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Mutations in Cytochrome c Oxidase Subunit VIa Cause Neurodegeneration and Motor Dysfunction in Drosophila

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ABSTRACT

Mitochondrial dysfunction is involved in many neurodegenerative disorders in humans. Here we report mutations in a gene (designated *levy*) that codes for subunit VIa of cytochrome c oxidase (COX). The mutations were identified by the phenotype of temperature-induced paralysis and showed the additional phenotypes of decreased COX activity, age-dependent bang-induced paralysis, progressive neurodegeneration, and reduced life span. Germ-line transformation using the levy$^+$ gene rescued the mutant flies from all phenotypes including neurodegeneration. The data from *levy* mutants reveal a COX-mediated pathway in Drosophila, disruption of which leads to mitochondrial encephalomyopathic effects including neurodegeneration, motor dysfunction, and premature death. The data present the first case of a mutation in a nuclear-encoded structural subunit of COX that causes mitochondrial encephalomyopathy rather than lethality, whereas several previous attempts to identify such mutations have not been successful. The *levy* mutants provide a genetic model to understand the mechanisms underlying COX-mediated mitochondrial encephalomyopathies and to explore possible therapeutic interventions.

MITOCHONDRIA are involved in several neurodegenerative diseases such as Parkinson's disease (Abou-Sleiman *et al.* 2006; Keeney *et al.* 2006), Alzheimer's disease (Atamna and Boyle 2006; Esposito *et al.* 2006), and Huntington's disease (Choo *et al.* 2004; Benchoua *et al.* 2006). In addition, several disorders arise directly from mutations that disrupt mitochondrial function. These include Leber hereditary optic neuropathy (LHON), mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibers (MERRF), chronic progressive external ophthalmoplegia and Kearns Sayre syndrome (CPEO/KSS), myoneurogastrointestinal encephalopathy syndrome (MNGIE), mitochondrial DNA depletion syndrome (MDS), Barth syndrome, and Leigh syndrome (LS) (McFarland *et al.* 2002; DiMauro and Schon 2003).

Complex I (NADH dehydrogenase) genes are responsible for the majority of LHON cases. Reduced activities of complexes I and IV (cytochrome c oxidase) are commonly associated with MELAS, and mutations in the thymidine phosphorylase gene on chromosome 22 and multiple mtDNA mutations are commonly associated with MERRF (DiMauro 2004; Gropman 2004). Deficiencies in a variety of factors such as pyruvate dehydrogenase complex (PDHC), cytochrome c oxidase (COX), and ATPase6 give rise to LS, a genetically heterogeneous disorder (Dahl 1998). Mitochondrial copy number is a central characteristic of MDS (Gropman 2004) and is linked to MNGIE (Giordano *et al.* 2006).

Mutations that disrupt molecular components of biochemical pathways allow identification of specific steps in such pathways and let us study the consequences of disrupting individual steps. Genetic tools available in Drosophila make it a useful model system for such studies. Drosophila has contributed significantly to our understanding of pathways involved in neuromuscular excitability and function under normal physiological conditions (Gu and Singh 1997; Bhattacharya *et al.* 1999; Ganetzky 2000; Bhattacharya *et al.* 2004; Ueda and Wu 2006). In addition, this model system is playing a significant role in analysis of pathways involved in neuromuscular dysfunction, especially in relation to neurodegenerative disorders (Fortini and Bonini 2000; Palladino *et al.* 2002; Bonini and Fortini 2003; Celotto *et al.* 2006; Gnerer *et al.* 2006; Pallanck and Greenamyre 2006). Here we report mutations that disrupted COX subunit VIa and resulted in several features of mitochondrial encephalomyopathies. Analysis

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of these mutations, of the phenotypes produced by them, and of the rescue from these phenotypes shows that COX plays a vital role in a pathway essential in maintaining neural integrity and that disruption of this pathway leads to encephalomyopathic effects including conditional paralysis, neurodegeneration, and early death.

MATERIALS AND METHODS

Fly strains and mutant isolation: Canton-S (CS) was used as the wild-type strain in all experiments. Flies were raised on a standard cornmeal medium at 21° and aged at 21° or 29° as described in RESULTS. Flies carrying compound chromosomes (CHOVNICK *et al.* 1970; SINGH 1983; SINGH *et al.* 1987) were fed ethylmethanesulfonate according to the mutagenesis procedure of Lewis and Bacher (LEWIS and BACHER 1968; ASHBURNER 1989), and the progeny were screened for paralysis on exposure to 38° for 5 min. This resulted in the identification of the $levy^1$ mutation. Additional *levy* alleles ($levy^2$, $levy^3$, and $levy^4$) were identified using *P*-element mobilization. Briefly, the *P* element in $Dcp\text{-}1^{k05606}$ was induced to mobilize by providing transposase ($\Delta 2\text{-}3$). F_1 flies with both *P* element and transposase were crossed with *w**; $levy^1$. The F_2 progeny were screened for temperature-induced paralysis. After screening ∼10,000 F_2 flies, 3 individual paralytic flies were isolated and the newly generated *P*-element mutations were balanced with *CyO*. Insertion sites for *P* elements were determined using inverse PCR according to the methods described by the Berkeley Drosophila Genome Project (BDGP) (BELLEN *et al.* 2004).

Behavioral assays: For paralysis test, a clear vial containing flies was submerged in water maintained at 38°. Time was recorded for flies to paralyze at 38° and to recover from paralysis after transfer to room temperature. Wild-type flies did not paralyze for at least 15 min under these conditions. For bang sensitivity, flies were placed in a clear vial and vortexed on a Vortex Genie-2 bench mixer (VWR) at its highest speed for 10 sec, followed by quantification of the time needed for paralyzed flies to stand up. To determine life span, flies were kept at either 29° or 21°. Flies were transferred to fresh food vials three times a week. Dead flies were counted every day and removed from the vials at the time of the next transfer.

Germ-line transformation: A 2-kb *CG17280* genomic DNA fragment was cloned into the *pCaSpeR4* vector and injected into early embryos produced by white-eyed $levy^1$ mutant flies (MCKAY *et al.* 1995). Transformants were identified on the basis of red eye color. In the control, the rescue construct was replaced with vector *pCaSpeR4* without an added insert.

Measurement of cytochrome c oxidase and citrate synthase activity: Microsomal fractions were prepared by grinding 30 adults in 2 ml of an extraction buffer containing 10 mM HEPES at pH 7.5, 200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 0.1 mM phenylmethyl-sulfonyl fluoride, 0.25 mM dibucaine, and 1 mM benzamidine, using five strokes on a motorized Potter-Elvehjem tissue grinder operating at 200 rpm. Homogenates were microfuged at 600 × *g* for 5 min at 4° and the resulting supernatants microfuged at 14,000 rpm for 20 min to collect microsomal pellets. Microsomal pellets were suspended in 200 μl of 10 mM HEPES at pH 7.5, 250 mM sucrose, 1 mM ATP, 80 μM ADP, 5 mM sodium succinate, 2 mM potassium phosphate, and 1 mM dithiothreitol and stored at −70° until used. Protein concentrations in microsomal preparations were determined using a bicinchoninic acid (BCA) protein assay kit from Pierce Biochemical. COX activity in microsomal preparations was determined using a COX assay kit (CYTOC-OX1) from Sigma-Aldrich. Citrate synthase activity in mitochondrial preparations was assayed according to instructions provided by Sigma-Aldrich.

Measurement of ATP levels: Extracts for ATP measurements were prepared by vigorously grinding a male and a female adult in 200 μl of 6 M guanidine hydrochloride using a small pestle that fits a microfuge tube, followed by collection of the liquid fraction after microfuging homogenates at 14,000 rpm for 20 min. Protein concentrations in extracts were determined using a BCA protein assay kit from Pierce Biochemical. ATP concentration was determined by measuring relative luminescence (RLU) of 1:50 dilutions of extracts in a luminometer using an ATP bioluminescence assay kit from Roche Diagnostics.

Characterization of neurodegeneration in *levy* and transformants: *Plastic sectioning:* Flies were decapitated and the heads fixed overnight in 1% paraformaldehyde and 1% glutaraldehyde. Fixed head samples were embedded in Epon 812 and then cut into 1-μm thick horizontal sections, stained with toluidine blue, and visualized using bright-field microscopy.

Paraffin sectioning: Standard paraffin embedding and sectioning techniques were followed on the basis of the protocol established by CRITTENDEN *et al.* (1998). Briefly, flies were fixed in Carnoy's solution (60% vol/vol ethanol, 30% vol/vol chloroform, 10% vol/vol glacial acetic acid) for 4 hr at room temperature. After four 100% ethanol washes of 30 min each, the tissue was immersed in methyl benzoate overnight, transferred to a 1:1 solution of methyl benzoate/paraffin for 1 hr at 56°, and then transferred to a 1:2 solution of methyl benzoate/paraffin for another hour at 56°. The flies were then immersed in pure paraffin, four times for 1 hr each at 56°, and finally embedded in paraffin at room temperature. The paraffin blocks were cut into 4-μm floating serial sections that were mounted on gelatinized glass slides. Prior to histological examinations, the tissue was deparaffinized with a 3-min wash in xylene and rehydrated through an ethanol series ending in either distilled water or Tris-buffered saline as appropriate. To characterize the presence or absence or neurodegeneration in *levy* mutants, rehydrated paraffin sections were stained with a 1% toluidine blue/10% bromophenol blue (BPB) solution for 10 min.

RESULTS

Isolation of *levy* mutations and identification of the gene: The $levy^1$ mutation was isolated in a screen in which a number of temperature-sensitive paralytic mutants were identified in Drosophila (HEGDE *et al.* 1999; SINGH and SINGH 1999; CHOPRA *et al.* 2000). The mutation was induced with EMS using compound chromosomes (SINGH *et al.* 1987). Mutant flies paralyzed on exposure to 38°. The flies paralyzed and recovered in an age-dependent manner as follows: 2-day-old flies took up to 5 min to paralyze and recovered within 30 min, while 30-day-old flies paralyzed within 30 sec and took up to 3 hr to recover.

The *levy* gene was localized to the right arm of chromosome 2 and mapped to 103 map units by recombination with visible markers L^2 and Pin^1 (Figure 1A). The chromosomal location of *levy* was further defined using several available chromosome deficiencies in the region. Two deficiencies that were useful in localizing

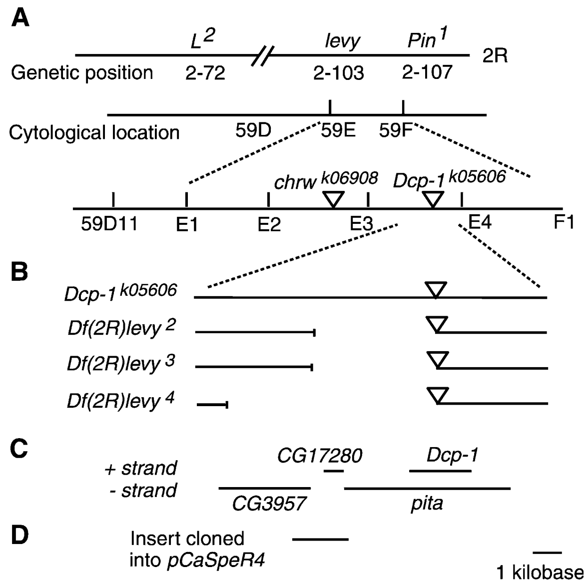

Figure 1.—Molecular identification of the *levy* gene. Schematic of DNA encompassing the *levy* gene. (A) The *levy* gene was first localized to genetic position 2-103 using recombination mapping of the *levy*^{1} mutation with visible markers L^2 and Pin^1 on the right arm of chromosome 2. Deficiency mapping localized the *levy* gene cytologically between bands 59D11 and 59E. Site-specific recombination mapping in males localized the *levy* gene between two *P*-element insertion sites, *chrw*^{k06908} and *Dcp-1*^{k05606} (represented by triangles). (B) Results of molecular analysis of three *levy* deficiencies (*levy*^{2}, *levy*^{3}, and *levy*^{4}) generated by *P*-element mobilization in *Dcp-1*^{k05606} flies suggested that the *levy* gene is *CG17280*, *pita*, or *Dcp-1*. Gaps in solid lines represent DNA deleted in these three *levy* deficiency strains. (C) The relative locations of genes in this region are shown as lines. The *CG17280* gene was identified as *levy* by transforming a 2-kb DNA fragment encompassing *CG17280* (represented by a line in D) into the germ line of *levy*^{1} mutants. These *levy* transformants did not exhibit temperature-induced paralytic behavior. The bar at the bottom represents 1 kb of DNA in B–D.

the *levy* mutation were *Df(2R)bw-HB132*, with breakpoints at 59D11 and 59F6-8, and *Df(2R)egl*^{2}, with breakpoints at 59E and 60A1. These deficiencies localized the *levy* mutation between 59D11 and 59E on polytene chromosomes (Figure 1A), a region encompassing 30 genes (http://flybase.bio.indiana.edu/).

Site-specific recombination mapping in males (Chen *et al.* 1998) placed the *levy* gene distal to the insertion site of the *chrw*^{k06908} *P* element and proximal to location of the *Dcp-1*^{k05606} *P* element (Figure 1A). Genetic crosses were conducted to mobilize the *Dcp-1*^{k05606} *P* element into the *levy* DNA and three new *levy* mutant alleles (*levy*^{2}, *levy*^{3}, and *levy*^{4}) were identified. All three alleles produced embryonic lethality in homozygous configuration and gave rise to temperature-induced paralysis when heterozygous with *levy*^{1}.

Inverse PCR and sequencing of DNA flanking the *P* element in *levy*^{2}, *levy*^{3}, and *levy*^{4} revealed that all three carried small deletions (Figure 1B). The smallest of the three deletions removed a 2.2-kb region that fully included the *CG17280* gene and parts of the *Dcp-1* and *pita* genes (Figure 1C). The most likely gene among these three to be *levy* was *CG17280* because the *P*-element insertion in *Dcp-1*^{k05606} complemented the *levy*^{1} mutation in genetic crosses and *Dcp-1*^{k05606} flies are known to have the insert within *Dcp-1* and *pita* DNA (Figure 1, B and C).

The *CG17280* gene is an ortholog of the human COX subunit VIa precursor (Fabrizi *et al.* 1989). Sequencing of *CG17280* DNA in the *levy*^{1} mutant revealed a G-to-A transition occurring at the 3′-splice junction of the single intron (Figure 2A). This base transition would presumably result in missplicing of *CG17280* RNA causing the deletion of a nucleotide, in turn leading to a reading frame shift in the second exon. This misspliced RNA would encode an aberrant protein of 54 amino acids, of which the first 32 match those in the nascent wild-type Levy protein of 109 amino acids (Figure 2B). RT–PCR amplification and DNA sequencing showed that a nucleotide was missing at the relevant position in the CG17280-encoded RNA (Figure 2C). The presence of this misspliced RNA suggested that *CG17280* is the *levy* gene.

To obtain proof that *CG17280* is the *levy* gene, a 2-kb DNA fragment encompassing the *CG17280* gene (see Figure 1D) was amplified from the wild-type (CS) genomic DNA and cloned into *pCaSpeR4* transformation vector at *Kpn*I and *Xho*I sites. The recombinant DNA construct was transferred into the genome of *levy*^{1} mutants using *P*-element-mediated germ-line transformation (McKay *et al.* 1995). As a control, transformation was performed with the *pCaSpeR4* vector without the *CG17280* gene. *levy*^{1} transformants carrying the wild-type *CG17280* transgene were not paralytic at 38°, showing that wild-type *CG17280* DNA comprises the *levy* gene. None of the seven independently generated transformants showed paralysis at 38° and all of the five transformant controls showed paralysis.

Reduction of cytochrome c oxidase activity in *levy* mutants: Subunit VIa plays a regulatory role in yeast and in mammalian COX (Taanman and Capaldi 1993; Taanman *et al.* 1994; Ludwig *et al.* 2001). To determine if the *levy* mutation affected COX activity, enzymatic assays were performed on age- and temperature-controlled flies. As shown in Figure 3 (A and B), *levy*^{1} mutants exhibited a reduction in COX activity compared to that in wild-type flies and *levy* transformants. Activity was reduced in *levy*^{1} mutant flies to 41% of that in the wild-type flies (Figure 3A). The reduction in COX activity was greater in 7-day-old flies (Figure 3B) than in 1-day-old flies. In this case the activity in *levy*^{1} was reduced to 17% of that in the wild type. Transformant flies carrying levy$^+$ transgene showed enzymatic activity similar to that in the wild-type flies, whereas activity levels in the transformant control flies were similar to those in the mutant. These data support the proposal that the *levy* gene encodes a COX subunit. There was no

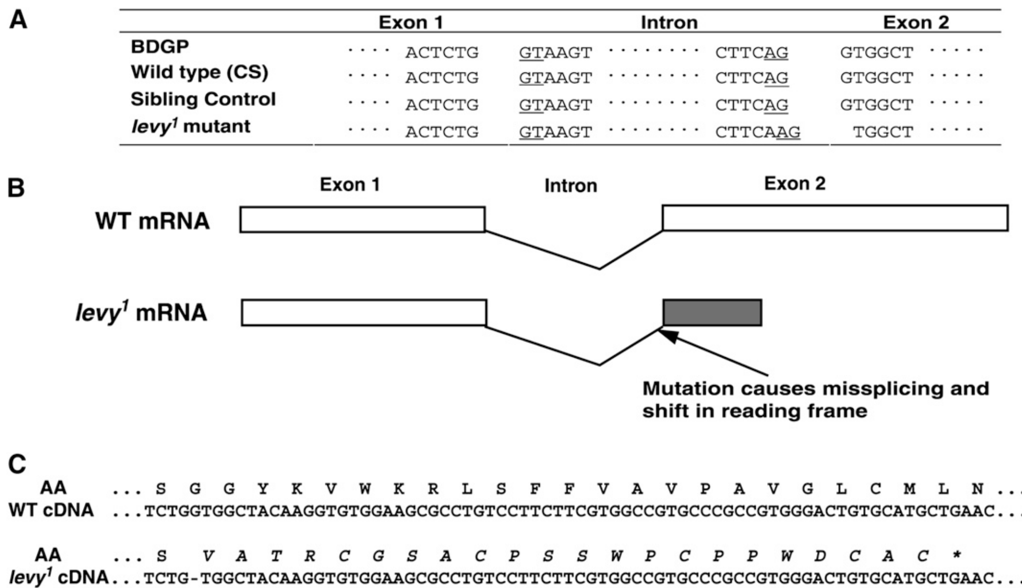

FIGURE 2.—Guanine-to-adenine transition in *levy*[1] DNA resulted in missplicing of *CG17280* RNA. (A) *levy*[1] DNA contained an adenine nucleotide at the 3′-splice junction within the intron of *CG17280* gene. This position was occupied by guanine in the wild-type gene as given in the BDGP database as well as in our own sequence determination of the region in CS and a sibling strain to *levy*[1]. The 5′- and 3′-intron splicing consensus sequences are underlined. The G-to-A transition caused the *levy*[1] RNA to be misspliced by 1 nucleotide and resulted in a reading frame shift in exon 2, as determined by RT–PCR analysis of mutant *levy* RNA. (B) A schematic of missplicing of *levy*[1] RNA. Translated regions are shown as rectangles with the wild type shown as open rectangles. The shift in reading frame would presumably lead to replacement of 77 amino acids encoded by exon 2 with 22 missense amino acids (shaded rectangle). (C) Sequencing of *levy*[1] cDNA confirmed the occurrence of missplicing in *levy*[1] RNA. A portion of the *levy*[1] cDNA sequence is shown with the deduced amino acid (AA) sequence. The wild-type cDNA sequence and deduced amino acid translation are shown for comparison to those of the *levy*[1] mutant. The aberrant amino acid sequence resulting from a shift in the reading frame is shown in italics.

further reduction in COX activity on exposing the flies to 38° for 5 min.

Heterozygous combinations of the *levy*[1] allele with two deletion alleles (*levy*[2], *levy*[3]) were tested for COX activity to test a prediction that these would also exhibit a reduction in COX activity compared to wild-type flies. Indeed, *levy*[1]/*levy*[2] and *levy*[1]/*levy*[3] flies, aged for 7 days, exhibited a reduction in COX activity (to 12% of that in the wild type in each case) that was comparable to the reduction seen in *levy*[1] homozygous flies (to 4% of the activity in the wild type) (Figure 3C). These data further corroborate our conclusion that the *levy* mutations lead to a reduction in COX activity and are in complete agreement with results showing the rescue of wild-type activity by levy$^+$ transgene.

To test if a reduction in COX activity might be due to a reduction in the number of mitochondria in *levy* mutants, fly preparations were examined by assaying for citrate synthase, an enzymatic marker for the mitochondrial matrix (Ferguson *et al.* 2005). No measurable differences were seen in levels of citrate synthase activity between *levy* mutants and wild-type flies (Figure 3F). This suggests that reduced COX activity observed in *levy* mutants was due to a decrease in COX enzymatic activity rather than to a reduction in the overall number of mitochondria in *levy* tissues.

Decreased levels of ATP in *levy* mutants: A defect in oxidative phosphorylation results in reduced levels of ATP in mitochondrial encephalomyopathies such as LS (Dahl 1998; Carrozzo *et al.* 2001). Similarly, *levy*[1] mutants exhibited reduced ATP (Figure 3, D and E). Compared to the wild type, 7-day-old *levy*[1] mutants that were close to dying showed 33% reduction in ATP after being subjected to temperature shock of 38° for 5 min (Figure 3E). ATP levels without the temperature shock were 103 ± 4 RLU/μg for the wild type and 95 ± 8 RLU/μg for the *levy*[1] flies. In flies given temperature shock, the levels were 102 ± 8 RLU/μg and 68 ± 5 RLU/μg in wild type and *levy*[1], respectively. ATP levels in *levy* transformants were comparable to those in wild-type flies. ATP reduction in *levy*[1] mutants appeared to be age dependent because there was a difference in ATP between 1-day-old and 7-day-old *levy*[1] mutants after temperature shock (Figure 3, D and E) (1-day-old *levy*[1]: 91 ± 6 RLU/μg; 7-day-old *levy*[1]: 68 ± 5 RLU/μg).

Reduced life span in *levy* mutants: *levy*[1] mutants displayed additional phenotypic characteristics that are similar to COX-deficient encephalomyopathies. A major characteristic of such disorders is early death (Leigh 1951; Rahman *et al.* 1996; Darin *et al.* 2001). This is paralleled by premature death of *levy*[1] mutants, which showed a median life span of 8 days at 29° compared to 28 days in wild type and 26 days in transformants (Figure 4A). The median life span of transformant control (9 days) was similar to that of *levy*[1] mutants, proving that *CG17280* DNA is essential to restore normal life span in *levy*[1] transformants. Life span of *levy*[1] mutants was also examined at 21° and had a mean value of 51 days compared to 100 days in the wild type, 93 days in *levy* transformants, and 55 days in transformant control flies.

Bang sensitivity in *levy* mutants: In another phenotypic parallel to mitochondrial encephalomyopathies,

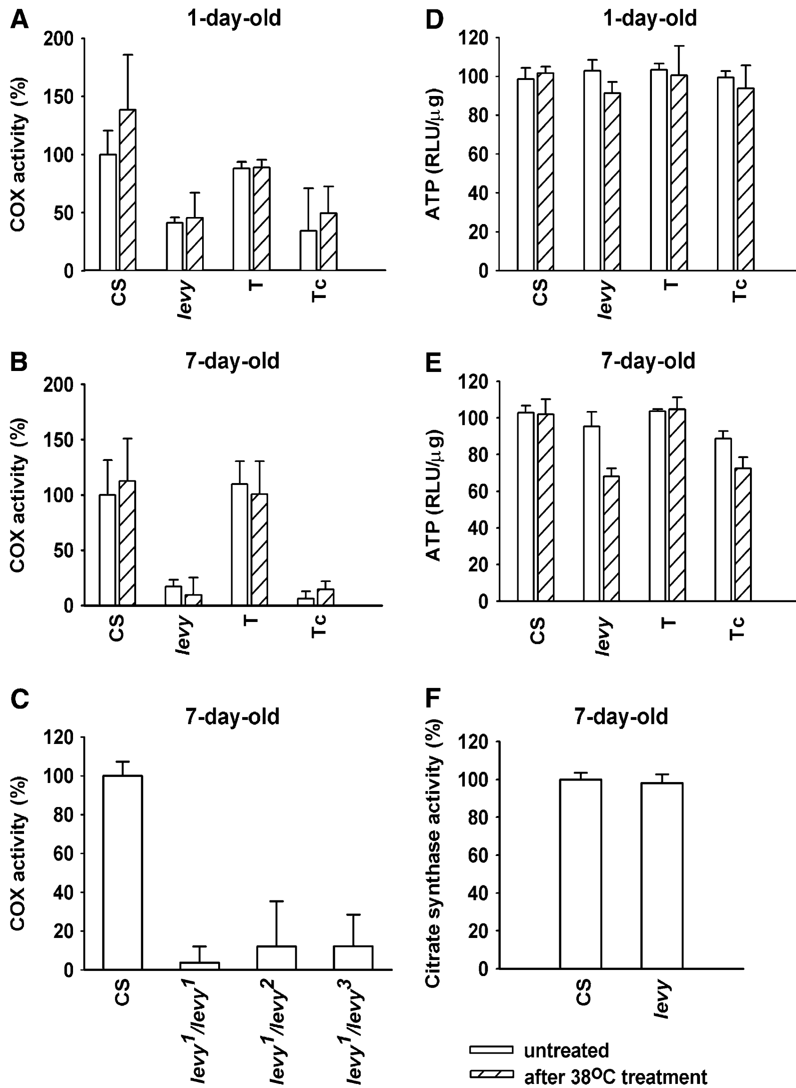

FIGURE 3.—*levy* mutants exhibited age-dependent reduction in COX activity and ATP, but not in a marker for the mitochondrial matrix, citrate synthase. COX activity and levels of ATP were measured in adult flies kept at 29° for 1 or 7 days. COX activity is set to 100% in untreated wild-type (CS) flies for comparison purposes. COX activity was reduced in *levy*^{1} mutants compared to that in wild-type flies (Student's *t*-test, $P < 0.025$ for 1-day old and $P < 0.050$ for 7-day old) and *levy* transformants ($P < 0.005$ for 1-day old and $P < 0.010$ for 7-day old) (A and B). Transformant flies carrying levy$^+$ transgene showed enzymatic activity similar to that in the wild-type flies ($P \approx 0.2$), whereas activity levels in the transformant control flies were similar to those in the mutant ($P \approx 0.2$). Heterozygous *levy* alleles exhibited a comparable reduction of COX activity with respect to wild-type (CS) flies (C). In comparison to the wild type, a reduction in ATP appeared in 7-day-old *levy*^{1} mutants ($P < 0.025$) and transformant controls ($P < 0.025$) after a temperature-shock regimen (E). Comparable reduction in ATP did not appear in 1-day-old (D) temperature-shocked *levy*^{1} mutants ($P \approx 0.2$) or in 7-day-old *levy*^{1} mutants prior to being subjected to temperature shock (E) ($P \approx 0.2$). There was no measurable difference between wild-type and *levy* mutants in regard to a classical enzymatic marker for the mitochondrial matrix, citrate synthase (F). Error bars show standard deviation from three or four measurements. T, transformant; Tc, transformant control.

levy^1 mutants exhibited motor deficiency by displaying age-dependent bang-induced paralysis (Figure 4B) (GANETZKY and WU 1982; PAVLIDIS *et al.* 1994). Two-day-old *levy*^{1} mutants were not paralytic in response to mechanical shock, but there was an increase in the number that showed such paralysis as they aged. In flies aged for 10 days at 21°, 73% of *levy*^{1} and 69% of transformant control flies paralyzed in response to a bang shock, whereas all 20-day-old mutant and transformant control flies exhibited bang-induced paralysis. With the onset and increase in bang-induced paralysis with age, there was also a concomitant increase in the severity of paralysis, prolonging the time required for recovery from paralysis. Ten-day-old paralyzed flies took up to 90 sec to recover from bang-induced paralysis while 20-day-old flies took up to 330 sec. Bang sensitivity was also examined in *levy*^{1}/*levy*^{2} and *levy*^{1}/*levy*^{3} heterozygous flies and they also showed age-dependent bang-induced paralysis (data not shown). Wild-type and *levy* transformant flies did not show bang-induced paralysis at any of the three ages at which flies were tested.

Characterization of neurodegeneration in *levy* mutants: The phenotype of temperature-induced paralysis has been shown to enrich for mutations that lead to neurodegeneration (PALLADINO *et al.* 2002). In addition, several mitochondrial encephalomyopathies lead to neurodegeneration (DIMAURO 2004; GROPMAN 2004). To determine whether or not *levy*^{1} mutants exhibited neurodegeneration, tissue sections taken through head capsules of mutant and the wild-type (CS) flies were fixed in Epon and examined microscopically. Neurodegeneration was not seen in wild-type flies (Figure 5A), but the brain and optic lobes of *levy*^{1} mutants showed Swiss cheese-like holes that are reminiscent of spongiform neurodegeneration (Figure 5B). Neurodegeneration was observed to be absent in *levy* transformants (Figure 5C), demonstrating that the phenomenon was produced by the *levy* mutation. As in the case of other phenotypes, neurodegeneration appeared to be age dependent. Paraffin sections from 20 flies each from the wild-type and the *levy*^{1} strains, aged for either 1 day or 6 days at 29°, were examined for holes in the brain. None

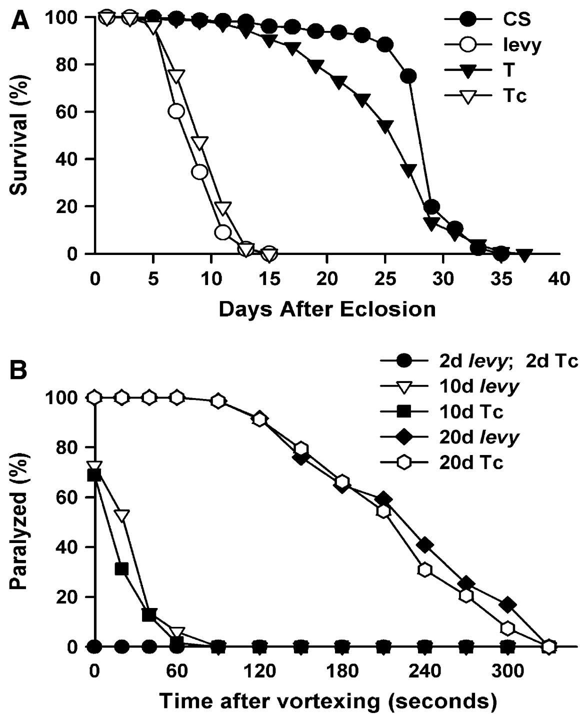

FIGURE 4.—Reduced life span and progressive bang-induced paralysis in *levy* mutants. (A) Percentage of surviving adults kept at 29° after eclosion. *levy[1]* flies ($n = 550$) died at a median age of 8 days at 29° compared to wild-type (CS) flies ($n = 669$), which lived to a median age of 28 days. Life span of *levy* transformants ($n = 955$) was similar to that of Canton-S flies, while transformant controls ($n = 682$) died about the same time as *levy[1]* mutants. (B) Time for recovery after testing for bang-induced paralysis for 2-, 10-, and 20-day-old (after eclosion) adults kept at 21°. At this temperature, the median life span was 100 days for wild-type flies (CS), 51 days for *levy[1]* mutants, 93 days for transformants, and 55 days for transformant controls. Two-day-old *levy[1]* mutants did not exhibit bang-induced paralytic behavior. Only a fraction of 10-day-old *levy[1]* mutants exhibited bang-induced paralysis, all of which recovered in $<$2 min. In 20-day-old *levy[1]* mutants, all flies paralyzed in response to bang stimulus and these took longer to recover from paralysis than their younger siblings. Numbers of 2-, 10-, and 20-day-old *levy* flies tested were 56, 66, and 71, respectively. Numbers of 2-, 10-, and 20-day-old transformant control flies tested were 69, 64, and 68, respectively.

of wild-type flies in either age category, and none of the *levy[1]* flies aged for 1 day, showed any signs of neurodegeneration (Figure 5D). On the other hand, 19 of 20 *levy[1]* flies aged for 6 days showed neurodegeneration.

Possible occurrence of neurodegeneration in additional *levy* alleles was examined in *levy[1]/levy[2]* and *levy[1]/levy[3]* heterozygotes. Heterozygous animals aged at 29° posteclosion for 6 days were stained using hematoxylin and eosin to determine the extent of vacuolization. The *levy[1]/levy[2]* (Figure 5E) and *levy[1]/levy[3]* (Figure 5F) flies exhibited *levy[1]*-type neurodegeneration in the lobula and extensive vacuolization within other neural structures. The phenotype of neurodegeneration overlapped that seen in the homozygous *levy[1]* mutants. The extent or the nature of neurodegeneration did not seem to be significantly different among the alleles examined. This is not unexpected since *levy[2]* and *levy[3]* are deletion mutations and *levy[1]* itself is likely to be a null allele because of a shift in the reading frame after 32 amino acids, followed by a premature stop codon.

DISCUSSION

Mitochondrial function is integral to the healthy function of cells, particularly in cells such as neurons and muscles that are critically dependent on an abundant energy supply. A spectrum of myopathic and neuropathic symptoms in humans have been correlated to lesions in mitochondrial respiratory chain. Use of genetically tractable model systems such as Drosophila can help in identifying individual steps in the pathways leading to such disorders. Animal models are particularly useful for diseases such as mitochondrial encephalomyopathies that are intransigent to treatment and about which relatively little is known. By generating mutations in a COX subunit, analyzing the resulting phenotypes, and obtaining rescue from these phenotypes by germ-line transformation, we provide direct evidence that disruption of COX VIa results in mitochondrial encephalomyopathic effects including neurodegeneration and motor dysfunction.

Mitochondrial encephalomyopathies related to COX are characterized by enzyme deficiency, reduced ATP production, motor difficulty, neurodegeneration, and shortened life span. Phenotypes associated with *levy* mutants mimic all of these symptoms. Consistent with the progressive nature of these symptoms in humans, all *levy* phenotypes including temperature-induced paralysis, bang sensitivity, reduction in COX activity and ATP level, and neurodegeneration show age-dependent increases in severity. For example, vacuolization in the brains of *levy* mutants is not detected in flies aged for 1 day. However, it is widespread in flies aged for 6 days. Importantly, germ-line transformation using wild-type *levy* gene rescues the mutant flies from all the phenotypes described above. However, the control vector—the same vector used in transformation rescue but without the *levy* gene—is unable to provide such a rescue. Thus all these phenotypes can be directly linked to the disruption of subunit VIa of COX. These data reveal a COX-mediated pathway in Drosophila, disruption of which leads to mitochondrial encephalomyopathic effects including neurodegeneration, motor dysfunction, and premature death. In addition, the transformation experiments provide direct evidence for a causal link between the disruption of COX and these phenotypes.

Human COX consists of 3 mitochondrial-encoded and 10 nuclear-encoded subunits. Defects in the 3 mitochondrial-encoded subunits COXI (also called MTCO1), COXII (MTCO2), and COXIII (MTCO3) as

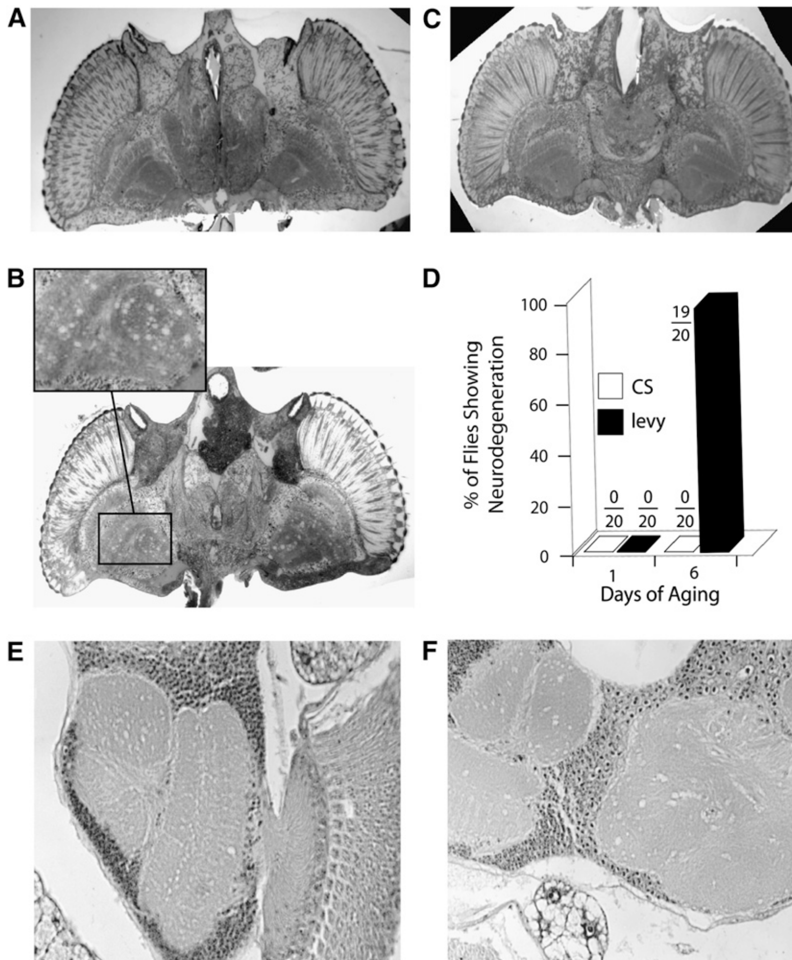

FIGURE 5.—*levy* mutants exhibited vacuolization of the optic lobes and brain, but were rescued by transformation with the wild-type gene. Epon-embedded horizontal brain sections were cut from head capsules of adults kept at 29° after eclosion. Slices through the heads of 7-day-old wild type (CS) (A) and *levy* transformant (C) are shown for comparison to that from the head of *levy*^{1} mutant (B). Swiss cheese-like holes appeared in the brain and optic lobes in 7-day-old *levy*^{1} mutants (inset). These cytological lesions also appeared in the brains and optic lobes of 7-day-old transformant control flies (data not shown). Neurodegeneration occurred in age-dependent manner (D), being absent in CS flies aged for 1 day or 6 days, and in *levy*^{1} flies aged for 1 day. On the other hand, 19 of 20 *levy*^{1} flies aged for 6 days showed clear neurodegeneration. Serial sections from paraffin processed flies were obtained from *levy*^{1}/*levy*^{2} (E) and *levy*^{1}/*levy*^{3} (F) flies after aging them for 6 days at 29°. Neurodegeneration was observed in flies from both strains, consistent with the phenomenon seen in *levy*^{1} mutants.

well as in several COX assembly factors such as COX10, COX15, SURF1, SCO1, and SCO2 have been correlated with encephalomyopathies (Barrientos *et al.* 2002; DiMauro and Schon 2003; DiMauro and Hirano 2005). For example, >30 distinct mutations in the SURF1 gene have been associated with COX-deficient LS (Pequignot *et al.* 2001). In light of this, it has been noted with intrigue that no mutations in any of the nuclear-encoded structural subunits of COX have been associated with such disorders and that attempts to find such associations have not been fruitful (Shoubridge 2001; DiMauro and Schon 2003; Schon 2004; Schapira 2006). This strongly suggests that mutations in the nuclear-encoded structural subunits of COX may be lethal (DiMauro and Schon 2003; Schapira 2006). This view is reinforced by results from an efficient screen conducted for mutations in nuclear-encoded mitochondrial proteins that yielded many such mutations in Drosophila (Liao *et al.* 2006). The only mutation in this set to target a structural subunit of COX, the *tenured* mutation in subunit Va, produces lethality. Similarly, a mutation in the *cyclope* gene, which codes for subunit VIc of COX, leads to lethality in Drosophila (Szuplewski and Terracol 2001). The *levy*^{1} mutation discussed here provides the first case of such a mutation leading to encephalomyopathic effects rather than lethality. However, the findings presented here raise additional questions. While subunit VIa is a structural component of COX, the role of this subunit is likely to be regulatory in nature (Taanman and Capaldi 1993; Taanman *et al.* 1994; Ludwig *et al.* 2001). This is likely to be the reason for the enzyme retaining some activity even with frame-shifted and truncated subunit VIa (Figure 3).

The *levy* mutant was identified for its temperature-induced paralysis. However, the primary biochemical effect of the mutation—reduction in COX activity—is not temperature dependent. It implies that the COX enzyme itself does not have to be temperature sensitive to produce temperature sensitivity (paralysis) in flies. This is a common feature among temperature-paralytic mutants of Drosophila where sensitivity to high temperature arises not from the temperature sensitivity of the primary biochemical target but from a constitutive change in a biochemical or a physiological parameter. For example, temperature-induced paralysis in the *para*^{ts} and the *nap*^{ts} mutants arises from a constitutive decrease in the number of sodium channels and not from channels that become temperature sensitive (Loughney *et al.* 1989; Kernan *et al.* 1991). Similarly, temperature

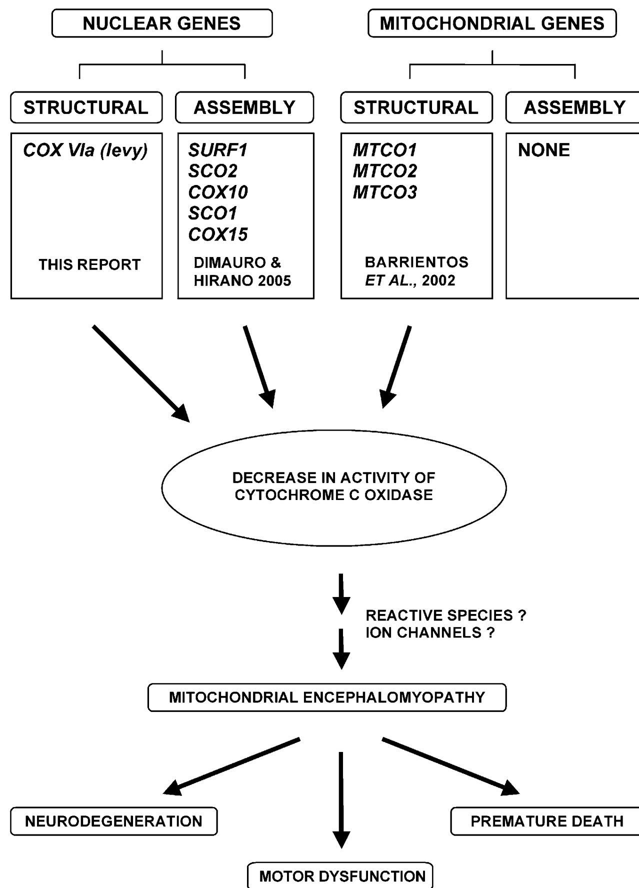

FIGURE 6.—From COX deficiency to neuromuscular dysfunction. This model shows nuclear and mitochondrial genes that code for structural components of COX or its assembly factors that have been shown to be involved in mitochondrial encephalomyopathies. Until now, it has been believed that mutations in the nuclear genes coding for structural subunits of COX would be embryonic lethal. The *levy*[1] mutation presents the first case where mutants are viable but show mitochondrial encephalomyopathic phenotypes. In addition, a general model of some possible subsequent steps via which COX deficiency could lead to neuromuscular dysfunction is presented. The data in this article establish a causal link between a reduction in COX activity and mitochondrial encephalomyopathic effects of neurodegeneration, paralytic behavior, and premature death in Drosophila.

sensitivity of ATP levels in old flies does not derive from temperature sensitivity of COX. Aged mutant flies showed a decrease in ATP levels after they were paralyzed by a temperature shock while COX activity was not affected by this treatment. While the mechanisms underlying an effect of temperature shock on ATP are unknown at this stage, it may possibly be related to the seizure activity that *levy* flies go through during temperature-induced paralysis. A reduction in ATP levels after seizure activity is known to occur in rat models of seizures (DeFrance and McCandless 1991; Yager *et al.* 2002; Darbin *et al.* 2005).

While the experiments reported here have identified one step in the pathway(s) leading to *levy* effects, and while further experimentation is needed to identify additional steps, the nature of the observed effects points to some likely mechanisms. Inhibition of COX has been shown to increase free radical generation in many systems including Drosophila (Smith and Bennett 1997; Duranteau *et al.* 1998; Ferguson *et al.* 2005). Excessive levels of free radicals can in turn lead to cell death via either apoptosis or necrosis (Beal 2000; Mattson and Kroemer 2003). There are other aspects of pathways involved in mitochondrial-mediated neurodegeneration. For example, ion channels have been implicated, either in the context of oxidative stress or outside of this context, in several types of neurodegeneration (Ueda *et al.* 1997; Liss *et al.* 2005; Burg *et al.* 2006; Chinopoulos and Adam-Vizi 2006). It remains to be seen if increased production of free radicals is involved in the effects observed in *levy*, whether *levy* brains show apoptotic or necrotic cell death, and if any ion channel dysfunction occurs in *levy* (Figure 6). Availability of *levy* mutations, genetic tractability of Drosophila, and the ease with which questions about oxidative stress (Ferguson *et al.* 2005; Dias-Santagata *et al.* 2007) as well as ion channel function can be explored in this model system (Chopra and Singh 1994; Gielow *et al.* 1995; Kraliz and Singh 1997; Kraliz *et al.* 1998) provide an excellent opportunity to address these questions.

Availability of *levy* mutations will particularly help in identifying steps in the pathway(s) leading to mitochondrial encephalopathy seen in the mutants. For example, it will be helpful to identify interacting genetic components by screening for suppressors or enhancers of a *levy* mutant phenotype. It is relatively easy to identify such modifier mutations in Drosophila, as thousands of mutagenized flies can be tested easily for phenotypes such as temperature-induced paralysis. Such modifier mutations can provide further leads into the pathways disrupted by the original mutations (the *levy* mutations in this case).

A significant level of our understanding on the structure, function, and regulation of COX has developed from identification and analysis of mutants in yeast (Barrientos *et al.* 2002). Mutations that lead to COX-related mitochondrial encephalomyopathies in organisms such as Drosophila and mice can similarly help us understand pathways leading to these disorders. Agostino *et al.* (2003) have generated a *Surf1* knockout mouse model of Leigh syndrome that lacks Surfeit-1, an enzyme involved in COX assembly. Similarly, a *Surf1* knockdown model has been generated in Drosophila by post-transcriptional silencing using dsRNA (Zordan *et al.* 2006). Studies comparing biochemical and physiological characteristics of various models of COX-related mitochondrial encephalomyopathies, including *levy* mutants and *Surf1* models in mice and Drosophila, can help in understanding the mechanisms underlying the effects of such disorders. This is particularly true in regard to neurodegeneration due to a fortuitous

difference between the *levy* flies and the *Surf1* models. Neither *Surf1* mice nor *Surf1* flies show neurodegeneration (Agostino *et al.* 2003; Zordan *et al.* 2006). Thus, studies of similarities and differences between *levy* mutants and *Surf1* models may enable us to address questions about what leads to neurodegeneration in one case but not in the other. These studies would lead to a better understanding of neurodegenerative processes in general and their occurrence in mitochondrial encephalomyopathies in particular. This information will also be useful in developing possible therapeutic approaches against such disorders.

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