Anesthetics Influence Mortality in a Drosophila Model of Blunt Trauma With Traumatic Brain Injury

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BACKGROUND: Exposure to anesthetics is common in the majority of early survivors of life-threatening injuries. Whether and to what degree general anesthetics influence outcomes from major trauma is unknown. Potential confounding effects of general anesthetics on outcome measures are usually disregarded. We hypothesized that exposure to isoflurane or sevoflurane modulates the outcome from blunt trauma with traumatic brain injury (bTBI).
METHODS: We tested the hypothesis in a novel model of bTBI implemented in *Drosophila melanogaster*. Fruit flies of the standard laboratory strain w^{1118} were cultured under standard conditions. We titrated the severity of bTBI to a mortality index at 24 hours (MI_{24}) of approximately 20% under control conditions. We administered standard doses of isoflurane and sevoflurane before, before and during, or after bTBI and measured the resulting MI_{24}. We report the MI_{24} as mean ± standard deviation.
RESULTS: Isoflurane or sevoflurane administered for 2 hours before bTBI reduced the MI_{24} from 22.3 ± 2.6 to 10.4 ± 1.8 ($P < 10^{-9}$, n = 12) and from 19.3 ± 0.9 to 8.9 ± 1.1 ($P < .0001$, n = 8), respectively. In contrast, administration of isoflurane after bTBI increased the MI_{24} from 18.5% ± 4.3% to 25.3% ± 9.1% ($P = .0026$, n = 22), while sevoflurane had no effect (22.4 ± 7.1 and 21.5 ± 5.8, n = 22).
CONCLUSIONS: In a whole animal model of bTBI, general anesthetics were not indifferent with respect to early mortality. Therefore, collateral effects of general anesthetics should be considered in the interpretation of results obtained in vertebrate trauma models. Invertebrate model organisms can serve as a productive platform to interrogate anesthetic targets that mediate collateral effects and to inform trauma research in higher organisms about the potential impact of anesthetics on outcomes. (Anesth Analg 2018;126:1979–86)

KEY POINTS

- **Question:** Do volatile general anesthetics modulate outcome from blunt trauma?
- **Finding:** Isoflurane and sevoflurane differentially affected 24-hour mortality in a *Drosophila melanogaster* model of blunt trauma.
- **Meaning:** The use of general anesthetics may affect the consequences of blunt trauma in mammalian experimental models.

The response to life-threatening injury activates cellular signaling cascades that trigger organism-wide, tightly regulated immune and inflammatory responses to limit damage and initiate repair.[1] Research aimed at predicting outcome from severe trauma strongly indicates a role for genetic background in molding the trauma-coping mechanisms.[2] However, linking genomic patterns to outcome from trauma has proven difficult in mammalian models[3] and in humans.[4] Leveraging the experimental flexibility of invertebrate model organisms may be a useful strategy to achieve a better understanding of the genetic influence on trauma outcome.

Although humans and *Drosophila melanogaster* (fruit flies) do not look very similar, evolutionary conservation has allowed findings initially made in flies to lead to clinically important discoveries in humans. These include how *Hox* genes control development of the human body plan, how Toll pathways mediate the human innate immune response, how the *period* and *clock* genes coordinate circadian rhythms, how chromatin-based mechanisms regulate epigenetic inheritance in humans, and how Wnt, Notch, and Ras signal transduction pathways contribute to cancer in humans (for overview[5]). Furthermore, results from flies translate to humans even when they were derived from studies of tissues that have no direct counterpart in humans.

We have shown that injuries induced by contact and inertial forces in fruit flies mimic characteristics of blunt trauma with traumatic brain injury (bTBI) in mammals.[6] Furthermore, we have shown that naturally occurring genetic polymorphisms substantially modulate the resilience to bTBI.[7] We and others have also demonstrated that cardinal pharmacodynamic and pharmacokinetic

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characteristics of the volatile general anesthetics (VGAs) isoflurane (ISO) and sevoflurane (SEVO) are conserved between flies and humans.[8] Here, we used flies to investigate the influence of exposure to VGAs on early mortality after bTBI because limited animal welfare concerns make it possible to isolate the effects of anesthesia in the context of bTBI. Future experiments will explore the genetic and genomic modifiers of VGA–bTBI interaction using the rich genetic toolbox available for fruit flies. The goal of this exploratory study was to test the hypothesis that VGAs modulate mortality from bTBI. We used the mortality index 24 hours after bTBI (MI_{24}) as our primary end point and found differences between ISO and SEVO. These findings are relevant for the interpretation of experimental work in trauma models that include anesthesia and suggests the existence of collateral effects of anesthesia in the heretofore unexamined context of blunt trauma.

METHODS

This manuscript adheres to the applicable Animal Research: Reporting of In Vivo Experiments (ARRIVE) reporting guidelines (preclinical animal research).

Approval from Institutional Animal Care and Use Committee has been waived.

Fly Lines and Culturing

All experiments used 0- to 7- or 1- to 8-day-old w^{1118} flies. All flies were maintained on molasses food at 25°C, as described in Katzenberger et al.[6]

Blunt Trauma With Traumatic Brain Injury

bTBI was inflicted with a high-impact trauma (HIT) device operated following standardized protocols[6,7,9] (for a visual demonstration see Katzenberger et al[7]). Eight vials were used to simultaneously expose 2 experimental conditions (Figure 1A), with each condition represented by 4 vials of 60 flies each. Results from 2 experimental or 2 control samples (eg, vials 1 and 2 or 5 and 6, respectively) were averaged and considered a single replicate, so n = 2 for the experiment illustrated in Figure 1A. The standard bTBI protocol took 20 minutes and consisted of 4 strikes from the HIT device with 5 minutes recovery between strikes and was administered either before, during, or after exposure to anesthetics as illustrated in Figure 1B (long bTBI rectangles). To maintain exposure to VGAs during the coexposure condition, foam plugs were used to contain flies in vials during the bTBI protocol. In the preexposure condition, to passively eliminate VGAs, which diffuse freely, cotton balls were used to contain flies in vials during the bTBI protocol. To maintain exposure to VGAs when bTBI was being inflicted in the 15-minute coexposure condition, 4 strikes from the HIT device were administered in quick succession within <2.5 minutes (Figure 1B, short bTBI rectangles). We have previously shown that the MI_{24} does not differ between the 20- and 2.5-minute bTBI protocols.[6] Two HIT devices were used that produced bTBI of slightly different severities under the same bTBI protocol; 1 device was used for Figures 2–4 and another device was used for Figure 5.

Experiments in Figure 5 used 0- to 7-day-old mixed-sex fly lines from the *Drosophila melanogaster* Genetic Reference Panel (RAL lines 161, 352, 381, 409, 427, 439, 774, 892, and 897) and w^{1118}, a standard laboratory strain of *Drosophila melanogaster*.[10]

For the primary outcome measure of mortality after bTBI, dead flies were counted 24 hours after bTBI. We defined the mortality index at 24 hours (MI_{24}) as the percentage of flies that died within 24 hours following bTBI minus the percentage of matching uninjured flies that died within the same 24-hour period. The overall average mortalities for uninjured flies were very low: 0.71% ± 0.11% for flies not exposed to anesthetic, 0.84% ± 0.13% for flies exposed to ISO, and 0.86% ± 0.1% for flies exposed to SEVO.

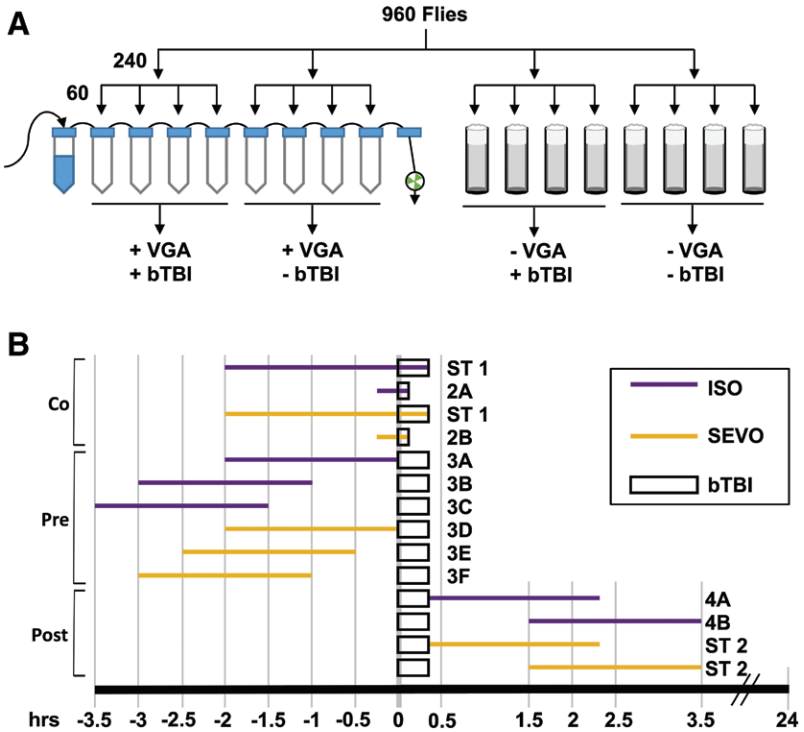

Figure 1. Diagrams that explain the workflow for experiments presented in Figures 2–4. A, A group of mixed-sex, 1- to 8-d-old w^{1118} flies was equally divided for examination under 4 conditions: anesthetic and trauma (+VGA [volatile general anesthetics] +bTBI [blunt trauma with traumatic brain injury]), anesthetic and no trauma (+VGA −bTBI), no trauma without anesthesia (−VGA +bTBI), and no anesthetic and no trauma (−VGA −bTBI). The +VGA −bTBI and−VGA −bTBI samples served to determine the percent mortality without trauma and were subtracted from the percent mortality of the +VGA +bTBI and −VGA +bTBI samples, respectively, to calculate the mortality index at 24-h (MI_{24}) values. B, Timelines of the co-, pre-, and postexposures to anesthetics relative to the infliction of either the standard bTBI protocol or the rapid bTBI protocol (long and short rectangles, respectively). Purple and yellow lines indicate the duration (short: 15 min, long 2 h) and timing of exposure to 2% isoflurane (ISO) or 3.5% sevoflurane (SEVO), respectively. Lettering at the end of each line indicates the figure panel showing the results. ST indicates that results are presented only in the Supplemental Digital Content 2, Table 1, http://links.lww.com/AA/C296. Timelines are drawn to scale in hours and the MI_{24} was determined 24 hours after initiation of the bTBI protocol.

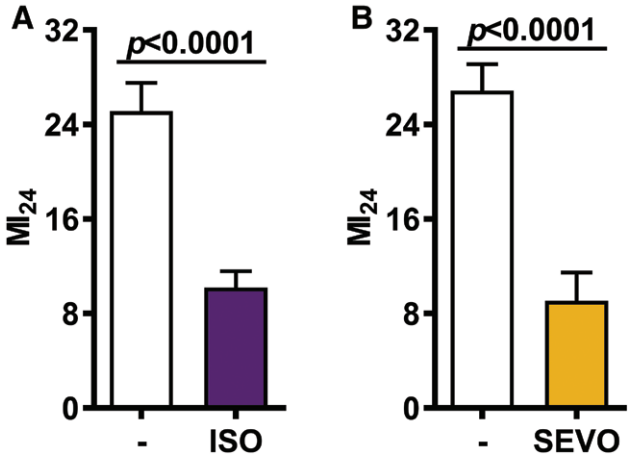

Figure 2. Coexposure to volatile general anesthetics reduces the risk of mortality after blunt trauma with traumatic brain injury (bTBI). The mortality index at 24 h (MI_{24}) was determined for mixed-sex, 0- to 7- or 1- to 8-d-old w^{1118} flies either not exposed to anesthetic (–) or exposed to isoflurane (ISO; A) or sevoflurane (SEVO; B) for 15 min before and during bTBI. Error bars represent the 95% confidence intervals.

Incapacitation

After a single strike from the HIT device, a fraction of flies was immobilized for varying periods of time. We defined those that remained immobile for a minimum of 1 minute and regained mobility (most within 5 minutes) as "incapacitated"[6] (Supplemental Digital Content 1, Video 1, http://links.lww.com/AA/C295; Figure 5A).

VIDEO+

VGA Administration

ISO and SEVO were delivered in air into the serial anesthesia array, as described previously.[11] In brief, commercial, agent-specific vaporizers and a custom-made serial anesthesia array were used to ensure rapid administration of equal doses to all 8 vials, resulting in equivalent exposure to anesthetics in all vials (Figure 1A).

Analogous to the commonly used quantification of anesthetic exposure in MAC-hours, we define "dose" as the product of agent concentration in air (in v%/v%) and exposure duration (in hours), that is, %hour. We examined 3 anesthetic regimens: preexposure (VGA administration discontinued before bTBI), coexposure (VGA present before and during bTBI), and postexposure (VGA administered after bTBI).

Non-VGA Immobilization

Groups of 60, mixed-sex, 0- to 7-day-old w^{1118} flies were immobilized with either CO_2 or exposure to cold (ie, a water-ice bath at 4°C) and subjected to the standard bTBI protocol while they were immobile. Immobility was maintained throughout the experiment by reexposure to CO_2 or water-ice between each of 4 strikes from the HIT device.

Statistical Analysis

The principal outcome measure is the MI_{24}. We tested the hypothesis that ISO and SEVO have an effect on MI_{24}. We measured the MI_{24} under control conditions and after application of ISO or SEVO at different time points and for different durations. The principal null hypothesis is that ISO and SEVO have no effect on the MI_{24}. Because control MI_{24}s were normally distributed and we had no a priori assumptions about the effect of our intervention, we tested the hypothesis by comparing the control MI_{24} with the MI_{24} after drug exposure using 2-tailed, unpaired t tests with the significance level set at $P = .05$. We considered an effect size of 25%, in either direction, as biologically significant.

Sample Size Justification. In previously published work comparing the MI_{24} between different fly strains, we found that an n of 8 was sufficient to reject the null hypothesis (no difference between 2 strains) with a power of 95% with a population mean MI_{24} of 25% ± 4% and an effect size (ie, difference in MI_{24}) of 20%. Therefore, we set the minimum number of replicates for rejecting the null hypothesis of no difference between anesthetic-exposed flies and anesthetic-unexposed flies to 8. However, we used a higher number of replicates in experiments when the sample standard deviation was higher to reduce the likelihood of falsely accepting the null hypothesis.

To test whether secondary events that lead to incapacitation also lead to mortality, we determined the incapacitation fraction and the MI_{24} after bTBI in separate experiments in 10 different strains. For incapacitation and the MI_{24}, each data point in Figure 5A is the mean of 8 replicates. The results for incapacitation are also based on 8 independent experiments, each with 3 vials of 20 flies (480 flies total for each experiment). For the MI_{24}, we report the mean values from 8 independent experiments, each consisting of 1 vial with 60 flies. The lower number of flies per vial for incapacitation was necessary for accurate scoring. We used the Pearson correlation coefficient and reported the 95% confidence interval.

Confounding Effects. We tested the hypothesis that immobilization by CO_2 or cold reduces the MI_{24} (Figure 5B, C).

We calculated the risk ratio for death comparing anesthetic-unexposed flies to anesthetic-exposed flies as described by Viera[12] and reported it, together with detailed numerical results, in Supplemental Digital Content 2–4, Table 1, http://links.lww.com/AA/C296, Table 2, http://links.lww.com/AA/C297, and Table 3, http://links.lww.com/AA/C298.

Unless otherwise stated, unpaired t tests were used to test for differences between mean values. We set the statistical significance criterion at $P < .05$. Prism 6.0 (GraphPad Software, Inc, La Jolla, CA) was used for graphing.

RESULTS

ISO and SEVO Present During bTBI Reduce Mortality

In vivo trauma models typically include exposure to anesthetics to instrument the preparation before the administration of trauma and, in most vertebrate models, during trauma as well. In fact, even a brief exposure to ISO, for example, for the purpose of euthanasia, influenced mRNA expression in both healthy mice and after TBI.[13] We examined whether exposure to ISO or SEVO at various time points before, during, or after the time of bTBI influenced mortality in 1- to 8-day-old w^{1118} flies (Figure 1B) Coexposure to ISO

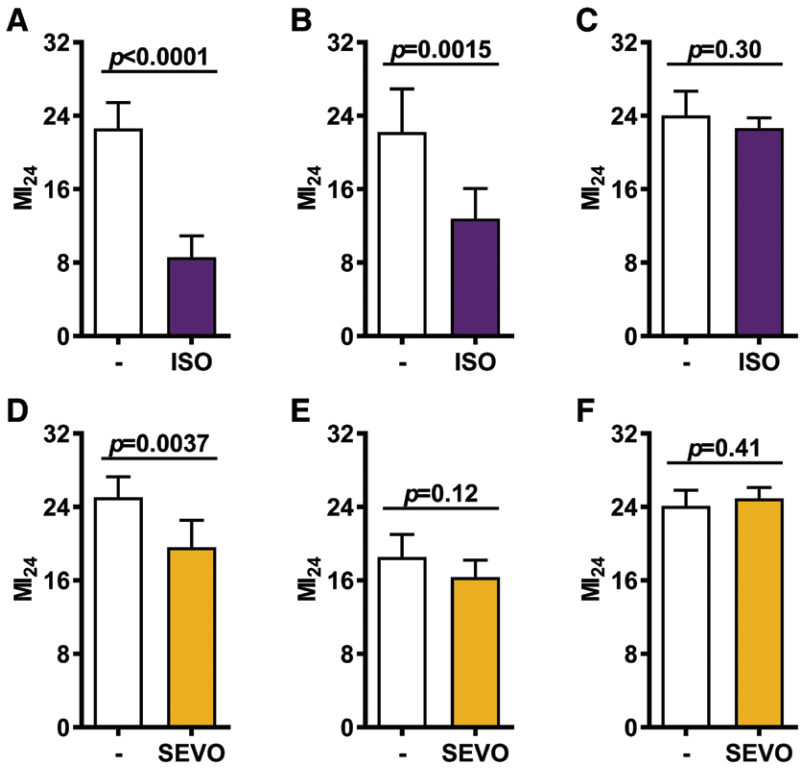

Figure 3. The time between preexposure to volatile general anesthetics and infliction of blunt trauma with traumatic brain injury (bTBI) differentially affects the ability of isoflurane (ISO) and sevoflurane (SEVO) to reduce the risk of mortality after bTBI. The MI_{24} was determined for mixed-sex, 0- to 7- or 1- to 8-d-old w^{1118} flies either not exposed to anesthetic (–) or exposed to ISO for 2 h before infliction of the standard bTBI protocol immediately (A), 1 h later (B), or 1.5 h later (C). Alternatively, the mortality index at 24 h (MI_{24}) was determined for mixed-sex, 0- to 7- or 1- to 8-d-old w^{1118} flies either not exposed to anesthetic (–) or exposed to SEVO for 2 h before infliction of the standard bTBI protocol immediately (D), 0.5 h later (E), or 1 h later (F). Error bars represent the 95% confidence intervals.

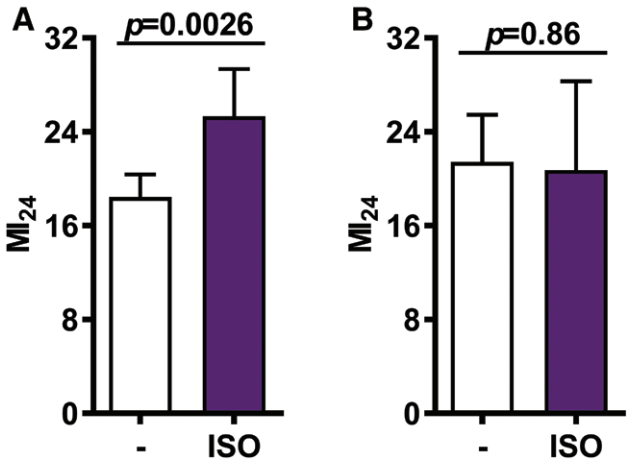

Figure 4. Exposure to isoflurane (ISO) shortly after blunt trauma with traumatic brain injury (bTBI) increases the risk of mortality due to bTBI. The mortality index at 24 h (MI_{24}) was determined for mixed-sex, 0- to 7- or 1- to 8-d-old w^{1118} flies that received the standard bTBI protocol and were either not exposed to anesthetic (–) or exposed to ISO for 2 h immediately afterward (A, $P = .003$) or 1.5 h later (B, $P = .86$). Error bars represent 95% confidence intervals. Sevoflurane had no effect (Supplemental Digital Content 3, Table 2, http://links.lww.com/AA/C297).

and SEVO for only 15 minutes reduced the MI_{24} by 59.4% from 25.3% ± 2.9% to 10.2% ± 1.6% ($P < 10^{-7}$) and by 68.5% from 26.9% ± 3.1% to 9.1% ± 3.4% ($P < 10^{-9}$), respectively (Figure 2A, B; Supplemental Digital Content 2, Table 1, http://links.lww.com/AA/C296). Longer exposures before bTBI to either 4%hour ISO or 7%hour SEVO did not further reduce the MI_{24}: 53.5% and 53.9% reduction of MI_{24} (from 22.3 ± 0.8 to 10.4 ± 0.5; $P < 10^{-10}$ and from 19.3% to 8.9%; $P < 10^{-5}$) for ISO and SEVO, respectively (Supplemental Digital Content 2, Table 1, http://links.lww.com/AA/C296).

We conclude that both ISO and SEVO exert protective effects in the context of bTBI that saturate within a short exposure time.

Protection by ISO Outlasts SEVO

To test whether the protective effect of VGAs is contingent on the presence of high concentrations of the agents during trauma, we discontinued anesthesia for various time intervals before the administration of bTBI. We chose the time intervals based on our previous finding that 50 minutes after 4%hour of ISO w^{1118} flies are behaviorally indistinguishable from nonanesthetized controls.[8] Flies reach the same degree of recovery about twice as fast after SEVO anesthesia of equal depth and duration.[11] Hence, bTBI was administered immediately after preexposures to both VGAs and at intervals of 60 and 90 minutes after 4%hour of ISO and 30 and 60 minutes after 7%hour of SEVO (Figure 3; Supplemental Digital Content 2, Table 1, http://links.lww.com/AA/C296).

Initiation of the standard bTBI protocol within 5 minutes after terminating anesthesia resulted in a differential reduction of the MI_{24}: 62.1% (from 22.6 ± 3.9 to 8.6 ± 3.3; Figure 3A) for ISO and 21.7% (from 25.0 ± 5.0 to 19.6 ± 6.6, Figure 3D) for SEVO. Increasing the time interval between anesthesia and bTBI widened the differential effects of ISO and SEVO. For ISO, a significant reduction of the MI_{24} was still detectable after an interval of 60 but not 90 minutes (Figure 3B, C). By contrast, for SEVO, no reduction of the MI_{24} was detectable after intervals of either 30 or 60 minutes (Figure 3E, F). We conclude that both ISO and SEVO reduce early mortality after bTBI, that this effect is time-limited, and may be contingent on the persistence of low VGA concentrations in the fly body. However, the effect of ISO persisted for a

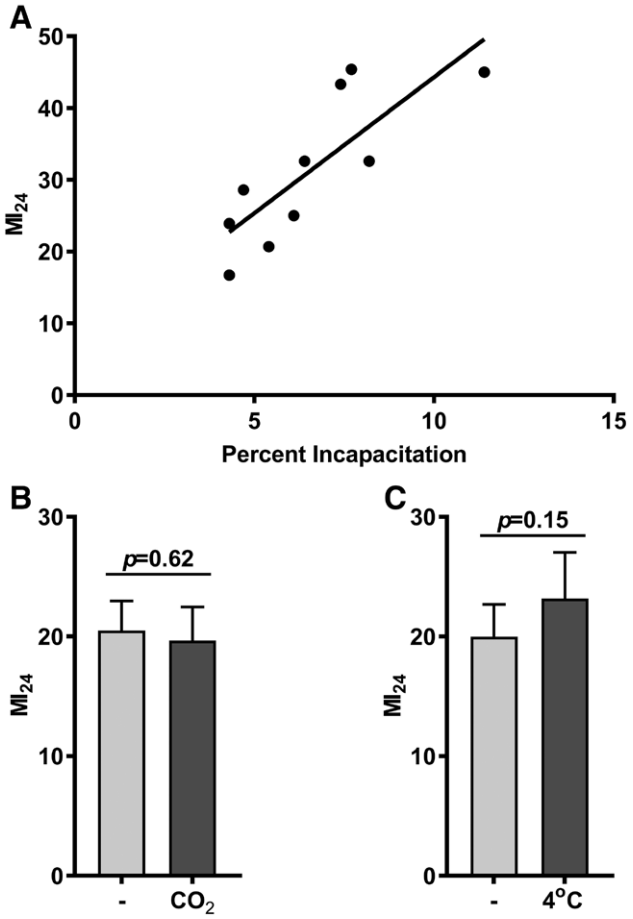

Figure 5. The risk of mortality after blunt trauma with traumatic brain injury (bTBI) is correlated with the risk of incapacitation after bTBI and is not affected by mobility at the time of bTBI. A, The mortality index at 24 h (MI_{24}) following the standard bTBI protocol versus the percent incapacitation following 1 strike from the high-impact trauma device for 10 mixed-sex strains (w^{1118} and 9 RAL lines) at 0- to 7-d old. Pearson correlation coefficient 0.80, 95% confidence interval 0.355–0.952, R^2 = 0.6476. B, The MI_{24} of mixed-sex, 0- to 7-d-old w^{1118} flies either mobile (−) or immobile due to exposure to CO_2 at the time of bTBI. No difference between the MI_{24}s of immobilized and mobile flies (20.5 ± 3.4 and 19.7 ± 3.9, mean ± standard deviation [SD]). C, The MI_{24} of mixed-sex, 0- to 7-d-old w^{1118} flies either mobile (−) or immobile due to exposure to low temperature (4°C) at the time of bTBI. The MI_{24}s of immobilized and mobile flies are not different (20 ± 3.8 and 23.2 ± 5.4, mean ± SD).

longer duration than that of SEVO, even after accounting for its slower elimination.

ISO Administered Shortly After bTBI Increases Mortality

VGA administration after ischemia/reperfusion injury modulates the extent of tissue damage in mammalian models. This effect may be agent- and tissue-dependent,[14–16] possibly less robust than preconditioning,[17] and has been studied in less detail than preconditioning. We examined whether exposure to VGAs after bTBI (postexposure) might influence the MI_{24}. We found that when the standard bTBI protocol was immediately followed (ie, within 5 minutes) by 4%hour ISO, the MI_{24} increased by 36.8% from 18.5 ± 4.4 to 25.3 ± 9.2 (confidence interval, 16.6–20.4 and 21.3–29.3; P = .0026, Figure 4A; Supplemental Digital Content 3, Table 2, http://links.lww.com/AA/C297). However, when ISO anesthesia was delayed by 90 minutes, the MI_{24} did not differ between unexposed and exposed flies (22.4% ± 7.1% and 21.5% ± 5.8%, Figure 4B; Supplemental Digital Content 3, Table 2, http://links.lww.com/AA/C297). Neither immediate postexposure to 7%hour SEVO nor exposure delayed by 90 minutes affected the MI_{24} (Supplemental Digital Content 3, Table 2, http://links.lww.com/AA/C297). We conclude that the effects of VGAs on the risk of mortality after bTBI are agent specific and fade within a limited time.

Mortality May Be Due to Brain Injury

To investigate the extent to which temporary incapacitation, a manifestation of brain injury, correlates with acute mortality after bTBI, we determined the percent incapacitation of 10 fly lines: w^{1118} (a standard laboratory strain) and 9 wild-type, inbred strains (RAL lines) from the *Drosophila melanogaster* Genetic Reference Panel that had different MI_{24}s ranging from 16.7 to 45.4 when injured at 0- to 7-day old. We found that the percent incapacitation was positively correlated with the MI_{24} (r = 0.80, 95% CI, 0.355–0.952; Figure 5A), suggesting that nervous system–based mechanisms leading to temporary concussion-like incapacitation also lead to mortality after bTBI.

Immobility and Intrinsic Toxicity Do Not Confound the VGA–bTBI Data

To investigate whether immobility at the time of bTBI affects mortality in the absence of anesthetics, we determined the MI_{24} of 0- to 7-day-old w^{1118} flies immobilized with either CO_2 or exposure to cold. We found that flies immobilized by CO_2 or cold had the same MI_{24} as fully mobile flies (Figure 5B, C, respectively; Supplemental Digital Content 3, Table 2, http://links.lww.com/AA/C297). Therefore, immobility by itself does not affect the risk of mortality after bTBI.

Flies are generally somewhat more sensitive to VGAs than mammals. Published anesthetic EC_{50}s for ISO in different strains of *Drosophila melanogaster* range from 0.21[18] to 1.3 v%/v%,[19] depending on the definition of anesthesia. Using a customized negative geotaxis-based assay, we determined that the EC_{50}s of 1- to 8-day-old w^{1118} flies were 0.41% and 0.68% for ISO and SEVO, respectively.[8] Because VGAs have narrow safety margins in many animals and because it is unknown whether VGAs administered in air can be lethal in flies, we determined the effect of incremental doses of ISO and SEVO on the percent mortality 24 hours after termination of exposure. We found that exposure of 1- to 8-day-old w^{1118} flies up to 18%hour with either agent did not cause mortality (Figure 6A). The highest ISO dose tested (24%hour, ie, 4% ISO for 6 hours) resulted in 8.6% ± 1.3% mortality, and the highest SEVO dose tested (39%hour, ie, 6.5% SEVO for 6 hours) resulted in 6.5% ± 0.5% mortality. Normalization of the ISO and SEVO data to their respective EC_{50}s that were determined in the same fly strain and at the same age revealed that ISO and SEVO had equivalent toxicity profiles (Figure 6B; Supplemental Digital Content 4, Table 3, http://links.lww.com/AA/C298). We conclude that flies tolerate concentrations of ISO and SEVO in the range of those commonly administered for anesthetic purposes for long time periods without obvious harm. The doses we used to examine VGA effects in bTBI (4 and

7%hour of ISO and SEVO, respectively) are well below the doses that increase mortality in the absence of trauma.

DISCUSSION

While virtually every vertebrate trauma model of severe injury involves the use of anesthetic/sedative drugs, potential interactions of anesthetics per se with the response to trauma are rarely the focus of analysis. In light of research conducted over the past 2 decades, the potential of VGAs to cause a plethora of effects beyond those resulting in the familiar clinical phenotype of anesthesia is undeniable.[20–24] Therefore, we specifically investigated the effect of ISO and SEVO on the risk of mortality in a fly model of blunt trauma with concomitant brain injury (bTBI). Trauma in flies clearly does not equal trauma in humans. However, basic molecular and cellular processes triggered by life-threatening tissue destruction are likely to overlap to some degree between flies and mammals. When seen from this perspective, flies offer a model with "tractable complexity" to examine, in an intact organism, the pathobiology of life-threatening injury. Our principal findings demonstrate that, at least in flies, VGAs modulate the outcome from bTBI.

VGAs Differentially Influence 24-Hour Mortality

The presence of ISO or SEVO before and during bTBI significantly reduced early mortality (Figures 2 and 3). When either VGA was present during bTBI, the reduction in the MI_{24} was similar between agents for both 15-minute and 2-hour exposures (Figure 2; Supplemental Digital Content 2, Table 1, http://links.lww.com/AA/C296). Interposition of a time interval between exposure to anesthetic and bTBI revealed a subtle but gradually increasing difference between ISO and SEVO (Figure 3). By the time the flies had recovered to preanesthetic levels of spontaneous activity (60 minutes for ISO and 30 minutes for SEVO),[8] only ISO reduced the MI_{24} (Figure 3B, E). Increasing the interval between anesthesia and bTBI to 90 minutes abolished the mortality-reducing effect of ISO (Figure 3C).

We examined whether immobility during bTBI has a protective effect in the absence of VGAs. Our experiments indicate that immobility alone is neither sufficient (as demonstrated by immobilization using CO_2 or exposure to cold, Figure 5B, C) nor necessary (as demonstrated by the 60-minute ISO preexposure experiment, Figure 3B) to reduce mortality. We conclude that the reduction in mortality is due to a "pharmacological" protective effect of ISO and, by extension, of SEVO as well.

What accounts for the longer duration of the protective effect of ISO relative to SEVO (Figure 3)? The most straightforward explanation is pharmacokinetics and is supported by the equally protective effect of ISO and SEVO in the coexposure conditions, that is, when anesthetics were intentionally maintained during trauma administration (Figure 2). As emergence is faster for SEVO than for ISO,[8] a delay between anesthesia and bTBI will result in a higher residual concentration of ISO than of SEVO at the time of trauma and hence more protection (Figure 3A, D). This purely pharmacokinetic explanation is, however, weakened by other preexposure experiments: only ISO effectively protected flies that had recovered long enough to become behaviorally indistinguishable from unanesthetized animals, that is,

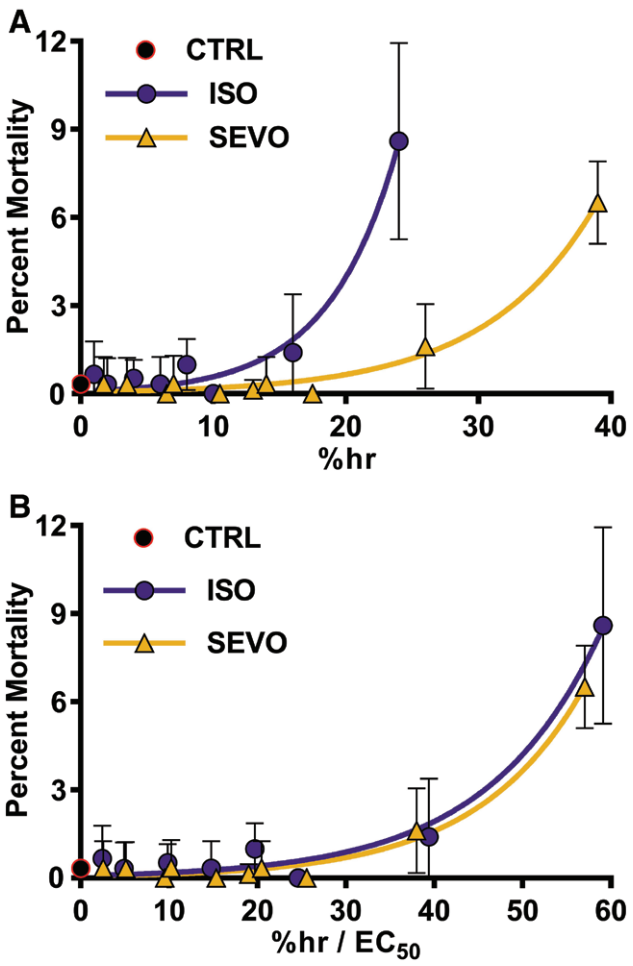

Figure 6. Isoflurane (ISO) and sevoflurane (SEVO) are well tolerated in flies. A, Mixed-sex, 0- to 7-, or 1- to 8-d-old w^{1118} flies were exposed to different doses (%h) of ISO or SEVO and the percent of flies that died within 24 h (percent mortality) was measured. Flies not exposed to anesthetic (control [CTRL]) were examined to establish the baseline percent mortality. B, %h values from A for ISO and SEVO were normalized to their respective EC_{50} values (ISO, 0.41 ± 0.03 and SEVO, 0.68 ± 0.05), as determined by Olufs et al.[8]

a recovery of 60 minutes for ISO and 30 minutes for SEVO (Figure 3B, E). These experiments suggest that, whatever the mechanism, ISO may be a more efficient protective agent than SEVO, that is, in addition to a pharmacokinetic difference there may also be a pharmacodynamic difference between the agents. A pharmacodynamic contribution is supported by our surprising discovery that ISO but not SEVO increased the MI_{24} when administered after bTBI (Figure 4; Supplemental Digital Content 3, Table 2, http://links.lww.com/AA/C297), an experiment where pharmacokinetic differences should not be of concern. The clear difference between agents in this condition suggests that despite their chemical similarity and the indistinguishable "anesthetic" phenotype, ISO and SEVO have overlapping but not identical cellular/molecular effect profiles. Importantly, this difference is revealed by a change in the physiological context of drug exposure: "anesthesia" was indistinguishable in uninjured and bTBI flies for both ISO and SEVO, but the expression of collateral effects differed dramatically between contexts only for ISO. Therefore,

concerns about the potential of VGAs to differentially confound outcome measures in trauma models are justified.

VGAs Differentially Modulate Molecular Effectors of Collateral Effects

The transient receptor potential (TRP) chemosensor family provides instructive examples of how differential modulation of molecular targets by chemically similar VGAs result in collateral effects with clearly different phenotypic presentations. ISO, but not SEVO, activates TRPA1, resulting in enhanced neurogenic inflammation in vivo.[25] The TRP family also provides an example of the importance of the biological context for VGA activity. A number of modern VGAs fail to activate the TRPV1 channel directly; however, they sensitize the channel to capsaicin, protons, and heat in vitro.[26] Under these conditions, ISO has a stronger effect than SEVO. For example, ISO directly activates TRPV1 only after stimulation of protein kinase C and under concomitant application of bradykinin, a situation that might be encountered in the wake of tissue trauma, indicating the importance of the biochemical context for VGA activity.[26] Analogous differential effects of ISO and SEVO have been reported for TRPA- and TRPV-mediated calcitonin gene-related peptide release from an ex vivo trachea model.[27] A further example of differing results of exposure to VGAs is provided by the differential effect of ISO, SEVO, and desflurane on cyclophilin-modulated mitochondrial H_2O_2 production[28] and the different phenotypes of ISO and desflurane on mitochondrial function and learning and memory.[29]

Anesthetic Modulation of Trauma

The most detailed experiments to date that are relevant to the present study were conducted in rodents using the controlled cortical impact (CCI) TBI model. Statler et al[30] compared 7 anesthetic/sedative drugs (including ISO) administered for 1 hour after CCI to a control group without post-CCI anesthesia. None of the anesthetic regimens improved any outcome measure when compared to anesthesia-free recovery from CCI, but among the tested anesthetics, post-CCI exposure to ISO resulted in better neuronal survival in the hippocampus than exposure to ketamine. By 5–16 days after CCI, no differences in behavioral measures were detectable among the treatment groups. Notably, CCI was administered under ISO anesthesia in all groups. This early exposure to ISO before and during CCI may have confounded any intrinsic differences between the subsequently administered anesthetics. This possibility is supported by the study of Luh et al,[31] who exposed mice to ISO, SEVO, or a combination of midazolam, fentanyl, and medetomidine (and their antagonists to reverse anesthesia) during the time required to prepare for and inflict CCI (analogous to our coexposure protocol). In contrast to Statler et al,[30] only short-term outcomes were assessed (maximum 24 hours). At this point, the lesion volume was smaller and the neurological function was better in the ISO group, indicating that even brief exposures to anesthetics modulate certain short-term outcome measures of CCI. Our model differs from those cited above in that the brain injury is but 1 component of a blunt polytrauma. What is the evidence that brain injury plays a decisive role for mortality? We believe that the most parsimonious explanation of the pathogenesis of incapacitation following contact- and inertia-induced trauma is a concussion-like brain injury. The correlation between incapacitation and the MI_{24} across 10 genotypes (Figure 5A) and the findings of progressive neurodegeneration and shortened lifespan in the absence of overt injury to other organs[6] suggest that brain injury not only occurs in our model but is also an important contributor to mortality.

Drosophila melanogaster as a Model for Discovery and Analysis of Collateral Anesthetic Effects in the Context of Trauma

Our results confirm that VGAs can profoundly affect the response of an organism to blunt trauma and that the effect of VGAs is context dependent, that is, it differs between naïve and traumatized organisms. Among the reasons contributing to the paucity of data in this area are, on one hand, the public's concerns about and regulatory agencies' requirements for humane treatment of laboratory animals and, on the other hand, the lack of awareness among trauma researchers of the broad spectrum of biological activities of VGAs beyond "anesthesia" per se. The delayed manifestation of such collateral effects (as suggested by findings in neurodevelopment and oncology) would require longer-term follow-up for either confirmation or refutation, further complicating the logistics and adding substantially to the cost of experiments.

Together with our previous demonstration of the reproducibility of key pharmacokinetic and pharmacodynamic properties of VGAs in flies,[8] these data indicate that flies can be used as relevant model organisms in trauma research: collections of inbred, near isogenic fly lines[10,32] can be used for unbiased phenotype to genotype screens of wild-type genomes, while collections of deletions covering virtually the whole genome[33] and a wide selection of mutants are available for genotype-to-phenotype screening. Future research may identify specific risk-conferring genetic variants, which then may be translated for research in higher animals.

In summary, we found that VGAs differentially modulate early mortality in an invertebrate model of blunt trauma.

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