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ORIGINAL RESEARCH ARTICLE

Isoflurane is an effective alternative to ketamine/xylazine/acepromazine as an anesthetic agent for the mouse electroretinogram

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Abstract The electroretinogram (ERG) is an essential measure of retinal function for studying mouse models of retinal disease. Ketamine, in combination with xylazine and/or acepromazine, is the most commonly used anesthetic agent. Although it works well in most situations, some fragile mouse strains have high mortality rates with this ketamine cocktail. We compared isoflurane with the ketamine cocktail in a longitudinal study of light-adapted and dark-adapted ERGs in *C57BL/6J* mice. Waveforms were averaged, oscillatory potentials (OPs) were extracted by digital filtration, and key ERG parameters were analyzed. The ERG waveforms were qualitatively similar with both anesthetics, and the male and female ERG parameters did not show significant differences. For light-adapted ERGs, b-wave amplitude and implicit time, and wavelet index were decreased under isoflurane anesthesia, whereas for dark-adapted ERGs, a- and b-wave implicit times were decreased and wavelet index was increased. The dark-adapted b-wave amplitude showed a significant inverse correlation with animal weight and age. Rod phototransduction gain and the Naka–Rushton n and R_{max} parameters were the same for both anesthetics, and only the Naka–Rushton log k parameter was significantly elevated for isoflurane anesthesia. We propose that isoflurane is a satisfactory alternative to the ketamine cocktail for anesthesia in the mouse ERG. Precise quantitative comparisons, however, should only employ study designs using isoflurane versus isoflurane, or ketamine versus ketamine. Moreover, in light of the effects of both isoflurane and the ketamine cocktail on blood glucose levels, it would be prudent to control the fasting state of the animals in quantitative ERG studies.

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Abbreviations
ERG Electroretinogram
OPs Oscillatory potentials

Introduction

The electoretinogram (ERG) is an essential clinical tool for assessing retinal function. In addition to its utility in defining retinal physiology, it is also useful in establishing clinical diagnoses when normal physiology is perturbed. Abnormal retinal function may occur as a result of injury, illness, or iatrogenic causes, such as drug exposure. Our interest and expertise in the ERG has been in its usefulness in diagnosing inherited retinal defects [1–3].

The principle behind the ERG is simple. A light stimulus of varying intensity, duration, or spectrum evokes a sequential response that delineates the function of the neural retina. The pupil is dilated, anesthetic drops are placed in the eye to allow the comfortable placement of a corneal electrode embedded in a contact lens, and reference and ground electrodes are placed. In the case of the dark-adapted ERG, an interval of dark-adaptation precedes the testing. In the cooperative individual, no other interventions are needed. However, if the subject cannot cooperate, such as the young child or an individual with developmental delay, sedation or anesthetics are needed to perform the examination.

With the explosion of mouse models for retinal disease over the past decade, the transition of the ERG from clinical diagnostic tool to laboratory method has provided remarkable opportunities to understand basic mechanisms of retinal function [4–8]. The mouse ERG does not differ in its substantial elements from the clinical ERG. What does differ, however, is the need for continuous anesthesia.

The current standard for anesthesia for the mouse ERG is the subcutaneous injection of ketamine, either alone or in combination with other agents such as acepromazine and xylazine [4, 9–11]. The application of this form of anesthesia can be cumbersome, especially if performed in the dark for dark-adapted ERGs or if re-dosing is required due to the duration of testing. The use of ketamine is also complicated by a prolonged recovery period, perhaps due to the inclusion of xylazine in the anesthetic cocktail. In especially fragile mouse models, such as those with inherited muscular dystrophies, there can be considerable morbidity and mortality amongst study subjects. In our experience, we have observed loss rates as high as 50% in a given experiment (unpublished observation). Given the expense and care involved in the breeding and maintaining of colonies of mutant mice, we were motivated to find an alternative method of anesthesia. Our goals were to provide a safe and effective method in which induction and recovery times are brief, the animal preparation is stable, with a consistent level of anesthesia, and which is easy to employ.

In this study, we compared ERGs performed using either isoflurane or ketamine under both light-adapted and dark-adapted conditions. We have examined the effects of gender, age, and weight on commonly measured parameters of the ERG. Our results suggest that the use of isoflurane anesthesia does not substantially alter the parameters for the mouse ERG and that it is a reasonable substitute for ketamine anesthesia.

Material and methods

Animals

Light-adapted and dark-adapted electroretinograms (ERGs) were studied in two cohorts of eight, age-matched *C57BL/6J* mice, consisting of four males and four females selected at random. The mice were obtained from The Jackson Laboratory (Bar Harbor, Maine) at 4 weeks of age and allowed to acclimatize in the Department of Comparative Medicine facility for 2 weeks prior to initiation of the study. The animals were maintained on a 12:12-h light–dark cycle in the Department of Comparative Medicine facilities and allowed free access to food and water. For the ERG studies one cohort received isoflurane inhalation anesthesia, whereas the other cohort was anesthetized by subcutaneous injection of a "ketamine cocktail" (see details below). Light-adapted and dark-adapted ERGs were collected in both cohorts of animals at 3–5 week intervals, with a 48-h recovery period between the light-adapted and the

dark-adapted ERG recordings. Handling and use of the animals conformed to the guidelines and policies on the ethical use of animals established by the National Institutes of Health (NIH), Oregon Health & Science University (OHSU), and the Association for Research in Vision and Ophthalmology (ARVO).

Anesthesia

Mice in the ketamine cohort were anesthetized with a subcutaneous injection of approximately 3.7 ml/kg body weight of a cocktail containing ketamine (13.2 mg/ml), xylazine (1.5 mg/ml) and acepromazine, (0.29 mg/ml) in sterile water, hereafter referred to as the "ketamine cocktail." Once the animals were sedated, they were transferred to the warming platform in the ERG apparatus (Fig. 1) and fitted with the nose cone described below. A steady flow of oxygen (flow rate: 0.9 LPM) was maintained via the nose cone for the animals anesthetized with isoflurane as well as the ketamine cocktail in order to control for the effects of oxygen delivery during the isoflurane anesthesia (Fig. 2). This level of ketamine anesthesia provided a stable animal preparation for the duration of both the light-adapted and the dark-adapted ERG testing. The light-adapted ERG testing lasted 22 ± 5 min (mean ± SD; max: 62; min: 10), whereas the dark-adapted ERG testing lasted 30 ± 3 min (max: 69; min: 17). Upon completion of an ERG test, the animals were removed from the ERG apparatus and were maintained on a heating pad until they were awake and mobile.

In the isoflurane cohort of animals, anesthesia was induced and maintained by inhalation of 2% isoflurane vapor (Isoflurane Vaporizer; Vaporizer Sales and Services, Rockmart, GA) in oxygen (flow rate: 0.9 LPM) in a holding chamber. Once the animals were sedated, they were transferred to the ERG apparatus, and isoflurane vapor was delivered via a specially constructed nose cone (Fig. 1). The nose cone consisted of a concentric outer tube (Tygon or Silastic; i.d. ~9 mm), which vented the expired air to an isoflurane scavenging cannister (Omnicon f/air; A.M Bickford, Wales Center, NY) and an inner, polyethylene tube (i.d. ~2 mm) which delivered the isoflurane/oxygen mixture from the vaporizer. Inasmuch as rodents are obligate nose breathers, this arrangement provided a stable, anesthetized animal preparation for the duration of the test, without interfering with the collection of ERG signals. Upon completion of the ERG recording, the isoflurane anesthesia was discontinued, and the animals were

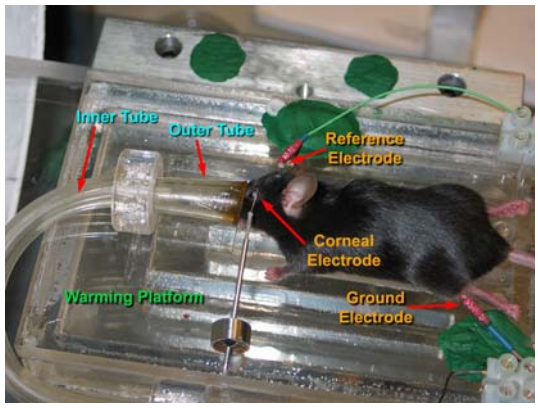

Fig. 1 Experimental Setup. The mouse is lying prone on the *Warming Platform*. The platform is maintained at 37°C using a circulating water bath. The anesthetic gas (or only O_2 if the animal is anesthetized with the ketamine cocktail) is delivered via an *Inner Tube* and administered to the animal through an *Outer Tube* which fits snugly over the animal's snout. The *Outer Tube* also serves to remove expired air, which then passes through a scavenging cannister to remove any isoflurane. The *Corneal Electrode*, which consists of a contact lens with an embedded platinum wire loop-electrode, is placed on the left eye. A *Reference Electrode* is inserted into the scalp and a *Ground Electrode* in the left hind limb. The *Warming Platform* is mounted on a sliding stage that can be positioned either in the center of the Ganzfeld apparatus for ERG testing or outside the apparatus for preparation of the animal

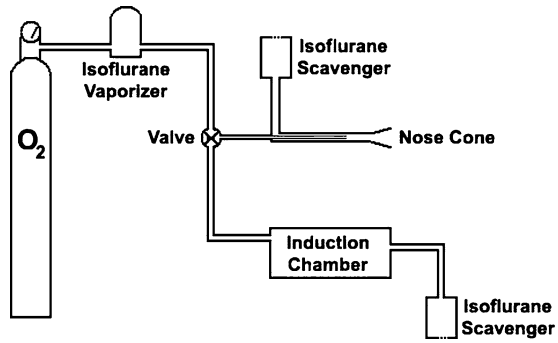

Fig. 2 Schematic of the Isoflurane Anesthesia Setup. A schematic diagram of the flow pattern of anesthetic gas for the mouse ERG. A 2% mixture of isoflurane vapor in oxygen flowing at a rate of 0.9 LPM can be directed either to an induction chamber, or to a nose cone on the ERG warming platform (see Fig. 1). Both the induction chamber and the nose cone are exhausted through scavenging canisters to remove isoflurane vapors before venting the air back into the general circulation

returned to cages with fresh bedding as soon as they were awake and mobile.

Preparation of the animals for electroretinography

For the light-adapted ERG studies, the mice were light adapted for at least 30 min prior to testing with 30 cd/m^2 incandescent illumination, whereas for the dark-adapted ERG studies, the mice were dark adapted for at least 4 h in a specially constructed light-tight box with a filtered air flow of 3 LPM.

Following induction of anesthesia, a drop of tropicamide (1%) was applied to the left eye to dilate the pupil, followed by a drop of the topical anesthetic, proparacaine (0.5%), to provide corneal analgesia. A teflon-coated platinum wire electrode (200 μm diameter wire coiled into a 1.0 mm loop with the teflon coating removed; Sigmund Cohn, Mount Vernon, NY) embedded in a cellulose acetate contact lens (1.5 mm diameter) was placed on the cornea with the aid of the demulcent, methylcellulose (2.5%).

Electroretinographic testing

The anesthetized mice were placed on a thermo-regulated, water-bath platform and positioned in the center of a custom-made Ganzfeld stimulator. During light-adapted ERG testing, data were collected with a background light intensity of 30 cd/m^2 generated by a LED panel consisting of a 5×5 array of white LEDs (Radio Shack) mounted on the surface of the Ganzfeld globe. The illumination intensity was adjusted by a potentiometer and monitored continuously by a photometer (350 Linear/Log Optometer; UDT Instruments, Baltimore, MD). A Grass Photostimulator (PS22, Quincy, MA) delivered 10 μs flashes, and neutral density filters were used to control flash illumination intensities, which were varied over a 4-log unit range (–3.2 to +0.8 log cd·s/m^2) for dark-adapted ERGs and over a 2-log unit range (–1.6 to +0.8 log cd·s/m^2) for light-adapted ERGs. The responses from the photostimulator included stimuli that conformed to the 1999 ISCEV standard [12]. For dark-adapted ERGs the interval between flash presentations for the lowest intensities was 5 s and for intensities greater than –0.47 log cd·s/m^2 the interval was increased with increasing flash intensity from 7 to 30 s. The inter-flash interval was 5 s for all the light-adapted ERG flash presentations. ERG responses were averaged for multiple flashes at each intensity level, and the ERG parameters for a given intensity level were calculated from the averaged waveform.

The ERG signal was recorded differentially between the corneal electrode and a platinum needle scalp electrode (Grass Instruments. Quincy, MA). A subcutaneous, left hind-limb platinum needle electrode served as the animal ground. The ERG signal was amplified (×1,000) by a Grass AC-coupled Amplifier (P511) with the 6 dB cutoff filters set at 0.1 Hz and 3 kHz. The amplifier output was digitized using an analog-to-digital conversion board (GW Instrument, Sommerville, MA) in a MacIntosh computer and custom software (ERGTool, developed by R. G. Weleber). The signal was recorded from the corneal electrode for a 128 ms epoch for light-adapted ERGs and for a 256 ms epoch for dark-adapted ERGs. After a 20 ms baseline recording, the software triggered a single flash from the photostimulator. The computer recorded 512 data points for each type of ERG recording, and each data point was the average of 10 data samplings. The oscillatory potentials (OPs) were extracted from the dark-adapted ERGs by digitally filtering the averaged waveform using a bandpass of 100–300 Hz and cursors placed on the major OPs in a 5–75 ms window after the stimulus presentation. The following ERG parameters were measured from the averaged waveform data at a given flash intensity: the a-wave amplitude and implicit time, the b-wave amplitude and implicit time; and for oscillatory potentials: the OP_3 implicit time (implicit time for the third and largest oscillatory potential wavelet), wavelet 2, 3 and 4 amplitudes, and the wavelet index (the summed amplitude of wavelets 2–5). The b-wave amplitudes were measured from the peak of the a-wave to the peak of the b-wave, after removal of the OPs, and implicit times were measured from the stimulus onset to the peak response.

Statistical analyses

The descriptive analysis and the non-linear curve fitting was performed in Prism 4 for Windows (GraphPad Software). We used a mixed-effects model for building multivariate models by using the

R statistical language [13]. The mixed-effects model can handle potential correlations of repeated measurements of a subject over time.

Results

General results

Weight gain

The animals were about 6 weeks old at the time of the initial ERG studies: the males weighed 18.3 ± 1.6 gm (mean ± SD) and the females weighed 16.1 ± 0.8 gm. The males gained weight more rapidly than the females until about 120 days, at which time the males weighed 28.0 ± 2.4 gm, whereas the females weighed 21.5 ± 0.9 gm. Thereafter the rate of weight gain by the animals slowed such that by 280 days the males weighed 31.8 ± 2.8 gm, and the females weighed 24.0 ± 1.3 gm.

Recovery from anesthesia

There was a significant difference in the time required for the ketamine-anesthetized animals to awaken from anesthesia compared to the isoflurane-anesthetized animals. The ketamine-anesthetized animals required 36.1 ± 9.6 min (mean ± SD) after completion of the ERG study before they were awake and freely mobile. During this time the animals were placed on a warming pad, and one of the investigators would monitor the animal until awake and mobile for any signs of distress. In contrast, animals anesthetized with isoflurane rapidly awakened from anesthesia (1.9 ± 0.7 min), and, therefore, did not require post-test monitoring of their body temperatures.

Deaths

The mortality rate during this study was 21.1% (4 of 19). Four male mice in the ketamine cohort died: one at age day 66, another on day 113 and two on day 184. All the deaths occurred during the period in which the animals were recovering from anesthesia, and the animals that died did not appear to be in any distress prior to expiration. None of the isoflurane-anesthetized animals nor any of the female mice anesthetized with the ketamine cocktail died during the course of the study. We have no explanation for the unexpectedly large loss of males in the ketamine cohort, except to note that ketamine anesthesia has in the past proved troublesome for us, especially with respect to anesthesia in mutant strains of mice. However, it should be noted that in the past we had never lost any *C57BL/6J* mice during the course of ERG studies. Three of the deceased animals were replaced by age-matched male mice that had been part of the original batch of animals and who had been maintained in the same quarters and under the same food and light regimen as the animals already participating in the study. The only difference between the replacement animals and those already in the study was that the former had not been anesthetized and studied prior to their insertion into the study.

Light-adapted ERGs

Typical light-adapted ERG waveforms for each of the groups are shown in Fig. 3. The differences in the light-adapted ERG parameters that we measured were not significant between males and females for either form of anesthesia; therefore we combined the male and female data for each of the anesthetic groups for analysis.

a-Wave

Neither the a-wave implicit time nor amplitude were significantly different between the two anesthetic groups. The a-wave implicit time was ∼11.1 ms for both anesthetic groups at all of the ages tested (data not shown). The a-wave amplitude was ∼13.4 μV for animals between the ages of 43–186 days, then rose by greater than 2-fold to ∼32.0 μV by 213 days (Fig. 4, upper panel). This abrupt rise in the light-adapted a-wave amplitude was seen in both males and females and for both isoflurane and ketamine cocktail anesthesia.

b-Wave

There was a statistically significant difference (∼5%) between the mean b-wave implicit time for the

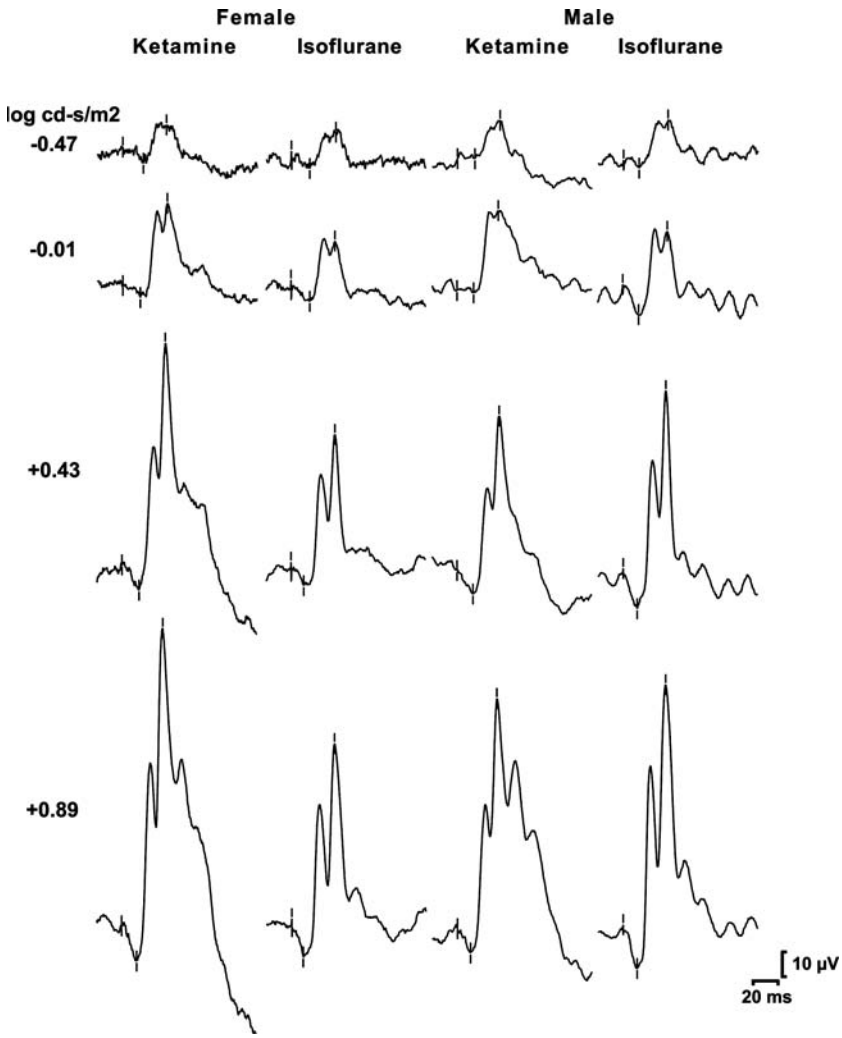

Fig. 3 Light-adapted ERG Waveforms. Representative light-adapted ERG recordings at four stimulus intensity levels from the left eye of female or male mice under ketamine cocktail or isoflurane anesthesia. The displayed waveforms are the average of waveforms for the four animals in each group. The individual animal waveforms were the average of 20 stimuli presentations at each stimulus intensity, as described in Methods. The mice were 113 days of age

isoflurane-anesthetized animals compared to the ketamine-anesthetized animals (34.3 ms vs. 32.6 ms; $P < 0.001$; mean difference: 1.8 ± 0.4 ms {95% confidence interval}, data not shown). There was a decrease of ~30% in amplitude for the b-wave in isoflurane-anesthetized animals compared to ketamine-anesthetized animals (91.7 µV vs. 129.4 µV; $P < 0.005$; mean difference: 37.8 ± 10.1 µV {95% confidence interval}, Fig. 4, lower panel).

Oscillatory potentials

There was a decrease of ~45% in the wavelet index for the light-adapted oscillatory potentials in the isoflurane-anesthetized animals compared to ketamine-anesthetized animals (33.5 µV vs. 60.0 µV; $P < 0.001$; mean difference: 26.5 ± 8.2 µV {95% confidence interval}; data not shown). There was, however, no difference in the implicit time for wavelet 3 of the oscillatory potentials for the two anesthetic groups (~26.9 ms, data not shown).

Increased background noise associated with ketamine cocktail versus isoflurane anesthesia

During the course of the light-adapted ERG studies, we observed a substantially larger amplitude (>200 µV), slow-wave (<1 Hz) background electrical activity associated with ketamine cocktail anesthesia compared to isoflurane anesthesia (Fig. 5). These slow-wave potentials complicated the extraction of the small amplitude light-adapted ERG

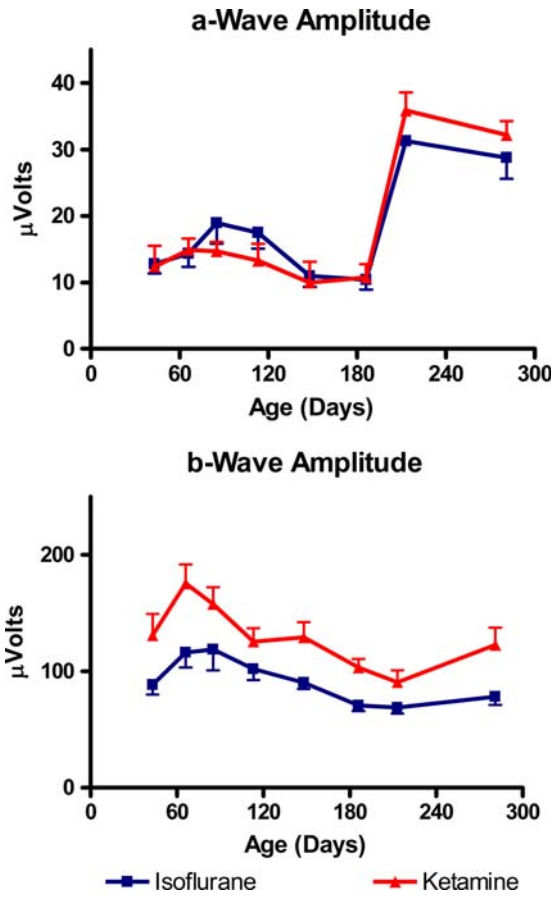

Fig. 4 Light-adapted a-Wave and b-Wave Amplitudes. The peak amplitudes of the light-adapted a-waves and b-waves for females and males at the +0.89 log cd·s/m^2 stimulus intensity were averaged for each anesthetic agent, and the means ± SEMs were plotted as a function of the age of the animals. The a-wave amplitudes (upper panel) were not different ($P = 0.92$), but the b-wave amplitudes (lower panel) for the isoflurane anesthetized animals were significantly lower than those for the ketamine anesthetized animals ($P < 0.005$)

waveforms but did not appear to be correlated with the stimulus onset. In order to prevent these large-amplitude, slow-wave potentials from masking the smaller-amplitude light-adapted ERG waveforms, we established a waveform acceptance criterion in which the peak-to-peak amplitude of the waveform trace for an individual flash presentation had to be less than or equal to 200 μV (approximately 10-fold greater than the average light-adapted b-wave amplitude at the dimmest flash intensity), and we presented flashes until 20 waveforms satisfied this criterion. This acceptance criterion was only applied at the two lowest flash intensities for light-adapted ERGs although the noise was observed at all flash intensities.

To examine and quantify this background electrical activity phenomenon, we modified the light-adapted ERG testing protocol. The recording epoch was expanded 4-fold, from 128 to 512 ms, and the baseline period prior to flash presentation was increased from 20 to 256 ms, such that the stimulus was presented midway through the recording epoch. The number of data points recorded was increased proportionally, such that the data sampling and recording rates were the same as for the standard light-adapted ERG protocol. We saved all individual waveforms until we had recorded 20 waveforms whose peak-to-peak amplitude satisfied our acceptance criterion of less than or equal to 200 μV peak-to-peak. We then analyzed the number of waveforms collected and the peak-to-peak voltages for the pre-stimulus, the post-stimulus and the entire epoch for each flash presentation.

The data from this study are presented in Table 1. The number of flashes required to satisfy our acceptance criterion was significantly greater for ketamine-anesthetized animals. Moreover, the peak-to-peak voltage ranges for the pre-stimulus period (0–255 ms), the post-stimulus period (257–512 ms) and the overall recording epoch (0–512 ms) were all 60–70% greater for the ketamine-anesthetized animals compared to isoflurane-anesthetized animals. However, the peak-to-peak voltage ranges were not significantly different for the pre-stimulus and post-stimulus periods (Table 1), and the voltage envelopes of the signals were symmetrical about the stimulus presentation (Fig. 5, 256 ms) for both isoflurane- and ketamine-anesthesia, suggesting that the background noise was not related to the flash stimulus for either form of anesthesia.

Dark-adapted ERGs

Typical dark-adapted ERG waveforms for each of the groups are shown in Fig. 6. The dark-adapted ERG parameters that we measured were not significantly different between males and females for either form of anesthesia; therefore we combined the male and female data for each of the anesthetic groups for analysis.

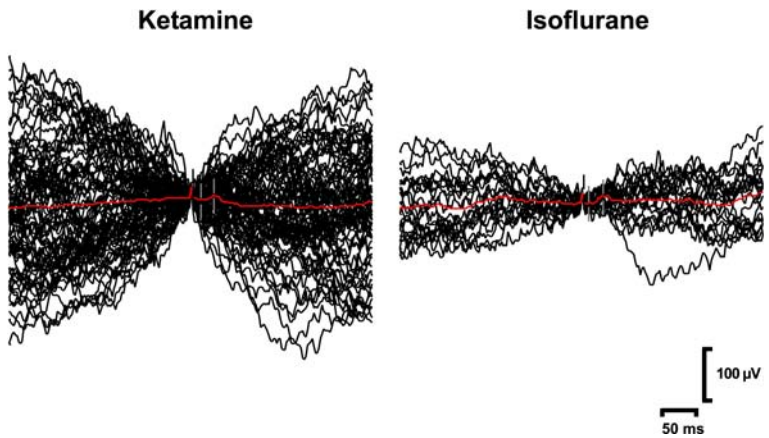

Fig. 5 Background Noise with Ketamine Cocktail and Isoflurane Anesthesia. Representative individual waveforms collected under light-adapted conditions at the lowest stimulus intensity level (–0.47 log cd·s/m^2) from a female mouse anesthetized with the ketamine cocktail and another female mouse anesthetized with isoflurane. The black lines are waveforms resulting from a single stimulus presentation, and the red lines are the averaged signal of all the individual waveforms, as generated by ERGTool. For the animal anesthetized with the ketamine cocktail, 77 stimulus presentations were required in order to satisfy the acceptance criterion of 20 waveforms with a peak-to-peak range ≤200 μV, whereas for the animal anesthetized with isoflurane only 21 stimulus presentations were required to satisfy the criterion. The recording epoch was 512 ms, and the stimulus presentation occurred after a delay of 256 ms. ERGTool normalized the traces to the signal level at the time of stimulus presentation. Note that the bandwidth of the noise is symmetrical before and after the stimulus presentation, indicating that this noise is not the result of the stimulus flash. The animals were 324 days of age

Table 1 Noise signals under ketamine cocktail and isoflurane anesthesia[a]

	Flashes[b]	Overall range (μV)[c]	Pre-stimulus range (μV)[d]	Post-stimulus range (μV)[d]
Isoflurane	21.0 ± 0.5	126.9 ± 10.6	93.1 ± 8.6	93.3 ± 7.5
Ketamine	53.0 ± 11.8	204.3 ± 20.8	152.6 ± 16.0	156.4 ± 17.2

[a] Light-adapted ERG data was recorded as described in the text at the lowest stimulus intensity (–0.47 log cd s/m^2)

[b] The mean ± SEM flashes required to obtain 20 waveforms with peak-to-peak amplitudes of less than 200 μV ($P < 0.05$)

[c] The mean ± SEM of the average peak-to-peak voltage range for the entire ERG waveforms (0–512 ms, $P < 0.005$)

[d] The mean ± SEM of the average peak-to-peak voltage range for the pre-stimulus (0–255 ms, $P < 0.005$) and post-stimulus (257–512 ms, $P < 0.005$) portions of the waveforms

a-Wave

There was a statistically significant difference (∼8%) between the mean a-wave implicit time for the isoflurane-anesthetized animals compared to the ketamine-anesthetized animals (7.5 ms vs. 8.1 ms; $P < 0.001$; mean difference: 0.6 ± 0.4 ms {95% confidence interval}, data not shown). The a-wave amplitude was not significantly different between the two anesthetic groups (Fig. 7, upper panel). The mean a-wave amplitude for the ketamine-anesthetized animals was ∼343.8 μV, and for the isoflurane-anesthetized animals was ∼360.1 μV.

b-Wave

There was a decrease of ∼20% in the implicit time of the b-wave for the isoflurane-anesthetized animals compared to the ketamine-anesthetized animals (40.0 ms vs. 50.2 ms; $P < 0.001$; mean difference: 10.3 ± 2.1 ms {95% confidence interval}, data not shown). There was, however, no significant difference in the b-wave amplitudes between the isoflurane-anesthetized and ketamine-anesthetized animals (mean amplitudes: 690.5 μV vs. 701.4 μV; Fig. 7, lower panel).

The amplitudes of the dark-adapted a- and b-waves declined with age in the animals (Fig. 7). By

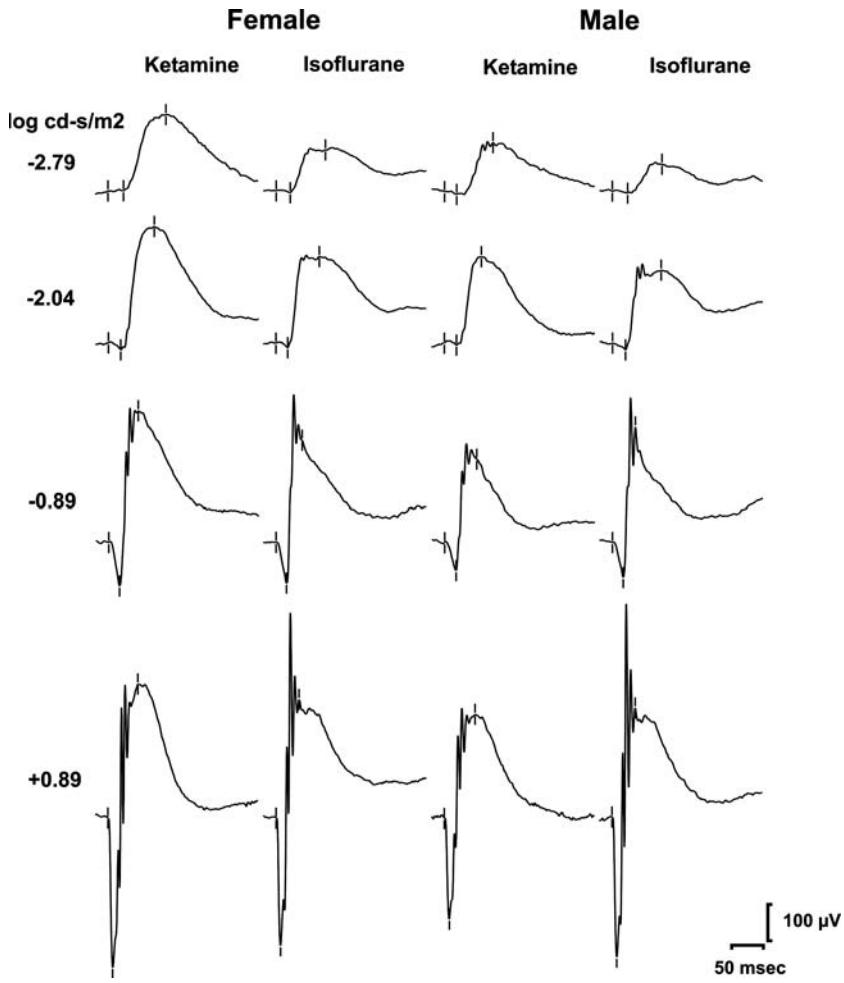

Fig. 6 Dark-adapted ERG Waveforms. Representative dark-adapted ERG recordings at four stimulus intensity levels from the left eye of female or male mice under ketamine cocktail or isoflurane anesthesia. The displayed waveform is the average of waveforms for the four animals in each group. The individual animal waveforms were the average of 20 stimulus presentations at the dimmest intensity levels (–2.79 and –2.04 log cd·s/m^2) and 14 and 6 stimulus presentations for the brightest intensity levels (–0.89 and +0.89 log cd·s/m^2, respectively), as described in the Methods. The mice were 115 days of age

linear regression we found that the decline in b-wave amplitude correlated best with animal weight (r^2 = 0.6653; –33.5 µV/gm, $P < 0.001$, Fig. 8, upper panel), although there was also a strong correlation with age (r^2 = 0.4622; –1.67 µV/day, $P < 0.001$, Fig. 8, lower panel).

Oscillatory potentials

Typical digitally filtered dark-adapted oscillatory potentials for ketamine cocktail and isoflurane anesthesia are shown in Fig. 9. There was an increase of ~16% in the wavelet index for the dark-adapted oscillatory potentials in the isoflurane-anesthetized animals compared to ketamine-anesthetized animals (1,139 µV vs. 982 µV; $P < 0.05$; mean difference: 157 ± 66 µV {95% confidence interval}; data not shown). It is of interest to note that the oscillatory potential wavelet index for dark-adapted ERGs increased with isoflurane anesthesia compared to ketamine cocktail anesthesia, whereas it decreased for light-adapted ERGs. There was a significant increase in the wavelet 2 amplitude in the isoflurane-anesthetized animals compared to ketamine-anesthetized animals (263 µV vs. 123 µV; $P < 0.001$; mean difference: 141 ± 20 µV {95% confidence interval}; Fig. 10, upper panel) and in the wavelet 3 amplitude (494 µV vs. 375 µV; $P < 0.001$; mean difference: 120 ± 44 µV {95% confidence interval}; Fig. 10, lower panel), but not in the wavelet 4 amplitude (~330 µV; data not shown). There was no difference in the implicit time for wavelet 3 of the oscillatory potentials for the two anesthetic groups (~22.0 ms, data not shown).

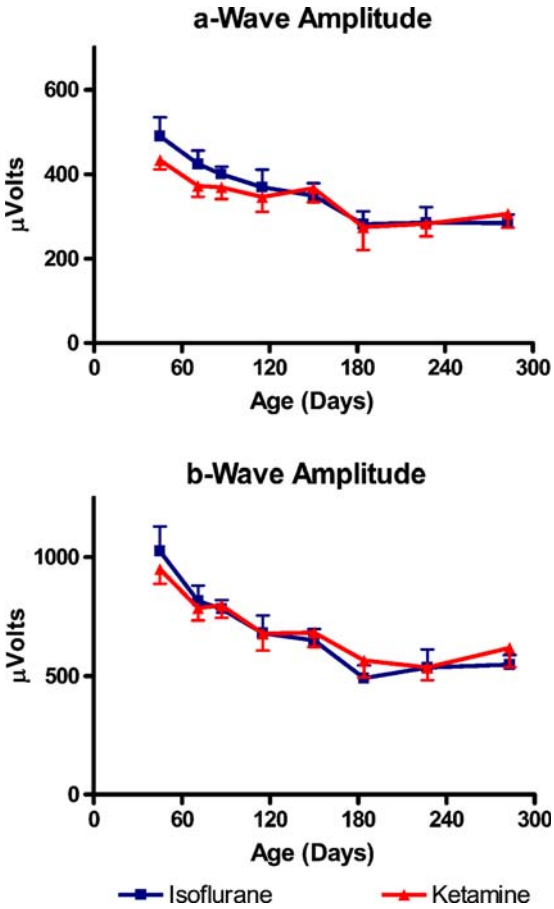

Fig. 7 Dark-adapted a-Wave and b-Wave Amplitudes. The peak amplitudes of the dark-adapted a-waves and b-waves for males and females for the +0.89 log cd·s/m^2 stimulus intensity were averaged for each anesthetic agent, and the means ± SEMs were plotted as a function of the age of the animals. Neither the a-wave amplitudes (upper panel) nor the b-wave amplitudes (lower panel) were different ($P = 0.15$ and $P = 0.56$, respectively) for the two anesthetic agents

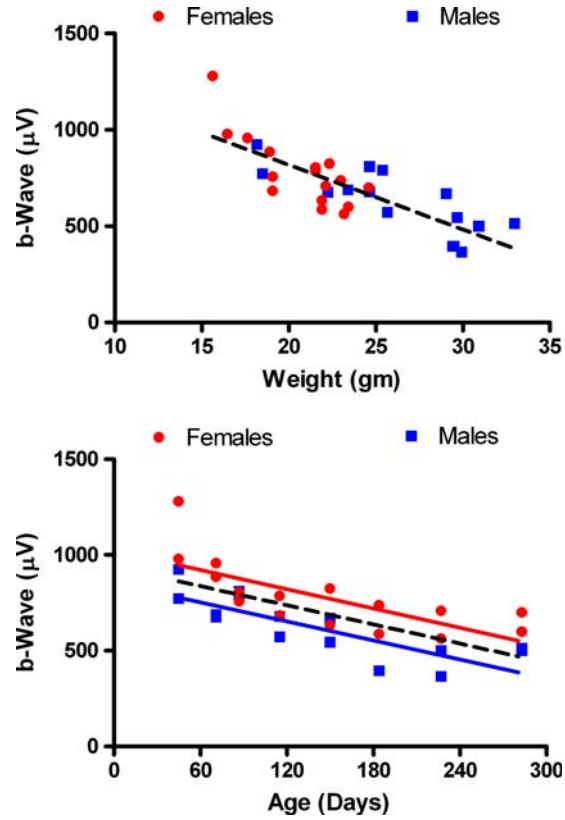

Fig. 8 Dark-adapted b-Wave Amplitude as a Function of Animal Weight and Age. The averaged b-wave amplitudes were plotted against the respective averaged weights of the males or females for both anesthetic agents. The dashed lines represent the linear regression fit through all the data points. There is a significant negative correlation between the b-wave amplitudes and the animals' weights (upper panel, $r^2 = 0.67$, $P < 0.001$) and the animals' ages (lower panel, $r^2 = 0.46$, $P < 0.001$). Notice that when the b-wave amplitude is plotted against weight (upper panel), the female data are clustered toward the left side of the graph, whereas the male data are clustered toward the right side. On the other hand, when the b-wave amplitude is plotted against age (lower panel), the female data are offset above the male data, and the slopes of the regression lines for the male data and female data are parallel (blue and red solid lines, respectively). The difference in weight between males and females at a given age accounts for the stronger correlation of b-wave amplitude with weight compared to age

Retinal sensitivity

Rod photoreceptor function can be assessed by fitting a model of rod phototransduction activation [14–18] to the leading edge of the a-wave of the dark-adapted ERG as described by Goto et al. [19] with the following modification: the maximum flash intensity used in these animals was 0.5 log unit below that needed to obtain a maximum a-wave, thereby producing a response that is about 50% of the maximum [5, 19]. Therefore the maximum a-wave amplitude for the photoreceptor model was fixed at 50% greater than the measured value for each of the groups. The model was then fitted by an iterative, non-linear curve fitting program (Prism 4) to the mean response for each of the groups at each age for the four highest flash intensities, using the following equation [19]:

$$P3(i,t) = \left\{1 - \exp\left[-iS(t - t_d)^2\right]\right\} Rm_{p3} \text{ for } t > t_d$$

where *P3* represents the mass response of the rod photoreceptors as a function of the intensity of the

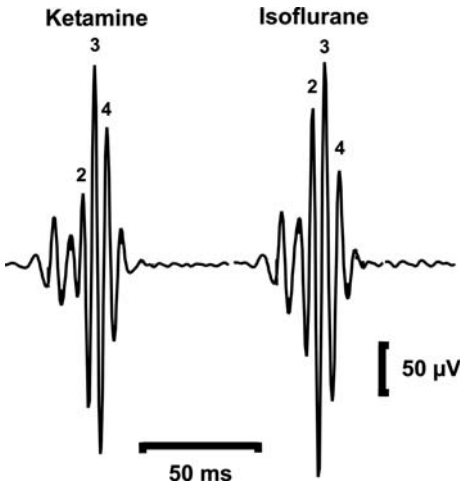

Fig. 9 Digitally Filtered Dark-adapted OP Waveforms. Representative dark-adapted OP waveforms that were recorded at the brightest stimulus intensity level (+0.89 log cd·s/m^2) from the left eye of female mice under ketamine cocktail or isoflurane anesthesia. The displayed waveforms were digitally extracted from the waveforms shown in Fig. 6 as described in the Methods. The numbers above the peaks are the wavelet numbers referred to in the text. Note that although there appears to be a difference in the amplitude of wavelet 4, this difference did not reach statistical significance (see Fig. 10)

flash (i) and time (t) after flash onset, t_d is the delay before onset of the response, S is the phototransduction gain, and Rm_{p3} is the maximum response.

The S values were analyzed by two-way ANOVA with the Bonferroni posthoc test for comparisons between groups when the between-groups F test indicated differences at a probability of $P < 0.01$. There were no significant differences in the S values between the isoflurane and the ketamine cocktail anesthesia.

The Naka–Rushton parameters [20], maximum response ($R_{\max}$), half-saturation coefficient (log k), and slope parameter (n), were determined by an iterative, non-linear curve fitting program (Prism 4) using the following equation [21]:

$$R = \frac{R_{\max} * I^n}{I^n + k^n}$$

where R is the dark-adapted ERG response amplitude in µV, $R_{\max}$ is the maximum ERG amplitude, I is the flash intensity in cd·s/m^2, k is the half-saturation constant in cd·s/m^2, and n is a dimensionless slope constant.

The Naka–Rushton parameters were calculated twice for the b-wave amplitude for each animal using

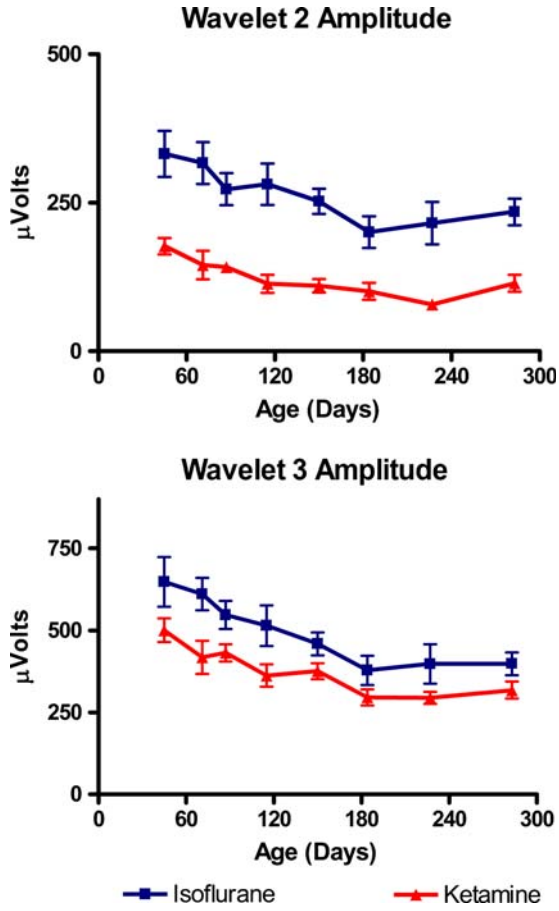

Fig. 10 Dark-adapted OP Wavelet 2 and Wavelet 3 Amplitudes. The peak amplitudes of OP wavelets 2 and 3 were digitally extracted from the averaged dark-adapted ERG waveforms for the +0.89 log cd·s/m^2 stimulus intensity for each anesthetic agent, and the means ± SEMs were plotted as a function of the age of the animals. The wavelet 2 amplitudes (upper panel) and wavelet 3 amplitudes (lower panel) for the isoflurane-anesthetized animals were significantly greater than those for the ketamine-anesthetized animals ($P < 0.001$ for both)

the five lowest flash intensities (–3.0, –2.6, –2.2, –1.7, and –1.1 log cd·s/m^2). For the first calculation n was determined uniquely for each animal. There were no significant differences in the value of n for isoflurane and ketamine cocktail anesthesia (0.54 ± 0.02 and 0.51 ± 0.03, respectively); therefore the Naka–Rushton parameters were recalculated while fixing n to the mean for all animals (0.53). The Naka–Rushton parameters were analyzed by a two-way ANOVA with the Bonferroni posthoc test for comparisons between groups when the between-groups F test indicated differences at a probability of $P < 0.01$.

Although there were no significant differences found in the R_{max} parameter between the isoflurane and the ketamine cocktail anesthesia, there was a significant decline in R_{max} with age (Fig. 11, upper panel; $P < 0.001$). On the other hand, although there was no significant effect of age on the half-saturation parameter for the two forms of anesthesia, the log k parameter was significantly greater for isoflurane anesthesia when compared to ketamine cocktail anesthesia (Fig. 11, lower panel; $P < 0.005$).

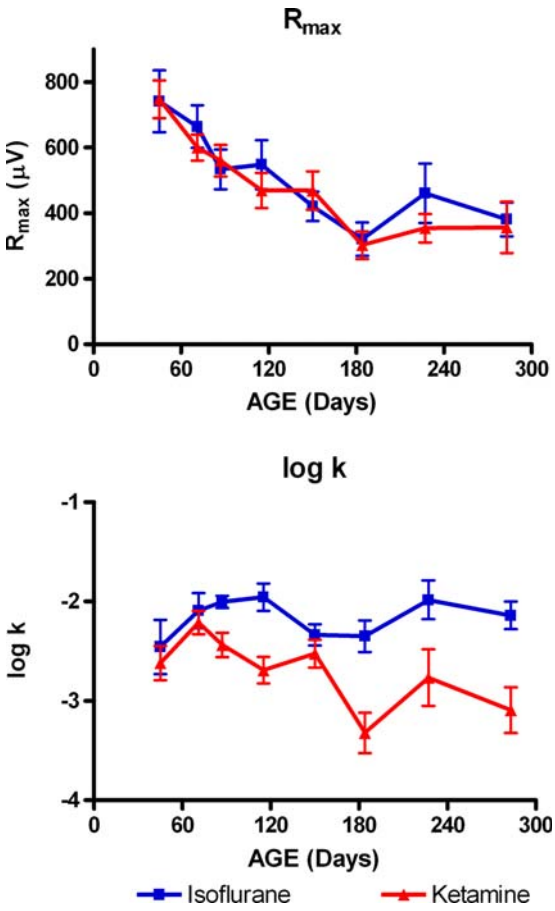

Fig. 11 Naka–Rushton R_{max} and log k parameters. The Naka–Rushton R_{max} (upper panel) and log k (lower panel) parameters were determined for each animal using a non-linear curve fitting algorithm described in the text after fixing $n = 0.53$. The means ± SEM of these parameters, for males and females combined, are plotted for each anesthetic as a function of age of the animals. The R_{max} declined significantly with age ($P < 0.001$) but did not differ between isoflurane and ketamine cocktail anesthesia. The log k was elevated for isoflurane anesthesia when compared to ketamine cocktail anesthesia ($P < 0.005$) but did not change with age

Discussion

We have compared ketamine cocktail and isoflurane anesthesia for recording mouse ERGs in an extended, longitudinal study. We have examined many of the commonly reported ERG parameters under both light-adapted and dark-adapted conditions, including implicit times and amplitudes for the a-waves, b-waves and oscillatory potentials. The impetus for this study was the increased mortality that we had experienced using ketamine, an NMDA receptor channel blocker, in certain mutant mouse strains, and our desire to find an easy-to-use, safe, and economical alternative. We chose the inhalation anesthetic, isoflurane, a halogenated ether compound [22] that is commonly used in veterinary anesthesia.

Isoflurane is a liquid at room temperature, is not flammable, making it relatively easy to store in most laboratory situations, and is not a drug of abuse. The Department of Comparative Medicine at OHSU developed a procedure for administering isoflurane anesthesia to rodents that did not require intubation and that protected the investigators from potentially adverse effects of prolonged exposure to isoflurane vapors. The level of isoflurane is controlled by an adjustable vaporizer, making it easy to achieve and maintain a stable depth of anesthesia for intervals up to 2 h (the longest period that we have used thus far).

Ketamine, generally in combination with xylazine and sometimes acepromazine, is typically administered by subcutaneous injection, making it difficult to maintain a stable depth of anesthesia for prolonged procedures and to supplement the anesthesia without disturbing the animal and/or risking an overdose. Induction of and recovery from anesthesia is very rapid with isoflurane, thereby eliminating the need for close monitoring of the animal's status during a prolonged post-procedure recovery period, as is required with the ketamine cocktail. Isoflurane is minimally metabolized, with nearly complete recovery of the drug occurring by exhalation. Since isoflurane is not patented, it is economical to use; however there are initial costs associated with the acquisition of an oxygen flow system, an isoflurane vaporizer and the tubing, valves and scavenging canisters. It has been our experience that the mortality rate has been significantly reduced with isoflurane anesthesia when compared with ketamine cocktail anesthesia, especially in our work with some fragile

mouse models for inherited muscular dystrophies. One minor constraint in using isoflurane is that it, like other halogenated anesthetics, is contraindicated in individuals at risk for malignant hyperthermia, making it undesirable for mouse models of that disorder [23]. For other mouse models of disease, it appears to be a suitable alternative anesthetic agent for the ketamine cocktail in rodent electroretinography.

We found that the a-wave and b-wave amplitudes for dark-adapted ERGs were no different between the two anesthetic agents. Inasmuch as many of the older rodent ERG studies focused on dark-adapted conditions, it is reassuring that results obtained with isoflurane will be qualitatively comparable to those obtained with the ketamine cocktail. There were, however, differences that investigators need to be aware of when designing and interpreting rodent ERG studies. There were decreases in the implicit times for the a- and b-waves under isoflurane anesthesia ($\sim$8% and $\sim$20%, respectively), although it is unclear whether the 600 μs difference between the a-wave implicit times has any biological significance. Somewhat more surprising was the increased OP wavelet index ($\sim$16%) under isoflurane anesthesia and the significant difference in the shape of the OP waveform with the two anesthetic agents. Although we currently cannot explain any of these differences, they are likely due to differences in the actions of the two anesthetic agents on membrane potentials, neurotransmitter receptors and/or possibly ionic channels. Clearly this is an area deserving of further investigation.

Although there were no differences between the two forms of anesthesia for the rod phototransduction gain (S) and the Naka–Rushton slope parameter (n); isoflurane elevated the Naka–Rushton log k parameter. This indicates that this agent is effectively decreasing retinal sensitivity, as measured by the dark-adapted b-wave. This may be because, as an inhalation anesthetic, isoflurane may affect the fluidity of membranes and thereby alter neurotransmitter release in photoreceptor inner segments, receptors or channels in bipolar cells, or otherwise elevate retinal thresholds, as reflected by the b-wave.

In contrast to the results in the dark-adapted ERGs, the light-adapted b-wave amplitude and the OP wavelet index are decreased under isoflurane anesthesia. Even so, there is no difference in the implicit time for the a-wave, and the b-wave implicit time is only slightly increased ($\sim$5%). It is unclear, however, whether the 1.8 ms difference between the b-wave implicit times has any biological significance. We conclude that there may be important differences in the shape of the OP waveforms in light-adapted ERGs with the two anesthetic agents (see Fig. 3); however, due to noise issues and the small sizes of the wavelet amplitudes, we have not been able to resolve this issue at this time.

Ketamine, a rapid-acting short-lasting anesthetic agent, is often used in combination with xylazine, a sedative and muscle relaxant, in order to counteract the side effects of ketamine such as tremors. However, xylazine, owing to its partial alpha-adrenergic agonist actions, also produces hyperglycemia [24, 25], which has been reported to increase the mouse b-wave amplitude [26]. Isoflurane has also been reported to produce hyperglycemia in rats [27], which may explain why we did not observe any differences in the dark-adapted ERG b-wave amplitudes between the two anesthesia regimens. It is of interest to note that neither ketamine/xylazine nor isoflurane anesthesia produces hyperglycemia in fasted animals [27], thus we strongly recommend that the fed or fasted state of the animals be given careful consideration when comparing ERG results from different studies, or in designing new studies.

There is a substantially lower background electrical noise associated with isoflurane anesthesia, probably due to the greater quieting of muscle activity. The noise is not correlated with the stimulus presentation, and, therefore, does not contribute to the ERG signal. This background noise, however, can be especially intrusive if the signal of interest is small, such as in the rodent light-adapted ERGs. In that situation, the collection of more waveforms is needed in order to extract the signal of interest by signal averaging, with all of the attendant problems that this introduces.

The amplitude of the human and mouse ERG declines with age [28, 29], a finding confirmed in the present study. A point worth noting, however, is that there is a better negative correlation of the dark-adapted b-wave amplitude with animal weight than with animal age. The reason for the relatively poorer correlation with age is that linear regression of the b-wave amplitudes with age for males and for females yields parallel curves, with the female curve offset 168 ± 61 μV (mean ± SEM) higher (Fig. 8,

lower panel). This suggests that in order to minimize data variability, at least in terms of measurement of b-wave amplitudes, it is important to compare both age and gender matched animals.

A final point that we would like to make is that, although we did not find gender differences in the light-adapted or dark-adapted ERGs, this might have been due to the small numbers of animals in each of the groups (i.e., 4), and this issue might merit closer examination.

This study validates the use of isoflurane as a substitute for ketamine cocktail anesthesia in rodent ERG studies. The light-adapted and dark-adapted ERG waveforms are qualitatively similar with the two anesthetic agents, and as long as the proper comparisons are made the quantitative conclusions should be valid. We caution, however, that comparisons between studies performed by isoflurane with those by the ketamine cocktail should be approached cautiously, and that statistical comparisons be limited to studies using the same form of anesthesia. However, given the ease-of-use, safety and cost of isoflurane, it is appropriate for use in mouse ERG studies, especially those that involve fragile mouse strains.

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