). The strict maternal inheritance and antibiotic resistance of the MATBS phenotypes led us to hypothesize that there was a mitochondrial mutation in this strain of flies.

**MATBS animals have a mitochondrial mutation in their ATP6 gene.** The mitochondrial genome was amplified in overlapping ~3 kb fragments using PCR and directly sequenced from our MATBS and two wild-type control strains. tRNA and rRNA sequences revealed no changes. One nonpolymorphic change in the coding regions was discovered that was unique to the MATBS strain. Interestingly, this G to A transition appeared homogeneous in the sequence chromatogram and results in a glycine to glutamate codon change at position 116 in the ATP6 gene (supplemental
ATP6\textsuperscript{1} animals have shortened lifespans and tissue degeneration

It is intriguing that the ATP6\textsuperscript{1} mutation was discovered in the sesB\textsuperscript{1} (ANT1) mutant genetic background and that each mutation results in a qualitatively similar conditional paralysis phenotype. Lifespan analyses revealed that both sesB\textsuperscript{1} (ANT1) and ATP6\textsuperscript{1} animals have a significant reduction in lifespan from wild-type controls (Fig. 1). Despite the similar paralysis phenotype, each mutant strain’s lifespan impairment was distinct: ATP6\textsuperscript{1} and sesB\textsuperscript{1} median adult lifespan was 12 and 25 d, respectively, whereas control animals’ median lifespan was \(\sim\) 50 d. The lifespan of the ATP6\textsuperscript{1}; sesB\textsuperscript{1} double mutants revealed an additional impairment of adult viability beyond each of the individual mitochondrial mutants (Fig. 1). It is important to note that ATP6\textsuperscript{1} does not appear to compensate for the sesB\textsuperscript{1} mutation and improve viability.

Mitochondrial dysfunction is known to manifest as neurological and muscular impairment in animals because of the intense metabolic demands of myofibrils and neurons. Furthermore, several Drosophila mutants with shortened lifespan have been characterized and have been found to result in neurodegeneration (Kretzschmar et al., 1997; Min and Benzer, 1997a, 1999; Rogina et al., 1997; Palladino et al., 2002, 2003). Thus, to test whether neuropathology may be causing the reduction in the ATP6\textsuperscript{1} median lifespan, mutant brains were examined for neurodegeneration using standard paraffin histology and H&E staining procedures. There were no apparent histologic abnormalities in aged ATP6\textsuperscript{1} mutants (Fig. 2). However, severe neuropathology was observed in aged ANT (sesB\textsuperscript{1}) and ATP6\textsuperscript{1}; sesB\textsuperscript{1} double mutant brains (Fig. 2). In all genotypes examined, young animals were free of pathology, demonstrating the neurodegeneration is strictly progressive (data not shown). Although ATP6\textsuperscript{1} mutants do not have obvious neurodegeneration, the mutation dramatically accelerates the onset of severe neuropathology in sesB\textsuperscript{1} (ANT1) mutants from days 23 to 12 (Fig. 2B, C). Day 12 sesB\textsuperscript{1} (ANT1) mutants did not exhibit neurodegeneration and were indistinguishable with age-matched ATP6\textsuperscript{1} mutants and aged wild-type flies (data not shown). NARP and MILS diseases are associated with profound neuromuscular impairment, prompting examination of muscle integrity in ATP6\textsuperscript{1} mutants. Histology was performed to examine the dorsal flight muscles for the presence of pathology in young and aged animals using standard paraffin histology and (H&E) staining procedures, which revealed muscle degeneration in ATP6\textsuperscript{1} mutants that was strictly progressive (Fig. 3A). Young animals revealed no evidence of myopathy in ATP6\textsuperscript{1}, sesB\textsuperscript{1} (ANT1), or wild-type controls. In contrast, ATP6\textsuperscript{1} mutants revealed striking pathology at day 10, a time consistent with the onset of poor viability (Figs. 1, 3A). Day 5 ATP6\textsuperscript{1} mutants have less severe myopathy. In contrast, sesB\textsuperscript{1} (ANT1) mutants revealed only minimal evidence of myopathy by day 10; however, this mutant strain had marked myopathy by day 22 (Fig. 3A). Examination of the thoracic ganglion, which comprises another significant portion of the fly’s CNS, revealed age-dependent neurodegeneration in sesB\textsuperscript{1} (ANT1) mutants (Fig. 3B). ATP6\textsuperscript{1} histopathology revealed some evidence of thoracic ganglion neurodegeneration,
namely individual large vacuolar structures that were not observed in age matched wild-type animals (Fig. 3B). As seen in the brain, the ATP6<sup>1</sup> mutant results in neural dysfunction and accelerates sesB<sup>1</sup> degeneration of the thoracic ganglion (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). It is intriguing that sesB<sup>1</sup> (ANT1) mutation results in severe neuromuscular pathology and ATP6<sup>1</sup> mutation results predominantly in muscle pathology, yet both mutants are predicted to disrupt mitochondrial ATP output and cause a similar locomotor impairment.

**Locomotor impairment in mitochondrial mutants**

One hallmark of mitochondrial encephalomyopathies is the progressive onset of symptoms, including locomotor impairment. Both sesB<sup>1</sup> (ANT1) and ATP6<sup>1</sup> mutants have reduced locomotor function and conditional paralysis brought on by mechanical stress. We characterized the time required for recovery from physical stress in both mitochondrial mutants. Wild-type flies are relatively unaffected by this stress and recover within \(\sim 2-3\) s at all time points examined. There is a small but significant decrement with age in wild-type animals observed using this assay, as seen by comparing day 2 and day 24 animals (Fig. 4) \((p < 0.05)\). In contrast, both sesB<sup>1</sup> (ANT1) and ATP6<sup>1</sup> mutants revealed a markedly progressive paralysis phenotype. Young mutants, day 2 and 4, are not more affected by mechanical stress than wild type \((p > 0.05)\), although there is greater variability in recovery time in the mutants than age-matched wild-type animals. However, by day 6, both mitochondrial mutants demonstrate significant locomotor impairment (Fig. 4). The trend continues for sesB<sup>1</sup> (ANT1) mutants at day 24, in which paralysis typically lasts \(\sim 3\) min. Interestingly, ATP6<sup>1</sup>; sesB<sup>1</sup> double mutants revealed enhanced locomotor impairment with an earlier onset than either of the separate mitochondrial mutants.

**Transmission electron microscopy analysis reveals mitochondrial pathology**

The F<sub>i</sub>F<sub>o</sub> ATP synthase (Complex V) uses the mitochondrial electrochemical potential in the form of a hydrogen ion gradient to drive rotary catalysis of ATP. Mutations affecting ATP6, the hydrogen ion channel through which dissipation of this gradient is coupled to ATP production, are thought to have a profound affect on mitochondria function (Mitchell and Moyle, 1967; Walker, 1995; Boyer, 1997; Stock et al., 2000). Mitochondrial dysfunction is known to manifest as neurological and muscular impairment in animals because of the intense metabolic demands of these tissues. The ATP6<sup>1</sup> mutation causes severe progressive muscle degeneration but not marked neurodegeneration. It does, however, enhance the neurodegeneration phenotype of sesB<sup>1</sup> (ANT1), demonstrating that the underlying dysfunction is present in this tissue. To examine the consequence of this dysfunction without risk of the results being confounded by tissue degeneration, thin sections of the cortical and central brain regions of day 10 ATP6<sup>1</sup> and age-matched control animals were examined using transmission electron microscopy (TEM). The analysis revealed mitochondria displaying highly abnormal internal compartmentalization that was not typical in age-matched wild-type flies (Fig. 5). The numerous small, round cristae \((\sim 50-80\) nm in diameter) give the mitochondria a honeycomb appearance. This was not an isolated or rare finding: the vast majority of mitochondria observed in numerous midbrain micrographs from independent ATP6<sup>1</sup> animals had abnormal internal membrane morphology \((n > 40\) micrographs from four independent animals). TEM micrographs were acquired using
Error bars represent SEM. Three populations of 15 animals/genotype were tested.

ATP61

TEM tomography analysis of graphic modeling has confirmed the functional importance of dria in the honeycomb regions of these mitochondria but also revealed a more complex three-dimensional structure than was evident by standard TEM analysis. Specifically, the round inner membrane compartments are not individual, unconnected vesicles (as in the case of Senger’s syndrome) but rather are part of a highly interconnected membrane network (Fig. 6). The tightly packed spherical compartments are connected by narrow tubular junctions to each other and to the inner boundary membrane (i.e., the region that parallels the outer membrane) (Fig. 6C, D, G). An even more intriguing feature of the tomographic reconstructions is that the roughly spherical compartments are clearly contiguous with the lamellar regions of the inner membrane (Fig. 6E, F). We were unable to find a single unambiguous example of a truly vesicular (unattached) inner membrane compartment within the tomogram, suggesting that the round compartments represent local dilations in the lamellae that remain physically coupled with the rest of the inner-membrane

stereological protocols allowing the frequency of this morphological abnormality to be quantified in a blinded analysis of randomly selected midbrain micrographs covering 711 μm² tissue from ATP6 mutants and wild-type control animals (Fig. 5C). The frequency of abnormally compartmentalized mitochondria was significantly higher in mutant brains and, correspondingly, the frequency of normal mitochondria was significantly reduced (p < 0.01). Although mitochondria are morphologically abnormal, the density (number of mitochondria per unit area) of mitochondria in ATP6 mutants (31 ± 3 per 100 μm²) is not significantly different from that of wild-type control animals (35 ± 2 per 100 μm²; Student’s t test; p = 0.45). Previous ultrastructural examination of mitochondria has revealed the dynamic nature of mitochondrial structure (Hackenbrock, 1966). More recently, tomographic modeling has confirmed the functional importance of the mitochondrial internal membrane, which can become compartmentalized in conditions associated with mitochondrial impairment (Frey and Mannella, 2000).

TEM tomography analysis of ATP6

The appearance of small, round cristae in mitochondria of ATP6 mutants is reminiscent of a morphological abnormality associated with a human mitochondrial myopathy, Senger’s syndrome (Huizing, 1998; Jordens et al., 2002). In the latter case, three-dimensional imaging of the mitochondria, using electron tomography of thick sections, demonstrated that the round cristae are, in fact, vesicles that are not attached to the inner boundary membrane by the usual crista junctions (Frey and Mannella, 2000). To elucidate the structure of the mitochondrial inner membrane in the Drosophila mutant, we generated tomographic reconstructions of the aberrant ATP6 mitochondria. The tomographic analysis verified the roughly spherical shape of the cristae in the honeycomb regions of these mitochondria but also revealed a more complex three-dimensional structure than was evident by standard TEM analysis. Specifically, the round inner membrane compartments are not individual, unconnected vesicles (as in the case of Senger’s syndrome) but rather are part of a highly interconnected membrane network (Fig. 6). The tightly packed spherical compartments are connected by narrow tubular junctions to each other and to the inner boundary membrane (i.e., the region that parallels the outer membrane) (Fig. 6C, D, G). An even more intriguing feature of the tomographic reconstructions is that the roughly spherical compartments are clearly contiguous with the lamellar regions of the inner membrane (Fig. 6E, F). We were unable to find a single unambiguous example of a truly vesicular (unattached) inner membrane compartment within the tomogram, suggesting that the round compartments represent local dilations in the lamellae that remain physically coupled with the rest of the inner-membrane

Mitochondrial function in ATP6 mutants

Mitochondrial encephalomyopathies are collectively an extremely prevalent and devastating class of diseases that can result from a variety of mitochondrial or nuclear mutations. Mitochondrial encephalomyopathies associated directly with ATP6 mutation, namely NARP, MILS, and FBSN, can result from numerous mutagenic events (Table 1) with the resulting disease onset and severity highly dependent on the percentage of homoplasmy of the mutation. The pathological basis of these diseases is uncertain, but it has been hypothesized that bioenergetic impairment and oxidative stress resulting from respiratory chain dysfunction contribute to the disease condition. To test whether the ATP6 mutation altered mitochondrial ATP synthase activity in vivo, luciferin-luciferase luminescence assays (Manfredi, 2001) were
used to biochemically measure the oligomycin-dependent component of ATP generation from ATP6\(^i\) mitochondria (Fig. 7A). Wild-type control mitochondria had robust ATP synthase activity (23.3 µmol/min/mg), whereas ATP6 mutant mitochondria never revealed significant ATP synthase activity in this assay (Fig. 7B) \((p < 0.01)\). We similarly examined ATP synthase activity from wild-type flies acutely treated with dinitrophenol (DNP). DNP-treated flies produced 6.9 µmol/min/mg ATP synthase activity, a significant reduction from untreated wild-type flies \((p < 0.05)\). These data demonstrate that ATP synthase function in ATP6\(^i\) mutants is markedly reduced and that the resulting bioenergetic impairment likely contributes to disease pathogenesis.

The abnormal morphology of ATP6\(^i\) mitochondria and the reduction in mitochondrial ATP synthase activity prompted us to investigate whether an altered rate of respiration in these animals may also be contributing to the disease pathogenesis. The rate of respiration of ATP6\(^i\) and age-matched wild-type control populations was measured in a fixed volume respirometer. Surprisingly, both genotypes produced an indistinguishable linear response (Fig. 7C). The respiration rate was derived from the slope of the best-fit line and was not different between the ATP6\(^i\) mutants and controls \((p = 0.68; n = 4)\). Although these data strongly argue that respiration rate is not affected in the mutant strain, an independent and extremely sensitive single fly assay was used to verify this finding (Van Voorhies et al., 2003, 2004). The resting metabolic rate of individual ATP6\(^i\) and age-matched wild-type control flies was measured and, in accord with population measurements, there was no significant difference in metabolic rate between these strains (Fig. 7D) \((p > 0.33)\). There was a significant decrease in the respiration rate from a positive control ATP6alphaDTS1R1 \((1R1)\) strain \((p < 0.001)\). These data suggest that our assay of respiration rate is sufficiently sensitive to detect differences in metabolic rate and that there is no apparent dysfunction in respiration rate contributing to the neuromuscular condition in ATP6\(^i\) mutant flies.

**Discussion**

Mitochondrial encephalomyopathies are a collection of devastating medical conditions that are poorly understood and for which therapeutic treatments remain extremely limited. Our understanding of these mysterious diseases is constrained by our limited ability to genetically manipulate mitochondria, difficulties associated with studying progressive conditions, and a lack of tractable in vivo models that recapitulate these diseases. This is especially true of conditions associated directly with mutation of the mitochondrial genome, such as NARP, MILS, and FBSN. We identified marked neuromuscular degeneration associated with mutations of the nuclear gene sekB that encodes the mitochondrial ANT1 protein. We also isolated a nearly homoplasmic mitochondrial mutation of the mitochondrial ATP6 gene that results in a marked shortening of lifespan, locomotor impairment, and progressive myodegeneration. These studies demonstrate that normal respiration rates are maintained despite a defect in mitochondrial ATP synthase activity. To our knowledge, this ATP6\(^i\) Drosophila mutant is the first isolation of an endogenous mitochondrial mutation that recapitulates key pathological features of human mitochondrial disease. This tractable genetic model of MILS disease will allow detailed studies to further elucidate the pathogenic basis of this disease and related mitochondrial encephalomyopathies.

**Mitochondrial morphology and neuromuscular pathology**

Examination of brain mitochondria in ATP6\(^i\) mutants revealed a striking morphological defect associated with the neural dysfunction in these mutants. The pathology resulted in mitochondria with a honeycomb appearance that was presumably caused by vesiculization of the inner mitochondrial membrane. More de-
The ultrastructural similarities among these three diverse systems (mitochondria) (Slautterback, 1965; Frey and Mannella, 2000). (Huizing, 1998) and an avian model system (canary myocardial human myopathy (i.e., vesicular cristae in Senger’s syndrome) (albeit not identical) morphological abnormalities are seen in a membrane. These findings are especially intriguing because similar tailed TEM tomography revealed that the roughly spherical inner membrane compartments were highly interconnected and contiguous with lamellar regions of the mitochondrial inner membrane. These findings are especially intriguing because similar (albeit not identical) morphological abnormalities are seen in a human myopathy (i.e., vesicular cristae in Senger’s syndrome) (Huizing, 1998) and an avian model system (canary myocardial mitochondria) (Slatterback, 1965; Frey and Mannella, 2000). The ultrastructural similarities among these three diverse systems suggest a conserved underlying pathophysiology and argue that our model system will provide valuable insight into the molecular basis of human mitochondrial diseases. In the case of Senger’s syndrome, there is evidence that the disorder is causally linked to a deficiency of ANT1 protein (Jordens et al., 2002). Such a defect could account for both a severe bioenergetic deficit in these patients and altered physical properties of the inner membrane. For example, an inhibitor-induced conformational change in ANT causes aberrant morphology of the inner membrane of isolated liver mitochondria (Scherer and Klingenberg, 1974). Likewise, a mutation in the subunit e of yeast ATP synthase that inhibits dimerization of the protein complex results in abnormal cristae morphology (Paumard et al., 2002). Whether the ATP6 mutation affects intermolecular interactions of the ATP synthase and whether this might account for the observed abnormality in inner membrane morphology of Drosophila mitochondria will need to be examined.

The rate of mitochondria with aberrant morphology was extremely high (>60% of the mitochondria in ATP6 mutants), suggesting widespread dysfunction throughout the brains of these animals. Significant neurodegeneration was not prominent in ATP6; however, neural dysfunction was evident and dramatically increased the onset of neuropathology in sesB animals. Severe myopathy was observed in ATP6 mutants coincident with the onset of morbidity in this strain, suggesting a causal relationship. It is curious that sesB (ANT) dysfunction results in neuromuscular pathology and ATP6 results primarily in myopathy. It is possible that the severe myopathy in ATP6 results in lethality before the onset of neurodegeneration and that the ultrastructural pathology observed is an early indicator of neuropathogenesis. Although the prevalence of the mitochondrial pathology is extremely high, it does not precisely match the near homoplasmy of the mitochondria in ATP6 mutants), suggesting widespread dysfunction throughout the brains of these animals. Significant neurodegeneration was not prominent in ATP6; however, neural dysfunction was evident and dramatically increased the onset of neuropathology in sesB animals. Severe myopathy was observed in ATP6 mutants coincident with the onset of morbidity in this strain, suggesting a causal relationship. It is curious that sesB (ANT) dysfunction results in neuromuscular pathology and ATP6 results primarily in myopathy. It is possible that the severe myopathy in ATP6 results in lethality before the onset of neurodegeneration and that the ultrastructural pathology observed is an early indicator of neuropathogenesis. Although the prevalence of the mitochondrial pathology is extremely high, it does not precisely match the near homoplasmy of the ATP6 mutation (98%). It is possible that the morphological defect represents a more advanced stage of organellar dysfunction and that a small subset of affected (mutant) mitochondria will maintain essentially normal morphology. Alternatively, if one assumes the ATP6 mutation is fully recessive, only mitochondria in which all of the mitochondrial genomes are mutated would exhibit abnormal morphology. Mitochondria are dynamic polyplloid organelles that regularly undergo morphological changes as well as fusion and fission. It is extremely difficult to determine the extent of the polyplloidy of mitochondria: estimates range from ~5–25 genomes per organelle (Satoh and Kuroiwa, 1991; Iborra et al., 2004; Ashley et al., 2005). If the ATP6 mutation was fully recessive, one would estimate ~60–90% of the mitochondria would exhibit the mor-

![Figure 6. Tomography TEM analysis of ATP6 mitochondrial inner membranes. Day 10 ATP6 (central brain mitochondria were examined using electron tomography. A, Projection image of an untilted thick section (0.02 mm) of a typical mitochondrion shows a honeycomb appearance similar to that in Figure 6B; numerous colloidal gold particles used for alignment are evident in the field. B, A representative slice, 2 mm thick, from the center of the tomogram reconstructed from 122 tilted views of the mitochondrion in A. C–G, Volume-rendered models of the tomogram reveal complex inner membrane compartmentalization. For clarity, the model is split into top (1–36, C and bottom (37–72, D) halves that each reveal an extensively interconnected network of inner membrane compartments. The round compartments are contiguous at numerous sites with lamellae and the intermembrane space. Removing the outer and peripheral inner membranes from the model and clipping it along various planes clearly reveals the transitions between the round, vesicle-like regions and the curving lamellar regions within the inner mitochondrial membrane (E, F, G). The interconnectedness of the compartments is revealed by regular forking membranous structures seen in the row of vesicle-like cristae. G, Note that the model is oriented in plates E–G such that “up” in these side views corresponds to the top surface shown in C. Also see supplemental movies (available at www.jneurosci.org as supplemental material) of the entire tomogram.](/image)

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Phenotypological defect (0.98n x 100), which is in good accord with experimental data. It is intriguing that mitochondria with a general honeycomb appearance were observed in age-matched wild-type brains, albeit at a very low rate. This suggests the dysfunction may result from a normal biological function occurring at an accelerated rate. Possibly the inner membrane vesiculazation is the result of excessive or poorly controlled mitochondrial fission, fusion, maturation, or a pathology associated with senescence that prematurely manifests with ATP6 dysfunction.

adPEO and ANT dysfunction

The discovery of a nearly homoplasmic mitochondrial mutation suggests that ANT dysfunction resulting from sesB* also have progressive myopathy, as do flies with the ATP6* point mutation (lacking the primary ANT dysfunction). These findings do not completely resolve, which is causing the myopathy in adPEO patients; however, our data demonstrate that ATP6 mutation alone causes myopathy independent of ANT dysfunction. Our data additionally suggest that point mutations result from ANT dysfunction and that these, unlike large-scale deletions, are heritable and likely selected for in the germ line.

Generating a nearly homoplasmic mitochondrial DNA (mtDNA) point mutation in a Drosophila strain would require selective pressure. This could result from any of the following conditions: (1) the ATP6 mutation improved the overall fitness of the animal (which appears unlikely given our lifespan, locomotor impairment, and histological data), (2) continued selection in the laboratory for the most affected animals in the strain, or (3) selective inheritance such as a meiotic drive system operating in the female germ line of the animals. The latter is supported by experimental data demonstrating selective maintenance of the variant ATP6* (T8993G) mtDNA (Vergani et al., 1999) and selective inheritance during oogenesis (Blok et al., 1997). Interestingly, in MIRS disease, a more severe variant of NARP manifests when the mutant heteroplasmy is above ~90%. Yet, these diseases are not extremely rare, as might be predicted. It is an enigma how such high heteroplasmy levels are achieved when the mutations that cause these diseases are predicted to have adverse effects on the cells bearing mitochondria with a high mutation load. The discovery and long-term maintenance of the nearly homoplasmic ATP6* mutation in vivo and the findings of Blok et al. (1997) support the hypothesis that meiotic drive contributes to the prevalence of these diseases through selection within the female germ cells for mitochondrial mutations.

Disease pathogenesis

Mitochondrial encephalomyopathies are widely recognized as an important and devastating class of diseases; however, the mechanism of pathogenesis remains controversial. Studies have reported severe oxygen consumption defects (Trounce et al., 1994; Mattiazzi et al., 2004), whereas other studies of the same T8993G mutation did not observe such a defect (Nijtmans et al., 2001). Additionally, studies of ATP synthase activity from cellular models nearly homoplasmic for the T8993G mutation revealed a ~50% reduction in activity, whereas an Escherichia coli model bearing the analogous mutation abolished all activity (Hartzog and Cain, 1993). More recent studies of the T8993G mutation in cell cybrid models revealed a ~95% reduction in ATP synthesis and described such a large reduction as a common feature associated with several respiratory chain disease mutations, with the notable exception of the T8993G mutation that revealed only a modest decrease (Pallotti et al., 2004). Nearly homoplasmic ATP6* mutant Drosophila clearly revealed severe neuromuscular impairment associated with marked decrease in ATP synthase activity similar to that reported by cellular models of human mutations (Hartzog and Cain, 1993). Respiration rates are main-
tained at normal rates in ATP6 variants similar to what was observed by Nijtmans et al. (2001). These data demonstrate that bioenergetic crisis is integral to the pathogenesis of ATP6 dysfunction. Additionally, the finding that mitochondrial respiration rates are unaffected in vivo suggests that the mitochondria may be functionally uncoupled, which may be integral to the pathogenesis in these mutants. Additional studies will be needed to test whether normal rates of respiration indicate normal reactive oxygen species generation to determine whether oxidative stress may also be contributing to the pathogenesis.

In humans, ATP6 dysfunction results in three different diseases with overlapping clinical manifestations; however, differences between these diseases and even between patients diagnosed with the same disease are evident. Thus, the genotype/phenotype relationship remains ambiguous. It is not evident why numerous mutations all affecting the same biochemical process, presumably resulting in bioenergetic impairment in the affected tissues, result in a veritable plethora of clinical manifestations that are not fully explained by the heteroplasy of the mutations (Schon et al., 1997; DiMauro and Andreu, 2000). Innovative techniques have been used to model mitochondrial diseases, such as cybrid cellular models derived from affected patient cells (Pallotti et al., 2004; Mattiazzi et al., 2004) and mouse models with mitochondrial DNA that was introduced via embryonic stem cells (Sligh et al., 2000). However, these model systems are difficult to study, and a complete understanding of disease pathogenesis has not emerged. This sentiment was exemplified in a review on mitochondrial encephalomyopathies: “thus far insurmountable problem of obtaining animal models for mtDNA mutations has prevented detailed studies of pathogenesis” (DiMauro and Andreu, 2000). The Drosophila strain reported here will enable additional such detailed studies of pathogenesis associated with ATP6 impairment in vivo.

References


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Vergani I, Rossi R, Brierley CH, Hanna M, Holt IJ (1999) Introduction of heteroplasmic mitochondrial DNA (mtDNA) from a patient with NARP into two human rho dgeless cell lines is associated either with selection and maintenance of NARP mutant mtDNA or failure to maintain mtDNA. Hum Mol Genet 8:1751–1757.


