ANESTHESIOLOGY

# Mitochondrial Complex I Mutations Predispose *Drosophila* to Isoflurane Neurotoxicity

Zachariah P. G. Olufs, B.S., Barry Ganetzky, Ph.D., David A. Wassarman, Ph.D., Misha Perouansky, M.D.

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## EDITOR'S PERSPECTIVE

### What We Already Know about This Topic

- Mitochondrial proteins are targets of general anesthetics
- The extent to which pathogenic mutations in mitochondrial proteins increase the risk of adverse reactions from anesthetics has not been previously explored

### What This Article Tells Us That Is New

- Isoflurane but not sevoflurane exposure increased mortality in *Drosophila* carrying homozygous mutations in mitochondrial complex I, and hyperoxia increased mortality associated with isoflurane administration
- In heterozygous flies, carrying mutations in mitochondrial complex I, age, and hyperoxia rendered flies susceptible to mortality after exposure to isoflurane
- These observations raise the possibility that heterozygous carriers of mitochondrial mutations may be more susceptible to perioperative complications after isoflurane exposure

## ABSTRACT

**Background:** General anesthetics influence mitochondrial homeostasis, placing individuals with mitochondrial disorders and possibly carriers of recessive mitochondrial mutations at increased risk of perioperative complications. In *Drosophila*, mutations in the ND23 subunit of complex I of the mitochondrial electron transport chain–analogous to mammalian NDUFS8–replicate key characteristics of Leigh syndrome, an inherited mitochondrial disorder. The authors used the *ND23* mutant for testing the hypothesis that anesthetics have toxic potential in carriers of mitochondrial mutations.

**Methods:** The authors exposed wild-type flies and *ND23* mutant flies to behaviorally equivalent doses of isoflurane or sevoflurane in 5%, 21%, or 75% oxygen. The authors used percent mortality (mean ± SD, n ≥ 3) at 24 h after exposure as a readout of toxicity and changes in gene expression to investigate toxicity mechanisms.

**Results:** Exposure of 10- to 13-day-old male *ND23* flies to isoflurane in 5%, 21%, or 75% oxygen resulted in 16.0 ± 14.9% (n = 10), 48.2 ± 16.1% (n = 9), and 99.2 ± 2.0% (n = 10) mortality, respectively. Comparable mortality was observed in females. In contrast, under the same conditions, mortality was less than 5% for all male and female groups exposed to sevoflurane, except 10- to 13-day-old male *ND23* flies with 9.6 ± 8.9% (n = 16) mortality. The mortality of 10- to 13-day-old *ND23* flies exposed to isoflurane was rescued by neuron- or glia-specific expression of wild-type *ND23*. Isoflurane and sevoflurane differentially affected expression of antioxidant genes in 10- to 13-day-old *ND23* flies. *ND23* flies had elevated mortality from paraquat-induced oxidative stress compared with wild-type flies. The mortality of heterozygous *ND23* flies exposed to isoflurane in 75% oxygen increased with age, resulting in 54.0 ± 19.6% (n = 4) mortality at 33 to 39 days old, and the percent mortality varied in different genetic backgrounds.

**Conclusions:** Mutations in the mitochondrial complex I subunit ND23 increase susceptibility to isoflurane-induced toxicity and to oxidative stress in *Drosophila*. Asymptomatic flies that carry *ND23* mutations are sensitized to hyperoxic isoflurane toxicity by age and genetic background.

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EXPOSURE to general anesthetics is very common but not without risks, and mutations in some anesthetic target proteins increase the risk of toxicity.[1] Of particular interest are mutations affecting mitochondria, because mitochondrial diseases are the most frequently inherited metabolic disorders, and mitochondrial proteins are targets of general anesthetics.[2,3] Because volatile general anesthetics inhibit the function of complex I of the mitochondrial electron transport chain,[4] they may increase the risk of perioperative complications for patients with disorders caused by mutations in complex I such as Leigh syndrome and other similar neurodegenerative disorders.[5] Indeed, Leigh syndrome patients are hypersensitive to volatile general anesthetics[6] and are challenging to manage in the perioperative setting.[7] Most of these disorders are caused by mutations in the nuclear genome rather than the mitochondrial genome and follow a Mendelian pattern of inheritance.[8] Leigh syndrome is rare, diagnosed in only 1:20,000 live births, but the prevalence of heterozygosity in the general population is substantially higher at

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1 to 2:100. The extent to which heterozygous carriers of pathogenic mutations are at increased risk of adverse reactions from anesthetics is unknown. Furthermore, it is not known how age, sex, and environmental factors modulate the penetrance of the underlying pathophysiology,[9] as they do for other more common diseases caused by complex I mutations.[10] The risk of complex I mutations can be explored in model organisms to generate testable hypotheses. Recently, two *Drosophila melanogaster* models of Leigh syndrome have been characterized in flies carrying mutations in the mitochondrially encoded ND2 subunit[11] or the nuclearly encoded ND23 subunit[12] of complex I. *ND23* is homologous to mammalian *NDUFS8* (*NADH:ubiquinone oxidoreductase subunit S8*), which is commonly mutated in mitochondrial diseases.[13]

We examined homozygous and heterozygous *Drosophila ND23* mutants. For most of the studies, we used the $ND23^{60114}$ allele, which contains a point mutation that results in a glycine-to-aspartic acid substitution at position 199 of the 217-amino acid protein.[12] $ND23^{60114}$ flies exhibit age-dependent neurodegeneration and a reduced lifespan. Neurodegeneration becomes apparent as spongiform lesions form in the neuropil at 10 to 12 days old and increase in severity with age. Mimicking children with Leigh syndrome, young $ND23^{60114}$ flies are more sensitive than wild-type flies to behavioral effects of isoflurane and sevoflurane.[14] Therefore, we tested whether $ND23^{60114}$ flies could serve as a sensitized model for anesthetic-induced toxicity to address whether heterozygous carriers are at risk and whether genetic background influences the risk of toxicity.

## Materials and Methods

Approval from the institutional animal care and use committee was waived. We followed ARRIVE guidelines to the degree applicable to invertebrate organisms.[15,16]

### Fly Lines and Culturing

All flies were cultured at 25°C on standard cornmeal-molasses food. The $ND23^{60114}$ and *UAS-ND23* lines are described by Loewen and Ganetzky.[12] $ND23^{G14097}$, which is homozygous lethal, contains a P-element insertion 259 base pairs downstream of the transcription start site for *ND23*. $ND23^{Del}$ (*i.e.*, *Df(3R)Exel8162*), which is homozygous lethal, deletes an ~125-kilobase pair region that contains 19 genes, including *ND23*. Canton S, $w^{1118}$, *Tub-Gal4*, *C155-Gal4*, *Repo-Gal4*, RAL352, and RAL774 lines were obtained from the Bloomington Drosophila Stock Center (Bloomington, Indiana). Canton S flies are referred to as wild-type throughout the article because the $ND23^{60114}$ mutation was generated in the Canton S genetic background. For overexpression of $ND23^{60114}$, flies were generated by crossing female $ND23^{60114}$ flies containing the *UAS-ND23* transgene to male $ND23^{G14097}$ flies containing the driver transgene. Heterozygous lines were generated by crossing *ND23* females to males of the other genotype.

### Anesthesia and Oxygen Exposure

On the day of the experiment, flies of different sex, age, and genotype were allocated to vials ensuring equal numbers for the different treatment groups. Flies were exposed to volatile general anesthetics and oxygen at specific concentrations using a device that permits simultaneous exposure of up to eight groups of flies.[14] Volatile general anesthetics were administered using an Aestiva/5 anesthesia machine equipped with commercial agent-specific vaporizers (Datex–Ohmeda Inc., USA). Compressed gas cylinders (Airgas, USA) containing 100% $O_2$, 100% $N_2$, or air (21% $O_2$/79% $N_2$) provided carrier gas of the desired composition. Anesthetic exposure consisted of either 2% isoflurane or 3.5% sevoflurane for 2 h at room temperature. Isoflurane and sevoflurane were obtained from Piramal Enterprises Ltd. (India). To control for effects of circadian rhythm, the flies were anesthetized at a similar time of day (between 10:00 AM and 2:00 PM).

### Percent Mortality Assay

Percent mortality was determined by counting the number of dead flies 24 h after the end of an anesthetic exposure and dividing by the total number of flies per vial. Mortality is a binary readout and was assessed by an unblinded observer. We interpret the observed mortality as due to anesthetic and/or oxygen toxicity. Each experiment was conducted in at least triplicate with 25 to 50 flies in each sample. The mortality time course was composed of independent experiments covering three time frames: 0 to 8, 8 to 16, and 16 to 24 h. All experiments followed the same basic structure. All flies were cultured at 25°C until the time of collection at 0 to 2 days posteclosion when they were separated by sex under carbon dioxide narcosis. Flies were then maintained at 29°C for the remainder of the experiment, with food changes every other day. For older heterozygous lines, food changing was paired with carbon dioxide narcosis.

### Gene Expression

We used quantitative real-time reverse transcription–polymerase chain reaction to measure the expression of select genes known to respond to oxidative stress. For each treatment, $ND23^{60114}$ flies were collected 30 min after the end of exposure to anesthesia. RNA isolation and processing have been described previously.[17] In brief, 25 flies per treatment were transferred to 1.5-ml tubes and frozen in liquid $N_2$, tubes were shaken vigorously by vortexing to shear heads from bodies, and heads were isolated from other body parts by sequential use of US Standard no. 25 (710 μm) and no. 40 (425 μm) sieves. Total RNA was isolated from heads using TRI reagent (Sigma, USA). The resulting RNA was resuspended in 40 μl of diethylpyrocarbonate-treated water

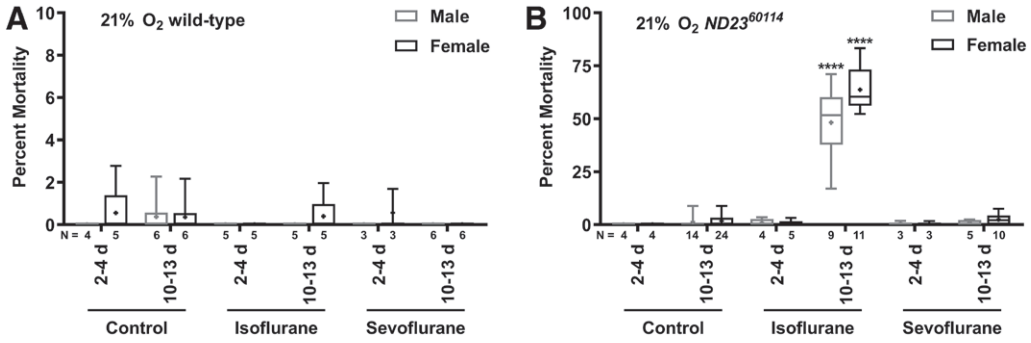


**Fig. 1.** Isoflurane, but not sevoflurane, is toxic to male and female *ND23*$^{60114}$ flies in an age-dependent manner. (*A*, *B*) Wild-type (*A*) and *ND23*$^{60114}$ (*B*) male and female flies at different ages were either not exposed (control) or exposed to 4%h isoflurane or 7%h sevoflurane in room air (21% $O_2$) at either 2 to 4 days old (*2–4 d*) or 10 to 13 days old (*10–13 d*), and the percent mortality was determined after 24 h. N = number of biologic replicates. Symbols indicate the following: *box*, second and third quartiles of data; +, mean; *horizontal bar*, median; and *whiskers*, minimum and maximum. The significance between control and experimental data was determined using the unpaired equal-variance two-tail Student's *t* test. ****$P < 0.0001$. Note the difference in *y*-axis scale between (*A*) and (*B*).

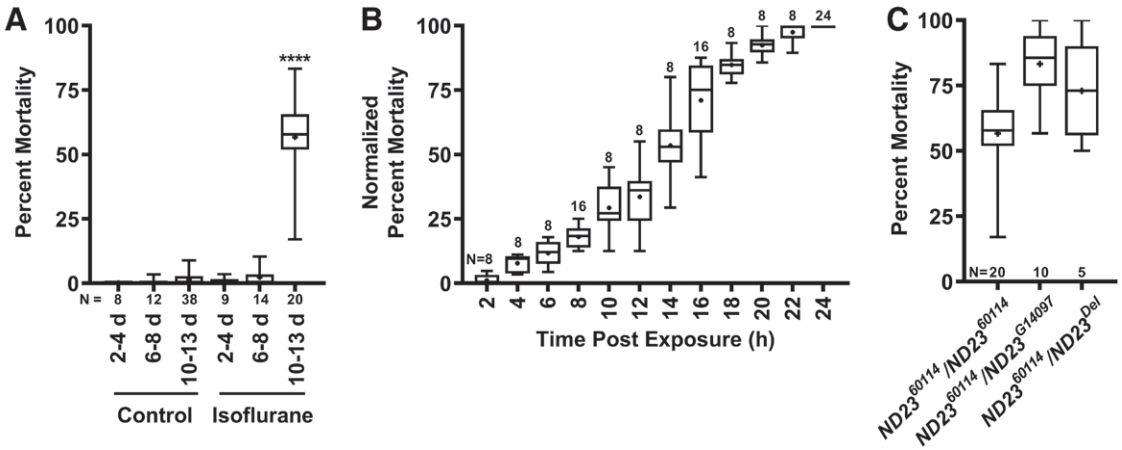


**Fig. 2.** Age of onset and time course of mortality in *ND23*$^{60114}$ flies. (*A*) Percent mortality was determined for mixed sex *ND23*$^{60114}$ flies not exposed (control) or exposed to 2 h of 2% isoflurane and 21% $O_2$ at the indicated ages. Significance between control and experimental data was determined using the unpaired equal-variance two-tail Student's *t* test. ****$P < 0.0001$. (*B*) The time at which 10- to 13-day-old, mixed-sex *ND23*$^{60114}$ flies died during the 24 h after exposure to 2 h of 2% isoflurane and 21% $O_2$. At 2-h intervals the percentage of dead flies was normalized to the percentage of flies that were dead at 24 h. (*C*) 10- to 15-day-old, mixed-sex *ND23*$^{60114}$, *ND23*$^{60114}$/*ND23*$^{G14097}$, and *ND23*$^{60114}$/*ND23*$^{Del}$ flies were exposed to 2 h of 2% isoflurane and 21% $O_2$, and the percent mortality at 24 h was determined. N = number of biologic replicates. Symbols indicate the following: *box*, second and third quartiles of data; +, mean; *horizontal bar*, median; and *whiskers*, (minimum and maximum.

and subjected to DNase treatment using the TURBO DNA-free kit (Invitrogen, Thermo Fisher Scientific, USA). The resulting RNA was ammonium acetate–precipitated and resuspended in 20 μl of diethylpyrocarbonate-treated water. cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad, USA). Real-time reverse transcription–polymerase chain reaction was carried out using a Bio-Rad CFX96 real-time system C1000 Touch thermal cycler (Bio-Rad). Primer sequences are provided in Supplemental Digital Content (http://links.lww.com/ALN/C454).

## Data Analysis

No *a priori* power calculation was conducted in this discovery-driven project. The unit for data analysis (replicate) was one vial containing at least 20 flies. The number of flies in a vial varied because of uneven fertility among mutant and wild-type strains. Independently tested vials were considered

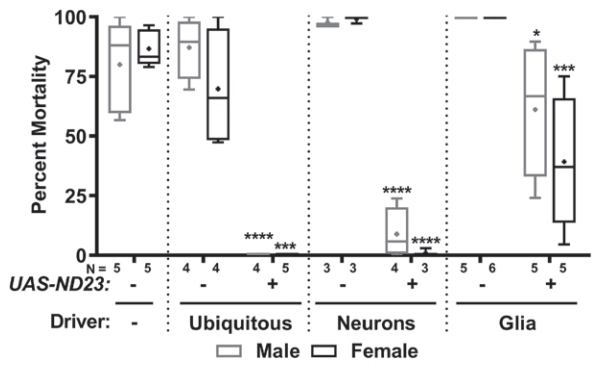


**Fig. 3.** Expression of *ND23* in the nervous system rescues the isoflurane toxicity of $ND23^{60114}/ND23^{G14097}$ flies. The percent mortality at 24 h after exposure to 2 h of 2% isoflurane in 21% $O_2$ was determined for 10- to 15-day-old, male and female $ND23^{60114}/ND23^{G14097}$ flies without (–) or with (+) a *UAS-ND23* transgene and without (–) or with (transgene) a Gal4 driver transgene: *Tub-Gal4* (ubiquitous), *C155-Gal4* (neurons), or *Repo-Gal4* (glia). Note the high control mortality (close to 100%) in *C155-Gal4* and *Repo-Gal4* crosses possibly caused by a more isoflurane-sensitive genetic background. N = number of biologic replicates. *Symbols* indicate the following: *box*, second and third quartiles of data; +, mean; *horizontal bar*, median; and *whiskers*, minimum and maximum. Significance between control and experimental data was determined using the unpaired equal-variance two-tail Student's *t* test. $^*P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

biologic replicates. Data analysis is based on biologic replicates and visualized using Prism 6 software (GraphPad, USA).

### Statistical Analysis

The box plots used to describe the data include the second and third quartiles of data. The mean (+) and the median (*horizontal bar*) are indicated, and *whiskers* extend to the minimum and maximum data point. We used parametric descriptive statistics because mortality rates for male and female mutants were normally distributed, and mean and median values were similar in all experiments. Statistical significance between two data points (control and experimental condition) was tested using an unpaired equal-variance two-tail Student's *t* test. Multiple groups were compared using one-way ANOVA unless otherwise indicated in the figure legend followed by the Dunnett's *post hoc* test for multiple comparisons. The significance level was set at 0.05. Outliers were included in all analyses.

## Results

### As $ND23^{60114}$ Flies Age, They Become Susceptible to Isoflurane but Not Sevoflurane Toxicity

To examine whether the $ND23^{60114}$ mutation sensitizes flies to toxic effects of volatile general anesthetics, we exposed wild-type and $ND23^{60114}$ flies to equipotent doses of isoflurane and sevoflurane (2% and 3.5% for 2 h, *i.e.*, 4%h and 7%h, respectively) in 21% $O_2$ (normoxia) and determined the percent mortality at 24 h after the exposure. We examined male and female flies at two age ranges, 2 to 4 and 10 to 13 days old, which for simplicity we refer to as young and old flies, respectively. For wild-type flies, no significant mortality was observed under any of the conditions (fig. 1A). In contrast, for old male and female $ND23^{60114}$ flies, isoflurane caused about 50% mortality (fig. 1B). No significant mortality was observed for young $ND23^{60114}$ flies exposed to isoflurane or for $ND23^{60114}$ flies exposed to sevoflurane at either age. These data indicate that the underlying age-associated isoflurane toxicity mechanism is not shared by sevoflurane, despite the fact that sevoflurane induces a state of anesthesia indistinguishable from that of isoflurane.[14]

To test the age dependence, the time course, and the *ND23* allele dependence of isoflurane toxicity, we conducted the experiments shown in figure 2. First, to narrow the age window for the onset of the toxic effect of isoflurane, we tested 6- to 8-day-old male and female flies. We observed no mortality in this age group (fig. 2A). We conclude that sensitization to isoflurane mortality develops in a narrow time window between 8 and 10 days of age. To examine whether mortality was due to an acutely lethal dose of isoflurane or a delayed effect, we determined when 10- to 13-day-old $ND23^{60114}$ flies died during the 24-h period after exposure. All flies initially regained mobility after exposure to isoflurane, and most flies that died did so between 12 and 24 h after exposure (fig. 2B). Therefore, the mechanism underlying isoflurane toxicity in $ND23^{60114}$ flies is likely to involve cellular and molecular signaling events that take several hours to build up. Finally, to test whether the age-dependent isoflurane toxicity of $ND23^{60114}$ flies was due to the *ND23* mutation, we repeated the mortality assay with other *ND23* alleles. We tested flies that were heteroallelic for $ND23^{60114}$ and $ND23^{G14097}$, which contains a P-element insertion in *ND23*, or $ND23^{Del}$, which deletes *ND23*. We found that similar to 10- to 13-day-old $ND23^{60114}$ flies, exposure to isoflurane caused greater than 50% mortality in 10- to 15-day-old $ND23^{60114}/ND23^{G14097}$ and $ND23^{60114}/ND23^{Del}$ flies (fig. 2C). Thus, mutations in *ND23* are a risk factor for age-dependent isoflurane toxicity.

### Isoflurane Mortality Is Due to Loss of ND23 Function in Neurons and Glia

To determine whether specific cell types control vulnerability to isoflurane toxicity, we attempted to rescue mortality in *ND23* mutants using the GAL4-UAS system to express wild-type *ND23* in various subsets of cells.[18] We found that ubiquitous expression of *UAS-ND23* (using the *Tubulin-Gal4* driver) completely rescued the mortality of old male and female $ND23^{60114}/ND23^{G14097}$

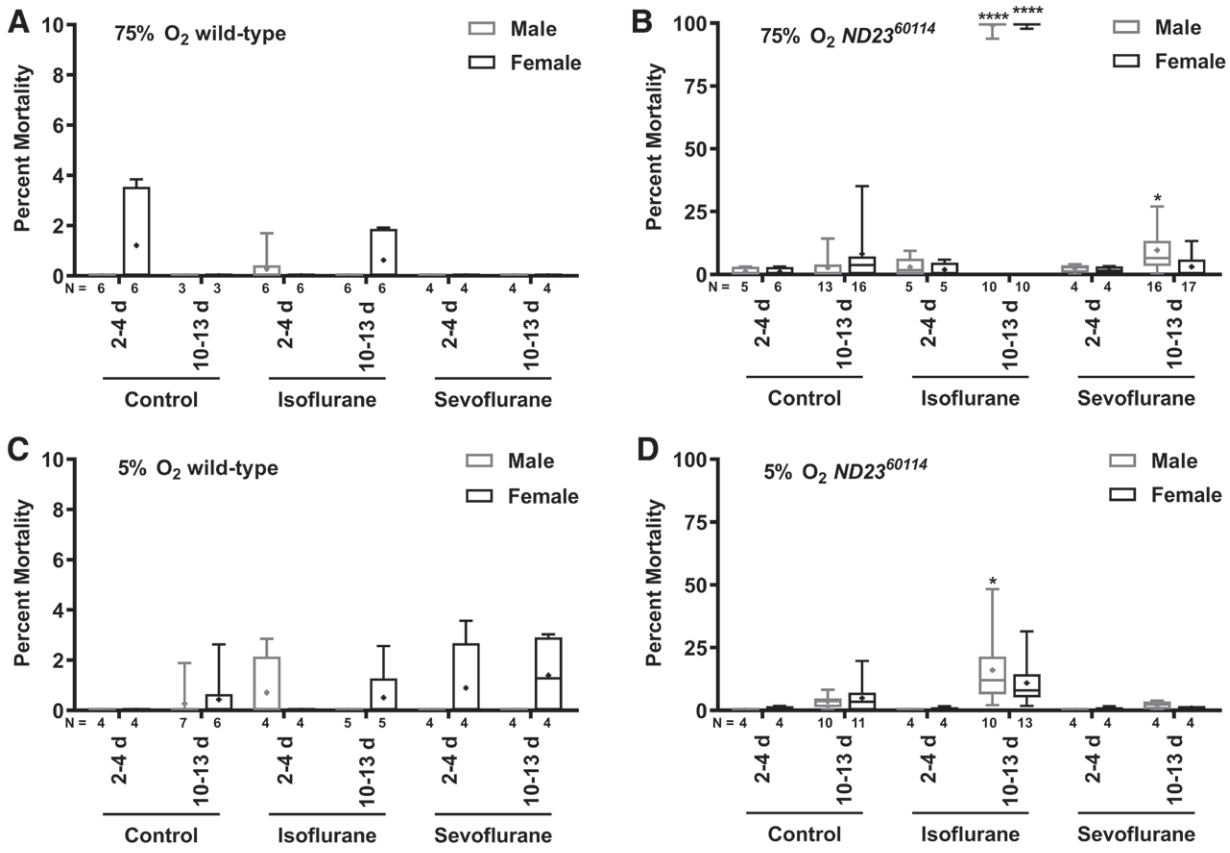


**Fig. 4.** Isoflurane toxicity in male and female *ND23*$^{60114}$ flies is oxygen-dependent. Male and female wild-type (*A* and *C*) and *ND23*$^{60114}$ (*B* and *D*) flies were either not exposed (control) or exposed to 2 h of 2% isoflurane or 2 h of 3.5% sevoflurane at either 2 to 4 days old (*2–4 d*) or 10 to 13 days old (*10–13 d*), and the percent mortality was determined after 24 h. The flies were exposed to 75% (*A* and *B*) or 5% (*C* and *D*) $O_2$ for 2 h, concurrent with the anesthetic. Note the difference in y-axis scale between (*A* and *C*) and (*B* and *D*). In addition, note almost 100% mortality in (*B*) for isoflurane in old animals. N = number of biologic replicates. Symbols indicate the following: *box*, second and third quartiles of data; +, mean; *horizontal bar*, median; and *whiskers*, minimum and maximum. Significance between control and experimental data was determined using the unpaired equal-variance two-tail Student's *t* test. **P* < 0.05.

flies exposed to isoflurane (fig. 3). This result provides additional evidence that isoflurane-induced mortality was due to the loss of *ND23*, as opposed to the loss of another gene. Neuron-specific expression of *UAS-ND23* (using the *C155-Gal4* driver) also resulted in almost complete rescue of the mortality from 97.6 ± 2.1 to 8.8 ± 10.7% and 99.1 ± 1.60 to 0.9 ± 1.7% in males and females, respectively. In addition, glia-specific expression of *UAS-ND23* (using the *Repo-Gal4* driver) partially rescued the mortality from isoflurane exposure from 100.0 ± 0.0 to 61.2 ± 27.7% and 100.0 ± 0.0 to 39.2 ± 27.7% in males and females, respectively. Taken together, these results indicate that isoflurane-induced mortality in *ND23* mutants is largely due to cell nonautonomous effects of loss of ND23 activity in the nervous system. Cell nonautonomous effects may reflect the close interconnection between glial and neuronal energetic homeostasis in neurodegenerative conditions.[19]

## The Severity of Isoflurane-induced Mortality of *ND23*$^{60114}$ Flies Is Oxygen-dependent

Because volatile general anesthetics are typically administered under hyperoxic conditions, we explored whether changes in oxygen concentration influenced the vulnerability of *ND23*$^{60114}$ flies to isoflurane and sevoflurane toxicity. Male and female wild-type and *ND23*$^{60114}$ flies were maintained in room air (~21% $O_2$) before and after a 2-h exposure to volatile general anesthetic in 5% $O_2$ (hypoxia) or 75% $O_2$ (hyperoxia). As with normoxia (fig. 1), no significant mortality was observed in young or old wild-type flies exposed to isoflurane or sevoflurane and hyperoxia or hypoxia (fig. 4, A and C). In contrast, old male and female *ND23*$^{60114}$ flies exposed to isoflurane and hyperoxia had significantly increased mortality compared with normoxia, whereas exposure to isoflurane and hypoxia had significantly reduced mortality compared with normoxia (fig. 4,

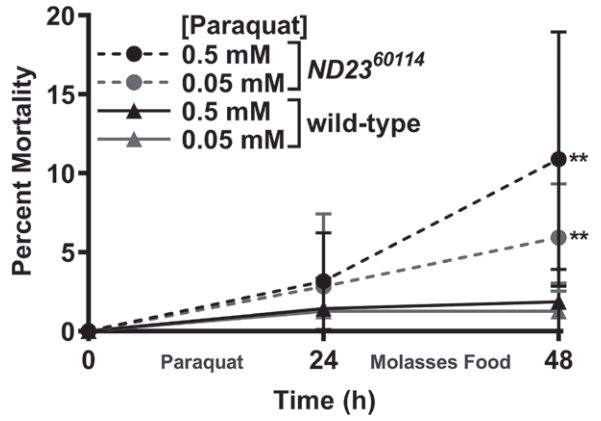


**Fig. 5.** *ND23*$^{60114}$ flies are more sensitive to paraquat than wild-type flies. After 10- to 13-day-old, mixed-sex *ND23*$^{60114}$ and wild-type flies were fed different concentrations of paraquat in 5% sucrose for 24 h, they were fed cornmeal-molasses food for the next 24 h. The percent mortality was determined at 24 and 48 h. Shown are the mean of 8 to 13 biologic replicates with *error bars* representing the standard deviations. For each dose and time point, comparisons were made between *ND23*$^{60114}$ and wild-type. Significance was determined using the unpaired, Student's *t* test assuming equal variances. $^{**}P < 0.01$.

B and D). The mortality from sevoflurane and hyperoxia or hypoxia was low, but male *ND23*$^{60114}$ flies exposed to sevoflurane and hyperoxia had significantly higher mortality than equivalently treated wild-type flies (9.63 ± 8.87% and 0.0 ± 0.0% for *ND23*$^{60114}$ and wild-type flies, respectively. $P = 0.0473$). Therefore, oxygen is an obligate cofactor for the manifestation of isoflurane toxicity in old *ND23*$^{60114}$ flies, and the susceptibility to inhalational agent toxicity may be greater in males than females. Rescue of isoflurane-induced mortality by hypoxia and aggravation by hyperoxia in *ND23*$^{60114}$ flies is comparable with the oxygen responsiveness of disease progression in *Ndufs4* mutant mice, which is also a model of Leigh syndrome.[20] The Ndufs4 subunit (*NADH:ubiquinone oxidoreductase subunit S4*) is, like ND23 (*NADH:ubiquinone oxidoreductase subunit S8*), part of the core of complex I of the mitochondrial electron transport chain. Our results are consistent with a potential role of therapeutic hypoxia in certain mitochondrial diseases.[21]

## Oxidative Stress May Contribute to Isoflurane Toxicity

A parsimonious explanation of these data is that an excessive increase in reactive oxygen species induced by a combination of isoflurane and hyperoxia[22] tilts the balance from redox homeostasis to oxidative stress[23] in the brains of old *ND23*$^{60114}$ flies. To test whether *ND23*$^{60114}$ flies have increased susceptibility to reactive oxygen species toxicity, we fed 10- to 13-day-old wild-type and *ND23*$^{60114}$ flies paraquat, which increases superoxide generation by complex I.[24] For the first 24 h of the experiment, after a 2-h starvation period, flies were fed paraquat in 5% sucrose, and for the second 24 h, they were fed standard cornmeal-molasses food. At 48 h after exposure to 0.05 or 0.5 mM paraquat, *ND23*$^{60114}$ flies had a significantly higher percent mortality than wild-type flies (fig. 5), indicating that the *ND23* mutation reduces resilience to oxidative stress.

To investigate the molecular pathways mediating the differential mortality of 10- to 13-day-old *ND23*$^{60114}$ flies to isoflurane and hyperoxia relative to sevoflurane and hyperoxia, we examined the expression of genes that are transcriptionally induced by oxidative stress. We examined the expression of *GstD1* and *GstD2* (encoding glutathione *S*-transferase D1 and D2, respectively, homologous to mammalian glutathione *S*-transferases), *Jafrac1* and *Jafrac2* (encoding thioredoxin peroxidase 1 and 2, respectively, homologous to mammalian peroxiredoxins 1 and 4),[25] *SOD1* and *SOD2* (encoding superoxide dismutase 1 and 2, respectively, homologous to mammalian superoxide dismutases), *Cat* (encoding catalase, homologous to mammalian catalases), and *IRC* (encoding immune-regulated catalase, homologous to mammalian COX2). We used real-time reverse transcription–polymerase chain reaction to measure mRNA amounts in the heads of 10- to 13-day-old *ND23*$^{60114}$ flies 30 min after a 2-h exposure to isoflurane or sevoflurane in hyperoxia or hypoxia. We examined this time point, which is before significant mortality (fig. 2B), because gene expression changes relevant to death should occur before its onset and because others have found that changes in gene expression become detectable as early as 30 min after initiation of isoflurane exposure.[26]

For almost all of the genes, the transcript amount was altered by exposure to either agent under both hyperoxic and hypoxic conditions, and the fold change was smaller in females than males (fig. 6, A and B). Moreover, in males, isoflurane and hyperoxia or hypoxia increased *GstD2* mRNA 5.6 ± 0.6- and 4.8 ± 0.3-fold, respectively (fig. 6A), whereas sevoflurane elicited a substantially larger increase in expression: 22.1 ± 0.4- and 25.2 ± 0.6-fold, respectively. Comparable differences between isoflurane and sevoflurane were observed in females (fig. 6B). Likewise, in both males and females, sevoflurane and hyperoxia or hypoxia led to a greater reduction in *Jafrac2* expression than isoflurane and hyperoxia or hypoxia (fig. 6). In contrast, in both males and females, isoflurane or sevoflurane and hyperoxia or hypoxia caused equivalent increases in *Sod1* and *Sod2* expression. Unexpectedly, oxygen concentration did not affect the expression of any of the genes. These data suggest that both isoflurane and sevoflurane activate reactive oxygen species–protective pathways, but they do so to different extents, which may contribute to the differences in mortality.

## Aging Sensitizes Heterozygous *ND23* Mutants to Toxicity from Isoflurane and Hyperoxia

To examine the dose dependence of *ND23* for isoflurane toxicity, we repeated the mortality assay with heterozygous

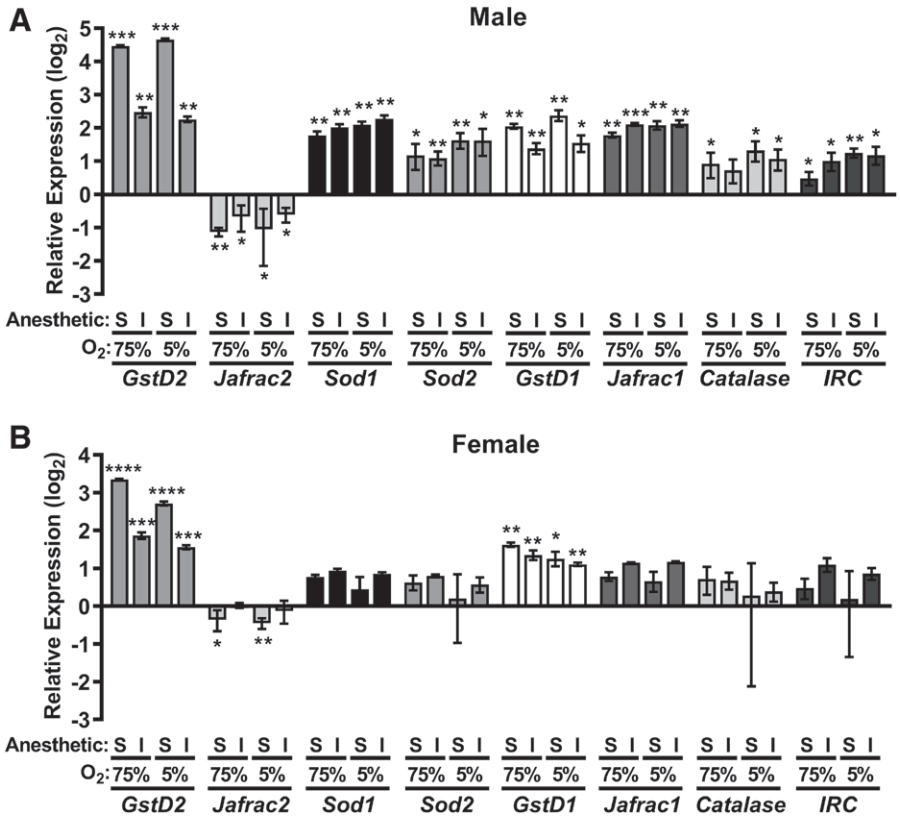


**Fig. 6.** Anesthetics modulate gene expression in reactive oxygen species-responsive pathways. Real-time reverse transcription–polymerase chain reaction was performed on the heads of 10- to 13-day-old male (*A*) and female (*B*) *ND23*$^{60114}$ flies collected 30 min after 2 h of 2% isoflurane (I) or 2 h of 3.5% sevoflurane (S) in either 5% or 75% $O_2$. Expression of the indicated genes was normalized to expression of *ribosomal protein L32* (*RpL32*) and to expression of the genes in heads of unexposed, 10- to 13-day-old *ND23*$^{60114}$ flies. Each experiment consisted of three biologic replicates. Significance was determined using the unpaired, Student's *t* test assuming equal variances. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001. *Error bars* represent standard deviations.

*ND23*$^{60114}$ flies that have approximately half the amount of ND23 protein and a slightly longer lifespan compared with wild-type flies.[12] At 15–22 days old, *ND23*$^{60114}$/wild-type flies (derived by crossing female *ND23*$^{60114}$ flies to male wild-type flies) were not vulnerable to mortality from exposure to isoflurane and hyperoxia (2 h of 2% isoflurane in 75% $O_2$; fig. 7). Because aging is associated with deteriorating mitochondrial function,[27] we also tested older *ND23*$^{60114}$/wild-type flies. We did not observe substantial mortality for *ND23*$^{60114}$/wild-type flies until they were more than 30 days old. At 33 to 39 days old, exposure to isoflurane and hyperoxia resulted in 54.05 ± 19.59% mortality in mixed-sex *ND23*$^{60114}$/wild-type flies. Thus, aging sensitizes old heterozygous *ND23* mutant flies to toxicity from isoflurane and hyperoxia.

In studies of 30- to 39-day-old flies, isoflurane and hyperoxia did not increase mortality in wild-type flies beyond natural attrition (fig. 8A); however, it significantly increased the mortality of female *ND23*$^{60114}$/wild-type from 3.9 ± 3.1 to 27.4 ± 15.9% (fig. 8B). Mortality was also increased in *ND23*$^{60114}$/wild-type male flies, but only the increase in isoflurane and normoxia from 25.7 ± 8.6 to 53.5 ± 24.5% was significant, probably because of the high rate of natural mortality. Similar results were observed with another *ND23* allele, *ND23*$^{Del}$/wild-type flies (fig. 8C). Because of their short lifespan, *ND23*$^{Del}$/wild-type flies were tested at 21 to 26 days old. Finally, we found that isoflurane and hyperoxia increased the mortality of 30- to 39-day-old *ND23*$^{G14097}$/wild-type flies, but the difference did not reach the significance cutoff (Supplemental Digital Content, http://links.lww.com/ALN/C455). This was unexpected because *ND23*$^{G14097}$ is a stronger allele than *ND23*$^{60114}$ in other contexts.[12] Thus, advanced age renders heterozygous carriers of some *ND23* alleles susceptible to toxicity from exposure to isoflurane and hyperoxia.

## Genetic Background Modulates the Toxicity of Isoflurane and Hyperoxia in Aged Heterozygous *ND23* Mutants

To test whether genetic background influences mortality from isoflurane and hyperoxia, we crossed *ND23*$^{60114}$ flies to

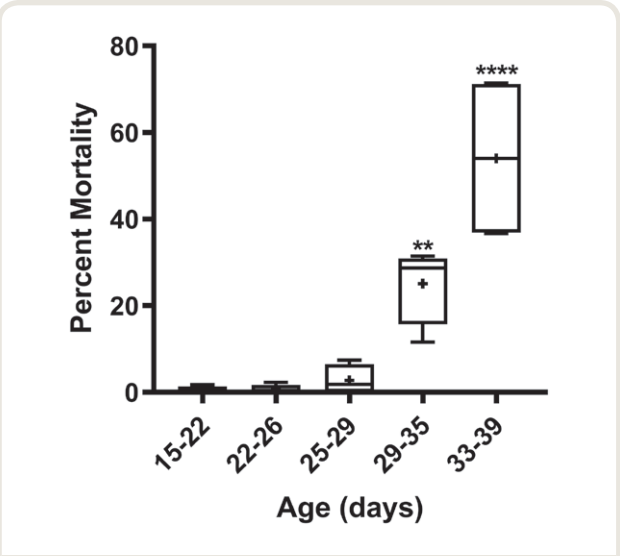


**Fig. 7.** Mortality after exposure to isoflurane increases with age in heterozygous *ND23*$^{60114}$ flies. *ND23*$^{60114}$/wild-type flies of the indicated ages were exposed to 2 h of 2% isoflurane in 75% $O_2$, and the percent mortality at 24 h was determined. Each experiment consisted of four biologic replicates. Symbols indicate the following: *box*, second and third quartiles of data; +, mean; *horizontal bar*, median; and *whiskers*, minimum and maximum. Significance was determined using an ordinary one-way ANOVA followed by the Dunnett's *post hoc* test for multiple comparisons. $^{**}P < 0.01$; $^{****}P < 0.0001$.

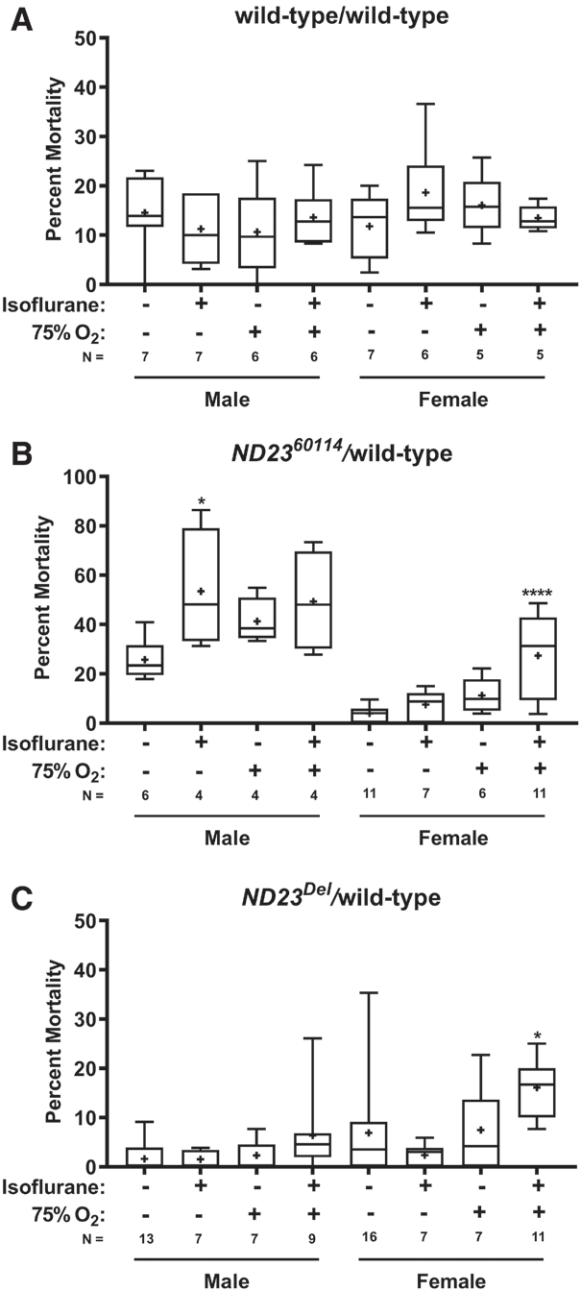


**Fig. 8.** Aging and hyperoxia sensitize heterozygous *ND23* mutants to isoflurane toxicity. The percent mortality at 24 h was determined for 30- to 39-day-old wild-type/wild-type (*A*) and *ND23*$^{60114}$/wild-type (*B*) and 21- to 26-day-old *ND23*$^{Del}$/wild-type (*C*) males and females exposed (+) or not exposed (–) to 2% isoflurane and 75% $O_2$ for 2 h. Symbols indicate the following: *box*, second and third quartiles of data; +, mean; *horizontal bar*, median; and *whiskers*, minimum and maximum. Significance between control and experimental data was determined using an ordinary one-way ANOVA followed by the Dunnett's *post hoc* test for multiple comparisons. $^{*}P < 0.05$; $^{****}P < 0.0001$.

$w^{1118}$ flies (a standard laboratory line) and to RAL774 and RAL352 flies, which are part of the Drosophila Genetic Reference Panel collection of inbred lines from a natural population.[28] RAL774 and RAL352 were selected for analysis because they are behaviorally more sensitive to isoflurane than $w^{1118}$ and wild-type and less sensitive than *ND23*$^{60114}$.[14] Exposure to isoflurane in hyperoxia significantly increased the mortality of *ND23*$^{60114}$/wild-type flies ($P < 0.0001$) and *ND23*$^{60114}$/$w^{1118}$ flies ($P < 0.05$). Interestingly, *ND23*$^{60114}$/RAL774 flies ($P = 0.667$) and *ND23*$^{60114}$/RAL352 flies ($P = 0.671$; fig. 9) were not significantly affected. We conclude that polymorphisms in the genetic background modulate the toxicity of isoflurane and hyperoxia in aged heterozygous *ND23* flies and that the tested RAL lines contain genetic polymorphisms in their background that apparently counteract the increased mortality that would otherwise be expected under the conditions tested. The nature of these polymorphisms could be explored with a genome-wide association study analysis of the Drosophila Genetic Reference Panel collection.

## Discussion

It is not known how age, sex, and environmental factors modulate the penetrance of the pathophysiology underlying Leigh syndrome,[9] as they do for other more common

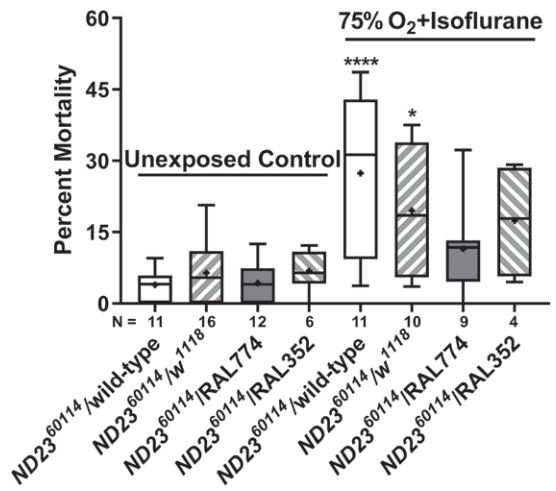


**Fig. 9.** Genetic background alters the sensitivity of heterozygous *ND23* mutants to mortality from isoflurane and hyperoxia. After 30- to 39-day-old females of the indicated genotypes were not exposed (unexposed control) or exposed to 2% isoflurane and 75% $O_2$ for 2 h (75% $O_2$ + isoflurane), the percent mortality was determined at 24 h. The percent mortality in exposed flies increased for $ND23^{60114}$/wild-type ($P < 0.0001$) and $ND23^{60114}/w^{1118}$ ($P = 0.021$) flies but not for $ND23^{60114}$/RAL 774 ($P = 0.667$) and $ND23^{60114}$/RAL352 ($P = 0.671$) flies. There was a significant effect of drug/genotype interaction ($P = 0.0435$, two-way ANOVA). Males were not examined because not enough survived for biologic replicates. Significance was determined using a two-way ANOVA followed by the Tukey *post hoc* test for multiple comparisons. *$P < 0.05$; ****$P < 0.0001$.

diseases caused by complex I mutations, such as Leber's hereditary optic neuropathy.[10] Obtaining information to answer these questions would thus be an important advance for the mitochondrial disease community and their medical teams. We used an invertebrate model of Leigh syndrome and of asymptomatic carriers to explore interactions with age, sex, volatile general anesthetics, and oxygen.

## Behavioral Sensitivity and Toxicity of Isoflurane and Sevoflurane Are Dissociated in *ND23* Mutants

Young, 2- to 4-day-old $ND23^{60114}$ flies are equally hypersensitive to isoflurane and sevoflurane, compared with six inbred strains, including the wild-type.[14] Notably, the relative potencies of isoflurane and sevoflurane (expressed as the ratio of $EC_{50}$ isoflurane/$EC_{50}$ sevoflurane) are nearly identical (0.42 and 0.40 for $ND23^{60114}$ and wild-type flies, respectively), indicating a proportional increase in the sensitivity of behavioral circuits to both agents without toxicity in animals lacking morphological signs of neurodegeneration.[12] Similarly, studies in mammals also report increased sensitivity to vapor anesthetics in young (23- to 27-day-old) mice harboring mutations in complex I at an age when the animals do not yet exhibit signs of neurodegeneration.[29] Whether advancing age uncovers toxic phenotypes in rodents is unknown. Furthermore, in the developing chemotactic system of worms, isoflurane neurotoxicity is linked to inhibition of mitochondrial function.[30]

In contrast with the similar behavioral sensitivity of *ND23* mutant flies to isoflurane and sevoflurane, *ND23* mutants exposed to isoflurane but not to sevoflurane abruptly developed a mortality phenotype between 8 and 10 days of age (figs. 1 and 2A). In this context, it is important to note that we do not expect the toxic phenotype (*i.e.* mortality) in the fly model to translate as mortality in higher animals. Hyperoxia (99% $O_2$), for example, dramatically shortens lifespan in fruit flies[31,32] but causes only impaired cognition without lethality in rats.[33] Analogously, mortality in flies serves as an indicator of interference with brain mitochondrial homeostasis that may present with cognitive ("neurotoxic") phenotypes in higher animals.

Isoflurane-induced mortality of *ND23* mutants is not the result of an acute anesthetic overdose because: (1) mortality increased over many hours after initial recovery from the behavioral effect of anesthesia (fig. 2B), (2) no death occurred in flies up to 8 days of age despite behavioral hypersensitivity (fig. 2A),[14] and (3) $ND23^{60114}$ flies were equally hypersensitive to isoflurane and sevoflurane, but only isoflurane caused high mortality. We interpret these results as indicating that anesthesia and mortality are dissociable: molecular targets mediating the conventional behavioral effects of vapor anesthetics ("anesthesia") are largely shared between isoflurane and sevoflurane, but targets contributing to mortality in mutant flies (and possibly to adverse neurocognitive outcomes in higher animals) may differ. The finding that isoflurane is more toxic than sevoflurane in cultured neuroblastoma cells injured by oxygen-glucose deprivation supports this notion.[34] However, only a few studies have directly compared toxic effects of isoflurane and sevoflurane administered *in vivo*. No differences were observed in the induction of apoptotic cell death by isoflurane and sevoflurane in the brains of neonatal mice,[35] but with a different experimental paradigm that used behavioral outcomes, isoflurane resulted in a more extensive memory impairment phenotype than sevoflurane.[36] Moreover, a clear difference between isoflurane and sevoflurane was found in their propensity to activate members of the transient receptor potential superfamily TRPA1 and TRPV1, resulting in an *in vivo* inflammatory response after exposure to isoflurane but not to sevoflurane,[37] thereby demonstrating that biologically significant differences exist between these interchangeably used agents.

## Production of Reactive Oxygen Species in the Nervous System May Mediate the Toxicity of Isoflurane in *ND23* Mutants

Our finding that isoflurane-induced mortality of $ND23^{60114}$ flies was rescued by expression of wild-type *ND23* in neurons or in glia indicates that mortality is driven by cell

nonautonomous consequences of ND23 activity in the brain (fig. 3). Notably, selective expression of *ND23* in neurons rescued mortality to a greater extent than expression in glia. Given that glial cells constitute only 10% of cells in the fly brain, the disproportionally high degree of rescue observed with glia-specific expression of *ND23* may be due to a higher rate of reactive oxygen species production by glia and/or to the energy support of neurons by glia.[38] Lopez-Fabuel *et al.*[39] found that complex I in neurons is mainly assembled into supercomplexes that produce reactive oxygen species at a lower rate than free complex I, which is more abundant in astrocytes. Interestingly, mice carrying a mutation of complex I only in astrocytes exhibit a subtle behavioral phenotype under isoflurane anesthesia.[40] Thus, isoflurane-induced production of reactive oxygen species in neurons and glia may mediate toxicity in *ND23* mutants.

In *Caenorhabditis elegans*, suppression of complex I function by isoflurane increases production of reactive oxygen species in wild-type and mutant mitochondria,[4] whereas complex I mutations confer increased sensitivity to oxidizing agents such as paraquat.[41] Further data from rodents[42] and from a mammalian cell culture model[43] suggest a role for reactive oxygen species in the toxicity of isoflurane. Our data regarding the effects of oxygen concentration on isoflurane toxicity (figs. 1 and 4), the increased sensitivity of *ND23* mutants to paraquat compared with wild-type flies (fig. 5), are also consistent with a role for reactive oxygen species in mortality. Additional support for the reactive oxygen species hypothesis of isoflurane toxicity is provided by our gene expression analysis showing that isoflurane and sevoflurane elicit qualitatively similar but quantitatively different patterns of transcriptional changes in some gene target of reactive oxygen species response pathways (*GstD2* and *Jafrac2*; fig. 6). *GstD2* expression in flies increases in response to hyperoxic stress[44] and to stress caused by blue light that results in neurodegeneration.[45] Jafrac2 has both antioxidant and signaling activity that regulates intracellular hydrogen peroxide levels.[25] Suppression of *Jafrac2* expression results in shortened lifespan in the face of oxidative stress with hydrogen peroxide and paraquat.[25] Reactive oxygen species are natural byproducts of electron transfer in mitochondria functioning as signaling molecules responsive to environmental cues.[46,47] Mitochondrial dysfunction and reactive oxygen species–induced damage accumulate with aging[32,48,49] and may sensitize flies to the acute oxidative stress–like insult from isoflurane. Therefore, although it is evident that exposure to both isoflurane and sevoflurane triggers responses in oxidative stress pathways, our data do not allow us to infer which changes are protective (*e.g.*, strong upregulation of *GstD2*), which are signs of a failure to adapt (*e.g.*, weak upregulation of *GstD2*), or how these changes in gene expression relate to mortality. A toxicity mechanism involving reactive oxygen species pathways is also consistent with the age dependence of isoflurane-induced mortality in *ND23* mutant flies (figs. 1, 2A, 4, and 7).

## Aging and Genetic Background Shape the Phenotypes of Isoflurane in Heterozygous *ND23* Mutants

About 5.9 in 100,000 individuals are at risk of nuclear DNA–related mitochondrial diseases,[8] the majority of which are due to recessive mutations. We found that age renders heterozygous carriers of *ND23* mutations vulnerable to the stress of isoflurane under normoxic and hyperoxic conditions (figs. 7 and 8). Polymorphisms in the nuclear genome, introduced either by other alleles of *ND23* (fig. 8) or by crosses into other fly lines (fig. 9), modified the genetic environment of the *ND23* mutation and resulted in varying degrees of vulnerability to the toxicity of isoflurane and hyperoxia. These results suggest that heterozygous mutations in complex I genes that underlie Leigh syndrome, the most common mitochondrial encephalomyelopathy,[5] can increase the risk for deleterious manifestations under the combined stresses of advanced age, anesthesia, and hyperoxia with the degree of risk dependent on genetic background. Importantly, our data from heterozygous flies indicate that under conditions of advanced age and environmental stress (*e.g.*, surgery under anesthesia with isoflurane and hyperoxia), an otherwise silent reduction in complex I function may translate into adverse outcomes. In the clinical scenario, the association of cardiopulmonary bypass with a high incidence of temporary central nervous system dysfunction[50] and with mitochondrial dysfunction and oxidative damage[51] may not be accidental.

In summary, our data raise testable predictions. Other mutations affecting complex I and mutations affecting other components of oxidative metabolism should result in a toxic phenotype under anesthesia. Furthermore, strategies aimed at mitigating oxidative stress should reduce anesthetic toxicity. A limitation of our data is that we have not yet established a direct causal relationship between oxidative stress and mortality. Other tests such as genome-wide transcriptome analysis are necessary to provide a more comprehensive overview of pathways involved in isoflurane toxicity. A better understanding of these pathways will reveal genetic risk factors and therapeutic approaches to anesthetic neurotoxicity. Our experiments demonstrate the value of *Drosophila* for understanding the multifaceted interactions of anesthetics, aging, genetic background, and mitochondrial function.

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## Competing Interests

The authors declare no competing interests.

## Correspondence

Address correspondence to Dr. Perouansky: School of Medicine and Public Health, University of Wisconsin–Madison, 600 Highland Avenue, B6/319 CSC, Madison, Wisconsin 53792-3272. mperouansky@wisc.edu. 

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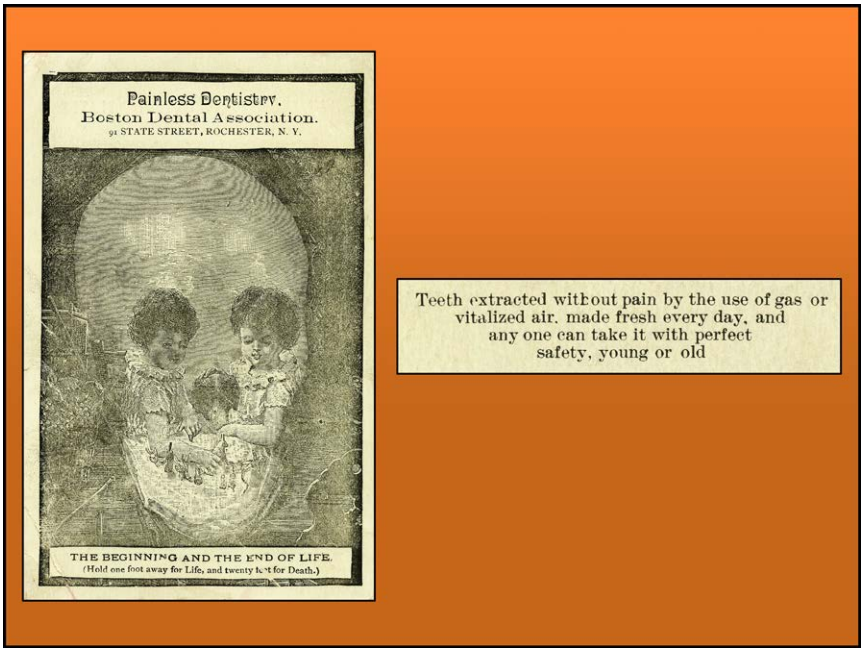
# Boston Skulling, Not Sculling: Anesthetic Advertising by Daring Dentists




Because this month is one in which Americans celebrate the haunting holiday of Halloween, this October Anesthesiology Reflection presents one of the more ghoulish trade cards used in advertising anesthesia. From their State Street office in Rochester, New York, the Boston Dental Association used a grinning skull on the obverse of their ill-advised advertisement to promote both pure and adulterated nitrous-oxide ("vitalized air") anesthetics for dental surgery. The association boasted that their anesthetic gases were "made fresh every day" and that "any one can take it with perfect safety, young or old" (*right*, extracted from the card's reverse). To underscore their claim, the association issued this trade card (*left*) as a "treat" depicting a "trick" image that symbolized Life (two young girls playing with a puppy) when viewed from "one foot away" and, in an optical illusion, depicted Death (a grinning skull) from 20 feet away. Ironically, the skull, advertising "the beginning and the end of life" on this 1890 trade card, may have foreshadowed the association's demise…that same year. (Copyright © the American Society of Anesthesiologists' Wood Library-Museum of Anesthesiology, Schaumburg, Illinois.)

*Melissa L. Coleman, M.D., Penn State College of Medicine, Hershey, Pennsylvania, and George S. Bause, M.D., M.P.H., Case Western Reserve University, Cleveland, Ohio.*