

Article

Expansion of Electron Transport Chain Mutants That Cause Anesthetic-Induced Toxicity in *Drosophila melanogaster*

Luke A. Borchardt^{1,2}, Zachariah P. G. Olufs¹, Philip G. Morgan^{3,4}, David A. Wassarman⁵
and Misha Perouansky^{1,*}

¹ Department of Anesthesiology, University of Wisconsin-Madison, Madison, WI 53792, USA; borcharl@ohsu.edu (L.A.B.); olufs@wisc.edu (Z.P.G.O.)

² Vollum Institute, Oregon Health & Science University, Portland, OR 97239, USA

³ Center for Integrative Brain Research, Seattle Children's Research Institute, Seattle, WA 98101, USA; pgm4@uw.edu

⁴ Department of Anesthesiology and Pain Medicine, University of Washington, Seattle, WA 98105, USA

⁵ Department of Medical Genetics, University of Wisconsin-Madison, Madison, WI 53706, USA; dawassarman@wisc.edu

* Correspondence: mperouansky@wisc.edu

Abstract: The mitochondrial electron transport chain (mETC) contains molecular targets of volatile general anesthetics (VGAs), which places individuals with mETC mutations at risk for anesthetic complications, as exemplified by patients with Leigh syndrome (LS). The *Drosophila melanogaster* homozygous mutant for *ND-23*, which encodes a subunit of mETC Complex I, replicates numerous characteristics of LS, including neurodegeneration, shortened lifespan, behavioral anesthetic hypersensitivity, and toxicity. The anesthetic phenotype of toxicity (lethality) is also observed in flies homozygous for mutations in other Complex I subunits. By contrast, mutations conferring sensitivity have not yet been identified for subunits of Complexes II–V. Furthermore, anesthetic phenotypes are thought to be recessive; that is, risk is not conferred by heterozygous mutations. However, at older ages, exposure of heterozygous mutant *ND-23* flies to the VGA isoflurane in 75% oxygen (hyperoxia) results in toxicity. It is also unknown whether combinations of heterozygous mutations in different subunits of the mETC can result in anesthetic toxicity. Here, we show that, following exposure to isoflurane in hyperoxia, flies carrying heterozygous mutations in two Complex I subunits, *ND-23* and *ND-SGDH* (NADH dehydrogenase (ubiquinone) *SGDH* subunit), had a level of anesthetic toxicity that exceeded the added toxicities of the individual heterozygous mutations. In addition, we show that flies heterozygous for two different alleles of the Complex II gene *SdhB* were susceptible to isoflurane/hyperoxia-induced anesthetic toxicity. Finally, a mutation in the *SdhC* subunit of Complex II of *Caenorhabditis elegans* resulted in isoflurane-induced mortality, supporting the role of Complex II in anesthetic toxicity. These data expand the landscape of mutations in the mETC that increase sensitivity to anesthetic toxicity.

Keywords: Complex I; Complex II; *Drosophila melanogaster*; *Caenorhabditis elegans*; electron transport chain; hyperoxia; isoflurane; mitochondria; succinate dehydrogenase; toxicity; volatile general anesthetics



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1. Introduction

Volatile general anesthetics (VGAs) in common use today are generally safe. The extent to which the genetic background can increase the risk of adverse effects from exposure to VGAs is only partially understood. Leigh syndrome (LS) is an example of a pharmacogenetic risk factor for the perioperative period [1]. The majority of LS cases are caused by mutations in Complex I of the mitochondrial electron transport chain (mETC). LS results in early severe neurodegeneration and a shortened lifespan, and it is accompanied by hypersensitivity to VGAs [2]. Additionally, individuals with LS are at increased risk of perioperative complications [3], which is attributed, at least partially, to

increased susceptibility to the depressant effect of VGAs on oxidative phosphorylation [4]. Unfortunately, no treatment is available for LS. The key to developing effective treatments is an improved understanding of the pathomechanisms of mitochondrial disorders, and to achieve that, animal models are essential.

In *Drosophila melanogaster*, the $ND-23^{60114}$ allele carries a point mutation in the nuclearly encoded Complex I subunit ND-23 (the *Drosophila* ortholog of mammalian NDUFS8). Flies homozygous for this allele ($ND-23^{60114}/ND-23^{60114}$) reproduce features of LS, including neurodegeneration and a shortened lifespan [5]. Moreover, these animals are hypersensitive to the behavioral effects of the VGAs sevoflurane and isoflurane [6] and, as young adults (i.e., 10–13 days old), they acquire a lethal anesthetic-induced toxicity phenotype, presenting as mortality at 24 h following exposure to the VGA isoflurane. Notably, mortality is increased by hyperoxia and suppressed by hypoxia [7], mimicking oxygen-dependent mortality in rodent models of LS [8]. By contrast, young, heterozygous $ND-23^{60114}$ flies ($ND-23^{60114}/+$) are phenotypically normal and do not show the anesthetic phenotypes. However, at 30–35 days old, they become susceptible to isoflurane-induced toxicity [7]. We also found that flies mutant for other Complex I subunits, including the nuclearly encoded $ND-SGDH^{G6447}$ (the *Drosophila* ortholog of mammalian *NDUFB5*), are susceptible to isoflurane-induced toxicity at 15–20 days old [9]. However, susceptibility is not conferred by alleles of other Complex I subunits (i.e., $ND-13B^{EY07792}$, $ND-39^{EY06323}$, $ND-15^{M110315}$, and $ND-B22^{KG08637}$) or alleles of Complex II–V subunits (Complex II: $SdhB^{C00364}$, Complex III: $UQCR-Q^{KG08923}$, Complex IV: $levy^{EY01834}$, and Complex V: $ATPsynB^{EY03793}$). Thus, the isoflurane-induced toxicity phenotype is specific to alleles of genes encoding particular mETC subunits and depends on the age at the time of isoflurane exposure as well as on the oxygen concentration during isoflurane exposure.

Here, we expand upon our prior studies by investigating the interaction of compound heterozygous mutations in different Complex I subunits in shaping the penetrance of the isoflurane-induced toxicity phenotype. We also examined mutations in Complex II subunits both in *Drosophila* and in the nematode *Caenorhabditis elegans* (*C. elegans*) to test for conservation of the isoflurane-induced toxicity phenotype across phyla.

2. Materials and Methods

2.1. Fly Lines and Culturing

All flies were raised at 25 °C on cornmeal–molasses food, as previously described [6]. $ND-SGDH^{G6447}$, $SdhB^{EY12081}$, and $SdhB^{C00364}$ were obtained from the Bloomington *Drosophila* Stock Center (Stock IDs 27208, 20343, and 10039, respectively). $ND-23^{60114}$, $ND-23^{def}$ (*Df*(3R)Exel8162), and Canton S were provided by Barry Ganetzky (UW-Madison). $ND-23^{60114}/ND-SGDH^{G6447}$ and $ND-23^{60114}/+$ flies were generated by crossing $ND-23^{60114}$ females with $ND-SGDH^{G6447}$ and Canton S males, respectively. $ND-SGDH^{G6447}/+$ flies were generated by crossing Canton S females with $ND-SGDH^{G6447}$ males. $ND-23^{def}/+$ and $ND-23^{def}/ND-SGDH^{G6447}$ flies were generated by crossing $ND-23^{def}/TM6B$ females with Canton S and $ND-SGDH^{G6447}$ males, respectively. $SdhB^{EY12081}/SdhB^{C00364}$ flies were generated by crossing $SdhB^{EY12081}/CyO$ females with $SdhB^{C00364}/CyO$ males.

2.2. Anesthetic and O₂ Exposure

Flies were raised at 25 °C for up to 5 days post-eclosion, transferred to vials of 35 mixed-sex flies using CO₂, and incubated at 29 °C until the age of anesthetic exposure. The temperature change from 25 °C to 29 °C served to remain consistent with prior studies [5–7]. Vials were placed on their sides to prevent flies from sticking to the food. Flies were transferred to fresh food vials three times per week. On the day of exposure, flies were transferred to 50 mL conical tubes without use of CO₂ and placed onto the serial anesthesia array (SAA) [6]. Each 50 mL conical tube represented a biological replicate. A mixture of O₂ and air (21% O₂, 79% N₂) (Airgas, Madison, WI, USA) was used to generate hyperoxic (75% O₂) conditions using a clinical anesthesia machine (Aestiva/5; Datex-Ohemda) and confirmed by an inline, internal O₂ sensor. Commercial agent-specific

vaporizers delivered 2% isoflurane for 2 h (i.e., 4% h ($2\% \times 2$ h)) at room temperature (Ohmeda Isotec 5). After exposure, the SAA was flushed with air for 5 min. Flies were transferred to fresh food culture vials and incubated at 29 °C for 24 h. Percent mortality was determined by counting the number of dead flies and dividing by the total number of flies per vial. Mortality is a binary readout and was assessed by an unblinded observer. Each experiment was conducted on at least three separate days. The change in mortality (Δ Mortality) was determined by subtracting the average percent mortality from unexposed controls from the percent mortality of each vial exposed to anesthetic. Data were analyzed using GraphPad Prism (version 10.0.2) software. Data were tested for normality using the Shapiro–Wilk test. *p*-values from comparisons of normally distributed data were generated from Bonferroni’s multiple comparisons test following an ANOVA. *p*-values from comparisons of non-normally distributed data were generated from Dunn’s multiple comparisons test following a Kruskal–Wallis test.

2.3. Nematode Culturing and Exposures

Behavior in Anesthetic—Nematode culture and anesthetic exposure was performed as previously described [10]. Briefly, 20–50 adult hermaphrodites were placed on agar plates fed with the bacteria OP50 and allowed to lay eggs for 4 h. The adults were removed, and the eggs were grown at 20 °C overnight. A total of 20–25 L1 animals were then transferred to new agar plates with OP50 as a food source. These plates were grown for 2–3 more days until the animals were first-day, egg-laying adults. The plates were then transferred into a glass chamber and exposed to 6.5% isoflurane for 3 h. The plates were removed, observed after 1 h to ensure all adults had regained mobility, and returned to a 20 °C incubator. Each plate was scored 24 h later for mortality. *p*-values for single comparisons made between non-normally distributed data were generated from two-tailed Mann–Whitney tests.

3. Results

3.1. Combining Heterozygous Mutations in Different Subunits of Complex I Significantly Increases Isoflurane-Induced Toxicity

To investigate the effects of combined heterozygous mutations in the mETC subunit on anesthetic toxicity, we examined mutations in the Complex I subunits *ND-23* (orthologous to mammalian *NDUFS8*) and *ND-SGDH* (NADH dehydrogenase (ubiquinone) *SGDH* subunit). We previously found that exposure of *ND-23*⁶⁰¹¹⁴/*ND-23*⁶⁰¹¹⁴ flies at 10–13 [7] or 8–13 [9] days old and *ND-SGDH*^{G6447}/*ND-SGDH*^{G6447} flies at 15–20 days old [9] to 4% h isoflurane in 75% O₂, hereafter referred to as hyperoxic isoflurane, caused a significant increase in percent mortality over unexposed flies, and exposure of wild-type Canton S flies at any of these ages did not affect mortality. Moreover, we found that multiple allelic combinations of *ND-23* mutants produced a similar phenotype.

To follow up on these findings, we examined heterozygous flies at an older age. Exposure of 30–36 day-old Canton S flies to hyperoxic isoflurane did not increase mortality at 24 h; however, it increased the mortality of *ND-23*⁶⁰¹¹⁴/*+* and *ND-SGDH*^{G6447}/*+* flies (Figure 1A). Moreover, we found that flies heterozygous for both *ND-23*⁶⁰¹¹⁴ and *ND-SGDH*^{G6447} had substantially higher mortality than the individual mutants. We then isolated the isoflurane-induced mortality by subtracting the unexposed from the exposed mortality for each genotype (Figure 1A'). We found that the isoflurane-induced mortality of *ND-23*⁶⁰¹¹⁴/*+* and *ND-SGDH*^{G6447}/*+* flies did not differ significantly (*p* > 0.99), but the mortality of double heterozygous *ND-23*⁶⁰¹¹⁴/*ND-SGDH*^{G6447} flies was higher than either mutation alone (*p* < 0.0001, for both). These results support our prior finding that aging increases the risk of isoflurane-induced toxicity for flies heterozygous for *ND-23* or *ND-SGDH*. Moreover, we conclude that the combination of mutations in distinct subunits of Complex I increases the susceptibility to isoflurane-induced toxicity in older flies.

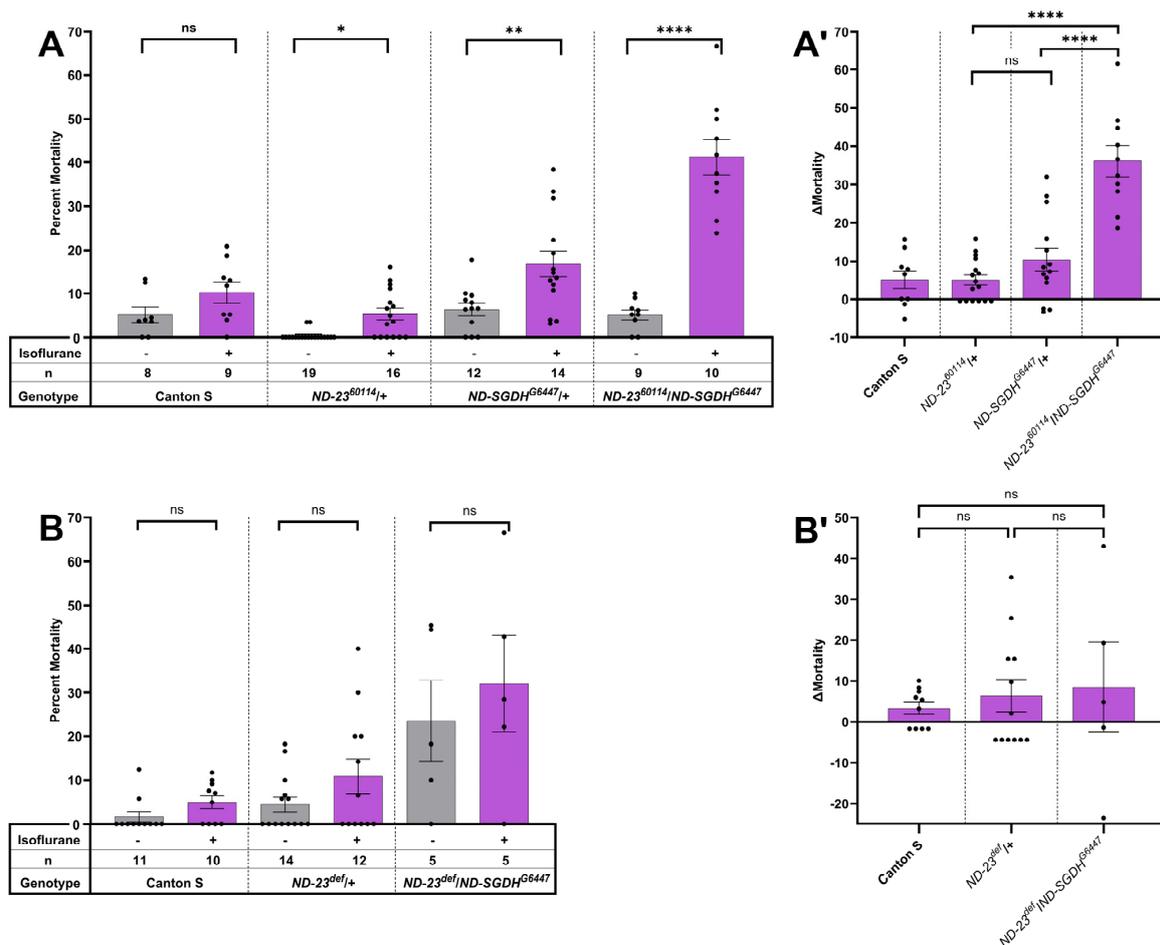


Figure 1. Mutations in Complex I of mETC increase isoflurane-induced mortality. (A,A') The 30–36 day-old Canton S, *ND-23*⁶⁰¹¹⁴/+, *ND-SGDH*^{G6447}/+, and *ND-23*⁶⁰¹¹⁴/*ND-SGDH*^{G6447} flies and (B,B') 24–30 day-old Canton S, *ND-23*^{def}/+, and *ND-23*^{def}/*ND-SGDH*^{G6447} flies were exposed to hyperoxic isoflurane, and percent mortality was determined 24 h after exposure. (A,B) Absolute mortalities. (A',B') Mortality differences between exposed and unexposed flies. Gray and purple bars indicate unexposed flies and flies exposed to hyperoxic isoflurane, respectively. Each dot represents a vial with 15–35 flies (A,A') or 5–20 flies (B,B'). Error bars are \pm standard error of the mean (SEM). ns: not significant, *: $p < 0.05$, **: $p < 0.01$, ****: $p < 0.0001$.

To test the allelic specificity of the toxicity phenotype, we combined a deficiency allele of *ND-23* (*ND-23*^{def}) with *ND-SGDH*^{G6447}. In this experiment, flies were exposed at a younger age, 24–30 days old, because *ND-23*^{def}/*ND-SGDH*^{G6447} flies had low viability at 30–36 days old. Hyperoxic isoflurane did not significantly increase the mortality of Canton S, *ND-23*^{def}/+, or *ND-23*^{def}/*ND-SGDH*^{G6447} flies, but there was a trend toward an increase for all three genotypes (Figure 1B). Isolating the isoflurane-induced mortality reflected this result (Figure 1B'). These data indicate that introducing a stronger allele of *ND-23* may uncover comorbidities that ultimately decrease the resolution of this acute toxicity phenotype.

3.2. Compound Heterozygous Mutations in the *SdhB* Subunit of Complex II Cause Isoflurane-Induced Toxicity in Older Flies

We previously found that 1–6, 8–13, 15–20, and 22–27-day-old flies homozygous for *SdhB*^{c00364} (*Succinate dehydrogenase, subunit B*), which encodes a Complex II subunit, showed no toxicity to hyperoxic isoflurane [9]. To determine if toxicity is conferred by other alleles, we tested *SdhB*^{EY12081} as a heterozygote (*SdhB*^{EY12081}/*CyO*) and as a compound heterozygote with *SdhB*^{c00364} (*SdhB*^{EY12081}/*SdhB*^{c00364}). Exposure of Canton S and *SdhB*^{EY12081}/*CyO*

flies to hyperoxic isoflurane at 1–6, 8–13 and 15–20 days old did not increase 24 h mortality over unexposed flies (Figure 2). However, exposure of 29–34 day old *SdhB^{EY12081}/SdhB^{c0036}* flies significantly increased mortality ($p = 0.0007$), whereas mortality was not affected for Canton S flies ($p = 0.57$) (Figure 3). These data indicate that aging sensitizes compound heterozygous *SdhB^{EY12081}/SdhB^{c0036}* flies but not heterozygous *SdhB^{c0036}* flies to isoflurane-induced toxicity.

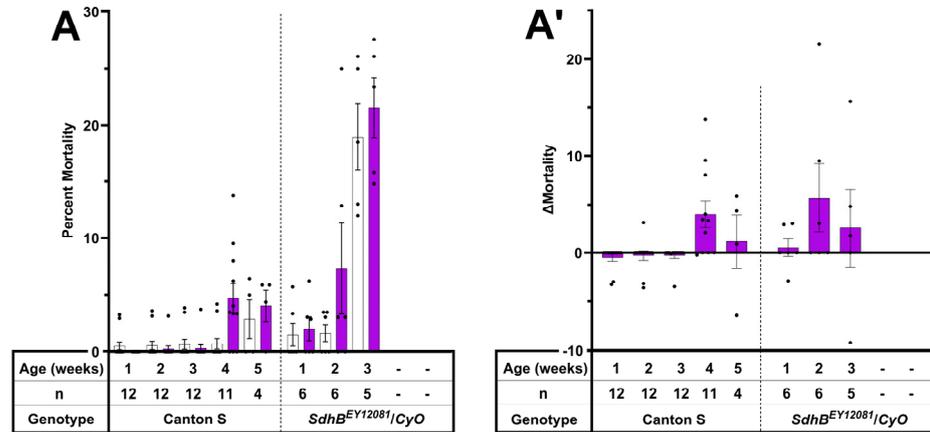


Figure 2. Flies heterozygous for *SdhB^{EY12081}* are not susceptible to isoflurane-induced mortality. Percent mortality was determined 24 h after exposure of the indicated flies to hyperoxic isoflurane. (A) Absolute mortalities. (A') Mortality differences between exposed and unexposed flies. White and purple bars indicate unexposed flies and flies exposed to hyperoxic isoflurane, respectively. Each dot represents a vial with 15–35 flies. Error bars are \pm SEM. Week 1 = 1–6 days old; Week 2 = 8–13 days old; Week 3 = 15–20 days old; Week 4 = 22–27 days old; Week 5 = 29–34 days old. No comparison was statistically significant ($p < 0.05$).

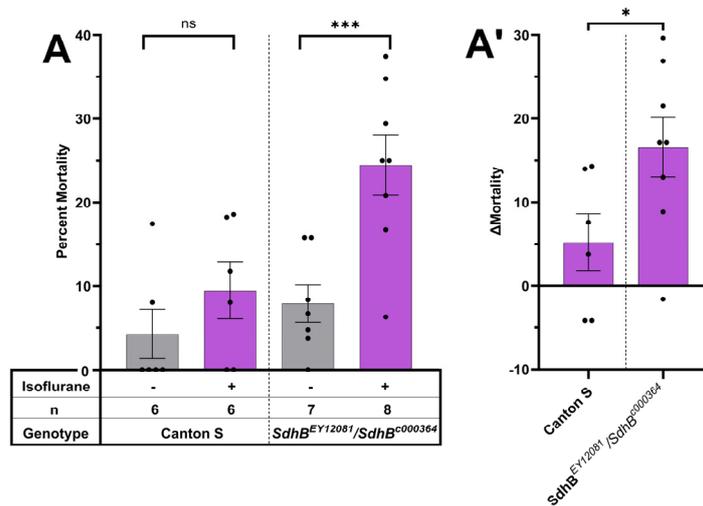


Figure 3. Compound heterozygosity for *SdhB* mutations increases isoflurane-induced mortality. Percent mortality was determined 24 h after exposure of the indicated flies to hyperoxic isoflurane. (A) Absolute mortalities. (A') Mortality differences between exposed and unexposed flies. Gray and purple bars indicate unexposed flies and flies exposed to hyperoxic isoflurane, respectively. Each dot represents a vial with 15–35 flies. Error bars are \pm SEM. ns: not significant, *: $p < 0.05$, ***: $p < 0.001$.

3.3. Mutations in the *SdhC* subunit of Complex II (*mev-1*) Sensitizes *C. elegans* to Isoflurane-Induced Toxicity

We investigated the organism specificity of the anesthetic toxicity phenotype using the nematode *C. elegans*. A missense mutation in *mev-1*, orthologous to the *SdhC* subunit

of Complex II in humans, has been shown to cause hypersensitivity to oxidative stress and premature aging [11]. We exposed wild-type N2 and mutant *mev-1(kn1)* worms to 6.5% isoflurane in room air (21% O₂) on the first day of adulthood. Mortality was scored 24 h following exposure. *mev-1(kn1)* mutants experienced a significant increase in mortality following exposure to isoflurane relative to unexposed ($p < 0.0001$), whereas N2 worms did not ($p > 0.99$) (Figure 4). These data indicate that mutations in Complex II can confer sensitivity to isoflurane-induced toxicity across phyla.

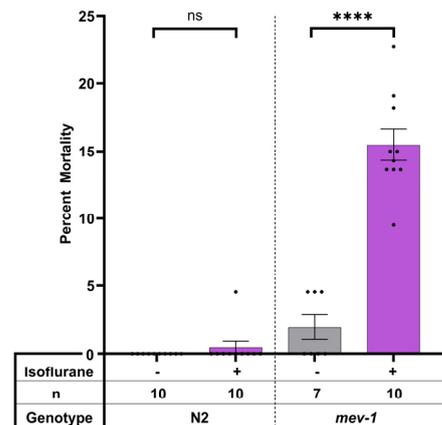


Figure 4. In *C. elegans*, a missense mutation in *SdhC mev-1(kn1)* results in an isoflurane-induced mortality phenotype. Animals were exposed on the first day of adulthood (DOL 3 for N2, DOL 4 for *mev-1*) to their isoflurane EC₅₀ for immobilization (6.5%) for 3 h on agar plates. All animals were grown, and all experiments were carried out at 20 °C. Percent mortality was determined 24 h after exposure of the indicated worms to isoflurane in room air (normoxic). Gray and purple bars indicate unexposed and exposed nematodes, respectively. Each dot represents an agar plate with 20–22 nematodes. Error bars are +/– SEM. ns: not significant, ****: $p < 0.0001$.

4. Discussion

Data presented here show that, following exposure to hyperoxic isoflurane, older flies carrying heterozygous mutations in two Complex I subunits, *ND-23* and *ND-SGDH*, had a level of mortality that was higher than the added mortalities of the individual heterozygous mutations. In addition, the data show that older flies compound heterozygous for two different alleles of the Complex II gene *SdhB* were susceptible to hyperoxic isoflurane-induced mortality. Thus, in older individuals, sensitization to anesthetic toxicity is not limited to homozygous mutations in Complex I genes.

We are not aware of experimental data directly comparable to ours. The suppressive effect of VGAs on oxidative phosphorylation was discovered in isolated mitochondrial preparations where the Complex I function was found to be particularly sensitive [12–14]. Behavioral experiments in *Caenorhabditis elegans* carrying homozygous mutations in single subunits of Complex I (*gas-1* analogous to *Nudfs-2*) but not in Complex II (*mev-1*, analogous to *SdhC*) agreed and assigned Complex I the role of the primary mediator of the behavioral endpoints of the VGAs halothane and isoflurane [4,15]. Furthermore, an observational study in young children also documented hypersensitivity of the central nervous system to the VGA sevoflurane only in carriers of Complex I mutations [2]. To provide context for our findings with data on behavioral sensitivity, it is important to compare the different models. In contrast to other in vivo studies, we used (i) anesthetic-induced mortality (i.e., toxicity) assessed at 24 h after exposure as an endpoint. Experiments testing behavioral sensitivity showed that Complex I mutants were hypersensitive to the VGAs sevoflurane and isoflurane [6] in accordance with behavioral findings in other models; (ii) aged flies as opposed to neonates typically used for experiments addressing toxicity; (iii) isoflurane in 75% O₂ as opposed to air; (iv) heterozygous mutations simultaneously present in different subunits of Complex I; and (v) compound mutations in the *SdhB* subunit of Complex II.

Therefore, our data represent a “proof of principle” that combinations of alleles in different subunits of mitochondrial Complexes I can act more than additively (Figure 1) to result in a phenotype of susceptibility to anesthetic toxicity in aged individuals. Furthermore, the results suggest that gene dose matters for its penetrance (Figure 3). This information is important because mutations in more than one gene coding for a subunit of the mETC are present in approximately half of all patients diagnosed with mitochondrial disease [16], but pharmacogenetic factors are rarely, if at all, considered to contribute to anesthetic complications in adult and aged individuals.

Complex II mutations are rare, even though, ironically, the *SdhA* subunit was the site of the first reported mutation causing a defect in the oxidative phosphorylation [17]. *SdhA* mutations preferentially present with an LS-like phenotype, while *SdhB* and *SdhC* mutations present with malignancies in adulthood, necessitating surgical intervention and, hence, exposure to anesthesia. To date, only 61 patients have been reported in the literature, harboring a total of 32 pathogenic variants in the Complex II genes [18]. This rarity could be due to the non-viability of most homozygous carriers of mutations in Complex II, possibly because of the unique role of Complex II as the only direct link between the Krebs cycle (located in the mitochondrial matrix) and oxidative phosphorylation (located at the inner mitochondrial membrane). Therefore, substantial functional disruption of *SdhB* via homozygosity of deleterious mutations will likely result in non-viability. In contrast, as all subunits of Complex II are nuclear encoded, minor alleles and compound mutations in the general population, when present, are likely to be asymptomatic.

We examined compound mutants of the *SdhB* subunit. Our findings that *SdhB*^{EY12081}/*SdhB*^{c00364} flies showed isoflurane-induced toxicity, but *SdhB*^{c00364}/*SdhB*^{c00364} did not, indicates that *SdhB*^{c00364} is a functionally weaker allele than *SdhB*^{EY12081}. This conclusion is supported by the fact that *SdhB*^{c00364}/*SdhB*^{c00364} flies are homozygous viable, but *SdhB*^{EY12081}/*SdhB*^{EY12081} flies are homozygous lethal. Furthermore, the fact that *SdhB*^{EY12081} and *SdhB*^{c00364} have similar mutations (i.e., transposons inserted near the 5' end of the gene, located 40 base pairs apart in opposite orientations) suggests that mutations compatible with survival into adulthood rarely meet the criteria of impairing Complex II function sufficiently to present an anesthetic toxicity phenotype. It is also compatible with the lack of a behavioral anesthetic phenotype in the *SdhC* subunit mutant *mev-1* [15]. Notably, we found that *mev-1* carries a distinct anesthetic toxicity phenotype even under normoxic conditions (*mev-1* was isolated as a strain hypersensitive to O₂ [19]) when assessed in the 24 h mortality assay (Figure 4), thereby mimicking the dissociation between behavioral sensitivity to and toxicity of the VGAs sevoflurane and isoflurane described previously [6,7].

It is tempting to speculate whether the simultaneous presence of two minor alleles—one in Complex I and the other in Complex II—would present with an anesthetic toxicity phenotype. We are not aware of experimental data addressing this issue, but clinical data can provide a basis for speculation. Generally, compound heterozygosity may lower the penetrance compared to homozygous mutants of one of the alleles. This is the likely cause for the viability of and lack of anesthetic-induced mortality in one of the *SdhB* alleles tested. Some compound heterozygous combinations may provide an evolutionary advantage under certain environmental conditions, as exemplified by beta-hemoglobinopathies [20]. However, at least one reported case of the Complex II compound heterozygosity affecting the *SdhA* subunit presented clinically with LS [21], which is the common clinical presentation of homozygous *SdhA* mutants. It is noteworthy that the *SdhA* subunit is the only Complex II subunit in which dominant pathogenic variants have been identified [18]. We have not yet tested *SdhA* subunits. However, our established experimental system would be a convenient platform to this end.

5. Limitations

SdhB heterozygous and compound heterozygous mutant flies were tested at different ages. Therefore, toxicity could be due to age or compound heterozygosity for *SdhB* mutations. Hyperoxia and isoflurane were not independently tested, so toxicity may be due to

hyperoxia, isoflurane, or a combination of the two. Flies heterozygous for Complex I and II mutations simultaneously were not tested; thus, it is not known if toxicity is limited only to mutations in two subunits of the same complex. Only one allelic combination each for Complex I and Complex II exhibited a significant increase in anesthetic-induced mortality, so sensitization to toxic effects due to these combinations may be allele-specific.

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