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Anaesthetics disrupt complex I-linked respiration and reverse the ATP synthase

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ABSTRACT

The mechanism of volatile general anaesthetics has long been a mystery. Anaesthetics have no structural motifs in common, beyond lipid solubility, yet all exert a similar effect. The fact that the inert gas xenon is an anaesthetic suggests their common mechanism might relate to physical rather than chemical properties. Electron transfer through chiral proteins can induce spin polarization. Recent work suggests that anaesthetics dissipate spin polarization during electron transfer to oxygen, slowing respiration. Here we show that the volatile anaesthetics isoflurane and sevoflurane specifically disrupt complex I-linked respiration in the thoraces of *Drosophila melanogaster*, with less effect on maximal respiration. Suppression of complex I-linked respiration was greatest with isoflurane. Using high-resolution tissue fluorespirometry, we show that these anaesthetics simultaneously increase mitochondrial membrane potential, implying reversal of the ATP synthase. Inhibition of ATP synthase with oligomycin prevented respiration and increased membrane potential back to the maximal (LEAK state) potential. Magnesium-green fluorescence predicted a collapse in ATP availability following a single anaesthetic dose, consistent with ATP hydrolysis through reversal of the ATP synthase. Raised membrane potential corresponded to a rise in ROS flux, especially with isoflurane. Anaesthetic doses causing respiratory suppression were in the same range as those that induce anaesthesia, although we could not establish tissue concentrations. Our findings show that anaesthetics suppress complex I-linked respiration with concerted downstream effects. But we cannot explain why only mutations in complex I, and not elsewhere in the electron-transfer system, confer hypersensitivity to anaesthetics.

1. Introduction

How do general anaesthetics work? Listed as one of the biggest unanswered questions in *Science* in 2005 [1], the answer remains mysterious. In terms of molecular structure, the only property that volatile general anaesthetics (GAs) have in common is lipid solubility – the Meyer-Overton rule observes that the potency of general anaesthetics can be predicted by their solubility in olive oil alone [2]. Yet the idea that anaesthetics affect membrane structure fails to explain why some lipid-soluble molecules with very similar structures do not cause anaesthesia, but act as convulsants instead [3,4]. The question is of pressing concern, as some of the safest anaesthetics contain halogens, which cause environmental damage [5]. Other clinically safe volatile GAs are flammable, posing a more immediate risk [6]. Those remaining are less safe, which is problematic in ageing populations, as even the safest volatile GAs can be hazardous for elderly patients [7,8]. Understanding how anaesthetics work will be critical to develop new approaches, while offering an invaluable experimental window into the basis of consciousness itself [9].

One of the most exciting recent avenues into the mechanism of volatile GAs comes from the work of Luca Turin and colleagues, who point to the efficacy of the inert gas xenon as a way of gaining traction on the question [10]. Xenon has no chemistry under relevant conditions in the brain, and it has no shape that could target any specific receptor – it is a perfect sphere of electron density. But it does have physics. Using electron spin resonance (ESR), Turin and colleagues have shown that xenon and other anaesthetics affect the spin signal deriving from O_2 in cell respiration in *Drosophila melanogaster* [10,11]. Anaesthetic-resistant mutants show an altered spin signal [10,11], which is largely abolished by cyanide [11], an inhibitor of electron transfer to O_2 in complex IV of

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the electron transfer system. These findings do not establish causality, but they strongly suggest that anaesthetics disrupt electron transfer in respiration. Recent work shows that volatile GAs interfere with electron spin directly [12]. Electron transfer through chiral molecules polarizes their spin, a process known as chiral-induced spin selectivity (CISS) [13]. Because proteins are composed of chiral amino acids, electron transfer through respiratory proteins also polarizes their spin. Volatile GAs seem to disrupt this respiratory electron spin polarization [12], which could slow down respiration because oxygen itself is a biradical, so it can only accept electrons in the same spin state.

But this beautifully simple picture is inconsistent with the details of electron transfer through respiratory complexes in respiration. There is an extensive literature on the effects of volatile GAs on respiration, mostly from the labs of Philip Morgan and Margaret Sedensky, who have demonstrated in a series of beautiful papers over three decades that complex I is a specific target of volatile GAs [14–19]. In several animal models, including mice [20–24], *Drosophila* [25] and *C. elegans* [26–31], mitochondrial mutations can cause hypersensitivity to volatile GAs. Patients with mitochondrial diseases are often hypersensitive to anaesthetics [32], and indeed prone to epileptic convulsions [33]. We compile these multiple observations into Table 1, and the pattern that emerges is striking. The puzzle is that *all* mitochondrial mutations that cause hypersensitivity to volatile GAs are in complex I of the electron transfer system. Mutations in other respiratory complexes are not known to

Table 1
Summary of the literature on the effect of mutations in respiratory complexes on the sensitivity to five volatile GAs. Blue denotes hypersensitivity to anaesthetics; green denotes no effect. There are no reported cases of resistance to volatile GAs caused by mitochondrial mutations (but see text).

Complex	Specific mutation	Anaesthetic				
Complex I		**Sevoflurane**	**isoflurane**	**halothane**	**enflurane**	**diethyl ether**
	Leigh's disease	[14]				
	Complex I in humans	[14,32]				
	Decreased pyruvate oxidation in humans	[14]				
	Decreased glutamate oxidation humans	[14]				
	Ndufs4(-/-) mouse mutants		[15,20–24]	[20–22]		
	Ndufs4 KO mice – specifically in GABAergic neurons		[18]	[18]		
	Ndufs4 KO mice – specifically cholinergic neurons		[18]	[18]		
	Ndufs4 KO mice – VGLUT2-+ve glutamatergic neuron		[18]	[18]		
	Ndufs4 KD mice – parietal association cortex region		[22]	[22]		
	Ndufs4 KD mice – central medial thalamus region		[22]	[22]		
	Ndufs4 KD mice – dorsal medial thalamus region		[22]	[22]		
	Ndufs4 KD mice – mesopontine tegmental region		[22] (NS)	[22] (NS)		
	gas-1 mutant in *C. elegans*		[19,26,27,29,30]	[16,26,28,30,31]	[26,27]	[26,27]
	nuo-1 mutant in *C. elegans*			[31]		
	seg-1;gas-1 in *C. elegans*		[27]	[27,31]	[27]	[27]
	seg-2;gas-1 in *C. elegans*		[27]	[27,31]	[27]	[27]
	mt:ND2^{del1} in *Drosophila*			[25]		
	ND-23^{60114} in *Drosophila*	[25,37]	[25,37]	[25]		
	ND-^{SGDHG6447} in *Drosophila*		[25]			
Ubiquinone	*clk-1* in *C. elegans* - Disrupts synthesis			[27,31]		
Complex II	Complex II in humans	[32]				
	mev-1 in *C. elegans*		[30]	[27,30,31]		
	SdhB^{c00364} in *Drosophila*		[25]			
Complex III	Complex III in humans	[32]				
	Complex III in *C. elegans*		[29]			
	isp-1 in *C. elegans*			[31]		
	UQCR-Q^{KG08923} in *Drosophila*		[25]			
Complex IV	Complex IV in humans	[32]				
	Levy^{EY01834} in *Drosophila*		[25]			
ATPase	*ATPsynB*^{EY03793} in *Drosophila*		[25]			

cause hypersensitivity to volatile GAs.

The paradox is as follows. If electrons are indeed polarized in spin as they are transferred through complex I, then this polarization must be lost upon their transfer to ubiquinone, as ubiquinol contains no unpaired electrons. If spin polarization of electrons in transit to oxygen is critical, then it would need to be reestablished in the Q cycle in complex III, or in electron transfer through the haem centres in complex IV. If that were the case, then mutations in complex III or complex IV should cause greater hypersensitivity to volatile GAs than those in complex I. They do not (Table 1). Likewise, if anaesthesia were induced by suppression of respiration in tissues with high aerobic demands, notably the brain, then respiratory inhibitors such as antimycin A should also act as anaesthetics. So should inhibitors of ATP synthase, and mutations in its genes, or respiratory uncouplers. We are not aware of any evidence for these effects. It is feasible that complexes II, III, IV and the ATP synthase are less sensitive to volatile GAs than complex I (arguably the rate-limiting gatekeeper of respiratory flux), but mutations in other complexes frequently cause mitochondrial diseases through respiratory disruption [34,35], so lowered sensitivity seems an unsatisfactory explanation.

Here we use tissue-specific high-resolution fluorespirometry (Oroboros O2k) to explore the effect of two volatile GAs, isoflurane and sevoflurane, on various respiratory parameters in permeabilised thoraces of *Drosophila melanogaster*. We confirm that these anaesthetics do indeed specifically suppress respiration with NADH-linked substrates, which feed electrons into complex I. The effect on maximal respiration is weaker, and can be explained entirely by the effect on complex I. Even with very high doses of anaesthetics, we find no anaesthetic effects on any of the other respiratory complexes. We show that the suppression of complex I-linked respiration leads to a counter-intuitive increase in mitochondrial membrane potential, probably produced by a reversal of the ATP synthase. That in turn collapses ATP availability and increases ROS flux, which may help explain the greater risk of oxidative damage in older people [36]. By using simultaneous measurements of multiple respiratory parameters, we show that anaesthetics have concerted effects on respiration. Our results corroborate the idea that anaesthesia is induced at least in part by falling ATP availability [17]. Intriguingly, our findings support the hypothesis that anaesthetics may disrupt electron spin polarization in complex I. The insensitivity of other respiratory complexes to volatile GAs, even at very high doses, combined with the fact that no other mutations, inhibitors or uncouplers affect anaesthetic sensitivity, all point to a specific interaction between volatile GAs with electron transfer in complex I as a primary cause of anaesthesia.

2. Materials and methods

2.1. D. melanogaster maintenance and strains

Wild-type LH_M strain of *D. melanogaster* were used. Female flies under 20 days old were used for all O2k fluorespirometry measurements and dose-response behavioural experiments.

The fly stock was maintained on a standard mix of molasses/cornmeal agar medium, with a 12:12 h light-dark cycle. The incubating room was kept at a constant temperature of 25 °C and relative humidity of 50 %. Lines were propagated using newly emerged parental flies. Live yeast was added to boost female fecundity. Egg density was maintained at ~80–100 per vial.

2.2. GA preparation and application

Two volatile general anaesthetics were explored, isoflurane ($C_3H_2ClF_5O$, Cayman, 26,675–46-7) and sevoflurane ($C_4H_3F_7O$, Cayman, 28,523–86-6). GAs are not soluble in water but miscible in ethanol, DMSO and organic oil. We diluted isoflurane and sevoflurane in ethanol for preliminary titrations. Pure ethanol was used as the control, as it is a common solvent for various O2k uncouplers and inhibitors. Volatile GAs evaporate easily, so their preparation required fast handling. Fresh aliquots were made on each day of use.

We chose an initial anaesthetic dose of 400 μM based on earlier fluorespirometry work [38]. This dose was also similar to that used by Federov et al. [39], which was published after we established our own concentration ranges. The stock (8.184 mol/L for isoflurane and 7.608 mol/L for sevoflurane) was diluted in pure ethanol with a ratio of 1:10, resulting in 0.8184 mol/L and 0.7608 mol/L of titration solutions for isoflurane and sevoflurane, respectively. During each titration step, 1 μL of the prepared titration solution was added into 2 mL O2k chambers containing 2 thoraces, making up final concentrations of 409.2 μM for isoflurane and 380.4 μM for sevoflurane. In pure anaesthetic titrations, a 10 μL Hamilton microsyringe was used to titrate aliquots of 0.1 μL isoflurane or sevoflurane (as neat oils supplied by the manufacturers) to make up final concentrations of 409.2 μM and 380.4 μM, respectively, in the 2 mL O2k chamber.

2.3. High-resolution tissue fluorespirometry

Two high-resolution O2k fluorespirometers (Oroboros Instruments, Innsbruck, Austria) were used to analyse mitochondrial respiratory parameters, including O_2 consumption, membrane potential, ATP synthesis and ROS flux. The instrument allows simultaneous recording of two respiratory parameters in various states. Female LH_M thoraces (permeabilised) or brains were examined at 25 °C. Preparation of permeabilised fibres (pfi) followed our established protocol [40]. We used female flies to reduce variance (compared with using both males and females), because they are larger (restricting the pooling of brains or thoraces) and because respiration in female flies is more dependent on complex I [41].

2.3.1. Fly thorax preparation

For thorax pfi preparation, female flies were dissected on ice, while soaked in a minimum volume of tissue preservation solution (BIOPS, 2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2$, 20 mM taurine, 15 mM Na_2 phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol, and 50 mM K-MES, pH 7.1). Dissection of thoraces involved removal of the head, abdomen, wings and legs. Tweezer tips were used to slightly open up the cuticle before transferring the tissue into a 24-well plate, in which each well contained 2 mL BIOPS and 81.25 $\mu g.mL^{-1}$ saponin (5 $mg.mL^{-1}$ stock solution in BIOPS). The plate was kept on ice and shaken for 20 min, at a speed of 300 rpm on a plate shaker. Tissues were then transferred to another well containing 2 mL MiR05 respiratory buffer (0.5 mM EGTA, 3 mM $MgCl_2.6H_2O$, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM D-sucrose, and 1 g/L BSA, pH 7.1) for a 5 min rinsing. The pfi were then weighed and transferred to O2k-chambers filled with 2 mL MiR05.

2.3.2. Fly brain preparation

For brain preparation, no permeabilization was needed. After removal of heads, the exoskeleton was pulled apart from the middle of the head using 5 mm tweezers. A neat dissection allowed the intact brain to fall out upon opening the exoskeleton. Care was taken to avoid damaging the eyes, as the red pigment complicates the dissection and interferes with fluorespirometry dyes. Each dissected brain was transferred into a 1.5 mL Eppendorf tube containing 50 μL MiR05. A minimum of 20 brains were added into O2k-chambers.

2.3.3. O2k protocol development for anaesthetic titration

A substrate-uncoupler-inhibitor-titration (SUIT) protocol was used to study the effects of anaesthetics on different respiratory states [40]. Anaesthetics were titrated during N-LEAK state, N-OXPHOS state (complex I-linked respiration) and NProSGp_P state (maximal respiration). For N-LEAK state (LEAK state), the NADH-pathway was primed by addition of NADH-linked substrates, pyruvate (10 mM) and malate

(2 mM). For N-OXPHOS state (complex I-linked respiration) ADP (5 mM) was added after 10 min of signal stabilisation in the N-LEAK state, to initiate the N-OXPHOS state (N_P), after which anaesthetics were titrated to the chamber. For NProSGp_P state (maximal respiration), proline (10 mM), succinate (10 mM), and glycerol phosphate (10 mM) were sequentially added after stabilizing N-OXPHOS state, to induce maximum coupled respiration. Reoxygenation was avoided by adjusting the number of thoraces used when necessary. As anaesthetics are volatile gases that evaporate easily, the system was kept air-tight to limit gas escape. The chamber was sealed except for needle injections, with minimal gas phase in the capillary of the chamber stopper, through which the syringe is inserted at each titration. The 2 mL volume of the chamber was entirely liquid except for the tissue.

Quality control through the cytochrome *c* test for outer mitochondrial membrane integrity could not be performed for all samples, as most membrane potential dyes interfere with cytochrome *c* [40]. Instead, we performed preliminary cytochrome *c* quality control tests on thorax pfi (10 μM cytochrome *c* during N-OXPHOS state) and obtained standard oxygen consumption values for undamaged samples. Experiments that failed to meet standard respiration values were discarded as damaged. Oxygen consumption was recorded by the oxygen electrode, and membrane potential, ATP synthesis and ROS flux were measured using the fluorescence module in parallel. GA titrations on brains were shown to behave in the same way as thoraces in O_2 flux and membrane potential responses (SI Fig. 1). For quicker sample preparation, most investigations were conducted using pfi thoraces.

2.3.4. O_2 flux control ratio (FCR)

The FCR was calculated as: FCR = titration step/N_P, where titration step corresponds to oxygen flux after anaesthetic titration, and N_P is maximum flux in that respiratory state. The FCR values provide an internal normalisation and indicate the lower and upper flux limits between 0.0 and 1.0. FCR values were corrected for drift as in preliminary titrations (diluted in ethanol) and pure anaesthetic titrations, a downward drift in respiratory rate was seen if the system was left running at maximum flux to run for >40 min. Median drift values were recorded at 10 min intervals, equivalent to each titration step.

2.3.5. Measurement of membrane potential

We used tetramethylrhodamine (TMRM) fluorescence to measure membrane potential (excitation 552 nm; emission at 574 nm). Higher concentrations of TMRM inhibit respiration [40], so the optimum concentration used for the study was determined as 0.5 μM [40]. At 0.5 μM, TMRM is in quench mode, meaning the fluorescence signal is inversely proportional to membrane potential [42].

Membrane potential measurements were analysed and corrected for baseline values to give relative fluorescence value. TMRM fluorescence values were normalised to complex I-linked or maximal respiration. Reciprocals of relative fluorescence were calculated to estimate the relative membrane potential. Drift correction was applied to membrane potential values for pure anaesthetic titration groups. Correcting for drift in the preliminary ethanol titrations gave a misleading visual impression, so we present raw data for these groups in SI Fig. 3. This method of membrane potential calculation was done for all datasets except oligomycin runs, where relative fluorescence collapsed to nearly zero following oligomycin titration. Here we took the reciprocal of raw TMRM fluorescence first (as reciprocals of 0 are meaningless) and normalised to that for upper and lower limits. This method retains the qualitative trends, but scales down the calculated values of relative membrane potential.

2.3.6. Measurement of ATP synthesis

For ATP synthesis measurements, we used magnesium green (MgG) fluorescence (excitation 505 nm; emission 531 nm) [43,44]. During calibration, MgG (1.1 μM) and $MgCl_2$ (1 mM) were injected to a special MiR05 without $MgCl_2$. MgG fluoresces when bound to Mg^{2+}. While both ADP and ATP bind to Mg^{2+}, decreasing MgG fluorescence, they have distinct binding affinities and dissociation constants (*Kd*) for Mg^{2+}. A decreasing MgG signal represents a decreasing ADP:ATP ratio caused by an increasing ATP synthesis, and *vice versa*. ATP synthesis and hydrolysis were calculated from the slope of signal change over time with predetermined *Kd* for ATP and ADP (manufacturer-supplied calculations). MgG fluorescence values were exported for individual timepoints; the values at specific titration events were analysed to determine ATP concentration.

2.3.7. Measurement of ROS flux

For H_2O_2 analysis, 15 μM DTPA, 5 U/mL superoxide dismutase (SOD), 1 unit horseradish peroxidase (HRP) and 10 μM Amplex UltraRed (AmR) were injected sequentially in the chamber before the samples were inserted. The AmR reaction with H_2O_2 is catalysed by HRP producing a red fluorescent compound with excitation and emission wavelengths of 563 and 587 nm, respectively, which is measured using the AmR sensor on the O2k. The changes in fluorescence intensity are directly proportional to the H_2O_2 produced [41,45].

2.4. Dose-response investigations

2.4.1. GA dose-response in O2k

For isoflurane dose-response titrations, O_2 flux and membrane potential were measured and compared between concentrations over five orders of magnitude. Five experimental groups were prepared, with concentrations ranging from 0.04 μM to 400 μM. Ethanol dilution was required to prepare doses lower than 400 μM. Titrations were done during complex I-linked respiration. Each titration corresponded to a final concentration of 0.04 μM to 400 μM in the 2 mL chamber. Five titration steps in 400 μM group therefore equated to final concentrations of 400 μM, 800 μM, 1200 μM, 1600 μM and 2000 μM.

2.4.2. Whole fly dose-response behavioural assay

Female flies were knocked down using CO_2 and transferred to 2 mL HPLC glass vials individually. They were left to recover for at least 30 min to avoid any effect of CO_2 on the response to GAs. The HPLC vials contained cotton pads fitted at the bottom to absorb moisture, the caps keeping the vials air-tight while allowing injection of anaesthetics using Hamilton syringes. Various concentrations of anaesthetic were used, from 0.04 μM to 400 μM, and the time it took for different states was timed following volatile GA injection. We recorded time to first fall from the wall; time to complete loss of movement (including loss of leg twitching); and time to climb back on the wall (recovery). Full anaesthesia was confirmed by tapping or inverting the vial.

2.5. Data extraction and statistical analysis

All fluorespirometry data were exported from DatLab 7.4 software, raw plots were saved and the metadata were processed using the manufacturer's data calculation templates, analysed by correcting by pfi mass (thorax) or unit (brain; pmol $O_2.s^{-1}.brain^{-1}$). Data were analysed in R (2022.12.0 + 353). The upper limit of respiratory rates (N-OXPHOS state, NProSGp_P state) and anaesthetic titrations were modelled as fixed effects; the FCRs and reciprocal of fluorescence signals were response variables. Unpaired *t*-test was used to analyse the statistical differences between each anaesthetic titration and the upper limit respiratory rates (control). For the behavioural states, anaesthetic treatments were modelled as fixed effects, and time taken to reach specific endpoint was the response variable. An unpaired t-test was used to analyse the differences in time taken between different treatments. O2k dose-response IC_{50} curve was generated using the online tool AAT Bioquest.

3. Results and discussion

3.1. Anaesthetics suppress complex I-linked respiration and increase membrane potential

Our first experiments examined the brains rather than thoraces of *Drosophila melanogaster*. These experiments required fine dissections with usually 20 freshly dissected brains in the chamber to achieve a suitable signal on the Oroboros O2k. In these first experiments, the anaesthetics were administered in ethanol at doses of 400 μM to fly brains. An example is shown in SI Fig. 1. We found that the responses to anaesthetics in fly brains were qualitatively similar, but much weaker, than comparable runs in the thoraces, which only required two dissected thoraces per run. We therefore switched to thoraces for the bulk of the work presented here. The thorax not only houses *Drosophila* flight muscle, but also contains the large thoracic ganglia, so the main results presented are on a composite neuromuscular tissue. We also added anaesthetic doses at various stages of the standard O2k runs, including

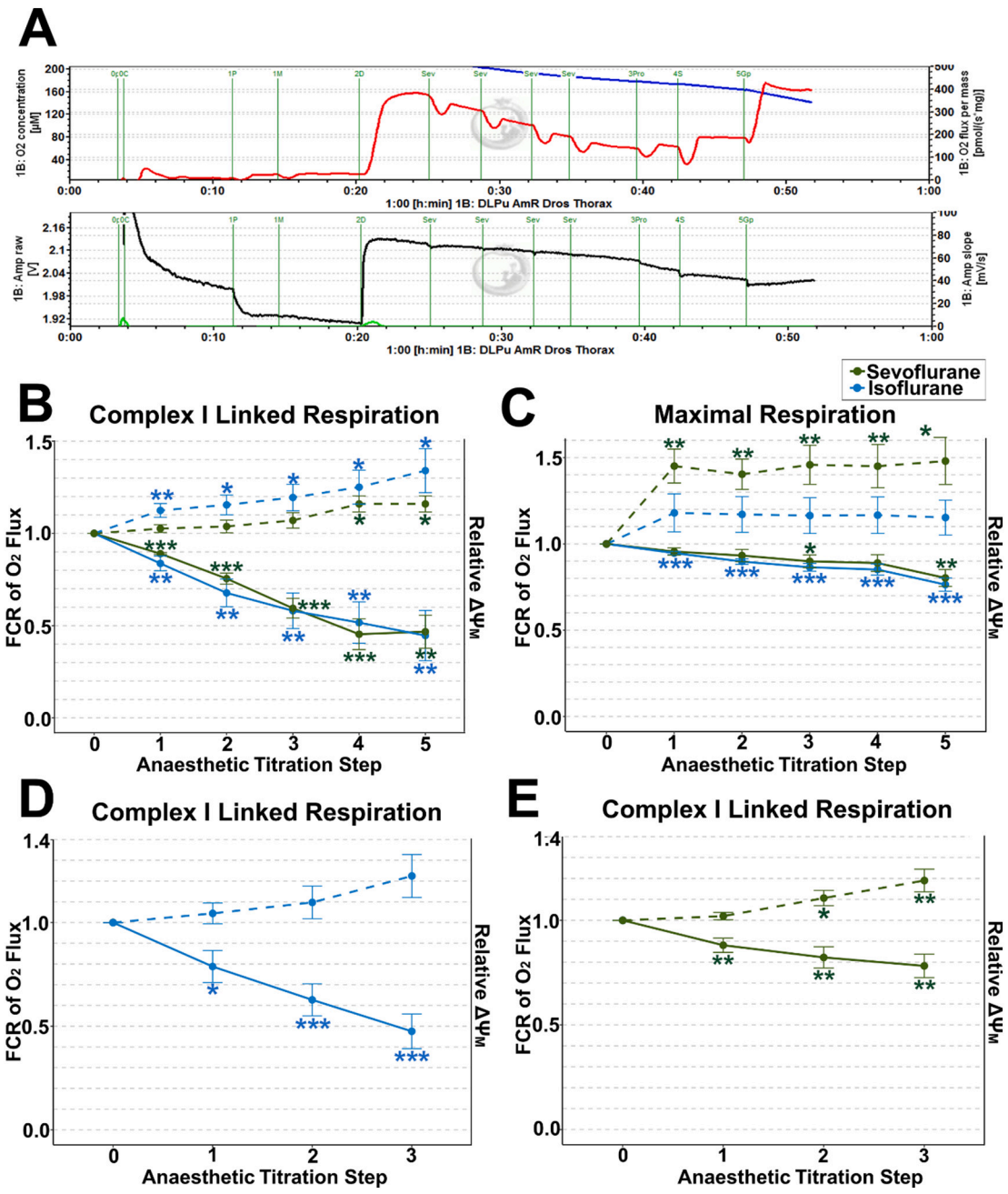


Fig. 1. Effect of isoflurane and sevoflurane on oxygen flux and membrane potential. (A) Example traces showing changes in oxygen flux (top) and TMRM fluorescent signals (bottom) induced by sevoflurane titration during complex I-linked respiration. 1P and 1 M refer to additions of pyruvate and malate, respectively, giving LEAK state respiration. 2D refers to addition of ADP to initiate complex I-linked respiration. Sev indicates sevoflurane titrations (400 μM per dose). 3Pro, 4S, 5Gp indicate additions of proline, succinate and glycerol phosphate, respectively. (B) Isoflurane (blue, $N = 6$) and sevoflurane (green, $N = 4$) titrations in complex I-linked respiration; both anaesthetics administered in ethanol. (C) Isoflurane ($N = 9$) and sevoflurane ($N = 5$) titrations in maximal coupled respiration; both anaesthetics administered in ethanol. (D) Pure isoflurane titration ($N = 7$) and (E) pure sevoflurane titration ($N = 6$). Solid lines indicate oxygen flux and dotted lines show membrane potential in all panels, with both normalised to complex I-linked respiration. Statistical significance of anaesthetic-titration steps all relative to normalised values at titration step zero. *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in LEAK state (before the addition of ADP), on NADH-linked substrates alone (pyruvate + malate), maximal respiration (pyruvate + malate + proline + succinate + glycerol phosphate), and on noncoupled tissue. Examples of these runs are shown in SI Fig. 2. We eventually settled on comparing the responses to anaesthetic in complex I-linked *versus* maximal respiration.

When respiring on NADH-linked substrates, administration of 400 μM anaesthetic in ethanol (as a final concentration in the chamber) to thoraces produced an immediate inhibition of oxygen flux by around 10–15 %, with similar responses to both isoflurane and sevoflurane (Fig. 1). Repeated dosing of 400 μM titres each gave a similar inhibition of respiration (Fig. 1A). We measured mitochondrial membrane potential simultaneously using TMRM in quenching mode (Fig. 1A). The top trace shows O_2 flux in various respiratory states, illustrating the expected rise upon addition of ADP (2D) and inhibition of O_2 flux on titrating with sevoflurane (Sev). The dips are disturbance from the injections. Addition of proline (3Pro), succinate (4S) and glycerol phosphate (5Gp) all increased respiratory rate as expected. The lower trace shows simultaneous TMRM fluorescence. Surprisingly, suppression of complex I-linked respiration by anaesthetics produced a small decrease in TMRM fluorescence, which in quenching mode corresponds to a small increase in membrane potential (Fig. 1A). The large increase in respiration produced by 5Gp corresponded to a modest fall in membrane potential (increase in TMRM trace), showing that complex III-linked respiration functioned as normal even in the presence of anaesthetics. Repeated doses of each anaesthetic produced a similar fall in oxygen flux, associated with a similar small rise in membrane potential, which began to flatten out by the fifth dose (Fig. 1B). This rise in membrane potential might in principle be caused by a reversal of the ATP synthase, which could also explain the discrepancy between our findings and earlier studies suggesting that anaesthetics collapse mitochondrial membrane potential [46,47]. These studies were not comparable to ours, as they stained tissue rather than applying fluorespirometry, hence did not provide exogenous substrates including ADP. Hydrolysis of ATP to maintain membrane potential could only be sustained for a limited period before ATP is exhausted, which would collapse membrane potential as observed by others [46,47]. Outcomes *in vivo* would be highly dose dependent, and many studies administer very high anaesthetic doses to facilitate measurements. Our findings suggest a possible causal mechanism for the collapse in membrane potential at higher anaesthetic doses that has been observed by others [46,47]: reversal of the ATP synthase hydrolyses ATP, and its eventual depletion collapses membrane potential.

Complex I-linked respiration was ultimately suppressed by ~50 % with each anaesthetic. However, control runs showed that the vehicle, ethanol alone, produced similar decreases in complex I-linked respiration, coupled to equivalent rises in mitochondrial membrane potential (SI Fig. 3). Surprisingly, further controls showed that adding water exerted similar effects, as did allowing respiration to continue over an extended period without adding anything at all, which we refer to as drift (SI Fig. 3). On NADH-linked substrates alone, respiration clearly declines over periods longer than 30 min on O2k runs, and this fall increases membrane potential. While suppression of complex I-linked respiration by anaesthetics was greater than either ethanol or drift, the effect was qualitatively similar (SI Fig. 3).

3.2. Anaesthetics suppress maximal respiration mainly at complex I

The effect of anaesthetics on maximal respiration is shown in Fig. 1C. In this case, the anaesthetics were added after the addition of all standard substrates. Maximal respiration showed less suppression. This had to include suppression of complex I as well. After five doses of anaesthetic, maximal respiration was suppressed by around 30 %. In the fly line used here, the contribution of complex I respiration to maximal respiration was nearly 70 %, which means that 50 % suppression of complex I equates to roughly 30 % suppression of maximal respiration. In other words, the observed suppression of maximal respiration was accounted for in full by the known suppression of complex I, so neither anaesthetic appears to have affected the other respiratory complexes, in agreement with the findings in Table 1. Ethanol alone had smaller effects on maximal respiration (SI Fig. 3). Intriguingly, ethanol had slightly larger effects than drift on complex I-linked respiration, but smaller effects than drift on maximal respiration (SI Fig. 3). While ethanol is reported to have multiple effects on respiratory function [48,49], and can also induce anaesthesia (see below), these effects do not completely overlap with volatile GAs. Clearly, we needed to distinguish between the effects of ethanol and those of volatile GAs in our system.

3.3. Anaesthetic suppression of maximal respiration increases membrane potential

Both anaesthetics increased membrane potential in maximal respiration, by more than their effect on complex I-linked respiration, and with a different dynamic: an immediate increase followed by plateauing. In this case, ethanol had considerably smaller effects than either of the anaesthetics (SI Fig. 3). If the raised membrane potential with anaesthetics is not an artefact (and the strong significance of the shift suggests it is not) then the question is: why would suppression of the complex I contribution to maximal respiration increase membrane potential so much? In the case of respiration on NADH-linked substrates alone (Fig. 1B) the most likely answer is reversal of the ATP synthase, as noted. But for maximal respiration, it is hard to see why a smaller suppression of respiration (equating to a lesser reversal of ATP synthase) would induce a larger increase in membrane potential. One possible explanation relates to redox span. Succinate feeds electrons into complex II, while glycerol phosphate feeds electrons into glycerol phosphate dehydrogenase. These substrates have a redox span to oxygen nearly as great as NADH, but are linked to the pumping of six rather than ten protons (at complex III and complex IV only). That gives more power per proton pumped, enabling them to generate higher membrane potentials (which can drive reverse electron flow into complex I). Suppression of complex I by anaesthetics would increase the contribution of succinate and glycerol phosphate to maximal respiration, which could raise membrane potential. This shift in substrate contribution in principle explains why membrane potential increases sharply before flattening, which implies saturation of substrate contribution. That cannot explain the increase in membrane potential observed with suppression of complex I-linked respiration, as NADH-linked substrates feed into complex I alone. But it is consistent with the effects of both ethanol and drift on membrane potential. Unlike the anaesthetics, neither ethanol nor drift had much effect on membrane potential in maximal respiration. This would be predicted if they did not suppress complex I directly, as substrate contribution would then be relatively unchanged.

3.4. Dosing pure anaesthetics distinguishes between isoflurane and sevoflurane

While this effect would be interesting to explore in future work, we considered it peripheral to the mechanism of anaesthetics, as neither succinate nor glycerol phosphate would normally contribute much to neural metabolism. Nor is succinate a major contributor to respiration in *Drosophila* [40,50]. Instead, we explored the effect of pure anaesthetics (without ethanol) on complex I itself. Addition of pure anaesthetics gave qualitatively similar results to those in ethanol, but now began to distinguish between isoflurane and sevoflurane in terms of their effects on complex I-linked respiration. Pure isoflurane reliably suppressed respiration to a greater extent than sevoflurane, with both anaesthetics producing a similar rise in membrane potential (Fig. 1D and E). It is perhaps surprising that isoflurane did not have a larger effect on membrane potential than sevoflurane, given its greater suppression of respiration, though our sample sizes may have been too small to

distinguish them. In fact, inferring mitochondrial membrane potential accurately is fraught with difficulties, and subject to artefact. TMRM itself can suppress respiration, although the low doses used here should have minimized that. But lower doses are also closer to the threshold for quenching mode fluorescence, which could produce artefacts in mitochondria that had taken up substantially less dye. These questions are beyond the remit of the current study, but worth exploring in future, not least because suppression of respiration has concerted downstream effects. The key point here is that pure titrations of isoflurane and sevoflurane suppress complex I-linked respiration to different levels, and both increase membrane potential. We next asked if this effect was indeed produced by reversal of the ATP synthase.

3.5. Oligomycin response is equivocal – cannot exclude ATP synthase reversal

Fig. 2 shows the effects of anaesthetics on membrane potential, ATP synthesis and ROS flux in complex I-linked respiration, using pure anaesthetics. We inhibited the ATP synthase using oligomycin, which prevents proton flux in either direction. Repeated anaesthetic doses before injection of oligomycin recapitulated our earlier results, with isoflurane again inducing a stronger inhibition of complex I-linked respiration than sevoflurane (Fig. 2A). Oligomycin collapsed respiration, as expected, as electron transfer was no longer possible, presumably the result of high membrane potential. That inference was confirmed by TMRM fluorescence, which showed an abrupt rise in membrane potential, back to the maximal LEAK state potential on addition of oligomycin (Fig. 2B).

This result does not confirm reversal of ATP synthase, but it is consistent with the possibility: electron transfer through complex I must be sufficient to regenerate membrane potential on inhibition of proton flux through ATP synthase, even with anaesthetic suppression. For comparison, IF1 (which inhibits reversal of the ATP synthase) is constitutively expressed [51,52]. Knocking it down tends to lower membrane potential, and *vice versa* [51], so there is heterogeneity between mitochondria and possibly cristae across cells. Because oligomycin inhibits the ATPase in both directions, electron flux through complex I only needs to match proton leak to maintain membrane potential. Complex I flux is greater than proton leak, so membrane potential will rise, albeit not by much, as noted; but anaesthetics only cause a mild suppression of respiration at complex I. In comparison, rotenone (even with 10-fold less than the standard dose used in O2k protocols) completely inhibits complex I, which in turn collapses membrane potential upon addition of oligomycin.

As an aside, our calculation of relative membrane potentials from the reciprocal of TMRM fluorescence differed slightly here from the previous figure, to avoid artefactual amplification from reciprocals of very low (quenched) fluorescence values for maximal membrane potential in LEAK state and after oligomycin inhibition (see Methods). This had the effect of minimising apparent changes in membrane potential upon dosing with anaesthetics, but the qualitative behaviour is exactly equivalent.

3.6. Anaesthetic suppression of complex I-linked respiration reverses ATP synthase

If indeed the ATP synthase does reverse upon dosing with anaesthetics, then magnesium-green fluorescence should reflect a shift in the ATP/ADP ratio towards ADP, meaning that ATP is hydrolysed to drive proton pumping. This is shown quite dramatically in Fig. 2C and D for isoflurane and sevoflurane, respectively. The zero time-point reflects mean ATP synthesis in complex I-linked respiration, and the collapse in ATP synthesis to negative values upon anaesthetic titration indicates reversal of the ATP synthase. ATP hydrolysis was especially strong with isoflurane compared with sevoflurane, which is as predicted based on its larger suppression of oxygen flux. Why the suppression of ATP synthesis is weaker after the third dose of anaesthetic for both isoflurane and sevoflurane is not clear, although in these runs respiratory rate was almost zero after the third anaesthetic dose. We cannot rule out weak interactions between the anaesthetics and dye (see below). Nonetheless, the key findings are clear: anaesthetics exert concerted effects on respiration: suppression of oxygen flux at complex I induces reversal of the ATP synthase, collapsing ATP availability and increasing mitochondrial membrane potential. Similar changes have been suggested before in rat and human synaptosomes using other fluorescence methods [53].

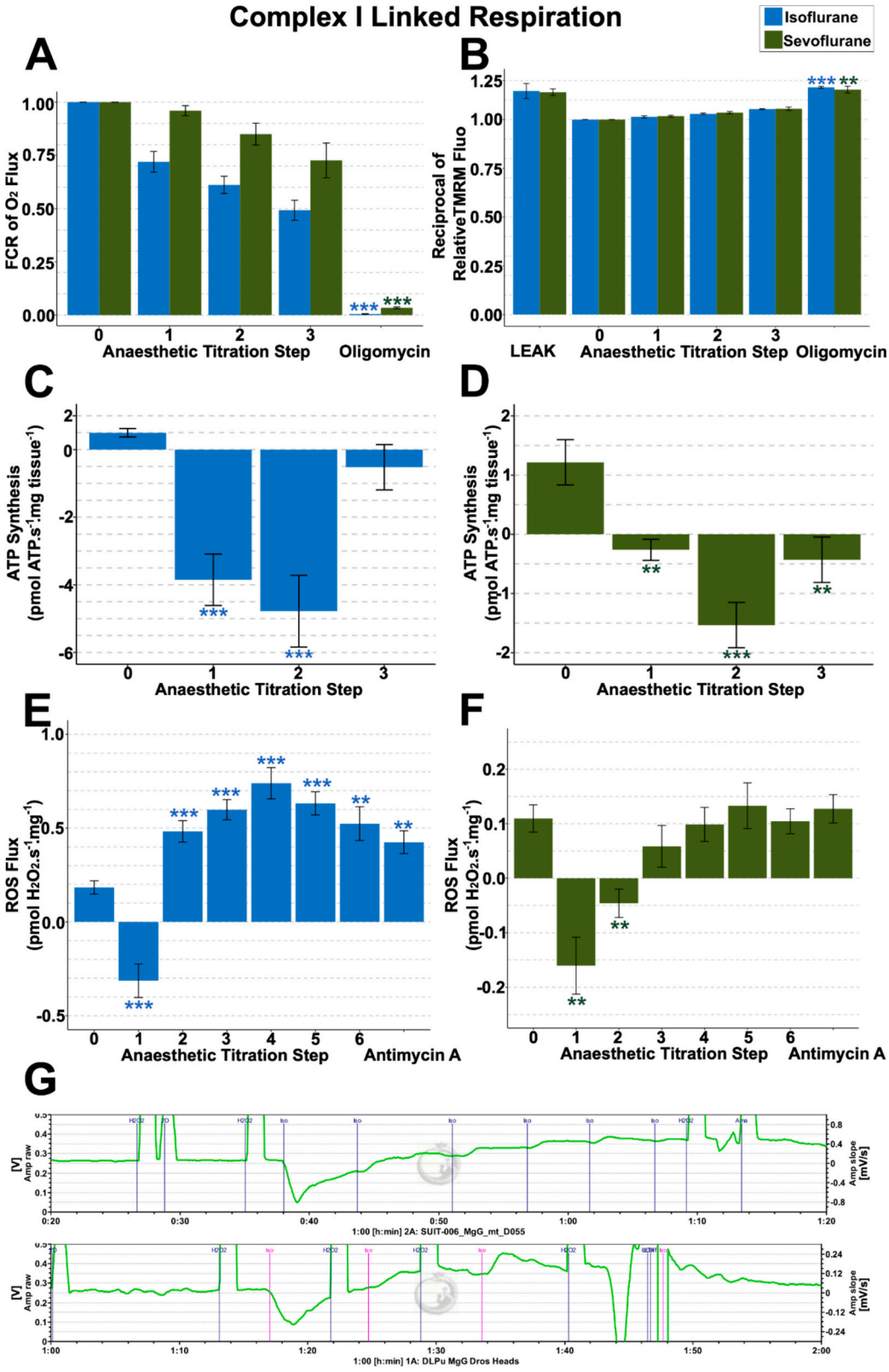
Importantly, the collapse in ATP availability seen here is not consistent with an inhibition of the ATP synthase followed by hydrolysis of ATP by other cellular processes. The immediate shift seen here is too fast to be simply consumption of ATP by other sinks. For comparison, inhibition of ATP synthesis by oligomycin in the presence of magnesium green (the dye used here) shows a slow decline in ATP availability, not the abrupt collapse seen here [54]. Likewise, if anaesthetics inhibit the ATP synthase, we should see slightly lower O_2 consumption in the LEAK state, linked with a slightly higher membrane potential. In fact, we see the reverse, as shown in SI Fig. 2A: O_2 consumption is stable, while membrane potential if anything falls. It is therefore implausible that the collapse in ATP availability we report was caused by anything other than reversal of the ATP synthase.

3.7. Anaesthetic suppression of complex I-linked respiration increases ROS flux

Raised membrane potential is typically associated with increased ROS flux, and this effect is confirmed in Fig. 2E and F for isoflurane and sevoflurane, respectively. In both cases, we recorded an initial fall in apparent ROS flux, which makes no sense in terms of the chemistry of H_2O_2. But this decrease in fluorescence was repeated in all runs, and seemed unlikely to be a pure artefact, as shown in the bottom raw trace of Fig. 2G. We therefore considered direct interactions between anaesthetics and dye alone. An example is shown in Fig. 2G, top trace, which confirms an initial large decrease in AmR fluorescence with isoflurane alone. This effect saturates on repeated dosing and so cannot simply be subtracted from later AmR fluorescence measurements in calculating ROS flux. We have therefore presented the ROS data in Fig. 2D and E with the initial decrease included, which is followed by a significant rise in the case of isoflurane, and a less marked rise in the case of sevoflurane.

While these direct interactions between anaesthetics and AmR fluorescence mean that our ROS data are not fully trustworthy, the apparent rise in ROS flux for isoflurane is consistent with its greater suppression of oxygen flux at complex I and higher membrane potential. It is also consistent with long clinical experience that isoflurane is less safe than sevoflurane [55], which is usually ascribed to greater oxidative damage [37]. To our knowledge, the findings reported here are the first to corroborate that difference in real time. On the other hand, we expected a greater increase in ROS flux on addition of antimycin A, which inhibits complex III, and should maximise ROS flux (albeit usually in the presence of rotenone). The fact that antimycin A did not increase ROS flux further means that either our data are unreliable, or ROS flux was already close to maximal after anaesthetic dosing. We note that others have reported suppression of ROS flux by anaesthetics using succinate as a sole substrate, and in the absence of rotenone, which is usually associated with high ROS flux [56,57]. These scenarios are not equivalent to that studied here, and are arguably less relevant, given the limited use of succinate by neurons, especially in fly tissue [40,50].

Altogether, our findings rely heavily on simultaneous high-resolution tissue fluorespirometry measurements, which has some disadvantages as well as advantages. The disadvantages relate to the lack of secondary validation of measurements made, in the face of some direct interactions between fluorescent dyes, anaesthetics and respiratory function. An important advantage is that measurements are made in real



(caption on next page)

Fig. 2. Effect of anaesthetics on oxygen flux, membrane potential, ATP synthesis and ROS flux. (A) Changes in oxygen flux relative to complex I-linked respiration after three titrations of 400 μM pure isoflurane ($N = 16$; blue) or 400 μM pure sevoflurane ($N = 18$; green) before addition of oligomycin. (B) Reciprocal of relative changes in TMRM fluorescence, indicating changes in membrane potential after the same three titrations of each anaesthetic followed by oligomycin ($N = 4$ for each of isoflurane and sevoflurane). (C) ATP synthesis in complex I-linked respiration, followed by three titrations of 400 μM pure isoflurane ($N = 8$), as inferred from magnesium green fluorescence. (D) ATP synthesis following the same protocol for sevoflurane (N = 8) titrations. (E) ROS (H_2O_2) flux inferred from amplex ultra red (AmR) fluorescence in complex I-linked respiration (time 0) followed by six titrations of 400 μM pure isoflurane before addition of antimycin A ($N = 10$). (F) ROS flux following the same protocol for sevoflurane (N = 8). (G) Example traces showing raw AmR fluorescence on addition of *Drosophila* thoraces (top trace) and anaesthetics alone (bottom trace), showing the direct effect of isoflurane on AmR fluorescence. Statistical significance of oligomycin titrations relative to anaesthetics titration step 3 (A-B). Significance of anaesthetic or antimycin titrations relative to titration step zero (C–F). *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

time, and can be interpreted in relation to simultaneous changes in other parameters that affect them directly. In the case of O2k measurements, inhibition of complex I by rotenone simultaneously decreases O_2 flux and increases ROS flux, while uncoupling increases O_2 flux and decreases ROS flux. Together, these measurements suggest that ROS flux really does derive from mitochondria and is not an artefact, because the signals behave appropriately in relation to other related respiratory parameters (and not always in the same way). In the case of anaesthetics, our findings are internally consistent in this way, giving us greater confidence in their veracity. Our results suggest that anaesthetic inhibition of oxygen flux in complex I reverses the ATP synthase, collapsing ATP availability and producing a corresponding rise in both membrane potential and ROS flux.

3.8. Dose response is consistent with causal effects on respiration

A final and important source of potential artefact relates to dose. The titrations used here were higher than those required to anaesthetize flies (Fig. 3A). Although we did attempt to measure tissue concentrations of anaesthetic in anaesthetized *Drosophila* thoraces, these measurements were very difficult, given the volatility of GAs, the air-filled tracheal system, and the need to homogenize whole flies in open Eppendorf tubes before making GC–MS measurements. It is therefore likely that most anaesthetic had evaporated long before we could make any measurements. *In lieu* of direct tissue measurements, we measured the times to fall, to lose movement, and to recover behaviour, over five orders of magnitude of anaesthetic dose, from 400 μM down to 0.04 μM, as well as ethanol alone (Fig. 3A and B). The initial dose used in our fluorespirometry studies, 400 μM, caused immediate falls and swift loss of movement, with, curiously, slightly faster recovery times than lower doses. Even the lowest doses induced anaesthesia, but they took much longer to do so, typically 15–20 min to induce loss of movement, coupled to slower recoveries. Ethanol caused very different behaviour, inducing falls within minutes but loss of movement taking an hour, and with enormously prolonged recoveries, taking around six hours. This striking difference could be ascribed in part to the great difference in volatility between GAs and ethanol: once anaesthesia had been achieved, flies were removed from the chamber, hence those that were anaesthetized most quickly were exposed to ethanol for a shorter period, presumably explaining their faster recovery times.

Finally, we measured oxygen flux at each of these concentrations in the O2k (Fig. 3C). While there was a large amount of scatter at lower concentrations, the IC_{50} for isoflurane was around 356 μM, broadly consistent with the doses that (rapidly) induced anaesthesia.

We therefore believe our findings are meaningful, in that the effects exerted on respiration were equivalent to those needed for anaesthesia.

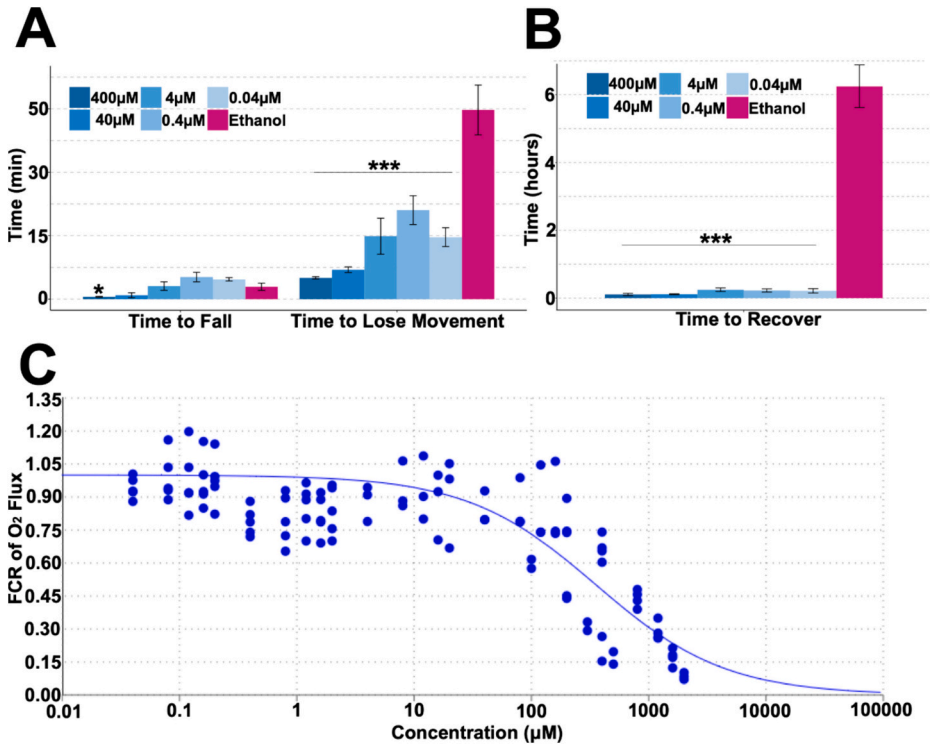


Fig. 3. Dose response of isoflurane titrations in whole flies. (A) Times to falling and loss of movement after injection of 1 μL isoflurane into 2 mL closed vials, giving the final vial concentrations indicated after evaporation. Pure ethanol (1 μL) was injected as control. (B) Times to recovery after removal of flies from vials containing isoflurane. Each bar is the mean of $N = 5$ or 6. *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$) relative to ethanol controls. (C) Dose-response curves using the O_2 flux data across different concentrations after correction for drift. An IC_{50} of 356 μM was predicted.

This was not very surprising, given that mutations in complex I cause hypersensitivity to anaesthetics in both patients and animal models (Table 1). While many of the findings presented here were produced by repeated anaesthetic titrations, adding up to much higher total doses, the effects were qualitatively very similar to initial single doses, with the effects on oxygen flux in complex I, especially, being close to linear. A single 400 μM dose of either isoflurane or sevoflurane suppressed both complex-I linked respiration and maximal respiration, increasing membrane potential, and collapsing ATP availability. The effects of a single anaesthetic dose on ROS flux were more equivocal, due to the interactions with the dye, but the abrupt increase in membrane potential suggests that a single anaesthetic dose would also increase ROS flux. We are therefore confident that our results are not an artefact of anaesthetic over-dosing. Overall, our findings are consistent with the possibility that the effects of anaesthetics on respiratory parameters could be the cause of anaesthesia, rather than a side-effect.

4. Conclusions

Our findings show that the volatile GAs isoflurane and sevoflurane suppress respiration, specifically at complex I, with concerted downstream effects on membrane potential, ATP availability and ROS flux. We analysed these respiratory parameters simultaneously using an Oroboros O2k, which allowed us to clarify the relationships between linked respiratory parameters. For example, increases in membrane potential immediately after anaesthetic titrations or oligomycin injection could be compared with simultaneous changes in O_2 flux, giving confidence that effects are genuinely coupled. The same applies to ATP availability and ROS flux. Our results offer a uniquely coherent view of the downstream consequences of complex I inhibition by anaesthetics.

Isoflurane exerts more marked effects than sevoflurane, including greater complex I suppression, a larger collapse in ATP availability and higher ROS flux, all of which are consistent with clinical findings suggesting that anaesthesia with isoflurane is less safe than sevoflurane [55]. Our results are also consistent with earlier work indicating that the respiratory effects of anaesthetics undermine ATP availability [39,46,47]. For example, expression of the yeast NADH dehydrogenase NDi1 in mice led to modest resistance to volatile GAs, which was interpreted as restoring electron flux to oxygen, enabling a partial rescue of ATP synthesis [17,58]. Likewise, a collapse in mitochondrial membrane potential observed by confocal microscopy in cell cultures could be interpreted as inhibition of complex I-linked respiration allied to the exhaustion of ATP needed to maintain membrane potential [46,47]. Consciousness is clearly energy-dependent, so any impact of anaesthetics on ATP availability makes sense.

But it is unlikely that ATP availability is the only factor governing anaesthesia. Our findings clearly show a direct effect of volatile GAs on electron transfer in complex I, yet they do not explain why *only* mutations in complex I cause hypersensitivity to volatile GAs (Table 1). This limited number of examples may be a biased sampling of a wider range of mutations in other respiratory complexes that eventually turn out to cause hypersensitivity to anaesthetics. But that also seems unlikely, as respiratory inhibitors including rotenone, cyanide and antimycin A should then cause anaesthesia, or at least hypersensitivity to anaesthetics, yet we are not aware of evidence that they do. Some commonly used drugs such as metformin also inhibit complex I, apparently binding close to the quinone-binding site, yet have not been reported to induce hypersensitivity to anaesthetics either. Nor is it clear why lipid-soluble molecules very similar in structure to anaesthetics should cause convulsions, rather than anaesthesia, if the effects related only to ATP availability. Mitochondrial diseases can be associated with epileptic tonic-clonic type or myonic seizures, presenting with characteristic EEG and MRI abnormalities [33], which are again difficult to explain purely in terms of ATP availability. Finally, the disparate molecular structures of volatile GAs from xenon to sevoflurane are not easily reconciled with a single binding site in complex I or any other protein.

These considerations bring us back to the recently discovered disruption of electron spin polarization by volatile GAs [12], most likely in complex I, with its nine FeS clusters spanning a distance of about 10 nm. Given that complex I is a specific target of volatile GAs, and that spin polarization is lost upon quinone reduction, then the question is: why would disrupting spin polarization in complex I cause anaesthesia? Spin polarization necessarily generates weak local magnetic fields, which could in principle interact with plasma membrane voltage-gated ion channels, for example in presynaptic dendrites, potentially influencing the EEG directly. Whether volatile GAs do in fact disrupt mitochondrial-derived magnetic fields is a question for future research.

CRediT authorship contribution statement

Enrique Rodriguez: Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Bella Peng:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Nick Lane:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Financial support was provided by the Bill and Melinda Gates Foundation and by the Biotechnology and Biological Sciences Research Council.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbabio.2024.149511.

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