Video Article

Assessing Changes in Volatile General Anesthetic Sensitivity of Mice after Local or Systemic Pharmacological Intervention

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URL: <http://www.jove.com/video/51079>

DOI: [doi:10.3791/51079](https://doi.org/10.3791/51079)

Keywords: Medicine, Issue 80, Anatomy, Physiology, Pharmacology, Anesthesia, Inhalation, Behavioral Research, General anesthesia, loss of righting reflex, isoflurane, anesthetic sensitivity, animal model

Date Published: 10/16/2013

Citation: McCarren, H.S., Moore, J.T., Kelz, M.B. Assessing Changes in Volatile General Anesthetic Sensitivity of Mice after Local or Systemic Pharmacological Intervention. *J. Vis. Exp.* (80), e51079, doi:10.3791/51079 (2013).

Abstract

One desirable endpoint of general anesthesia is the state of unconsciousness, also known as hypnosis. Defining the hypnotic state in animals is less straightforward than it is in human patients. A widely used behavioral surrogate for hypnosis in rodents is the loss of righting reflex (LORR), or the point at which the animal no longer responds to their innate instinct to avoid the vulnerability of dorsal recumbency. We have developed a system to assess LORR in 24 mice simultaneously while carefully controlling for potential confounds, including temperature fluctuations and varying gas flows. These chambers permit reliable assessment of anesthetic sensitivity as measured by latency to return of the righting reflex (RORR) following a fixed anesthetic exposure. Alternatively, using stepwise increases (or decreases) in anesthetic concentration, the chambers also enable determination of a population's sensitivity to induction (or emergence) as measured by EC₅₀ and Hill slope. Finally, the controlled environmental chambers described here can be adapted for a variety of alternative uses, including inhaled delivery of other drugs, toxicology studies, and simultaneous real-time monitoring of vital signs.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51079/>

Introduction

General anesthetics are defined by their ability to cause a reversible state of hypnosis in a wide variety of species, yet an explanation as to how such a diverse class of drugs can all elicit a singular endpoint remains elusive. A number of theories have been posited over the years, starting with the Meyer-Overton correlation between anesthetic potency and lipid solubility, which suggested general membrane disruptions as the basis for hypnosis^{1,2}. More recent evidence suggests that protein targets affecting neuronal signaling contribute to anesthetic effects. Mice have proven to be an indispensable model for exploring these theories because of the homology between murine and human anesthetic responsiveness. Though a mouse cannot be asked about its subjective awareness under general anesthesia, certain primitive reflexes serve as useful surrogate measures of rodent hypnosis. In the first few days following birth, mice develop a reflexive righting response that prevents them from being passively placed in a supine position³. The dose of anesthesia at which a mouse loses its righting reflex correlates well with human hypnotic doses⁴.

Assessment of loss of righting reflex (LORR) has become a widely used laboratory standard for testing anesthetic sensitivity in mice as well as a variety of other species including rat, guinea pig, rabbit, ferret, sheep, and dog⁵⁻⁸. The dose of a given anesthetic at which LORR will occur for members of a species is extremely consistent, but it can be shifted significantly by environmental factors. For example, sleep-deprived rats are more sensitive to both volatile and intravenous anesthetics⁹ and rats with high aerobic capacity are less sensitive to isoflurane¹⁰. Hypothermia has also been shown to decrease the dose of numerous anesthetics required for hypnosis in a large spectrum of species¹¹⁻¹⁴. In order to reliably identify the anesthetic dose at which LORR occurs in a group of experimental animals, it is critical that the assessment environment be carefully controlled to minimize stress, maintain euthermia, and deliver equal amounts of drug to all subjects. Not surprisingly, genetic factors are also known to alter anesthetic sensitivity¹⁵⁻¹⁸. Consequently, careful consideration should also be given to controlling for genetic background¹⁹.

We have developed an apparatus that ensures identical gaseous anesthetic delivery to each of 24 mice while maintaining a constant 37 °C environment. The transparent cylindrical design of our exposure chambers allows for fast LORR assessment and easy integration of telemetric physiological measurements. This system has been shown to accurately measure isoflurane, halothane, and sevoflurane induction EC₅₀ and time to emergence in wild-type mice²⁰. We have also used this system to observe changes in anesthetic sensitivity in mice with genetic

mutations and targeted hypothalamic lesions²¹⁻²³. Here we describe two ways in which anesthetic sensitivity may be assessed after a pharmacological intervention using our controlled environment apparatus. Steady-state phenotyping of volatile anesthetic induction and emergence sensitivity requires 8-10 hours and is consequently best tailored for studies in which experimental conditions do not change, such as in chronic or long-acting pharmacological interventions. However, for short-acting treatments whose effects dissipate significantly over time we also present a simple procedure to evaluate changes in righting reflex following stereotactically-targeted microinjections or intravenous drug treatments that significantly impact anesthetic emergence. These tests represent a small subset of the potential applications for this controlled environment system, which could be adapted for any number of subjects of a variety of species to receive any type of inhaled therapeutic.

Protocol

All procedures involving animals outlined herein have been approved by the University of Pennsylvania's Institutional Animal Care and Use Committee.

1. Overview of the Testing Apparatus

1. The testing apparatus consists of 24 clear acrylic cylindrical chambers 10 cm in length and 5 cm in diameter (total volume of 200 ml). This size is appropriate for a typical 25 g adult mouse. Chambers have ports at each end for gas inlet and outlet. The outlet end is removable so that animals may be easily loaded into the chamber. Gas port openings are carefully sealed with Teflon tape, while rubber o-ring gaskets are used to seal the removable end of the cylindrical chambers.
2. Each chamber is mounted on a rack that sits inside a water bath. The rack is fitted so that only the lower portion of the chambers (below the gas inlets) is submerged. For stability, the back end of the chamber rests on a support so that the entire chamber sits horizontally. This ensures even contact of the entire chamber with the bath.
3. Polyethylene tubing connects an oxygen tank to an anesthetic vaporizer, and then passes through a 10 L/min flow meter. The tubing splits into 25 small-diameter resistors of equal length to ensure equal flow is delivered to each of the 24 chambers and to an agent analyzer.
4. Vacuum lines exit each chamber at the opposite end of the gas inlet. This promotes unidirectional flow that eliminates rebreathing of exhaled carbon dioxide. The vacuum lines combine at a manifold to connect to an in-house suction line. A pop-off valve along the main vacuum line ensures atmospheric pressure conditions within each chamber.
5. The bath is filled with enough water to fully contact the bottom of each chamber. The water is circulated through the bath and maintained at a constant 37 °C by a pump.

2. Check the System Prior to Exposure

1. Check that the temperature of the water bath is 37 °C throughout the bath.
2. Flow oxygen at a rate of 5 L/min (200 ml/min per chamber + agent analyzer). Submerge each chamber under water and look for bubbles or entry of water into the chamber, both of which are indicative of leaks. Seal any leaks before beginning the experiment.
3. For each chamber, connect a 500 ml/min flow meter in line after the chamber to make sure that flows are balanced across each of the 25 gas lines. This ensures that the input 5 L/min flows will be distributed evenly so that each chamber receives 200 ml/min flow. Any chamber not receiving the expected flow should have its inflow and outflow tubing checked for obstructions.
4. Calibrate the agent analyzer to ensure a reading of 0.00% isoflurane when 100% oxygen is flowing.

3. Implant Temperature Transponder

1. One week prior to habituation, anesthetize each mouse with 2% isoflurane.
2. Sterilize the dorsal neck area with betadine.
3. Inject a temperature transponder subcutaneously between the shoulder blades using the sterile, prepackaged injector needle.
4. Monitor the injection site daily for infection and migration of the transponder.

4. Habituate Animals to Testing Chambers

1. Four days before the first assessment, place all mice into individual chambers for 2 hr with 100% oxygen flowing.
2. Repeat step 4.1 daily for the four days prior to assessment to avoid the confounding effects of stress due to a new environment.

5. Perform the Pharmacological Intervention that You Wish to Test for Effects on Anesthetic Sensitivity

1. This intervention may be a stereotaxic injection into a specific part of the brain²⁴, an intravenous or intraperitoneal injection²⁵, or delivery of a drug to a specific brain area via cannula²⁶.
2. Because these procedures themselves may change anesthetic sensitivity compared to a naïve animal, a proper control group should undergo the same procedure with vehicle injections.
3. Ensure that the pharmacological intervention has an appropriately long duration of action if you are planning to do a stepwise increasing and/or decreasing determination of anesthetic sensitivity as shown in step 6 below; otherwise, skip to step 7.

6. Assess Anesthetic Sensitivity using the Stepwise EC₅₀ Determination for Induction and Emergence

1. Place each animal into individual chambers with 100% oxygen flowing.
2. Set the isoflurane concentration to 0.4%* for 15 min. During the last 2 min of this period, assess each animal's righting reflex by gently rolling the chamber until the mouse is placed on its back. The righting reflex is considered to be intact if and only if the mouse is able to restore all of its paws to the floor of the chamber within 2 min.
 1. *Note that 0.4% isoflurane is a subhypnotic dose in C57BL/6J mice. If any mice lose their righting reflex at the first step, the initial dose was too large and should be reduced on subsequent days.
3. Record the state of righting reflex for each mouse and scan each mouse for temperature data. A template record is shown in **Table 1**.
4. Increase the isoflurane concentration by ~0.05% for 15 min and repeat step 7.2. Continue to do this until all animals have lost their righting reflex.
5. Optional: repeat the same procedure for decreasing step-wise isoflurane doses until all animals have regained their righting reflex (see step 6.3).
6. To end the experiment, turn off the isoflurane and flush the entire system with 100% oxygen for 15 min. This will help to prevent hypoxia as the mice recover before being returned to their home cages and will protect the experimenter from any anesthetic exposure.
7. Optional: if the number of animals or the number of anesthetic concentrations are limited due to resource or time constraints, the curve-fit parameter estimates-particularly the Hill slope-may have underappreciated, falsely low error estimate. In such cases, it may be necessary to repeat the anesthetic sensitivity measurement described in steps 6.1-6.6 on up to two additional experimental days to fully obtain the true Hill slope's parameter and its corresponding error estimation.

7. Assess Short-term Changes in Anesthetic Sensitivity with Time to Emergence

1. Place each animal into individual chambers with 100% oxygen flowing.
2. Set the isoflurane concentration to 1.2%, which corresponds to the induction ED₉₉ for wild-type C57BL/6J mice²⁰. Maintain for 30-60 min depending upon the expected duration of action of the acute intervention.
3. Confirm LORR in all animals by gently rolling each chamber until mice are placed on their backs.
4. Turn off isoflurane and flow 100% oxygen. Measure the time until each animal regains its righting reflex. This is defined by placement of all four paws on the floor of the chamber and confirmed by the presence of three consecutive tests with an intact righting reflex.

Representative Results

Figure 1 demonstrates the utility of the step-wise LORR assay for determining long-term effects of a pharmacological intervention. Ibotenic acid (IBA) is an agonist of the glutamatergic N-methyl-D-aspartate (NMDA) receptor that is often used as an excitotoxin to cause permanent neuronal lesions. Here we injected 10 nl of 1% IBA bilaterally into the ventrolateral preoptic area (VLPO) of C57BL/6J mice one week prior to testing. The majority of neurons in this nucleus exhibit low rates of firing during wakefulness and specifically increase their activity during non-rapid eye movement sleep, rapid eye movement sleep, and with exposure to hypnotic doses of general anesthetics^{23,27-29}. Successful lesions in the VLPO should cause resistance to isoflurane-induced hypnosis. At each increasing level of isoflurane, the fraction of mice that had lost the righting reflex was plotted against anesthetic concentration on a log₁₀ scale. Data for each group of mice (vehicle-injected and IBA-injected) was then fit with a sigmoidal dose-response curve. Because this assay always starts with all animals upright and always ends with all animals having lost the righting reflex, bottom and top constants were constrained to 0 and 1, respectively. The remaining free parameters of the curves are the EC₅₀, or the concentration of anesthetic at which 50% of mice have lost their righting reflex, and the Hill slope, which reflects the population variance during their hypnotic state transition. An F-test was used to query whether a single induction curve with shared EC₅₀ and Hill slope parameters best fit both vehicle and IBA groups or whether separate induction curves with distinct parameters better fit the data. The degrees of freedom in this test arise from the raw data points underlying the curve fit and consequently depend upon the number of anesthetic concentrations tested and the number of parameters being fit-EC₅₀ and Hill slope in this case. Step-wise emergence data were analyzed and modeled identically to data for induction. Note that the EC₅₀ for emergence is almost always lower than that of induction due to anesthetic hysteresis also known as neural inertia³⁰. Contrary to anticipated results, animals that received IBA in the VLPO showed no significant differences in the EC₅₀ or Hill slope for induction or emergence compared to vehicle-injected controls ($F_{2,80} = 1.73$ and $p = 0.184$ for induction, $F_{2,88} = 2.89$ and $p = 0.061$ for emergence). This indicates that mouse VLPO neurons are resistant to lesion with 1% IBA, a fact confirmed with post-mortem histology (not shown). Lu *et al.* have previously demonstrated that a dose of 10% IBA is required to lesion the rat VLPO³¹, but histological examination of the mouse VLPO following injection of 10% IBA also showed no significant cell loss (not shown). The rat VLPO is known to express NMDA receptors³². Since 10% IBA is able to exert an acute effect on anesthetic sensitivity when injected into the VLPO (see **Figure 2**, discussion below), this argues that the mouse VLPO must also possess the NMDA receptors necessary for IBA's actions. Thus the reason for the discrepancy between species remains unclear. Successful mouse VLPO lesions have been achieved using a targeted galanin-saporin²³.

Though IBA does not have a long-term effect on isoflurane sensitivity when injected into the VLPO, the acute excitatory nature of this drug would be expected to stimulate VLPO neurons and transiently increase anesthetic sensitivity. In **Figure 2**, we have used the time to emergence test to demonstrate a large acute shift in isoflurane sensitivity immediately following bilateral IBA microinjection into the VLPO as evidenced by markedly prolonged hypnosis after cessation of anesthetic delivery ($p < 0.001$). Conversely, microinjection of IBA into the nearby medial septum caused no change in time to emergence compared to vehicle-injected controls ($p > 0.05$). This finding adds an interesting facet to previous work

showing that inactivation of this nucleus extends time to emergence^{33,34}. Data for experimental and control groups in the time to emergence test was averaged and compared with a one-way ANOVA.

Time	Isoflurane (% atm)	Mouse #1	Mouse #2	Mouse #3	...
12:00 PM	0.4	-	-	-	-
12:15 PM	0.45	-	X	-	-
12:30 PM	0.5	-	X	X	-
12:45 PM	0.55	-	X	X	-
...	0.6	-	X	X	X

Table 1. Example of a Log Sheet for Long-term Anesthetic Sensitivity Assessment: Every 15 min the anesthetic dose was increased by 0.05% and righting reflex was assessed for each animal. "X" denotes animals that had lost their righting reflex for a given time point and "-" denotes those that maintained their righting reflex.

Figure 1. Assessment of the Righting Reflex One Week After Ibotenic Acid Injection in the Ventrolateral Preoptic Nucleus: The long-term anesthetic sensitivity assay was conducted on mice with either vehicle or ibotenic acid (IBA) injected into the ventrolateral preoptic area (VLPO) one week prior to testing. Induction and emergence data for each group was fit with a sigmoidal dose-response curve (induction in solid lines, emergence in dashed lines) along with the 95% confidence interval bracketing the best-fit curves (shaded bars). Anesthetic concentration was plotted on a $\log_{10}$ scale. Overlapping 95% confidence intervals are shown in purple. The sigmoidal dose-response fits for both vehicle and IBA groups suggest no evidence for distinct best-fit curves based on EC_{50} and Hill slope. [Click here to view larger image.](#)

Figure 2. Time to Emergence After Local Microinjection of Ibotenic Acid: Immediately prior to assessment, mice received a microinjection of the N-methyl-D-aspartate (NMDA) receptor agonist ibotenic acid (IBA) into the ventrolateral preoptic nucleus (VLPO). This area is known to be activated during isoflurane-induced hypnosis. IBA injection led to an acute increase in the time to return of righting reflex compared to vehicle-injected controls ($p < 0.001$). Time to emergence for animals with IBA injected into medial septum did not differ from controls ($p > 0.05$). [Click here to view larger image.](#)

Discussion

Though assessment of LORR in a single mouse is a seemingly straightforward task, it is nevertheless essential to maintain identical physiological conditions between subjects in order to collect reliable data from a group of animals. The tightly regulated, high-capacity LORR

apparatus presented here offers a way to standardize experiments and maximize efficiency. By following the basic tenets of thermoregulation and equal flow distribution, this system can be easily recreated and customized to fit individual experimenters' needs. Chamber size may be scaled for other species, such as rats, and additional chambers may be accommodated by attaching more branch points to the inflow and vacuum. All subjects are easily visible through the clear acrylic chambers, which makes it possible to video record experiments for secondary post-hoc confirmation of results. The acrylic is also compatible with radio frequency telemetry systems, which can be used to monitor temperature, blood pressure, and biopotentials.

We present two different methods for assessing anesthetic sensitivity following a pharmacological intervention. Both the time to emergence and the stepwise induction tests require the experimenter to score the presence or absence of the righting reflex. Even with an explicit definition of LORR, such as "unable to place all four paws on the chamber floor within two minutes of being rolled onto its back", assessment can be somewhat subjective. It is best to have the same treatment-blinded individual score each animal for the duration of the experiment to ensure consistency. When choosing which test to use for anesthetic sensitivity assessment, the anticipated length of the effect from the pharmacological intervention should be the deciding factor. Many drugs have a short duration of action, for which the acute time to emergence paradigm can provide useful information on anesthetic sensitivity in a limited period of time. However, a drug may preferentially affect an animal's sensitivity to induction of hypnosis rather than emergence; changes in time to induction are often difficult to detect because induction occurs rapidly and thus requires continuous assessment. The longer stepwise test for EC_{50} of induction and emergence can give information on both entrance to and exit from hypnosis. The total length of the experiment will depend on the size of the increment by which anesthetic concentration is altered at each step, with typical induction + emergence tests lasting about 8 hr. Decreasing the anesthetic step size around the anticipated EC_{50} and increasing the number of animals in each group will give a better fitted dose-response curve but would also lengthen the time required to complete the assay.

Some pharmacological interventions may differentially alter the minute ventilation of experimental animals when compared to their controls. This could cause one group to exhale the volatile anesthetic in the time to emergence test more quickly than the other, thus confounding the results.

Solt *et al.* describe a good alternative method for testing anesthetic sensitivity in this scenario³⁵. In their experiment, systemic methylphenidate is delivered during constant isoflurane exposure in animals that have already equilibrated with anesthetic. Potential confounding effects on the minute ventilation are thus excluded during continuous anesthetic exposure as anesthetic uptake and distribution during steady-state conditions are precisely balanced by metabolism and elimination. The chambers we describe could be easily modified with an additional gas-tight port to allow passage of tubing for intravenous or intracerebral drug delivery. It should also be noted that the described 15 min of equilibration to each concentration of anesthetic in the step-wise assay might not be sufficient in certain cases. Anesthetics with a higher solubility than isoflurane, such as halothane, will take longer to reach their full concentrations in the tissue. Larger animals and animals that undergo larger steps in anesthetic concentration may also require more time to equilibrate. To determine if 15 min is truly adequate for equilibration, anesthetic tissue levels at the same concentration of anesthetic on both the ascending and descending limbs of exposure should be measured.

In cases where an animal's ability to move is physically or pharmacologically hindered, LORR may not serve as a good surrogate measure of hypnosis. The most reliable and widely used alternative is cortical electroencephalographic (EEG) recordings. Though EEG may be better able to pick up more subtle changes in anesthetic sensitivity, it is significantly more expensive to set up than the apparatus we describe. Implanting EEG electrodes is an invasive and time-consuming procedure, and the ability to obtain data from multiple mice simultaneously is often limited by equipment availability. Moreover, analysis of EEG recordings is conceptually more abstract and difficult to interpret than the simple binary output of LORR assessment. For these reasons, behavioral tests like those described here are often more feasible methods for rapidly screening anesthetic sensitivity. Note that EEG patterns suggestive of arousal and hypnosis may not correlate well with behavior. LORR and EEG are distinct endpoints which both likely provide useful information regarding anesthetic sensitivity.

In addition to potential drug-induced changes in minute ventilation and mobility, there are several other limitations to the methods described herein. Though LORR is a standard surrogate for hypnosis across the field, the criteria and methodology used for its measurement differ across laboratories. Some advocate that mice should be rotated at a constant speed to assess the righting reflex. Continuous assessment logically narrows the precise timing with which the righting reflex is lost and/or returns; however, the act of being turned supine may be more stimulating than simply remaining supine. In addition, step-wise LORR assessment is a time-consuming assay that may be further extended if 15 minutes of equilibration at each step is found to be insufficient.

Despite these limitations, the potential applications for this protocol extend far beyond the specific instances we have presented. Clearly, pharmacological interventions are not the only method by which anesthetic sensitivity might be altered; targeted lesions, anatomic abnormalities, and genetic mutations may all be tested using the same stepwise EC_{50} determination. The controlled environment system presented here can be used to deliver any kind of inhaled drug, such as corticosteroids, antibiotics, or experimental therapeutics. The ability to expose many mice to the same amount of drug at once makes this setup ideal for toxicology studies. Additionally, chambers serve as an ideal post-surgical recovery environment with regulated ambient temperature and fresh oxygen flow. This apparatus is useful for any instance in which basic animal vital signs need to be monitored and controlled.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work was supported by R01 GM088156 and T32 HL007713-18. We would like to thank Bill Pennie and Michael Carman from the University of Pennsylvania Research Instrumentation Shop for their help in assembling our righting reflex apparatus.

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