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Anesthetic Preconditioning of Traumatic Brain Injury Is Ineffective in a Drosophila Model of Obesity

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ABSTRACT

We tested the hypothesis that obesity influences the pharmacodynamics of volatile general anesthetics (VGAs) by comparing effects of anesthetic exposure on mortality from traumatic brain injury (TBI) in lean and obese *Drosophila melanogaster*. We induced TBI with a high-impact trauma device. Starvation-selection over multiple generations resulted in an obese phenotype (SS flies). Fed flies served as lean controls (FC flies). Adult (1–8-day-old) SS and FC flies were exposed to equianesthetic doses of isoflurane or sevoflurane either before or after TBI. The principal outcome was percent mortality 24 hours after injury, expressed as the Mortality Index at 24 hours (MI_{24}). TBI resulted in a lower MI_{24} in FC than in SS flies [21 (2.35) and 57.8 (2.14), respectively $n = 12$, $P = 0.0001$]. Pre-exposure to isoflurane or sevoflurane preconditioned FC flies to TBI, reducing the risk of death to 0.53 (0.25 to 1.13) and 0.82 (0.43 to 1.58), respectively, but had no preconditioning effect in SS flies. Postexposure to isoflurane or sevoflurane increased the risk of death in SS flies, but only postexposure to isoflurane increased the risk in FC flies [1.39 (0.81 to 2.38)]. Thus, obesity affects the pharmacodynamics of VGAs, thwarting the preconditioning effect of isoflurane and sevoflurane in TBI.

SIGNIFICANCE STATEMENT

Inadvertent preconditioning in models of traumatic brain injury (TBI) is a recognized confounder. The findings in a fruit fly (*Drosophila melanogaster)* model of closed-head TBI indicate that anesthetic pharmacodynamics are profoundly affected by obesity. Specifically, obesity thwarts the brain-protective effect of anesthetic preconditioning. This finding is important for experimental studies of TBI and supports the versatility of the fruit fly as a model for the exploration of anesthetic pharmacodynamics in a wide parameter space.

Introduction

Preconditioning, i.e., the capacity of anesthetics to induce tolerance to injury when administered prior to ischemia, is a potentially valuable property of volatile general anesthetics (VGAs). Anesthetic preconditioning effectively protects the brain (Kitano et al., 2007) and the heart (Stadnicka et al., 2007) from ischemic damage. However, its effectiveness in protecting the myocardium is suppressed in obese rodents (Song et al., 2011) and in models of diabetes (Ge et al., 2018). Although the cause of this failure is not fully understood, it may be due to metabolic abnormalities linked to excessive accumulation of lipids in the heart (Nakanishi and Kato, 2014). This pathologic entity is termed "lipotoxic cardiomyopathy" or "fatty heart syndrome" (Szczepaniak et al., 2007). Rodents develop this syndrome in experimentally induced diabetes and obesity (Zhou et al., 2000). Whether these common comorbidities also influence anesthetic preconditioning of nervous tissue remains unknown as no analogous "lipotoxic" brain phenotype has been yet described.

Examining anesthetic interactions with experimental brain injury is notoriously complicated because nonanesthetized control groups are impossible, as experiments require exposure to anesthesia for technical (e.g., immobility for surgery) and/or animal welfare reasons. VGAs, however, profoundly influence almost all aspects of brain physiology (Statler et al., 2006b; Tétrault et al., 2008; Staib-Lasarzik et al., 2014; Semple et al., 2016). As a result, in both focal and diffuse brain damage, injury and intervention always occur on the background of a brain exposed to anesthetics, and inadvertent preconditioning may confound the interpretation of experiments and

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ABBREVIATIONS: CI, confidence interval; FC, fed control (lean control fly line from A. Gibbs laboratory – UNLV); MI_{24}, Mortality Index at 24 hours; SS, starvation-selected (obese phenotype fly line from A. Gibbs laboratory – UNLV); TBI, traumatic brain injury; VGA, volatile general anesthetic.

interventions. Further complicating matters, the extent and molecular mechanisms of preconditioning differ among anesthetic agents (Statler et al., 2006a). This long-standing problem has been explicitly recognized by many research groups and may contribute to the frequent failure in translating findings from mammalian models to human patients.

To address some of these limitations, we have tested the effect of VGAs on mortality in a traumatic brain injury (TBI) model implemented in the fruit fly (*Drosophila melanogaster*). This model reproduces key characteristics of TBI in mammals, including temporary incapacitation (concussion), ataxia, death, neurodegeneration, and shortened lifespan (Katzenberger et al., 2013; Putnam et al., 2019; Saikumar et al., 2020), while also faithfully mimicking the behavioral effects of VGAs (Olufs et al., 2018). Crucially, as flies pose no animal welfare concerns, control experiments without exposure to anesthetics are feasible. We have found that exposure of a standard laboratory fly line (w^{1118}) to the VGAs isoflurane and sevoflurane prior to TBI effectively preconditioned the brain, as indicated by suppression of 24 hour mortality (Fischer et al., 2018). By contrast, exposure to isoflurane after TBI increased mortality. To test the hypothesis that obesity modulates anesthetic pharmacodynamics, we inflicted TBI in a fly model of obesity acquired by starvation selection (see *Methods* for details), which mimics many of the phenotypic characteristics of obesity in mammals, including increased weight and triglyceride storage as well as behavioral, anatomic, and metabolic abnormalities (Reynolds, 2013; Masek et al., 2014; Hardy et al., 2015). We found that obesity thwarts anesthetic preconditioning by isoflurane and sevoflurane in TBI. These data indicate that preconditioning with VGAs in fruit flies is responsive to biologic variables and reproduces the effect of obesity on anesthetic pharmacodynamics in mammalian ischemia. Although this information complicates the design of experiments that require the use of anesthetics, it can be instrumentalized to improve our understanding and treatment of TBI.

Materials and Methods

The experiments adhere to applicable ARRIVE (Animal Research: Reporting of In Vivo Experiments) reporting guidelines (preclinical animal research). Approval from the Institutional Animal Care and Use Committee has been waived.

Fly Husbandry. Unless otherwise indicated, experiments were conducted on flies generously provided by Dr. Allen Gibbs (School of Life Sciences, University of Nevada, Las Vegas, NV). The original founding populations for these flies were *D. melanogaster* collected from Terhune Orchards, Princeton, NJ in 1999 and maintained as outbred stocks at 25°C on cornmeal medium. One population underwent starvation-selection (SS population) over multiple generations by subjecting sequential generations of flies to severe starvation on 1% agar until only 15%–20% of the original population survived. Surviving flies were then placed on food to lay eggs. The next generation of adults was selected for starvation resistance in the same manner. The obese phenotype that developed in the SS population was characterized by increased lipid storage of nearly two times the amount of total lipids as the unselected control population, including a 30% increase in weight and high fat stores along with high whole body triglyceride levels (Reynolds, 2013). SS flies also had a depressed metabolic rate, low activity levels, dilated cardiomyopathy, and excess sleep (Reynolds, 2013; Masek et al., 2014; Hardy et al., 2015). The control population was cultured under the same conditions as the SS population but was provided ad libitum food and water and is referred to as the FC (for fed control) population. We received one subpopulation each of SS and FC flies that had undergone at least 120 generations of selection. Once in our laboratory, all flies were maintained on cornmeal-molasses food at 25°C and used at 1–8 days post eclosion. As evolutionary pressure of starvation-selection is removed, SS flies gradually start losing the obese phenotype. Therefore, experiments reported in Figs. 1, 3, and 4 were performed on generations 2–4. For Figs. 2 and 5, later generations of SS flies were also used. Fly lines 2P9, *D. virilis*, and *D. funebris* were generously provided by Bob Kreber and Dr. Barry Ganetzky (Department of Genetics, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, WI). The w^{1118} line is a standard line maintained in our laboratory. All experiments were conducted using mixed sex samples except fly mass that was determined using males. Fly mass was determined by averaging the results of 12 replicates of 30 flies for each line using an analytical balance with 0.1 mg accuracy (Mettler Toledo XSE104, Columbus, OH).

TBI. TBI was induced using a high-impact trauma device as described previously (Katzenberger et al., 2013). On the day prior to an experiment, eight vials containing 20 mixed sex flies were incubated at 25°C with cornmeal-molasses food. On the day of the experiment, flies were rapidly transferred into empty vials. TBI was induced with four strikes from the high-impact trauma device with the spring deflected to 90 degrees and 5 minutes between strikes. Anesthetics were administered either before or after TBI. After injury and anesthesia, flies were transferred to vials with cornmeal-molasses food and incubated at 25°C.

The primary outcome was mortality expressed as the Mortality Index determined 24 hours after TBI (MI_{24}). We define the MI_{24} as the percentage of flies that are dead at 24 hours after TBI minus the percentage of matching uninjured flies that died within the same 24 hour period. Because mortality after TBI does not differ between male and female flies, we performed all experiments on mixed sex groups. Unless otherwise indicated, at least six independent replicates were performed for each experimental condition.

Anesthesia. We used a custom-built Serial Anesthesia Array to simultaneously expose up to eight samples of 20 flies each to precise doses of VGAs in air, as described previously (Fischer et al., 2018; Olufs et al., 2018). VGAs were administered through the Serial Anesthesia Array using a Datex-Ohmeda Aestiva/5 anesthesia machine equipped with commercial agent-specific vaporizers (Datex-Ohmeda Inc., Madison, WI). Compressed gas cylinders (Airgas USA, LLC., Radnor, PA) containing air (21% O_2/79% N_2) provided the carrier gas. To test the effect of obesity on anesthetic pharmacodynamics, we exposed the flies to anesthetics either immediately before or after inflicting TBI, mimicking pre- and postconditioning, respectively. We used either 2% isoflurane or 3.5% sevoflurane for both exposure protocols. These anesthetic concentrations are behaviorally equivalent and do not affect median and maximum lifespans (Olufs et al., 2018). The dose of anesthetic administered is reported as concentration (%) multiplied by duration (hours), e.g., 2% isoflurane for 2 hours equals 4%h. All flies resumed movement within less than 1 hour after discontinuing isoflurane or sevoflurane (i.e., no flies died immediately after TBI with or without anesthetic exposure, indicating that the doses were safe). A typical assay simultaneously tested two control conditions (i–ii) and two experimental condition (iii, iv): (i) no treatment, (ii) anesthesia alone, (iii) TBI alone, and (iv) TBI and anesthesia. All experiments were conducted under normobaric conditions. Mortality under control conditions (i and ii) was less than 1%.

Statistical Analysis. This study was exploratory with respect to examining the obese phenotype. The sample sizes were based on our experience with previous experiments testing the effect of anesthetics on the MI_{24}. Data are presented as mean (± standard deviation), number of biologic replicates (n), and [95% confidence interval]. Each replicate included 20 individuals except for the determination of weight. To test for significance between treated and untreated FC and SS flies, we used the unpaired two-sample student's t test and ANOVA with Bonferroni's multiple comparison test. To compare the MI_{24} between FC and SS flies subjected to the same treatment, we used the independent two-sample t test. We quantified the effect of the VGAs on the MI_{24} using relative risk of death, calculated as the relative risk (Altman, 1991). Data underlying the calculations is plotted in box [interquartile range (IQR) of P_{25} to P_{75}, i.e., 25th to 75th percentile] and whiskers to maximum and minimum values with '+' = mean and 'horizontal line' = median. Dots indicate individual replicates. We used Graphpad© Prism for all statistical calculations. We used Hedge's *g* to calculate effect size for comparing different sample sizes and four benchmarks to accommodate the range of our data (0 no effect; 0.2 small effect, cannot be discerned with the naked eye; 0.5 medium effect; and 0.8 large effect, can be seen with the naked eye) (https://www.statisticshowto.com/hedges-g). A negative value indicates an increase and a positive value indicates a decrease in the MI_{24}.

Results

SS Flies Are Heavier and at Increased Risk of Mortality after TBI. Under our culture conditions, SS flies exhibited an easily recognizable obese phenotype weighing 40%–50% more than FC flies (1.25 versus 0.84 mg/fly), in agreement with previously reported data (Reynolds, 2013). To investigate whether the obesity phenotype alters the risk of early mortality after TBI, we determined the MI_{24} of FC and SS flies after four strikes with the high-impact trauma device (Fig. 1). The MI_{24} of FC flies was 20.1 (±2.35) $n = 6$, confidence interval (CI) [17.6 to 22.5], close to that previously reported for w^{1118} flies of the same age (Fischer et al., 2018). By contrast, the MI_{24} of SS flies was 57.8 (±2.14), $n = 6$, CI [55.5 to 60.0], which is in the top decile of MI_{24} values reported for inbred and outbred collections (Katzenberger et al., 2015) (Fig. 1). In summary, changes associated with obesity induced by experimental evolution increased the relative risk of TBI-induced early death 2.85-fold CI [1.86 to 4.37] ($P < 0.0001$).

Early Mortality Is Positively Correlated with Fly Weight. To examine whether the high MI_{24} of SS flies was attributable to their increased weight, we examined male flies from four other fly lines whose weights varied over a 3-fold range from 0.5 to 1.7 mg/fly, bracketing the weight of FC and SS flies (Fig. 2). In addition to FC and SS flies, we included two *Drosophila melanogaster* lines with the following weights: w^{1118} 0.55 (±0.02) mg/fly, and 2P9 0.73 (±0.02) mg/fly as well as lines of *Drosophila virilis* 1.57 (±0.03) mg/fly and *Drosophila funebris* 1.62 (±0.05) mg/fly. w^{1118} is a standard laboratory line and 2P9 is an uncharacterized P-element insertion line. FC flies weighed 0.84 (±0.09) mg/fly. SS flies from the 2nd to 10th, i.e., early (E) generations maintained an obese phenotype (GenE) and weighed 1.25 (±0.1) mg/fly. The late (L) generation SS flies that were losing the obese phenotype (SS GenL) weighed 0.97 (±0.1) mg/fly by generation 20. All lines were tested at 1–8 days old. We found that fly weight was highly correlated with the MI_{24} (R^2= 0.96) (Fig. 2). This might be expected because both the force imparted on the flies and the energy they were subjected to should be proportional to their mass [i.e., force = mass × acceleration ($F = ma$) and energy = 1/2 mass × velocity of impact squared ($E = 1/2\ mv^2$)]. The close, but not perfect, correlation between the weight of a fly and the MI_{24} leaves room for other factors to affect the MI_{24} (e.g., genetic background). We previously found that when tested at 0–7 days old, inbred fly lines from the Drosophila Genetic Reference Panel, whose males vary in weight from 0.58 to 0.87 mg/fly (Unckless et al., 2015), had MI_{24} values that varied from 8 to 58 (Katzenberger et al., 2015), which exceeds the expected variability based exclusively on weight of 23 to 37 predicted by the data in Fig. 2. These data indicate that the weight plays a major role in increasing TBI-induced mortality of SS over FC flies, but it remains possible that increased

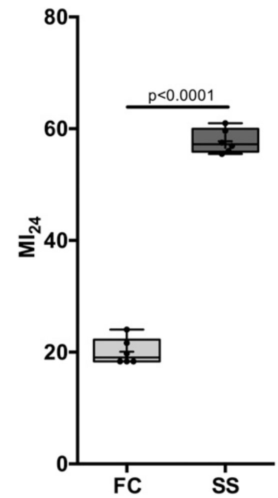

Fig. 1. Obesity is associated with increased mortality. The Mortality Index at 24 hours after TBI (MI_{24}) was determined in 1–8-day-old lean FC (fed control) and obese SS (starvation-selected) flies. The relative risk of death was 2.85 (CI [1.86 to 4.37], $n = 6$ per group). + indicates the mean, the horizontal line indicates the median, the box indicates 25th to 75th percentile, the whiskers extend to minimal and maximal values, and dots indicate individual replicates.

mortality of SS flies results from severe secondary injuries due to cellular and molecular effects associated with obesity.

Anesthetic Pretreatment Does Not Precondition SS Flies. To test the hypothesis that obesity influences anesthetic pharmacodynamics, we assayed the effect of anesthetic exposure prior to TBI. In FC flies, pretreatment with equianesthetic doses of isoflurane (4%h) or sevoflurane (7%h) reduced the MI_{24} from 17.5 (5.1, $n = 24$) to 9.4 (4.1, $n = 16$) and to 14.3 (3.5, $n = 8$) for isoflurane and sevoflurane ($P <$ 0.0001 and 0.18), respectively (Fig. 3). Thus, exposure to isoflurane preconditioned flies to TBI, whereas sevoflurane trended toward this phenotype. Preconditioning resulted in a reduction of the relative risk of death to 0.53 [CI 0.25 to 1.13] and 0.82 [CI 0.43 to 1.58] for isoflurane and sevoflurane, respectively. By contrast, pre-exposure of SS flies with the same doses of isoflurane or sevoflurane did not precondition to

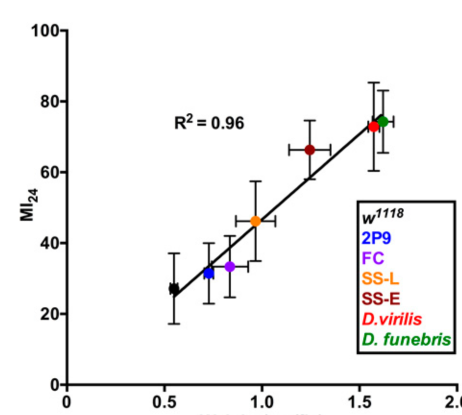

Fig. 2. Early mortality (MI_{24}) is correlated with weight for six fruit flies lines. Weight (independent variable) and the MI_{24} were determined for four lines of *D. melanogaster* (w^{1118}, FC, SS, 2P9) and for *D. funebris* and *D. virilis*. The data were fitted with a simple linear regression. Tested SS flies were pooled into two groups determined by the time dots of their reproduction cycles in the laboratory: early (2nd–10th generation, SS-E) and late (up to generation 20, SS-L). For the determination of MI_{24}, $n = 14$ except for SS-E where $n = 24$. The MI_{24} for the tested lines positively correlated with weight (R^2= 0.96).

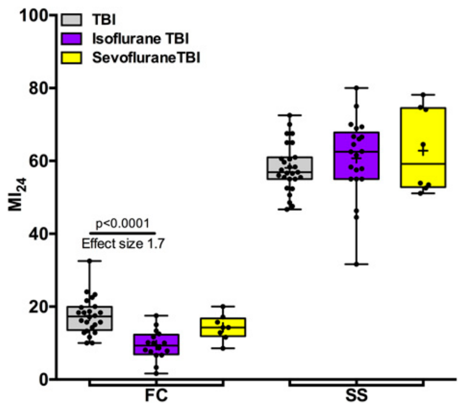

Fig. 3. Obese flies are resistant to the preconditioning effect of pre-exposure to VGAs. Exposure of FC flies (fed control, i.e., lean flies) to the isoflurane prior to TBI (left) reduced the MI_{24}. The effect was large for isoflurane (Hedge's g 1.7; $P < 0.0001$, ANOVA with Bonferroni's test) and medium (Hedge's g 0.7; ($P = 0.18$, ANOVA with Bonferroni's test) for sevoflurane. Exposure of SS flies (starvation-selected, i.e., obese flies) to either VGA did not appreciably affect the MI_{24} (right). + indicates the mean, the horizontal line indicates the median, the box indicates 25th to 75th percentile, whiskers extend to the minimal and maximal values, and dots indicate individual replicates.

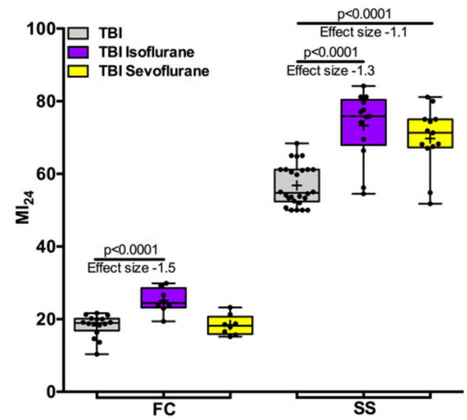

Fig. 4. Exposure to VGAs after TBI increases mortality in obese flies. Exposure of FC flies (fed control, i.e., lean flies) to isoflurane but not to sevoflurane after TBI increased the MI_{24} (left). By contrast, exposure of SS flies (starvation-selected, i.e., obese flies) to both isoflurane and sevoflurane increased the MI_{24} (right). Hedge's g effect size of isoflurane in FC and SS flies was large (-1.5 and -1.3, respectively, $P < 0.0001$ for both comparisons, ANOVA with Bonferroni's multiple comparisons test). The effect of sevoflurane in SS flies was large (Hedge's g −1.1, $P < 0.0001$), whereas there was no effect in FC flies (Hedge's g 0). + indicates the mean, the horizontal line indicates the median, the box indicates 25th to 75th percentile, whiskers extend to the minimal and maximal values, and dots indicate individual replicates.

TBI. The MI_{24} without pre-exposure was 58.1 (±6.6, $n = 27$) and with pre-exposure was 60.7 (±10.9, $n = 21$) and 62.8 (±11.4, $n = 8$) for isoflurane and sevoflurane, respectively. We conclude that although the effectiveness of preconditioning in FC flies is comparable to the previously reported protective effects of these agents in w^{1118} flies (Fischer et al., 2018; Schiffman et al., 2020), the obese phenotype generated by starvation-selection is associated with changes that thwart molecular mechanisms underlying anesthetic preconditioning.

Anesthesia after TBI Selectively Increases the MI_{24}. In contrast to the unambiguous effectiveness of preconditioning, the results from exposure to VGAs after ischemia (i.e., postconditioning) are mixed (Lucchinetti et al., 2005; Li and Zuo, 2011). We tested the effect of exposure to VGAs after TBI using the same doses of isoflurane and sevoflurane as used for pre-exposure. Exposure of FC flies to isoflurane after TBI increased the MI_{24} from 18.2 (±3.1, $n = 16$) to 25.1 (±6.5, $n = 8$), also increasing the risk ratio for death to 1.38 [CI 0.81 to 2.38), whereas the MI_{24} was not appreciably affected by sevoflurane (18.4 ± 4.3, $n = 8$). (Fig. 4). These results replicate our findings in w^{1118} flies, in that postexposure with isoflurane but not sevoflurane revealed a toxic potential of VGAs when administered after TBI (Fischer et al., 2018; Schiffman et al., 2020). The outcomes differed somewhat in SS flies where exposure to both isoflurane and sevoflurane increased the MI_{24} from 56.8 (±8.1, $n = 26$) to 73.3 (±18.4, $n = 13$) and to 69.7(±17.3, $n = 13$), respectively. Postconditioning hence increased the risk of death to 1.28 [CI 1.04 to 1.58] and 1.23 [CI 0.99 to 1.52] for isoflurane and sevoflurane, respectively. These results in SS flies resemble the increase in MI_{24} from post-treatment with both agents reported for old w^{1118} flies (Schiffman et al., 2020). We conclude that metabolic changes associated with obesity lower the threshold for VGA toxicity and reveal a toxic potential for sevoflurane in the context of an injured brain.

Pre-Exposure and Postexposure Phenotypes Normalize after Many Generations in the Absence of Starvation-Selection. Figs. 3 and 4 show that early generation (≤ 5th generation) SS flies are distinct from FC flies in their resistance to preconditioning of TBI by isoflurane and sevoflurane and toxicity from postexposure to sevoflurane. To test whether these distinct phenotypes persist after SS flies lose the obese phenotype, we examined flies up to the 10th generation. The MI_{24} declined proportionally to the loss of weight (Fig. 2, SS-E and SS-L) but remained higher than that of FC flies (Fig. 5). After the eighth generation, pre-exposure to isoflurane suppressed the MI_{24} (Fig. 5A, SS gen 9–10). Concomitantly, postexposure to sevoflurane lost its toxic effect (Fig. 5B, SS gen 9–10). We conclude that pathways mediating the molecular mechanisms of the effects of VGAs on survival after TBI recover after prolonged absence of starvation-selection.

Discussion

The principal finding of this work is that obesity resulting from starvation-selection interferes with preconditioning of a TBI outcome by VGAs. To reach this conclusion, we combined two fly models (TBI and obesity) that reproduce many features of their counterparts in higher animals, and we took advantage of the fact that key pharmacokinetic and pharmacodynamic properties of VGAs (Fischer et al., 2018; Olufs et al., 2018) are also evolutionarily conserved.

Preconditioning by VGAs in mammals is well documented but its mechanisms are not fully understood. Diverse injurious stimuli can precondition the brain but all of them have exceedingly narrow therapeutic indices rapidly resulting in injury when a certain, largely ill-defined threshold is exceeded (Stenzel-Poore et al., 2004; Gidday, 2006; Obrenovitch, 2008; Yokobori et al., 2013). VGAs are exceptional in that preconditioning is induced rapidly, but even sustained exposure will not injure the adult "healthy" brain. Therefore, the mechanism underlying anesthetic preconditioning must differ qualitatively and/or quantitatively from other preconditioning stressors that have to be administered either very briefly (e.g., hypoxia and

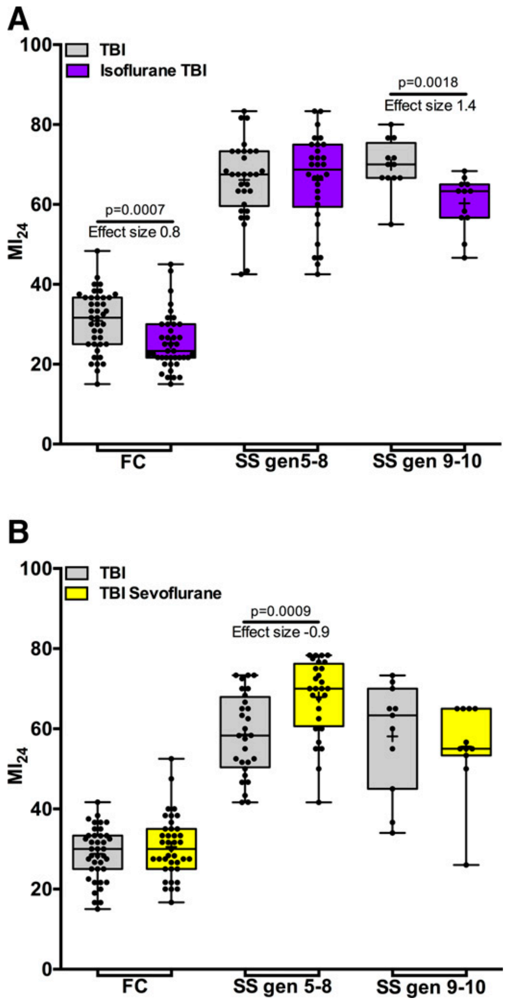

Fig. 5. Anesthetic pharmacodynamics gradually normalize after termination of starvation-selection. (A) SS flies became susceptible to isoflurane preconditioning after the 8th generation. (B) Postexposure toxicity of sevoflurane disappears in SS flies after the 8th generation. Gray bars indicate the MI_{24} without anesthetic exposure, purple bars indicate preconditioning with 15 minutes of 2% isoflurane, and yellow bars indicate postexposure to 15 minutes of 3.5% sevoflurane. SS gen 5–8 and SS gen 9–10 indicate the number of generations that the flies reproduced on cornmeal-molasses food. FC are fed control, i.e., lean flies. Note: the MI_{24} of SS flies remains higher than that of FC flies. + indicates the mean, the horizontal line indicates the median, the box indicates 25th to 75th percentile, whiskers extend to the minimal and maximal values, and dots indicate individual replicates.

oxidative stress) or for prolonged periods of time [e.g., hyperthermia (Shohami et al., 1994; Su et al., 2009)].

The principal, not mutually exclusive, mechanisms of anesthetic preconditioning under investigation are: (i) signaling from an early increase of intracellular Ca^{2+} (Gray et al., 2005; Weber, 2012), (ii) triggering of proteostatic responses (e.g., the unfolded protein response) (Baker et al., 2011; McClintick et al., 2011), (iii) isoflurane-induced mitochondrial reactive oxygen species-mediated signaling cascades leading to ischemic tolerance (Hirata et al., 2011) by modulation of the AMPK (AMP-activated protein kinase) signaling pathway (Song et al., 2011), (iv) inducible nitric oxide synthase, implicated in both ischemic and anesthetic preconditioning (Kapinya et al., 2002) with possibly different sources of NO in the heart versus the brain (Kapinya et al., 2002; Amour et al., 2009), (v) modulation of the immune-inflammatory system, possibly via VGA-induced modulation of the transcription factor NF-kappaB (Zhang et al., 2013), and (vi) modulation of the mitochondrial inner membrane permeability transition pore (mPTP) (Sedlic et al., 2010). The mPTP serves as a rescue pathway for excessive mitochondrial Ca^{2+} accumulation and its opening is a critical, irreversible step committing a cell to apoptosis. The state of the mPTP is controlled by numerous upstream and downstream targets and even its exact molecular composition is under debate (Baines and Gutiérrez-Aguilar, 2018), but delays in its opening have been suggested with various types of preconditioning (Pravdic et al., 2009).

Obesity modulates some of these pathways. For example, the failure of preconditioning was attributed to interference with sevoflurane-induced phosphorylation of AMPK and activation of eNOS (endothelial nitric oxide synthase) in the myocardium of obese rats (Song et al., 2011) and with misregulation of microRNA 21 and NOS by isoflurane in the hearts of diabetic mice (Ge et al., 2018). The degree to which similar processes play a role in the brain remains to be investigated, and experiments presented in this paper are a first step.

The use of *Drosophila* as a model for clinical conditions is only possible because of extensive evolutionary conservation. For example, over 70% of human disease-causing genes have orthologs in the fly (Reiter et al., 2001) and, as basic cellular processes are conserved between flies and humans, both share secondary molecular and cellular events triggered by injury (Chow and Reiter, 2017). For example, oxidative stress is a major molecular driver of obesity-related complications (Furukawa et al., 2004) and plays similar role in obesity models in the fruit fly (Trindade de Paula et al., 2016).

Our previous work has shown that pretreatment with VGAs effectively protected flies from death due to TBI (Fischer et al., 2018; Schiffman et al., 2020), indicating that some molecular mechanisms by which anesthetics precondition are operational in flies. Here we expand on these findings by showing that, in agreement with data from the rodent myocardium (Song et al., 2011; Ge et al., 2018), obesity interferes with isoflurane preconditioning in brain injury. We cannot make a statement regarding sevoflurane in this context because the reduction in MI_{24} by sevoflurane preconditioning, despite a moderate effect size, did not reach the threshold for statistical significance ($P = 0.18$). These findings are particularly relevant for experimental studies of TBI. For example, in TBI induced in rodents either by fluid percussion (Wu et al., 2003) or controlled-cortical impact (Hoane et al., 2011), diet-induced obesity resulted in worsened outcomes. Both research groups attributed their findings to the effect of diet and/or obesity on biochemical alterations such as brain BDNF (brain derived neurotrophic factor) levels. It is notable though that all animals were exposed to general anesthetics around the time of injury. Therefore, although a role for BDNF is possible, the alternative explanation of differential preconditioning between experimental groups by anesthetics cannot be excluded, illustrating the value of unconventional approaches using invertebrate models to complex, multifactorial pathologies like TBI.

The high MI_{24} and the lack of preconditioning in SS flies resemble the phenotypes of aged laboratory flies (Schiffman et al., 2020). Aging increases vulnerability to TBI in humans (Maas et al., 2008) and flies (Katzenberger et al., 2013) and also reduces the effectiveness of preconditioning in the human myocardium (Mio et al., 2008). Because starvation-selection does not shorten lifespan (Archer et al., 2003), FC and SS flies were injured at the same point in their lifespan. One explanation for our findings may be that obesity associated changes result in a premature aging-like phenotype revealed under stress caused by TBI.

In summary, although neither TBI nor obesity in flies equals their human counterparts, flies are a useful tool to inform research in higher animals by exploring parameters that would be difficult, expensive, or simply unethical to examine in higher animals.

Authorship Contributions

Participated in research design: Wassarman, Perouansky.

Conducted experiments: Fischer, Schiffman.

Performed data analysis: Johnson-Schlitz, Olufs, Scharenbrock.

Wrote or contributed to the writing of the manuscript: Olufs, Wassarman, Perouansky.

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