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The Serial Anesthesia Array for the High-Throughput Investigation of Volatile Agents Using *Drosophila melanogaster*

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Abstract

Volatile general anesthetics (VGAs) are used worldwide on millions of people of all ages and medical conditions. High concentrations of VGAs (hundreds of micromolar to low millimolar) are necessary to achieve a profound and unphysiological suppression of brain function presenting as "anesthesia" to the observer. The full spectrum of the collateral effects triggered by such high concentrations of lipophilic agents is not known, but interactions with the immune-inflammatory system have been noted, although their biological significance is not understood.

To investigate the biological effects of VGAs in animals, we developed a system termed the serial anesthesia array (SAA) to exploit the experimental advantages offered by the fruit fly (*Drosophila melanogaster*). The SAA consists of eight chambers arranged in series and connected to a common inflow. Some parts are available in the lab, and others can be easily fabricated or purchased. A vaporizer, which is necessary for the calibrated administration of VGAs, is the only commercially manufactured component. VGAs constitute only a small percentage of the atmosphere flowing through the SAA during operation, as the bulk (typically over 95%) is carrier gas; the default carrier is air. However, oxygen and any other gases can be investigated.

The SAA's principal advantage over prior systems is that it allows the simultaneous exposure of multiple cohorts of flies to exactly titrable doses of VGAs. Identical concentrations of VGAs are achieved within minutes in all the chambers, thus providing indistinguishable experimental conditions. Each chamber can contain from a single fly to hundreds of flies. For example, the SAA can simultaneously examine eight different genotypes or four genotypes with different biological variables (e.g., male vs. female, old vs. young). We have used the SAA to investigate the pharmacodynamics of VGAs and their pharmacogenetic interactions in two experimental fly models

associated with neuroinflammation-mitochondrial mutants and traumatic brain injury (TBI).

Introduction

The existence of collateral anesthetic effects (i.e., effects that are not immediately observable but may have delayed behavioral consequences) is generally accepted, but the understanding of their mechanisms and risk factors remains rudimentary[1,2]. Their delayed manifestation and subtleness limit the number of potentially important variables that can be investigated in mammalian models within reasonable time frames and at an acceptable cost. The fruit fly (*Drosophila melanogaster*) offers unique advantages in the context of neurodegenerative disease[3] and for toxicologic screening[4] that have, to date, not been applied to the study of anesthetic collateral effects.

We developed the serial anesthesia array (SAA) to facilitate the use of fruit flies in the study of anesthetic pharmacodynamics and pharmacogenetics. A key advantage of the SAA is the simultaneous exposure to identical experimental conditions of multiple cohorts. When paired with the experimental flexibility of fruit flies, the high throughput of the SAA allows the exploration of biological and environmental variables on a scale impossible in mammalian models.

In principle, the SAA is simply a series of connected anesthetizing locations (chambers made of 50 mL vials) through which a carrier gas delivers volatile agents. The system's first chamber contains distilled water through which the carrier gas is humidified (flies are sensitive to dehydration), and it terminates with a simple flow indicator that indicates the gas flow through the system. Fine nets placed on the openings of the connecting tubing separate the chambers to prevent the migration of flies between the chambers. The number of locations "in series" is limited by the resistance to the unpressurized gas flow (tubing, nets).

We characterized the kinetics of this SAA prototype in a previous publication[5]. Although the exact pharmacokinetic properties will vary between SAAs, the relevant basics that have been tested experimentally are as follows: (i) an initial flow of 1.5-2 L/min equilibrates all the chambers (total volume of ±550 mL) with the desired concentration of anesthetic within 2 min; (ii) the concentration of anesthetic vapor delivered to the chambers does not change appreciably between the first and the last location because the amount of anesthetic contained in the volume of gas in an individual chamber (50 mL) far exceeds the amount taken up by any number of flies; and (iii) once the chambers have equilibrated, the carrier gas flow can be reduced (50-100 mL/min or less) to avoid waste and contamination of the environment (volatile anesthetics have greenhouse gas properties). The minimal flow necessary to maintain a steady-state concentration of vapor depends primarily on the leakiness of the SAA, as the uptake of vapor by the flies is negligible. Under these standard conditions (2% isoflurane and 1.5 L/min carrier gas flow), flies are anesthetized (i.e., immobile) in all positions of the array within 3-4 min, with unnoticeable differences between positions. VGAs can be administered for minutes to hours, and our typical exposure paradigms are in the range of 15 min to 2 h. To flush the system, the vaporizer is turned off, and flow is maintained to exchange approximately 10x volumes

of the array (1.5 L/min for 5 min). The speed of anesthetic elimination will vary with the set rate of flow.

Volatile anesthetic agents interact with numerous still unidentified targets, including the immune-inflammatory system[6]. The contribution of individual molecular targets to primary versus collateral outcomes (the "anesthetic state" vs. long- and short-term "side effects") is poorly understood. Therefore, a sensitive, high-throughput fly system is valuable to inform experiments in higher animals, despite the obvious differences between flies and mammals[7]. Some differences can, in fact, be advantageous; for example, the fly's immune system differs from that of higher animals in that it lacks the adaptive arm of the response[8]. While this may seem like a limitation for understanding disease in humans, it offers a unique opportunity to study the interaction of VGAs with the innate immune-inflammatory response in isolation from the adaptive response[9]. This allows studies of the pharmacologic effects of VGA on inflammation and their modulation by the varied genetic backgrounds present in a population.

Protocol

NOTE: See the **Table of Materials** for details about all the materials used in the protocol.

1. Construction of the SAA

1. Make the frame by cutting wood and assembling the frame using the dimensions in **Figure 1A**.
2. Modify 50 mL conical tube caps.
    1. Drill two holes in each cap with a 9/32 in drill bit. Sand the holes to clean up the ragged plastic. Sand the top of the cap to roughen the surface (this helps with glue adhesion).
    2. Cut 5 mL serological pipettes to size (3 in for inflow and 1.5 in for outflow) by scoring the plastic and then breaking it clean at the scored line. Sand the ends of the cut/broken pipettes.
    3. Glue netting to the tubes (allow proper drying time for the adhesive). Cut the netting to the size of the tube after the adhesive dries.
    4. Insert the tubes into the holes of the conical caps with both tubes extending (3/4 in) above the cap; ensure that the inflow tube extends longer into the tube than the outflow (**Figure 1B**).
    5. Apply glue to the tops of the caps around the tubes to secure the parts together (allow proper drying time for the adhesive before continuing).
3. Attach the caps to the frame, and route the tubing (**Figure 1C**).
    1. Attach adhesive cable tie-downs to the frame (3.25 in apart, center to center).
    2. Affix the caps to the frame using zip ties; cut the zip tie tag ends short.
    3. Cut and connect lengths (9 in) of Tygon tubing to the inflow/outflow tubes on each modified cap (**Figure 1D**). Starting at the upstream end, attach first to the inflow, and then subsequently attach tubing from the outflow to the inflow of the next position.
    4. Add a flow indicator to the most downstream "inflow" (position 10, **Figure 1E**).
    5. Put a 50 mL conical tube on the first position, and fill it with water to just below the inflow tube (**Figure 1F**).
4. Prepare the interfacing for the vaporizer. Remove the plungers, cut notches out of two 10 mL dispensing syringes (1/2 in deep x 1/4 in wide, **Figure 1G**), and

insert them into the vaporizer inflow and outflow with the notches facing directly toward the front of the vaporizer to align with the holes (**Figure 1H**). Optional: Glue the modified syringes into place. If affordable, use a commercial manifold (see the **Table of Materials** for one option).

5. Hook up the entire system. Use Tygon tubing to attach the components together in the following order: carrier gas tank with regulator > gas specific flowmeter > vaporizer > SAA (**Figure 1C**).

6. Fill empty positions on the array with empty 50 mL conical tubes. Turn on the gas tank, open the **flowmeter** to **~2 L/min**, and turn on the **vaporizer** to **0%**. Confirm gas flow through the system by checking the flowmeter upstream of the vaporizer and the flow indicator downstream of the last chamber of the SAA for flow. Alternatively, insert the downstream tubing end into water, and look for bubbles.
NOTE: As the system is not pressurized, a water column higher than a couple of centimeters will stop the flow. If there is no flow at the downstream end of the array, check the following: the vaporizer needs to be on to allow flow; check that the tank regulator and flow meters are allowing flow; check the array positions to make sure the tubes are screwed on tightly; and check for leaks around the adhesive on the modified caps.

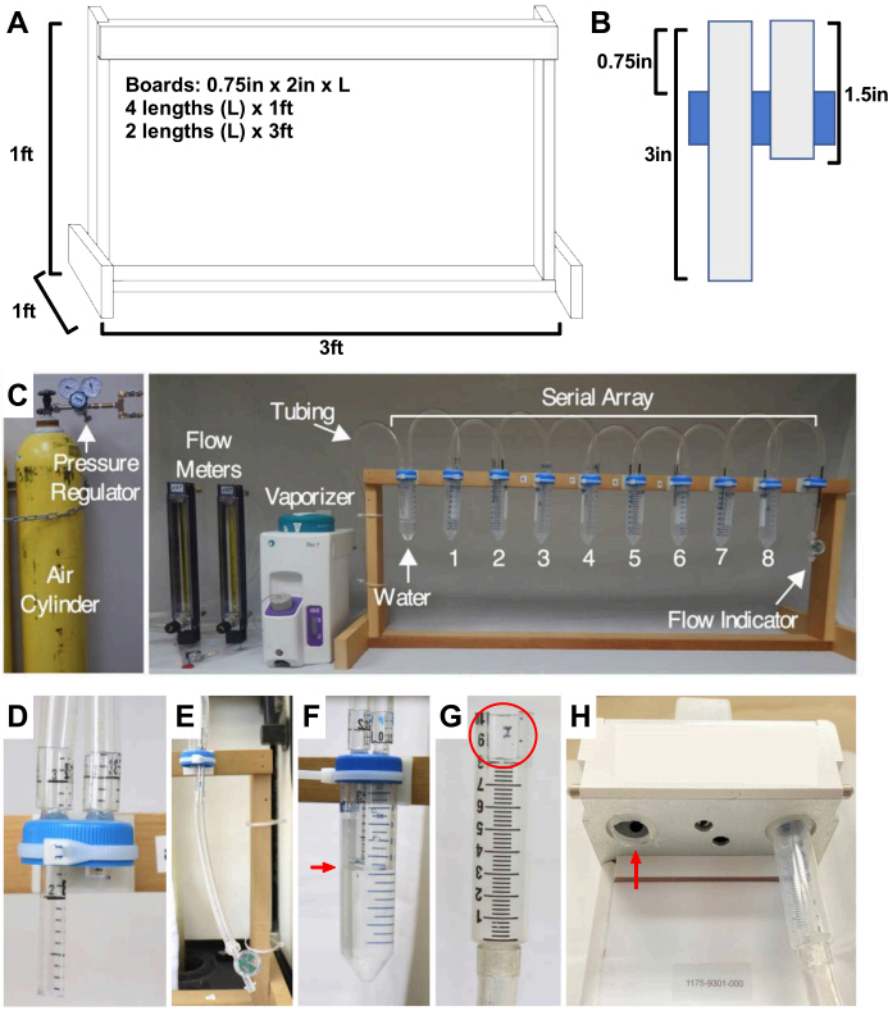

Figure 1: Construction of the SAA. (**A**) Schematic, with measurements, of the wooden frame that supports the SAA. (**B**) Schematized cross-section, with measurements, of a modified cap with inflow and outflow tubes made of 5 mL serological pipettes. (**C**) Assembled SAA (reproduced from Olufs et al.[5]) (**D**) Details of a modified 50 mL conical cap showing inflow and outflow tubes. (**E**) Downstream (position 10) outflow with the flow indicator. (**F**) Upstream (position 1) water-filled tube to humidify the carrier gas. The red arrow indicates the water level. (**G**) Modified 10 mL dispensing syringe for the makeshift manifold. The red circle highlights the cut-out notch located between the 8 mL and 10 mL marks (or 1/2 in x 1/4 in). (**H**) Rear view of the Tec7 vaporizer showing the insertion and orientation of the modified syringes. Only one syringe is in place in this view to show, on the left, the hole (red arrow) that needs to be aligned with the notch of the modified syringe. Note: Misalignment of this cut-out notch and the outflow opening will disrupt anesthetic administration. This part is a potential weak spot in this custom-made system. If funds are available, a commercial manifold should be used. Abbreviation: SAA = serial anesthesia array. Please click here to view a larger version of this figure.

2. Prior to anesthetic exposure

1. Twenty-four hours or more prior to the anesthetic exposure, sort the fly cohorts as needed for the experiment using the preferred method (e.g., CO_2 or ether).

3. Operation of the SAA

1. Transfer flies from food vials into empty 50 mL conical tubes (without CO_2).
    1. Count and record any dead flies prior to exposure.
2. Uncap and screw 50 mL conical tubes with flies onto the SAA.
3. Turn on the carrier gas, and set to the desired flow rate.
   NOTE: We typically use 1-2 L/min.
4. Set the anesthetic vaporizer to the desired concentration.
   NOTE: We typically use 2% for isoflurane and 3.5% for sevoflurane, which are equipotent doses in mammals[10].
5. Expose the flies for the desired duration (min: 15 min).
   NOTE: A minimum exposure time of 15 min is recommended to avoid possible variability in equilibration across the positions of the SAA. In this system, it takes 2-3 min for the anesthetics to equilibrate across all the positions.
6. At the end of the exposure, flush the system with fresh gas flow (vaporizer set to 0%) at 1.5 L/min for 5 min corresponding to approximately 10x volumes of the total SAA volume.

4. Checklist prior to starting an experiment

1. Open the high-pressure regulator (on top of the air tank) completely, and then close it half a turn to ensure carrier gas flow.
2. Follow tubing for each line to the i) flowmeters and ii) vaporizer (make sure the inflow/outflow are connected correctly), and iii) check the anesthetic level in the vaporizers.
3. After loading the chambers with subjects, check that the air/gas is flowing with the bubble test or the flow indicator.
   NOTE: Some vaporizers do not allow air flow when the dial is in the off position.
4. When gas is flowing, confirm that both the flowmeter and the downstream flow indicator indicate flow.
5. At the end of the experiment, allow for 4-5 min of airflow to wash out the anesthetic.

Representative Results

An SAA video link is provided here: Perouansky Research Methods - Department of Anesthesiology - UW-Madison (wisc.edu) (https://anesthesia.wisc.edu/research/researchers/perouansky-laboratory/perouansky-research-methods/) Our lab has used the SAA to (i) study the effect of genotype on behavioral sensitivity to anesthetics[5]; (ii) screen mitochondrial mutants for the collateral effects of anesthetics[11]; and (iii) investigate the pharmacodynamics of isoflurane and sevoflurane on outcomes in traumatic brain injury (TBI)[12,13,14,15,16,17]. The published results clearly demonstrate that the genetic background influences the pharmacodynamics of clinically used VGAs with respect to both the conventional phenotype of anesthesia and the

collateral effects of anesthetic toxicity, as well as tissue protection[5,11,13,14,15].

Representative example 1 (Figure 2):Genetic drift in resilience to isoflurane toxicity detected by reliably reproducible experimental conditions

The discovery of a gradual quantitative change in VGA-induced mortality among separately cultured *ND23*^{60114} flies is an example of the usefulness of reliable comparisons of anesthetic pharmacodynamics across experimental groups using the SAA. *ND23* is a gene encoding a subunit in the core of Complex I of the mETC (analogous to *Ndufs8* in mammals)[18]. Mutations in this subunit are a cause of Leigh syndrome, a lethal mitochondrial disease. We observed a gradual weakening of the isoflurane-induced mortality phenotype over time in various homozygous *ND23*^{60114} stocks cultured simultaneously under standard laboratory conditions (i.e., without exposure to VGAs). This evolutionary adaptation to isoflurane toxicity occurred in the absence of any exposure to VGAs and is probably a collateral effect of "survival of the fittest" within the mutant stocks. This gradual change in isoflurane sensitivity would have remained unrecognized without our confidence that the experimental conditions were identical across the assays and over time. We conclude that selection favors modifiers of the effects of *ND23*^{60114}, with coincidental increased resilience to isoflurane toxicity. As inflammation in the central nervous system plays an important role in the pathogenesis of Leigh syndrome, the witnessed evolution of resistance may be due to adaptive changes in the innate immune-inflammatory response, with resistance to isoflurane toxicity being an accidental byproduct.

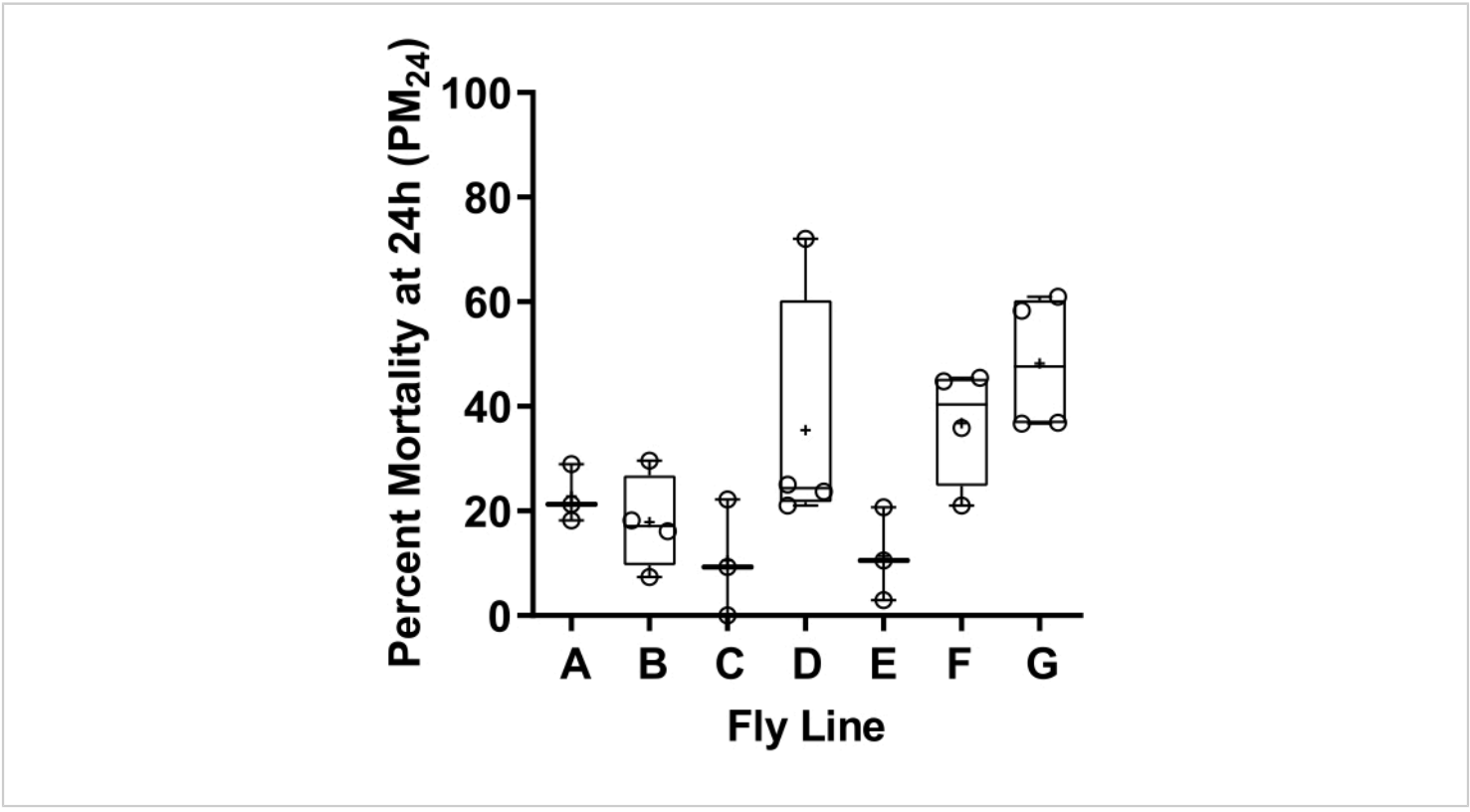

Figure 2: Variation in isoflurane toxicity-induced mortality as a result of evolutionary pressure in *ND23*^{60114} flies. Seven lines (**A-G**) isolated from a single population through single-pair matings, expanded, and tested for 24 h mortality (PM_{24}) following a 2 h exposure to 2% isoflurane (at 10-13 days old) show variability in the phenotype arising from a single population. Data shown as box and whisker plots. The boxes represent the second and third quartiles of the data, with the whiskers extending to the minimum and maximum data points. The mean and median are indicated by "+" and horizontal lines, respectively. The percent mortalities of the individual replicates (N) are shown as circles. N = 3-4 vials of 20-50 flies/vial. *P*-value for an ordinary one-way ANOVA; $p = 0.012$ indicates a significant difference among the means. Please click here to view a larger version of this figure.

Representative example 2 (Figure 3): Illustration of a high-throughput application of the SAA to reveal genetic background effects on isoflurane pharmacodynamics

As an example of the high throughput of the system, **Figure 3** illustrates the effects of identical exposures to isoflurane (15 min of 2% isoflurane) prior to traumatic brain injury (TBI)[16], a protocol testing anesthetic preconditioning (AP) in this fly model[13,15,19]. The readout is mortality 24 h after TBI corrected for natural attrition (MI_{24}). In this model, all the flies regained mobility (i.e., were alive) within 30 min after TBI, and the mortality recorded in the MI_{24} was a result of secondary brain injury (sBI). In the four fly lines, AP with isoflurane reduced the MI_{24} to various degrees, indicating that responsiveness to AP is a quantitative trait. As the inflammatory response is an important factor in morbidity from sBI, AP may involve modulation of the immune system[20].

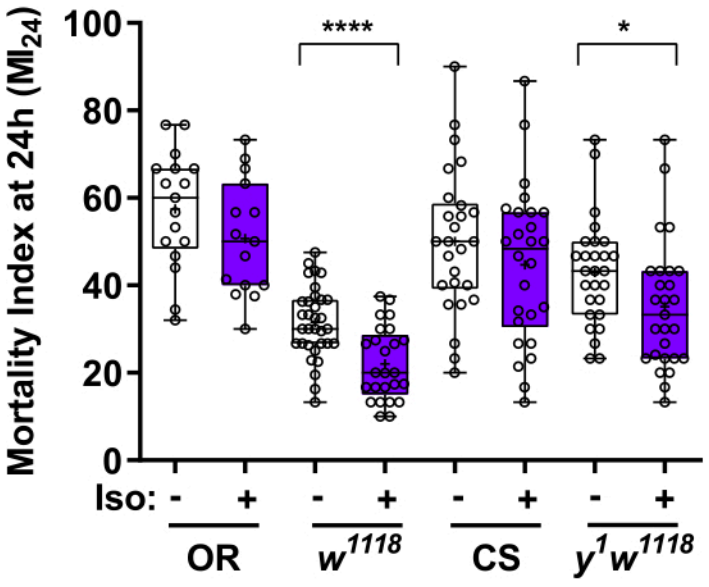

Figure 3: Influence of genetic background on the suppression of mortality (MI_{24}) by preconditioning with isoflurane. Preconditioning flies with 15 min of 2% isoflurane (purple) reduced the mortality index at 24 h (MI_{24}) in w^{1118} and y^1w^{1118} strains ($p < 0.0001$ and $p = 0.036$, respectively). The MI_{24} was not significantly lower in the preconditioned Oregon R (OR)

and Canton S (CS) lines (p = 0.16 and p = 0.27, respectively). Data shown as box and whisker plots. The boxes represent the second and third quartiles of the data, with the whiskers extending to the minimum and maximum data points. The mean and median are indicated by "+" and horizontal lines, respectively. The MI_{24} values of the individual replicates (N) are shown as circles. N = 15-33 vials of 30-40 flies/vial for TBI-treated flies. N = 2-15 vials of 30-40 flies/vial for untreated controls. *P*-values from an unpaired, two-tailed Student's *t*-test. Please click here to view a larger version of this figure.

Discussion

Critical steps in the construction of the SAA include ensuring tight fittings to avoid leakage of the anesthetic mixture of gases. The SAA must be housed in a fume hood to avoid contamination of the laboratory space. All the elements from the carrier gas cylinders to the flow indicator downstream of the SAA should be checked as outlined in the checklist.

Other methods of administering VGAs to flies are complicated to operate (the inebriometer)[21], have low throughput[22], do not allow the simultaneous exposure of multiple populations[23], do not allow precise control of the anesthetic concentration[21], or have a readout that is difficult to translate into clinically accepted terms[24].

The current version of the SAA relies on a commercial vaporizer, and hence, toxicologic studies are limited to volatile anesthetics. If used with other volatile substances, a vaporizer could be used "off label" after calibrating the output. Alternatively, a different method of vaporizing the volatile substances could be applied, which would require dedicated measurements to titrate the drug concentrations, as described previously[25].

Apart from the flow indicators, there are no alarms (i.e., if the tanks empty, the flow through the SAA will be interrupted). Depending on the intensity of the use, the SAA may need cleaning, tightening, and possibly replacement of the Tygon tubing. We have performed "maintenance" on our original SAA twice in 7 years of use.

This method for anesthetizing fruit flies allows the use of the genetic toolbox available to *Drosophila* researchers in a high-throughput system. Multiple cohorts of flies of different populations (e.g., genotype, age, sex) can be simultaneously exposed to identical anesthetic concentrations and the desired combination of carrier gas (air, O_2, N_2O, noble gases) suitable to the research question at hand.

We show here that the SAA has been useful for revealing unexpected changes in resilience to isoflurane toxicity in the *ND23*^{60114} fly line and that standard laboratory fly lines differ in their responsiveness to AP. Identifying these findings was possible because of the tight control of the experimental conditions and the high throughput of the SAA.

The SAA can be adapted to study the effects of other volatile organic compounds (VOCs) on insects (e.g., honeybees). For VOCs with vapor pressures close to those of volatile anesthetics (isoflurane: 240 mmHg at 20 °C), conventional vaporizers could be used, but the output would have to be calibrated. The commercial vaporizer for desflurane is heated, potentially offering additional flexibility.

Disclosures

The authors have no conflicts of interest to declare.

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