

Protective Effect of Halothane Anesthesia on Retinal Light Damage: Inhibition of Metabolic Rhodopsin Regeneration

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PURPOSE. To determine whether the volatile anesthetic halothane protects against light-induced photoreceptor degeneration in the rodent retina.

METHODS. Albino mice and rats were anesthetized with halothane and exposed to high levels of white or blue light. Non-anesthetized animals served as controls. Retinal morphology was assessed by light microscopy, and apoptosis of photoreceptor cells was verified by detection of fragmented genomic DNA and in situ staining of apoptotic nuclei (TUNEL assay). Rhodopsin regeneration after bleaching was determined by measuring rhodopsin levels in retinas of mice or rats at different time points in darkness.

RESULTS. Halothane anesthesia reversibly inhibited metabolic rhodopsin regeneration and thus prevented rhodopsin from absorbing high numbers of photons during light exposure. Consequently, photoreceptors of mice and rats anesthetized with halothane were completely protected against degeneration induced by white light. In remarkable contrast, however, halothane anesthesia did not protect against blue-light-induced photoreceptor cell death.

CONCLUSIONS. After the initial bleach, halothane impeded photon absorption by rhodopsin by inhibiting metabolic rhodopsin regeneration. Apparently, the rhodopsin-mediated uptake of the critical number of photons to initiate white light-induced retinal degeneration was prevented. In contrast, halothane did not protect the retina against blue light. Blue light can efficiently restore functional rhodopsin from bleaching intermediates through a process termed photoreversal of bleaching. This process does not depend on the visual cycle via the pigment epithelium but nevertheless enables rhodopsin molecules to absorb the critical number of photons required to induce retinal degeneration. (*Invest Ophthalmol Vis Sci.* 2001;42:476–480)

Visible light can induce photochemical lesions in the retina of vertebrates¹ (for reviews, see refs. 2–4). Furthermore, light exposure may also accelerate some forms of retinitis pigmentosa (RP) and possibly age-related macular degeneration (AMD)^{5–8} in humans. Similarly to the human diseases,

progression and severity of the degenerative process is enhanced by light in several animal models, some of which carry gene mutations identical with those found in human patients.^{9–11} After exposure to high levels of visible light, photoreceptors die by apoptosis, the mode of cell death recognized as the final common death pathway in many human retinal diseases (for review, see ref. 2). Therefore, light exposure of mice and rats represents an experimental system to study apoptotic signaling and execution pathways that may also be involved in human diseases.

Light-induced retinal degeneration depends on the presence of bleachable rhodopsin, which is the primary chromophore mediating light damage.^{1,12,13} Susceptibility to light damage is at least partially determined by the genetic background.¹⁴ A genetic locus associated with light-damage susceptibility has been mapped recently to *Rpe65*,¹⁵ a gene expressed in the pigment epithelium (PE) and required for the (re)generation of rhodopsin in the visual cycle.¹⁶ A critical determinant of light-damage susceptibility appears to be the rate at which rhodopsin is metabolically regenerated after bleaching: BALB/c mice have a regeneration rate constant of 0.036/min, whereas C57/Bl6 mice have a constant of 0.009/min.¹⁷ Correspondingly, BALB/c mice have a high susceptibility to light damage, C57/Bl6 have a low susceptibility.¹⁴ F1 mice of a BALB/c x C57/Bl6 cross have an intermediate rate constant of 0.017/min¹⁷ and show an intermediate susceptibility to light damage.¹⁴ Furthermore, mice completely devoid of rhodopsin regeneration are completely protected against light damage.¹²

Anesthesia with halothane slows cone dark adaptation,^{18,19} and recent in vitro work suggests that halothane may also affect restoration of the visual pigment in rods by competing with retinal for binding to opsin (Ishizawa Y, Liebman PA, Eckenhoff RG, personal communication, June 2000). We therefore tested the effect of halothane anesthesia on rhodopsin regeneration in vivo during white-light and blue-light exposure and analyzed its consequences for the retinal sensitivity to light damage.

METHODS

Anesthesia and Light Exposure

All experiments conformed to the ARVO statement for care and use of animals in research and to the guidelines of the Veterinary Authority of Zurich. Albino mice (BALB/c; 6 to 10 weeks of age) were reared in a light-dark cycle (12:12 hours) with 60 lux at cage level. Mice were dark-adapted overnight (16 hours) and were anesthetized with halothane (1.5% in O₂) during exposure to diffuse, white fluorescent light (TLD36 W/965 tubes, Philips Light GmbH, Hamburg, Germany; ultraviolet-impermeable diffuser) in cages with reflective interior. Nonanesthetized mice served as controls. Mice were exposed to white light for 20 minutes using 5,000 lux (5 klux) or for 60 minutes using 13,000 lux (13 klux). Immediately after light exposure, anesthesia was discontinued and mice were either kept in darkness for additional 24 hours or were put back into the normal light-dark cycle for 10 days before

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retinal morphology was analyzed. Albino rats (Sprague-Dawley) were reared in a 12:12 hour light-dark cycle with 5 lux at cage level. Dark adaptation was as for mice, and anesthesia was maintained at 1.8% halothane in oxygen. Rats were exposed to 3,000 lux of white fluorescent light for 60 minutes. For exposure to blue light, dark-adapted rats were anesthetized with halothane (1.8% in O₂) and exposed for 30 minutes to 3.5 mW/cm² of blue light (403 ± 10 nm) as described.²⁰ In control experiments, rats were anesthetized with ketamine (75 mg/kg), xylazine (23 mg/kg), or a mixture of both.

Microscopy, TUNEL Staining, and DNA Fragmentation Analysis

For morphologic analysis of retinal tissue, enucleated eyes were fixed in 2.5% glutaraldehyde and embedded in Epon 812. Terminal transferase-mediated dUTP nick end labeling was performed 24 hours after light exposure essentially as described.²¹ Briefly, retinal tissue was fixed in 2% paraformaldehyde for 2 hours at 4°C and embedded in paraffin. The in situ cell death detection kit (Roche Diagnostics, Rotkreuz, Switzerland) was used with minor modifications to perform staining on 0.5 μm sections. Genomic DNA was prepared 24 hours after light exposure from isolated retinas by phenol-chloroform-isoamylalcohol extraction.²⁰ Total DNA (20 μg) was separated on 1.5% agarose gels and stained with ethidium bromide.

Rhodopsin Regeneration

Dark-adapted mice (16 hours) were exposed to 5 klux of white light for 10 minutes, either killed immediately thereafter or allowed to regenerate rhodopsin for different time intervals in darkness. Three groups of mice were analyzed: mice nonanesthetized with halothane, mice anesthetized with halothane during bleaching, and mice anesthetized with halothane during bleaching and the subsequent regeneration period in darkness. Rhodopsin was extracted from isolated retinas and quantified as described.²² Both retinas from one mouse were combined for determination of rhodopsin contents,²² and values were divided by 2 to give pmol rhodopsin per eye. Significance of the results was tested using an unpaired *t*-test.

Rhodopsin regeneration in Sprague-Dawley rats was determined in dark-adapted (16 hours, overnight) animals after bleaching by an exposure to 3,000 lux of white light for 10 minutes. Photoreversal was analyzed after exposure to 700 μW of green light (550 ± 10 nm) for 5 minutes followed by a 1-minute exposure to 300 μW of blue light (403 ± 10 nm).

RESULTS

Light Damage in Mice

Albino mice exposed to white light without halothane anesthesia (control mice; Fig. 1A) showed severe photoreceptor cell death at 24 hours after light exposure (Fig. 1B, 1D). Ten days after illumination, the outer nuclear layer (ONL) was almost completely destroyed (Fig. 1F). In contrast, mice anesthetized with halothane did not show any signs of light damage (Fig. 1C) even 10 days after exposure (Fig. 1E). Protection by halothane was significant: the threshold for light damage in nonanesthetized BALB/c mice was below 20 minutes of exposure to 5 klux (Fig. 1B), at least 8 times lower than the light dose (exposure time × intensity) used in the experiments shown in Figure 1C-F. Biochemical markers for apoptosis such as positive TUNEL staining and the presence of internucleosomally fragmented genomic DNA confirmed that white light exposure induced photoreceptor apoptosis exclusively in nonanesthetized control mice (Fig. 2A, 2C). Both markers were negative in mice that were anesthetized with halothane during light exposure (Fig. 2A, 2B).

Rhodopsin Regeneration in Mice

Rhodopsin regeneration was analyzed after a complete bleach in nonanesthetized mice and mice anesthetized with halothane

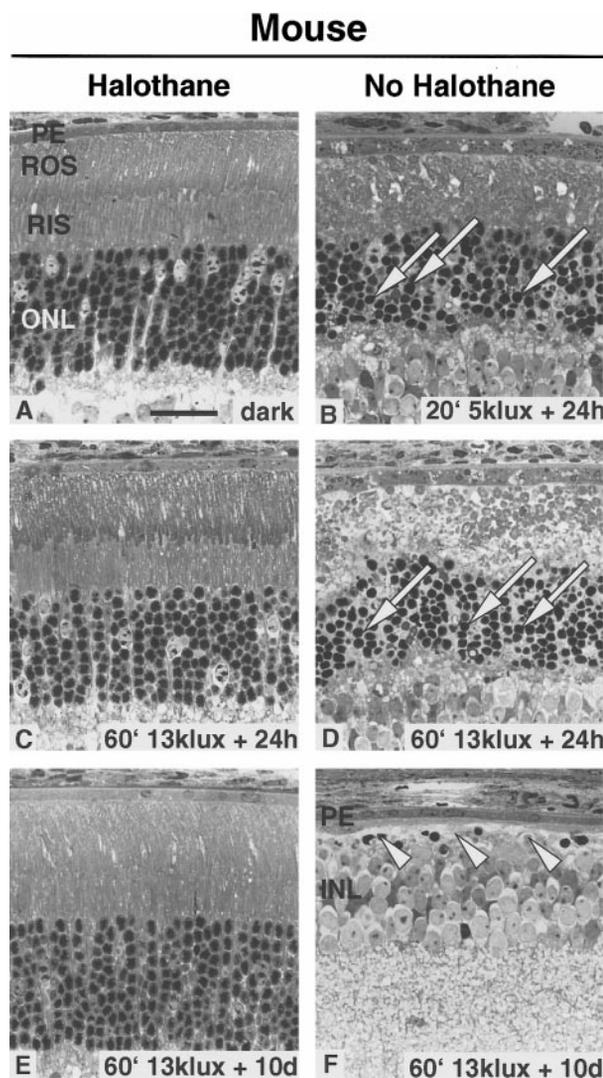


FIGURE 1. Halothane anesthesia protects against retinal damage by white light. Dark-adapted BALB/c mice were either kept in darkness (A) or exposed to white, fluorescent light for the times and intensities indicated (B through F). After exposure, mice recovered in darkness for 24 hours (A through D) or were kept in a 12:12 hour light-dark cycle for 10 days (E, F). (C, E) Mice anesthetized with 1.5% halothane in oxygen for 60 minutes during exposure. (B, D, F) Mice nonanesthetized with halothane. (A) Retina of a halothane anesthetized mouse kept in darkness. Pigment epithelium (PE), rod outer segments (ROS), rod inner segments (RIS), and outer nuclear layer (ONL) appear intact and well organized. (B) 20 minutes of exposure with 5,000 lux (5 klux) induced photoreceptor apoptosis as indicated by the condensed nuclear chromatin in the majority of the nuclei in the ONL (arrows). RIS and ROS are disorganized and not clearly distinguishable. (C) Retinal morphology appears intact in halothane anesthetized mice even after exposure to 13,000 lux (13 klux) for 60 minutes, a light dose (exposure time × intensity) approximately 8 times higher than used in (B). (D) The same light exposure as in (C) but without halothane anesthesia caused severe photoreceptor degeneration (arrows). (E) Mice anesthetized with halothane show normal retinal morphology 10 days after light exposure. (F) In contrast, photoreceptor cells of mice nonanesthetized with halothane were almost completely removed from the retina 10 days after exposure (arrowheads). Cells of the PE had recovered and were morphologically indistinguishable to dark controls. Representative sections (lower central retina) of three independent experiments are shown. Scale bar, 25 μm.

(Fig. 3). Both anesthetized and nonanesthetized mice had comparable levels of rhodopsin after dark adaptation (ca. 430 pmol/eye). Exposure to 5 klux for 10 minutes resulted in an almost complete bleach of rhodopsin in both groups of mice.

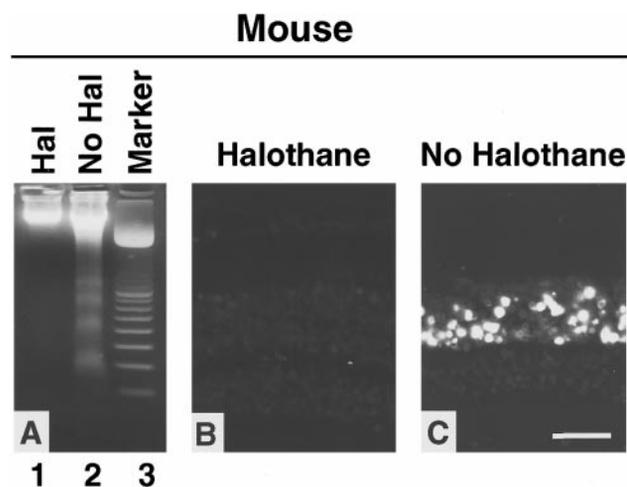


FIGURE 2. Absence of light-induced retinal apoptosis in mice anesthetized with halothane. Dark-adapted BALB/c mice were or were not anesthetized with halothane and exposed to 13 klux of white light for 60 minutes. After a recovery period of 24 hours in darkness, retinas were isolated and genomic DNA was analyzed by agarose gel electrophoresis. (A, lane 1) Genomic retinal DNA of halothane anesthetized mice did not show internucleosomal DNA fragmentation after light exposure. (A, lane 2) DNA of a control mouse nonanesthetized with halothane displayed a DNA ladder characteristic for apoptotic cells. The marker lane 3 shows a 100 bp DNA ladder. (B) TUNEL staining of a retina from a mouse anesthetized with halothane. No positive signals were detectable demonstrating that light exposure did not induce retinal apoptosis. (C) TUNEL staining of a retina from a mouse nonanesthetized with halothane. Positive staining of photoreceptor nuclei indicates ongoing apoptosis. Scale bar, 50 μ m.

Strikingly, metabolic regeneration was significantly ($P < 0.05$; unpaired *t*-test) and almost completely inhibited in mice anesthetized with halothane (Fig. 3). Whereas nonanesthetized mice regenerated rhodopsin to completion within 1 hour (Fig. 3, white bars), mice under halothane anesthesia had regenerated the visual pigment only to 15% to 20% of the dark value, even after 240 minutes in darkness (Fig. 3, black bars). This inhibition of regeneration was reversible. When halothane anesthesia was terminated immediately after bleaching, rhodopsin started to regenerate as soon as 15 minutes thereafter (Fig. 3, gray bars). Although only approximately 15% of the dark value was regenerated after 15 minutes, compared to 50% in controls, regeneration was almost complete after 60 minutes. The delay at 15 minutes might reflect the time needed to sufficiently clear the halothane from the mouse retina.

Light Damage and Rhodopsin Regeneration in Rats

Exposure of albino rats (Sprague-Dawley) to 3 klux of white light for 1 hour resulted in many pycnotic photoreceptor nuclei indicating apoptosis (Fig. 4B). Furthermore, the PE appeared swollen, irregular, and with many inclusions, demonstrating that cells of the PE were severely affected by the light exposure with subsequent death of the PE.²³ However, when rats were anesthetized with halothane, exposure to white light of the same intensity did not induce photoreceptor cell death (Fig. 4C) showing that the protective effect of halothane against white-light damage was not species specific. Notably, cells of the PE were not affected, suggesting that the changes observed in nonanesthetized rats after light exposure might either be related to rhodopsin regeneration or might be effects secondary to the changes in photoreceptor cells. This is in marked contrast to mice where light exposure did not induce cell death in the PE (Fig. 1D, 1F). The reason for this species difference is not clear at this time and is the subject of ongoing

studies in our laboratory. Measurements of rhodopsin revealed that halothane efficiently prevented metabolic rhodopsin regeneration also in rats. In animals nonanesthetized with halothane, $37 \pm 4.4\%$ rhodopsin ($n = 5$ retinas) was regenerated after 30 minutes and $83.3 \pm 6.1\%$ after 120 minutes ($n = 5$ retinas) in darkness (dark value: 100% = 2.3 ± 0.086 nmol; $n = 4$ retinas). Rats anesthetized with halothane, however, regenerated rhodopsin to only $7.6 \pm 1.8\%$ ($n = 4$ retinas) after 30 minutes and to $8.35 \pm 2.3\%$ ($n = 4$ retinas) after 120 minutes in darkness.

Blue-Light Effects in Rats

Blue-light exposure induces the photochemical reversal of rhodopsin bleaching intermediates *in vitro*²⁴ and *in vivo*,²⁰ which is independent of the metabolic regeneration via the PE. Therefore, in blue-light conditions, rhodopsin and its bleaching intermediates can absorb large numbers of photons in a short period.²⁰ When anesthetized rats were exposed to blue light, photoreceptor apoptosis was efficiently induced as shown by the formation of pycnotic nuclei 24 hours after light exposure (Fig. 4D). As for white light in nonanesthetized animals, exposure to blue light induced severe changes in the PE. Cells appeared irregularly swollen and contained many inclusions suggesting that these cells would eventually die. Rhodopsin measurements revealed that halothane did not prevent photoreversal of bleaching by blue light; when anesthetized rats were illuminated for 5 minutes with green light (550 ± 10 nm; 700μ W), rhodopsin was bleached to 5.9% of the dark value. However, when blue light (403 ± 10 nm; 300μ W) was given for 1 minute immediately after the bleach with green light, rhodopsin was photoreversed to $13.7\% \pm 1.2\%$ ($n = 3$) of the dark value. We used green light for the bleaching process because at this wavelength no photoreversal of bleaching is occurring.²⁰ In contrast, the blue-light component of white light could interfere with the analysis of photoregeneration by photoreversing some of the rhodopsin molecules.

DISCUSSION

Halothane anesthesia suppressed metabolic rhodopsin regeneration in mice and rats and completely protected against photoreceptor apoptosis induced by white light. Inhibition of metabolic rhodopsin regeneration rendered retinas virtually devoid of rhodopsin during light exposure

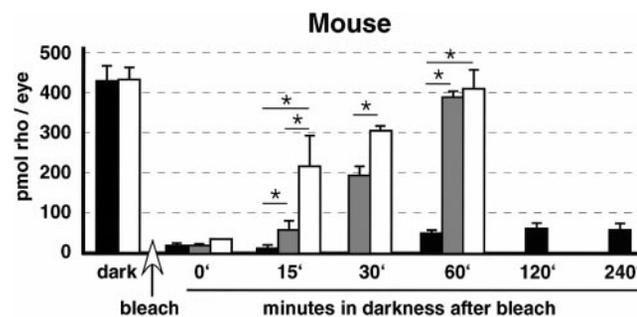


FIGURE 3. Rhodopsin regeneration is inhibited in mice anesthetized with halothane. Rhodopsin was bleached by exposing mice to 5 klux of white light for 10 minutes (arrow). Regeneration was determined by measuring rhodopsin after different time intervals in darkness. **Black bars:** Mice anesthetized with halothane during bleaching and recovery in darkness. **Grey bars:** Mice anesthetized with halothane during bleaching only but not during regeneration in darkness. **Open bars:** control mice nonanesthetized with halothane. All statistically significant (unpaired *t*-test; $P < 0.05$) differences between rhodopsin values within a particular time point are marked (*). **Error bars:** Standard deviations. Numbers of eyes per condition and time interval, $n = 4$ to 8.

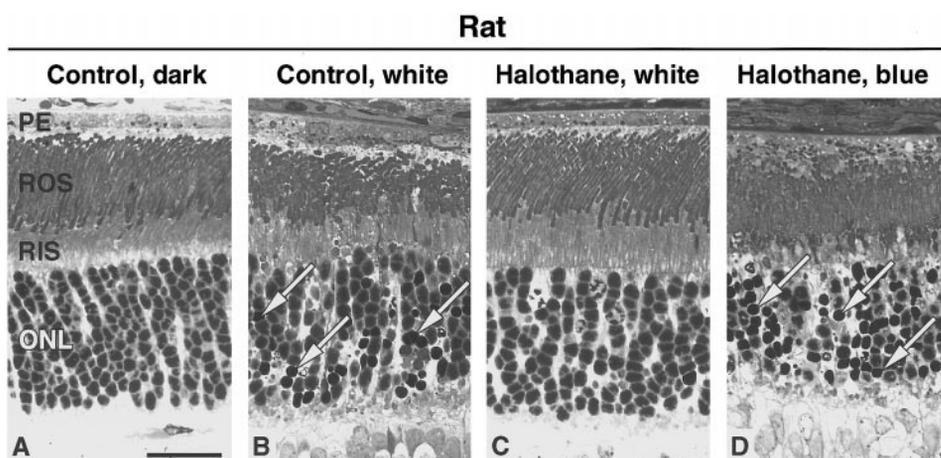


FIGURE 4. Halothane protects against white light damage but not against blue light damage in albino rats. Retinal morphology of rats exposed to white light (**B, C**) or blue light (**D**) was analyzed 24 hours after light exposure. (**A**) Dark control. Retina of a rat not exposed to light. (**B**) Many photoreceptor nuclei with condensed chromatin (*white arrows*) were detected in the ONL of non-anesthetized rats exposed to 3 klux of white light for 60 minutes. ROS are disorganized, PE cells appear swollen. (**C**) No signs of light damage in the retina of a rat anesthetized with halothane during exposure to 3 klux for 60 minutes. (**D**) Rat anesthetized with halothane and exposed to blue light (403 nm, 3.5 mW/cm²) for

30 minutes. Retinal damage was evidenced by the detection of numerous pycnotic nuclei containing condensed chromatin (*white arrows*). Representative sections from the lower central retina of 2 to 3 independent experiments are shown. Abbreviations as in Figure 1. Scale bar, 25 μ m.

after the initial bleach. As a result, the absorption of photons per time—an essential element in the chain of events leading from light exposure to photoreceptor apoptosis—was strongly reduced. In contrast, blue light causes photoreversal of bleaching and enables absorption of photons by rhodopsin independent of metabolic rhodopsin regeneration.²⁰ Consequently, halothane anesthesia did not prevent blue-light damage to the retina.

Recent *in vitro* work by Ishizawa and coworkers suggests that halothane directly interacts with rhodopsin²⁵ and competes with retinal for the opsin binding site (Ishizawa Y, Liebman PA, Eckenhoff RG, personal communication, June 2000). Such a competition would explain the almost complete inhibition of metabolic rhodopsin regeneration after light exposure. Previously it was proposed that halothane reduces the light-induced uptake of protons by rhodopsin in rod disc membranes²⁶ thereby inhibiting the transition from metarhodopsin I to metarhodopsin II during the process of bleaching. Although our present data do not fully exclude this possibility, they suggest that such an effect might be minor *in vivo*. Bleaching of rhodopsin in mice anesthetized with halothane is at least as efficient as in control mice (Fig. 3), and no accumulation of metarhodopsin I could be detected spectrophotometrically immediately after exposure to 10 minutes of white light in both anesthetized and control mice (data not shown). Furthermore, rhodopsin could be regenerated by blue light, indicating that photoreversal was occurring under halothane anesthesia. As metarhodopsin II is the most probable intermediate that is photoreversed by blue light (for discussion, see ref. 20), it is unlikely that halothane blocked the metarhodopsin I to metarhodopsin II transition in the living eye. However, photoreversal was less efficient in rats anesthetized with halothane than in rats anesthetized with ketamine/xylazine (reversal to 13.7% in halothane compared to 28% in ketamine/xylazine²⁰). Therefore, halothane might influence rhodopsin metabolism by several mechanisms that may act both upstream and downstream of MII. Halothane did not affect unactivated rhodopsin as dark values were similar in anesthetized and nonanesthetized animals.

Because halothane almost completely blocked metabolic rhodopsin regeneration, but did not or only marginally impair photoreversal, halothane anesthesia strongly inhibited further photon absorptions by rhodopsin after the initial bleaching by white light but not by blue light. Because rhodopsin is the photon receptor needed for the induction of light damage,¹² halothane anesthesia protected against photoreceptor degeneration induced by white but not by blue light. The photoreceptor cell death induced by blue light also indicates that

halothane did not interfere with the execution of the apoptotic program in general. This conclusion is further supported by the induction of the DNA-binding activity of the transcription factor AP-1 by blue light in rats anesthetized with halothane (data not shown). Induction of *c-Fos* containing AP-1 is essential for the induction/execution of light-induced photoreceptor apoptosis.^{21,27}

The protective effect of halothane was not a general effect of anesthesia: BALB/c mice anesthetized with ketamine or xylazine, for example, showed severe photoreceptor degeneration after exposure to white light (data not shown). Furthermore, anesthesia with a mixture of ketamine/xylazine slowed but did not block metabolic rhodopsin regeneration in rats: 120 minutes after a complete bleach, anesthetized rats regenerated rhodopsin to $54\% \pm 6\%$ ($n = 4$ retinas) of the dark value. In comparison, rats that were nonanesthetized regenerated rhodopsin in the same time to $84\% \pm 6\%$ ($n = 5$) and halothane anesthesia allowed regeneration only to $8.35 \pm 2.3\%$ ($n = 4$ retinas). We therefore suggest that the observed protective effect is specific for halothane due to the block of rhodopsin regeneration. However, we cannot exclude that other volatile anesthetics such as isoflurane might have effects similar to halothane.

CONCLUSIONS

Halothane anesthesia prevented metabolic regeneration of rhodopsin after bleaching. This led to a retina with very little bleachable rhodopsin after the initial bleaching, and, therefore, to an almost complete prevention of photon absorption in white light. Because the rate of photon absorption by rhodopsin is a critical parameter for light damage, halothane anesthesia led to protection against photoreceptor apoptosis. In contrast, when rhodopsin molecules could repeatedly absorb photons under blue-light exposure, halothane did not protect against light damage.

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