Flash Responses of Mouse Rod Photoreceptors in the Isolated Retina and Corneal Electroretinogram: Comparison of Gain and Kinetics

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Purpose. To examine the amplification and kinetics of murine rod photoresponses by recording ERG flash responses in vivo and ex vivo from the same retina. We also aimed to evaluate the two available methods for isolating the rod signal from the ERG flash response, that is, pharmacology and paired flash method on the isolated retina.

Methods. Dark-adapted ERG responses to full-field flashes of green light were recorded from anesthetized (ketamine/xylazine) C57BL/6N mice. ERG flash responses to homogenous light stimuli arriving from the photoreceptor side were then recorded transretinally from the same retinas, isolated and perfused with Ringer's or Ames' solution at 37°C. The responses were analyzed to determine the a-wave kinetics as well as the estimated flash sensitivity and kinetics of the full rod responses derived with the paired flash protocol. The analysis was complemented with pharmacologic blockade of glutamatergic transmission in the isolated retina.

Results. The a-waves were of comparable size, sensitivity and kinetics in vivo and in the isolated retina, but the onset of the b-wave was delayed in the isolated retina. The Lamb-Pugh activation constants determined for the a-waves were similar in both preparations. The kinetics of the derived photoreceptor responses were similar in both conditions, although the responses were consistently slightly slower ex vivo. This was not explicable as a direct effect of ketamine or xylazine on the photoreceptors or as their indirect effect through hyperglycemia, as tested on the isolated retina.

Conclusions. Through comparison to the corneal ERG, the transretinal ERG is a valuable tool for assaying the physiologic state of isolated retinal tissue. The rod photoreceptor responses of the intact isolated retina correspond well to those recorded in vivo. The origin of their faster kinetics compared to single cell recordings remains to be determined. (*Invest Ophthalmol Vis Sci.* 2012;53:5653–5664) DOI: 10.1167/iovs.12-9678

While various in vivo imaging and electrophysiology techniques have gained ground in brain research, neurophysiologic studies are still routinely performed on mechanically isolated neural structures or individual neurons. Under these artificial conditions, the neurons and neural networks are easily amenable to various experimental manipulations. However, the physiologic state of the cells relative to their function in vivo is difficult and sometimes impossible to validate.

The vertebrate retina is easy to isolate and its layered structure makes it well suited for detailed studies of neural circuits and signal processing. It can be stimulated with an exceptional spatial and temporal precision with its natural stimulus, light. The excised, perfused vertebrate retina has been used for several decades in vision research and as a more general model of signal processing by a well-defined neural circuit. In vision research, recordings from the retina ex vivo complement studies of visually guided behavior and recording of the electroretinogram (ERG) or visually evoked potentials (VEP) from live animals. In clinical sciences, the ERG is the electrophysiologic tool of choice for diagnosis and study of retinal malfunction. The electrophysiologic repertoire available for studies on the excised retina ranges from intracellular recording and patch clamp of the individual cells to recording field potentials (ERG) either across retinal layers or transretinally.[1–5] The electrical function of photoreceptors is routinely studied by recording electrical responses from individual cells,[6] or isolated cell parts.[5]

Electroretinogram recorded either across cell layers or transretinally from the isolated retina serves as a sort of intermediate between single cell level recordings and studies from live animals.[7–11] Photoreceptor function can be readily studied with minimal mechanical stress to the cells while they maintain their contacts to the rest of the retinal network. Compared to both corneal ERG and single cell studies, transretinal ERG presents superior signal to noise ratio. It enables long and complex experiments and allows free usage of pharmacologic and experimental manipulations that are either challenging or difficult to apply in vivo.

Although much of the foundational work in retinal research is built on amphibian tissues, the main interest of the field currently lies in mammalian vision. The mouse has emerged as the most important model species in phototransduction study, due to its easy husbandry and amenability to genetic modifications. The rod-dominated retina of this nocturnal animal has been deemed a reasonably good model for human

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peripheral retina.[12] Especially, the rod photoreceptor of the mouse retina has become the model of choice for phototransduction research. The rate constants and catalytic efficiencies of individual molecular factors of mouse rod phototransduction and adaptation have been clarified in high detail through ingenious experimental protocols and using various genetically manipulated mouse strains.[13–16] However, different experimental conditions may lead to quite different response kinetics, amplification and recovery rate constants in mouse rods.[17,18] A debate still remains over which experimental conditions and methods best represent the physiologic function of these cells.

Our aim was to use the corneal ERG as a benchmark for rod physiologic function. We recorded dc-ERG flash responses corneally from dark-adapted anesthetized mice. The retinas were then isolated and the same experimental protocol was repeated with ERG over the same retina ex vivo, perfused either with our standard nutrient solution or Ames' solution. We could thus rule out differences due to the mouse strain or rearing conditions, and address directly the consequences of isolating the retinal preparation for electrophysiologic studies. The questions we asked were: (1) How well do the rod flash response amplification and kinetics correspond each other in the isolated retina and in vivo?, (2) Do the two available methods for isolating rod photocurrent responses from the ERG signal-paired flash protocol and pharmacologic isolation yield similar results ex vivo?, and (3) How different experimental conditions, specifically the perfusant medium, affect the measured rod photoresponse parameters ex vivo?

We found that with suitable experimental conditions, mouse rod photoreceptors can be maintained in a stable state for hours ex vivo, with minor compromising of their sensitivity or response kinetics associated with isolating the retina. The rod flash responses obtained ex vivo with paired flash protocol and by pharmacologic isolation corresponded well to each other. In addition, we did not find major differences between the amplification and kinetics of the rod flash responses in the two perfusant solutions tested.

Methods

Animal Handling and the In Vivo Recording

Adult C57BL/6N mice were obtained from the Laboratory Animal Centre (LAC) of the University of Helsinki. They were maintained on a light dark cycle of 12 h light/12 h dark and dark adapted for $\geq$ 3 hours before the experiments. Preparations of the animals for the recordings as well as handling of the isolated tissues were done under dim red light. The procedures in experiments adhered to the European Union statement for the use of laboratory animals as well as the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the National Animal Experiment Board of Finland.

The mouse was anesthetized prior to an in vivo experiment by an intraperitoneal (i.p.) injection of ketamine (60 mg $\cdot$ kg^{-1} bodyweight, obtained from Intervet International B.V. Boxmeer, The Netherlands) and xylazine (6 mg $\cdot$ kg^{-1}, Bayer Animal Health GmbH, Leverkusen, Germany). Anesthesia was maintained by subcutaneous injections of the same substances with a 62% dose of the initial one. The first maintenance dose was given approximately 30 minutes after the initial i.p. injection, and thereafter at approximately 40 minute intervals. The left pupil was dilated using 10 mg $\cdot$ mL^{-1} atropine sulphate solution (Chauvin Pharmaceuticals Ltd., London, UK) followed by 100 mg$\cdot$mL^{-1} phenylephrine HCl (Bausch & Lomb, London, UK) solution 3 to 4 minutes later. The animal was placed on its abdomen in a small bed of black fabric glued to a plywood frame. The bed had a headrest, which resulted in the mouse's head being level with its neck and trunk. The bed containing the mouse was then placed inside a copper heating element (opening to the side), which enabled the animal's rectal temperature to be maintained between 37.4°C and 38.0°C.

The corneas of both eyes were anesthetized with 4 mg$\cdot$mL^{-1} oxybuprocaine HCl eyedrops (Santen Ltd., Tampere, Finland) before application of a 0.2 mm thick clear acrylic contact lens (left eye) fitted with a short peripherally sitting acrylic shaft containing a sintered Ag-AgCl pellet electrode, the tip of which was in close contact with the nasal corneal surface bordering the limbus. Electrical contact between the pellet and cornea as well as adhesion between the contact lens and cornea was facilitated by methylcellulose 50 mg soluted in 1 mL 0.9% NaCl. The right eye served as a reference: it was fitted with an identical lens with three coatings of black matte paint. No light evoked responses were recordable from the right eye following photic stimulation of the left eye (fitted with the clear lens electrode). As a ground electrode, a 0.37 mm diameter Teflon coated Ag wire with uncoated chlorided tip looped and twisted around itself, was moistened with the described methylcellulose solution and placed in the mouse's mouth immediately behind and to the left of its upper incisor teeth.

Stimuli were delivered to the left eye by a spherical (40 mm inner diameter) aluminum full-field stimulator, the inner surface of which was coated by Kodak White Reflectance Coating (part 6080) and fitted by 4 LEDs (Luxeon 1W cyan, Lumileds LLC, San Jose, CA). The LEDs were fitted equidistant to each other in holes in the stimulator wall close to a 12 mm diameter aperture in the sphere and directed in a 30° angle from the plane of the aperture. Direct light to the animal's eye from the LEDs was prevented by small curved aluminum shields spray painted with the reflective coating. The stimulator was brought close to the mouse's head so that the left eye was in the center of the 12 mm aperture, with the rim of the aperture approximately in the plane of the eyelids. The head of the mouse was tilted approximately 5 degrees to the right side thus directing the optical axis of the left eye towards the (inner) pole of the sphere opposite to the center of its eye aperture. The flash strength from the LEDs was current controlled through a custom made control unit driven by computer controlled command voltages. The photon flux in the center of the aperture was measured with a factory calibrated FDS100 silicon photodiode (Thorlabs Ltd., Cambridgeshire, UK) separately for each command voltage.

The ERG signals were amplified 1000×, low-pass filtered (eight-pole Bessel filter, $f_c = 1$ kHz) and digitized at 10 kHz with 0.25 to 2.5 μV resolution for further analysis.

After recordings the mouse was immediately euthanized, the eyes were enucleated and the retinas isolated for the ex vivo experiment (see below).

Transretinal (Ex Vivo) Recordings

The protocol for the ex vivo experiments has been described before.[19,20] In brief, the retinas were isolated in cooled Ringer's solution under dim red light and placed in a specimen holder with an active recording area of 0.5 mm (diam.) at the flat-mounted central retina. The upper (photoreceptor) side was superfused with a constant flow of Ringer's solution containing (mM): NaCl, 115.7; KCl, 3.3; $MgCl_2$, 2.0; $CaCl_2$, 1.0; glucose, 10.0; EDTA, 0.01; HEPES 10.0; NaOH, 4.8 mM; $NaHCO_3$, 20 mM. Leibovitz culture medium L-15 (0.72 mg/mL; Sigma-Aldrich Ltd., Helsinki, Finland) was added to improve photoreceptor viability. The solution was pre-heated to approximately 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2, which maintained its pH at 7.5. In a set of paired flash control experiments (indicated in the results), bicarbonate buffered Ames' medium (Sigma, pH $\approx$ 7.4) was used both for perfusion and isolation of the retina. Ketamine HCl was received as a gift from CU Chemie Uetikon GmbH (Lahr, Germany).

The retinas were stimulated from the photoreceptor side with 2 ms homogenous full-field flashes approximately parallel to the long axis of the rod outer segments. The stimuli were obtained from a dual-beam optical system with a 543.5 nm HeNe laser (Melles Griot 05 LGR 173,

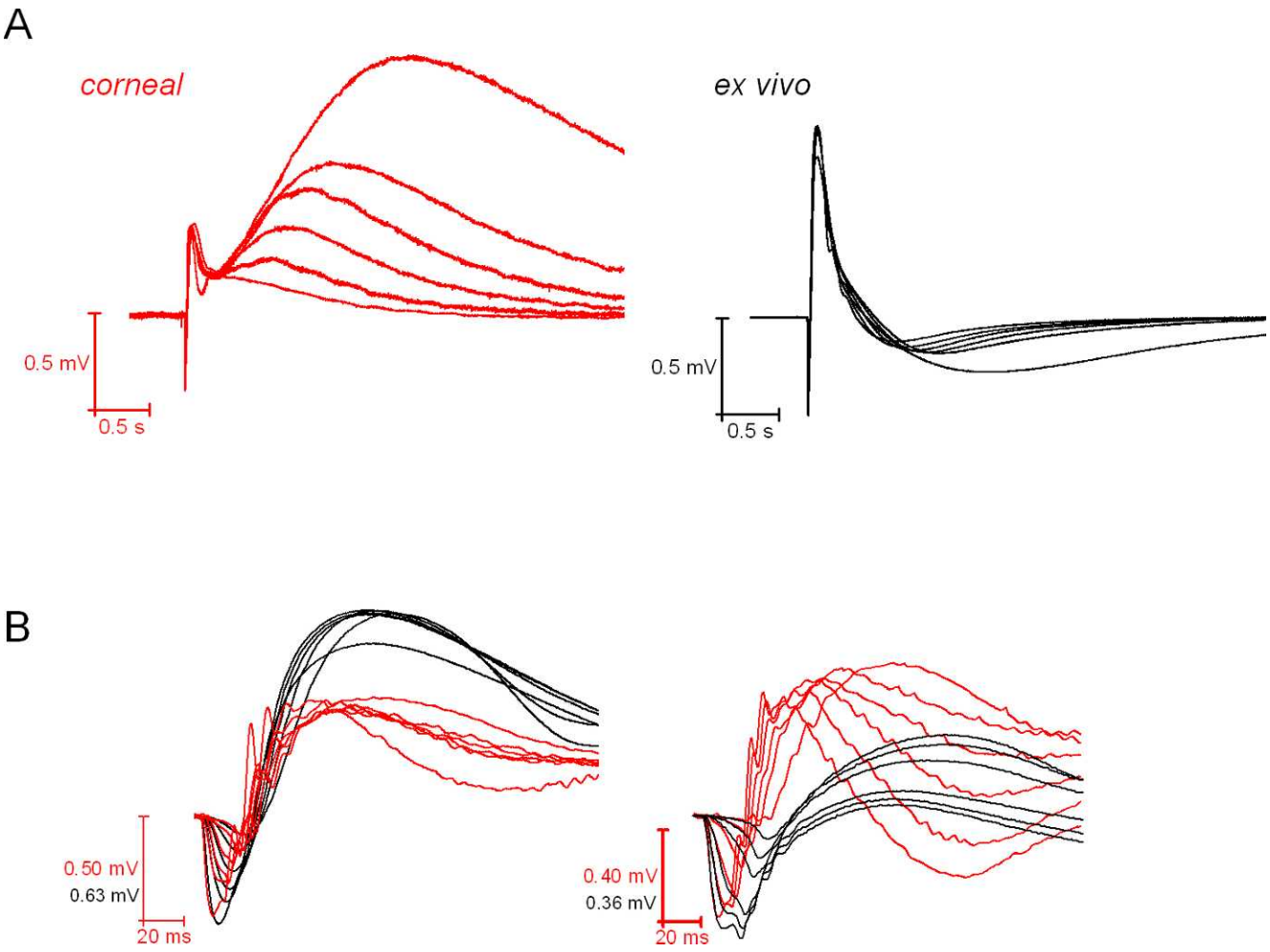

Figure 1. ERG flash response families from the same retina in vivo and ex vivo. (**A**) Scotopic flash response families recorded with corneal electroretinogram (*red traces* on the *left*) and ex vivo with transretinal electroretinogram (*black traces* on the *right*) from the retina isolated from the same eye immediately after the corneal recordings. The flash strengths, which were selected to produce approximately equal amounts of Rh*/rod in the two setups, range from 100 to 10,000 Rh*/rod with 0.5 log unit intervals for the five weakest stimuli, while the strongest stimuli elicited approximately 100,000 Rh*/rod. (**B**) Corneally (*red*) and transretinally (*black*) recorded flash responses superimposed for the retina of panel A (*left*) and another representative retina (*right*). The expanded time scale reveals the time course of the a- and b-waves. To facilitate comparison of the data, the ex vivo responses are scaled by factor 0.8 in the left panel and by 1.1 in the right panel to approximately match the rising edge of the a-waves recorded in response to the strongest stimulus in the two setups.

0.8 mW, Melles Griot, Carlsbad, CA), a 532 nm laser (Power Technology IQ5C[532–100]L74, ~ 130 mW; Power Technology, Alexander, AR) with Oriel (model #76992; Newport) shutters, the midpoint of the flash indicating the zero-time for the recordings. The light intensity of each source was controlled separately with calibrated neutral density filters and wedges. The absolute intensity of the unattenuated laser beam (photons μm^{-2} s^{-1}) incident on the retina was measured in each experiment with a calibrated photodiode (HUV1000B; EG&G Inc., San Francisco, CA; calibration by the National Standards Laboratory of Finland).

ERG light responses were recorded with Ag/AgCl pellet electrodes on either side of the retina. The DC signal was handled similarly to the corneal ERG data, that is, amplified 1000×, low-pass filtered (eight pole Bessel) with f_c = 1 kHz and sampled at 10,000 Hz with a voltage resolution of 0.25 μV. The electronics of the transretinal setup caused an additional 0.5 ms delay to the signal compared to the corneal ERG. This was taken into account during the data analysis.

Conversion of Flash Stimuli to Isomerized Photopigments in Rods (Rh*/rod)

In vivo. The photon flux incident on the cornea was converted to photoisomerizations (Rh*/rod),[21] that is, by calculating an effective collecting area for the rods regarding corneally incident photon flux, $a_{c,\,cornea} = 0.098\ \mu m^2$ at λ_{max}. The slight discrepancy to the previous estimate $a_{c,\,cornea} = 0.11\ \mu m^2$ arrives from the somewhat lower value for the optical density of the photoreceptors,[22] that is, 0.016 o.d. units μm^{-1} applied in our calculations. The collecting area at the cornea was further adjusted for the spectrum of the Luxeon 1W LEDS using the A1 nomograms,[23] leading to a stimulus-specific collecting area $a_{c,\,cornea} = 0.083\ \mu m^2$.

Ex vivo. The conversion of photons incident to the rod outer segments axially from the photoreceptor side of the retina was done as described previously,[19] leading to an effective collecting area of $a_{c,\,retina} = 0.73\ \mu m^2$ at λ_{max}, and for our stimulus lights (532 nm and 543.5 nm) 0.50 and 0.37 μm^2, respectively.

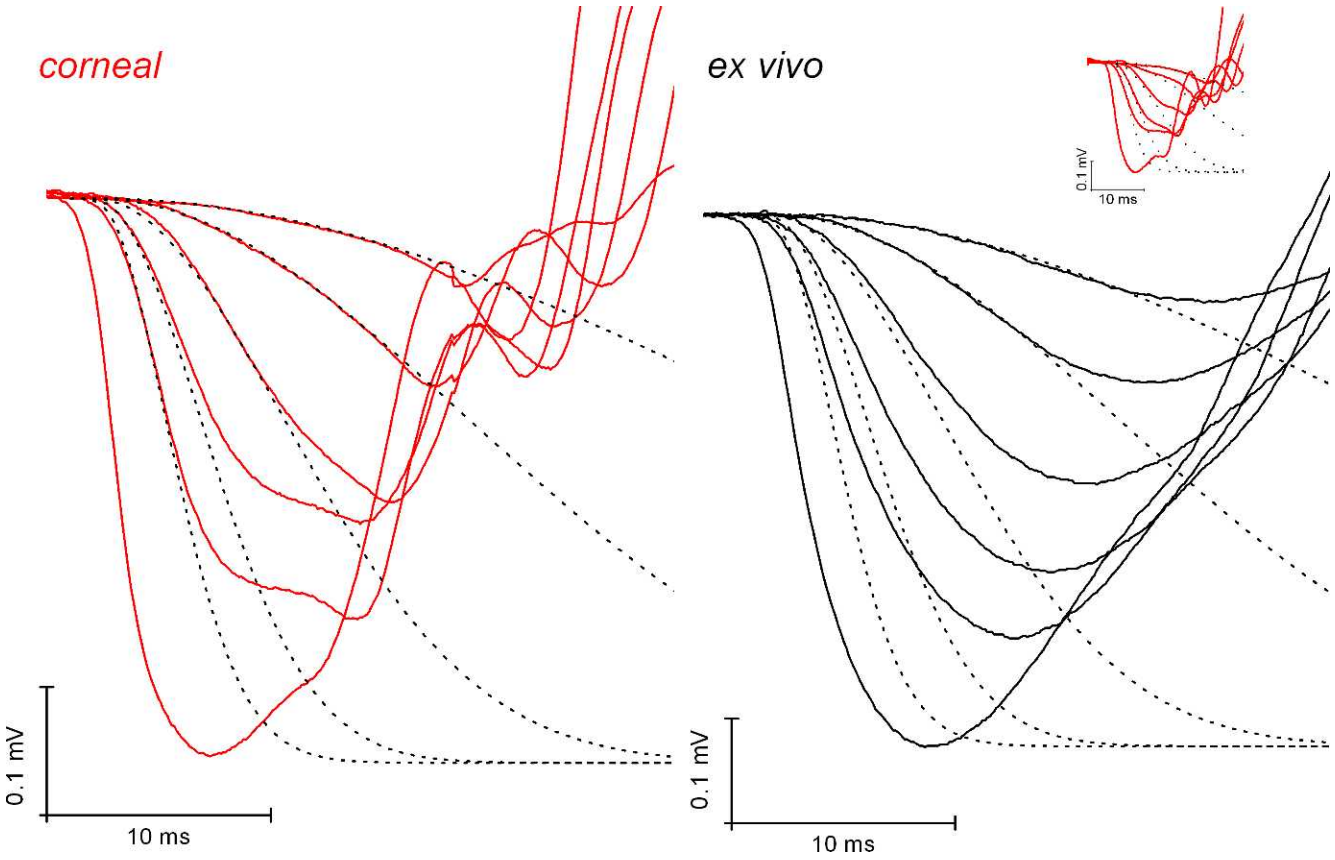

Figure 2. The activation efficiency of the rod flash responses is similar in corneal ERG and in the isolated retina. The phototransduction activation model (Equation 2) fitted to the a-waves of corneally and transretinally registered scotopic flash responses. For illustrative purposes, the corneally registered data consist of the averaged responses of 6 retinas to flashes producing estimated 100, 350, 1400, 4400, 10,000, and 98,000 Rh*/rod. The rightmost figure presents a flash response family of one representative retina ex vivo, flash strengths 50, 130, 420, 1700, 5300, and 110,000 Rh*/rod, with the corneally registered response family from the same retina shown in the inset for comparison. The model traces were fitted to the five smallest responses, while the amplitude of the largest response was used to determine the saturation level for the fit. The activation coefficients were 10.1 s^{-2} for the averaged response family on the left and 9.5 s^{-2} for the individual retina of the rightmost figure (10 s^{-2} for the same retina in vivo). The mean of activation coefficients from individual fits to the set of retinas used for the corneal average was $A = 11 \pm 1\ s^{-2}$ and $11 \pm 2\ s^{-2}$ for the same set of retinas ex vivo.

In both cases, the number of isomerizations per rod per stimulus of length Δt (Rh*/rod) was calculated from the measured photon flux I incident either on the cornea or on the retinal surface for respective collection areas a_c

$$\phi = I \cdot a_c \cdot \Delta t. \quad (1)$$

Experimental Protocols and Data Analysis

The retinas were allowed to dark-adapt for 20 to 30 minutes at the beginning of each experiment before 2 ms light stimuli, "flashes" were delivered. Interflash intervals (ranging from 15 seconds for the dimmest stimuli to 3 minutes with the saturating stimuli) were adjusted to allow the retinas to recover from previous stimulation prior to delivering the next one.

To analyze the activation kinetics of the a-wave, response families to flash stimuli ranging from ca. 1 to 100,000 Rh*/rod were collected. The early negative edge of the responses R(t) to stimulus range of ca. 20 to 40,000 Rh*/rod was fitted with the Lamb and Pugh activation model

$$R(t) = R_{sat}\left(1 - \exp\left(-\frac{1}{2}A\Phi(t - t_d)^2\right)\right). \quad (2)$$

where t_d is a delay parameter, which accommodates for the small delays both in phototranduction and the measurement electronics. The saturation level (R_{sat}) in each fitting was chosen as the peak of the a-wave in response to the strongest, saturating stimulus (~100,000 Rh*/rod). t_d and A were allowed to vary freely between retinas but were held constant for responses obtained from the same retina. The averaged activation coefficients A determined from fits to individual retinas are expressed as mean ± SE, similarly to other parameter values reported in the paper.

The time course of the rod photocurrent responses to an approximately half-saturating test flash was estimated in both setups with the paired flash paradigm.[24,25] In this protocol, the rod response to a test flash is probed with the reduction of the a-wave of the response to the second, saturating flash. A set of flash pairs with an intense probe flash following weak test flash with intervals varying from 10 ms to 600 ms was delivered to the retinas. A point-by-point presentation of the full time course of the rods' flash response can then be constructed from the reduction of the probe flash amplitude in the individual pairs (see Fig. 4 in Results for details and illustration). The paired-flash data were analyzed by subtracting the response to the test stimulus alone from the combined response to a test flash/probe flash-pair and determining the amplitude of the residual response. The amplitudes of the corneal responses were determined at a fixed time, selected in each experiment near the peak of the probe flash response, that is, approximately 7 ms from the initiation of the 2 ms light pulse. The transretinal responses peaked later (Fig. 1), and their amplitudes were determined both at the fixed time of 8 ms and another chosen near the a-wave peak, typically at 13 to 14 ms from the onset of the

probe flash (Fig. 5). To account for the gradual decline sometimes observed in the overall response amplitudes, the data were scaled with the saturated a-wave amplitude, as interpolated linearly from amplitudes to the probe flash delivered alone with 15 to 20 minute intervals during the experiment. The set of flash pairs was repeated two to three times. The individual points of the rod photoresponse were then constructed by averaging data over trials. To quantify the kinetics of thus derived responses, they were fitted with a model previously shown empirically to describe the mouse rod photoresponses isolated with the paired flash method in vivo:[26]

$$r(t) = 1 - \exp(-k \cdot u(t)), \quad (3)$$

in which the sensitivity parameter k scales with the stimulus intensity. The normalized response waveform

$$u(t) = [1 - \exp(-\alpha(t - t_d)^2)]\exp(-t/t_\omega). \quad (4)$$

The response kinetics are defined by two parameters (together with the sensitivity parameter k): α determines the response activation efficiency and τ_ω is a time constant dominating the response recovery. When Equation 3 is fitted to an individual pointwise response obtained with the paired flash protocol, both of these are affected by the choice of sensitivity parameter and are thus rather ambiguous in the absence of more response-intensity data. Thus the individual parameters were not assigned specific physiologic significance in this study, but the continuous response obtained from the fitting was rather used for reproducing the overall time course of the derived response.

The in vivo experiments, which lasted 1.5 to 2 hours after reaching stable anesthesia, were either "flash response family experiments" or "paired flash experiments." A typical transretinal recording consisted of both experimental protocols, after which the rod flash responses were pharmacologically isolated (Fig. 3 in Results).

Results

Scotopic Flash Response Families Recorded In Vivo and from the Isolated Retina

The dark-adapted flash response families recorded in vivo and across the same retina following isolation can a priori be expected to differ at least in one respect: the detachment from the pigment epithelium should abolish the c-wave. Indeed, this is the most noteworthy difference between the two response families of Figure 1A, which have been recorded from the same retina corneally and following subsequent isolation. The a- and b-waves appear similar, although in a closer look of the superimposed corneal and transretinal responses from this and another retina (panel B) it is evident that the b-wave, and thus apparently also the synaptic transmission, have been delayed in the isolated retina. In line with data published elsewhere on the isolated rodent retina,[11,27] the oscillatory potentials typical to the corneal ERG have also been greatly attenuated. This is not surprising in our recording geometry, as the setup is optimized for photoreceptor performance. The retina is efficiently perfused with the nutrient solution on the photoreceptor side and the layout of the experimental chamber is designed to support outer retinal function while the inner retina is laid on a filter paper, in contact with only a static reservoir of solution. The experimental conditions chosen for the present work will be further considered in the Discussion.

The a-wave Is Well Conserved in the Isolated Retina

To ease visual comparison, transretinally registered response families of Figure 1B have been scaled to approximately match the leading edge of the a-waves of the corneal (red) and transretinal (black) responses to the strongest stimulus (producing equal amount of Rh*/rod). Although there is variability in the postreceptoral components, the a-waves to the stimuli estimated to evoke comparable amounts of Rh*/rod in each case are approximately superimposed, suggesting that at least the activation phase of rod phototransduction is well conserved in the ex vivo recordings.

For a quantitative measure of the a-wave survival, the activation model by Lamb and Pugh[28] was fitted to the early stages of the a-waves recorded in both setups (Fig. 2). Due to the lower signal to noise ratio of the corneal ERG, an average over six response families from different retinas was selected for illustration, while the ex vivo response family has been recorded from one representative retina. Peak amplitudes of the responses to the brightest flashes, sufficient to saturate the a-wave, were used as the reference level for the saturated response. The model fitted well to the a-waves of flash responses from approximately 100 to 3000 Rh*/rod with constant A.[29] The average activation constant obtained from fits to 18 retinas did not differ statistically between in vivo ($A = 11.9 \pm 0.7$ s^{-2}, mean $\pm$ SE) and transretinal ERG ($A = 10.6 \pm 1.0$ s^{-2}), as judged by the paired *t*-test ($P = 0.24$). However, the comparison is subject to uncertainties in converting incident light to Rh*/rod in the two recording geometries (see Discussion).

Flash Response of Rod Photoreceptors Isolated with Two Methods Ex Vivo

Except for the very early activation phase, the photoreceptor flash responses in the ERG are masked under the prominent b-wave and other components arising from the inner retina. If the full gain and kinetics of the photoresponses are to be estimated, they must be revealed either pharmacologically or with the less invasive, although time consuming paired flash stimulation protocol (see Methods). Both methods are readily applicable ex vivo. We first compared these two widely used approaches in the excised retina, and then proceeded to compare the isolated rod photoresponse data in vivo and in the excised retina.

Figure 3 shows scotopic flash response families of a representative retina ex vivo before and after application of 50 μM DL-APB and 70 μM $BaCl_2$ to the standard perfusion. Under perfusion containing the agents, the b-wave as well as the glial component (slow PIII) are abolished and the photoreceptor component, fast PIII remains. The inset illustrates the pronounced glial contribution (slow PIII) to the flash ERG in another isolated retina after the b-wave had been removed with 20 μM DL-APB. The integrative nature of the glial component completely hinders analysis of the rod response recovery.[22] It also masks the typical plateau behavior of saturated rod responses, which is revealed following application of $BaCl_2$ to the bath solution (here 100 μM, red traces). In a subset of six retinas where the activation model was fitted before and after pharmacologic isolation, the activation coefficient was $A = 9.6 \pm 1.8$ s^{-2} in the control Ringer's solution and 8.9 ± 1.7 s^{-2} in the presence of 20 to 50 μM DL-APB and 70 to 100 μM BaCl2. The difference was not significant, as judged by the paired *t*-test ($P = 0.39$), confirming the observation that the early phase of the responses coincided in the two cases (Fig. 3).

Pharmacologic agents can be applied to the retina also in vivo, via intravitreal injections, but these methods do not eliminate the contribution of glial and pigment epithelial cells to the electroretinogram. We thus chose the paired-flash protocol[26] as another method for extracting the full waveform of the subsaturated rod flash response. The rod component can be enticed apart from the combined retinal response if a linear relationship holds between the a-wave and the

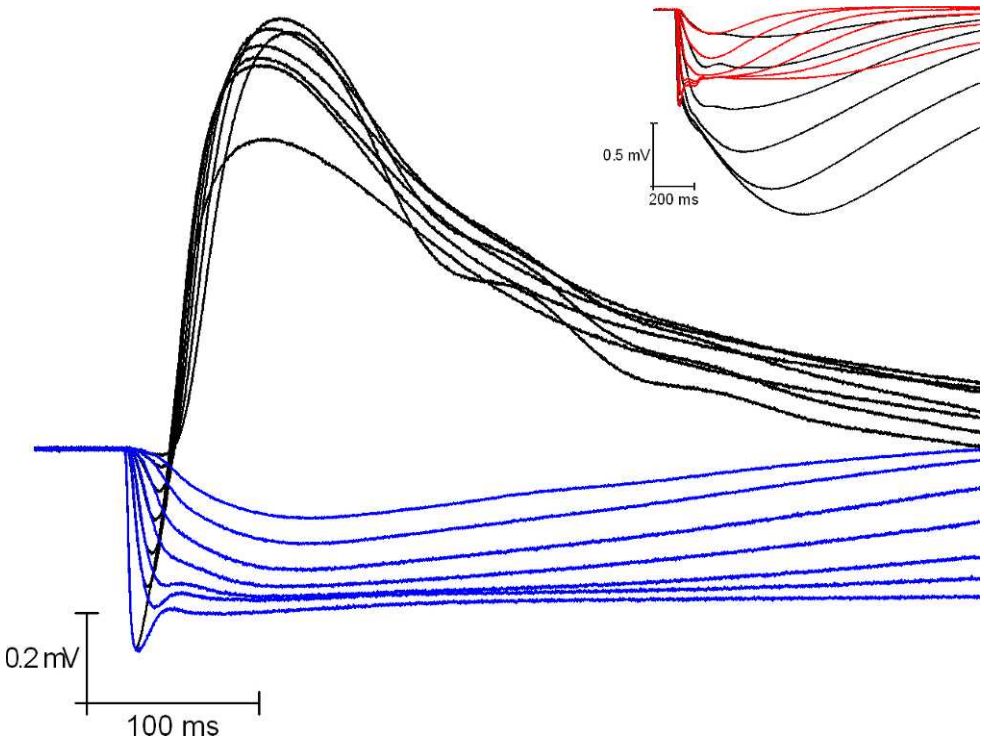

Figure 3. Pharmacologic isolation of the photoreceptor flash responses ex vivo. Flash responses from a single isolated retina to stimuli ranging from 20 to 600,000 Rh*/rod with 0.5 to 1 log unit intervals before (*black traces*) and after application of 50 μM DL-APB and 70 μM $BaCl_2$ (*blue traces*) to the perfusing medium. The inset demonstrates the removal of the glial component (slow PIII) by $BaCl_2$ in another retina: first a flash response family (*black traces*) to flash strengths ranging from 7 to 2300 Rh*/rod was recorded in the presence of 20 μM DL-APB to remove the b-wave, then 100 μM $BaCl_2$ was applied and the responses to identical stimuli were recorded (*red traces*).

circulating, light-sensitive current of the rods. The fraction of the light-sensitive current that has been turned off in response to a test flash at a given moment can be probed with a second, intense flash that quickly quenches the rest of the light-sensitive current. Panels A and B of Figure 4 demonstrate this method on one representative retina ex vivo. The retina of this illustration was perfused with Ames' medium to better preserve the b-wave during the intense stimulation (see Discussion). The response to the test flash delivered alone (red trace) is drawn superimposed to responses to test/probe flash pairs with varying time interval between flashes. In panel B the response to test flash alone has been subtracted from the paired responses. The reduction of the response to the latter flash reveals the relative amount of photocurrent extinguished by the test flash at the time of determination, t_{probe}. The full point-by-point photoreceptor response to the test flash (I_{flash} = 38 Rh*/rod, red continuous trace) is shown in Figure 4C (stars). Given the 310 μV amplitude of the saturated a-wave, this response is nearly half-maximal, indicating 2% to 3% fractional single photon response amplitude. For illustrative purposes, the derived responses shown in Figure 4 are determined as the reduction in the a-wave peak amplitude, although the fractional reduction of the probe flash response can be determined also at an earlier (constant) point in time. To better compare the flash responses of the isolated retina to those recorded in vivo, the analysis was performed at two time points: near the peak of the a-wave and at the fixed time of 8ms following the light flash onset. Comparison to the pharmacologically isolated response (black continuous trace) reveals excellent correspondence between the two isolation methods in this retina, even though the return phase of the pharmacologically isolated response is somewhat rounded and delayed. Panel 4D shows similar illustration on a typical retina perfused with Ringer's solution.

The Rod Photoresponse In Vivo and in the Isolated Retina

The pair flash protocol was applied to a set of retinas in vivo, and immediately repeated on the isolated retinas. Panel A of Figure 5 compares the averaged derived responses determined in vivo (three mice) to those recorded from the same retinas in Ringer's solution. The gray symbols denote rod flash responses determined from the probe amplitudes read near the a-wave peak (13–14 ms) and black symbols represent data points determined from the same data at 8 ms following the probe flash onset. As the b-waves tended to decay during the rather intensive stimulation required in the paired flash protocol, we did a control set of experiments in the Ames' solution, which is specifically known to support inner retinal function.[11] Figure 5B shows the respective data averaged from three other mice, compared to the paired flash responses determined in Ames' solution from the same retinas (in two mice) or a littermate's retina (one mouse) on the same day. The averaged transretinal and corneal responses are compared in the insets of panels A and B, showing higher sensitivity for the Ames' responses when determined at earlier times compared to the response determined near the probe response peak, but no significant difference in the standard solution. The flashes eliciting approximately half-saturated response in vivo were estimated to deliver 20 Rh*/rod. The isolated retina required approximately 20 to 50 Rh*/rod for corresponding flash responses, in line with previously published data.[30] Assuming Michaelis-Menten relation for the response amplitude versus intensity data, these would

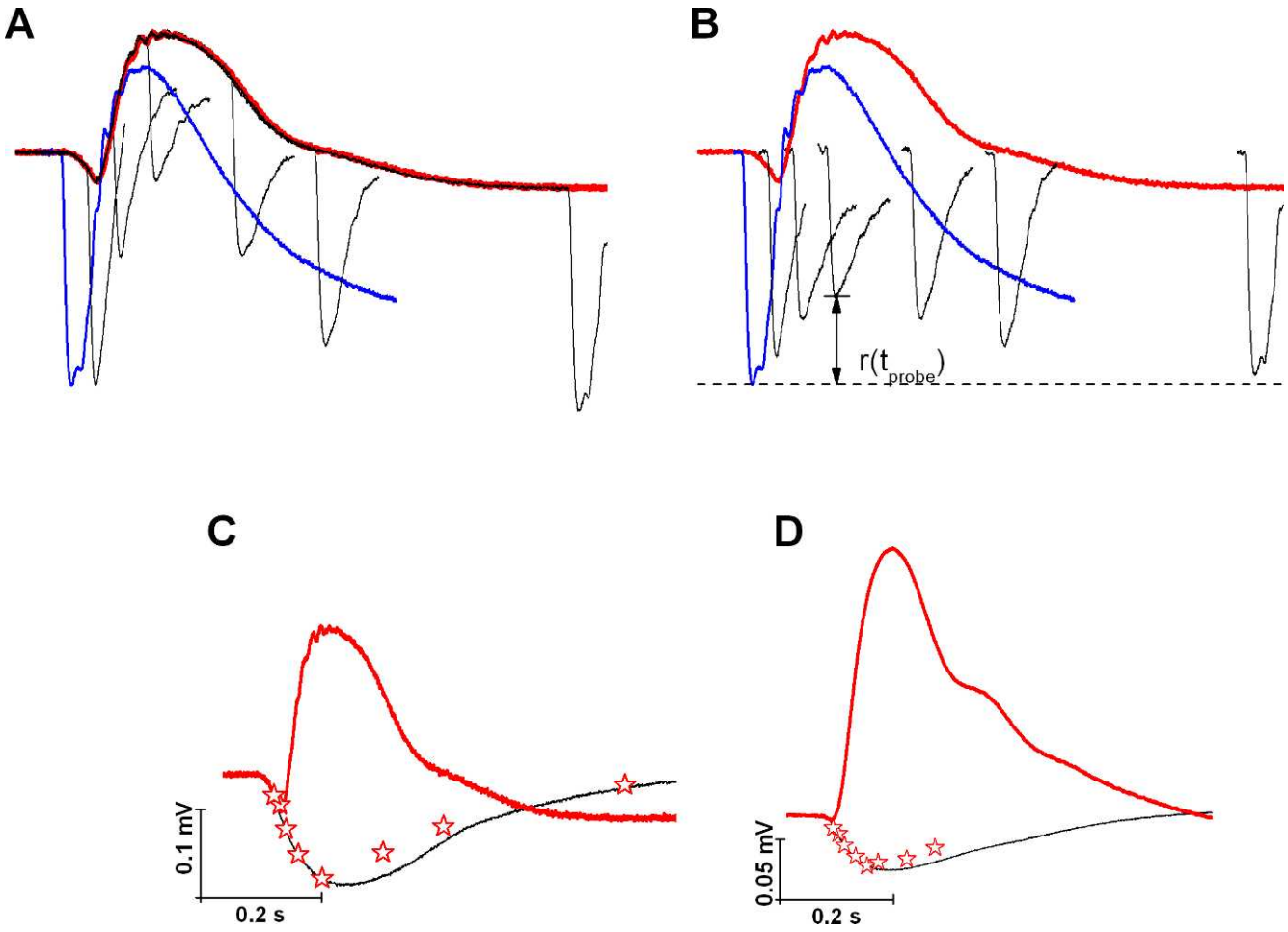

Figure 4. Extraction of the rod response to a test flash with the paired flash method ex vivo. (**A**) Responses to test flash (*red*) and probe flash (*blue*) presented alone at time zero, superimposed with responses recorded to paired stimuli, probe flash following the test flash with varying intervals (*black*). Individual traces represent single trials. (**B**) After the response to the test flash presented alone is subtracted from responses to the paired stimuli, reduction in the residual responses to the probe flashes, $r(t_{probe})$, reveal a pointwise presentation of the fractional photocurrent response to the test flash. (**C**) The photoreceptor response derived from the full set of test/probe flash pairs averaged over 3 trials on the retina of (**A**) and (**B**) (*red stars*), compared with the test flash response before (*red*) and after pharmacological isolation with DL-APB and $BaCl_2$ (*black*). (**D**) The photocurrent response isolated with the same two methods on another retina. The retina in **A–C** was superfused with the Ames' medium, the retina of (**D**) with the standard Ringer's solution. The test flash delivered approximately 38 Rh*/rod in both experiments. The saturated a-wave amplitude was 310 μV in the retina of (**A–C**) and 155 μV in the retina of (**D**).

signify approximately 5% fractional single photon response in vivo and 2% to 5% ex vivo. Yet, again, this comparison is subject to uncertainties in the photon-Rh*/rod conversion between the two setups.

A notable consequence of the isolation was the slight but consistent delay of the response peak and slowed deactivation. To quantify the delay, we fitted the empirical flash response model previously shown to fit the paired flash responses of mouse rods in vivo[26] to the pointwise photocurrent responses. The time-to-peak determined for averaged half-saturated responses fitted thus was 85 in corneal ERG vs. 113 ms ex vivo under perfusion with the standard solution (three retinas), determined near the a-wave peak and 109 ms as determined at 8 ms after the probe flash onset. For the three retinas perfused with Ames the averaged responses peaked at 113 vs. 110 ms, respectively, compared to 85 ms in vivo.

As there was clearly more scatter in the transretinal data than in the corneal responses, and especially one of the retinas of Figure 5A had pronouncedly slowed kinetics, we compared the responses determined for selected individual retinas in Figures 5C, D. The kinetics of these rod responses, which we interpreted to have preserved their physiological function well after isolation, were remarkably close to their corneally determined counterparts, especially in the retina perfused with Ringer's solution (Fig. 5D).

The Effect of Xylazine and Ketamine on the Photoreceptor Responses Ex Vivo

To test for any direct effects of the anesthetics on the photoreceptor function during the corneal recordings, ketamine (1–10 μM) and xylazine (0.3–3 μM) were each administered separately to isolated retinas. At these concentrations we observed relatively minor changes in the rod flash responses (Fig. 6A). The pharmacologically isolated rod responses to weak flash stimuli accelerated slightly upon direct application of 10 μM ketamine: the time to peak determined from near-linear range responses decreased 12%, from 196 ms to 171 ms (four retinas). This acceleration was consistent, although not statistically significant in this limited sample (paired *t*-test, $P = 0.109$). The saturated amplitudes (as measured from the peak of the a-wave) were also reduced by

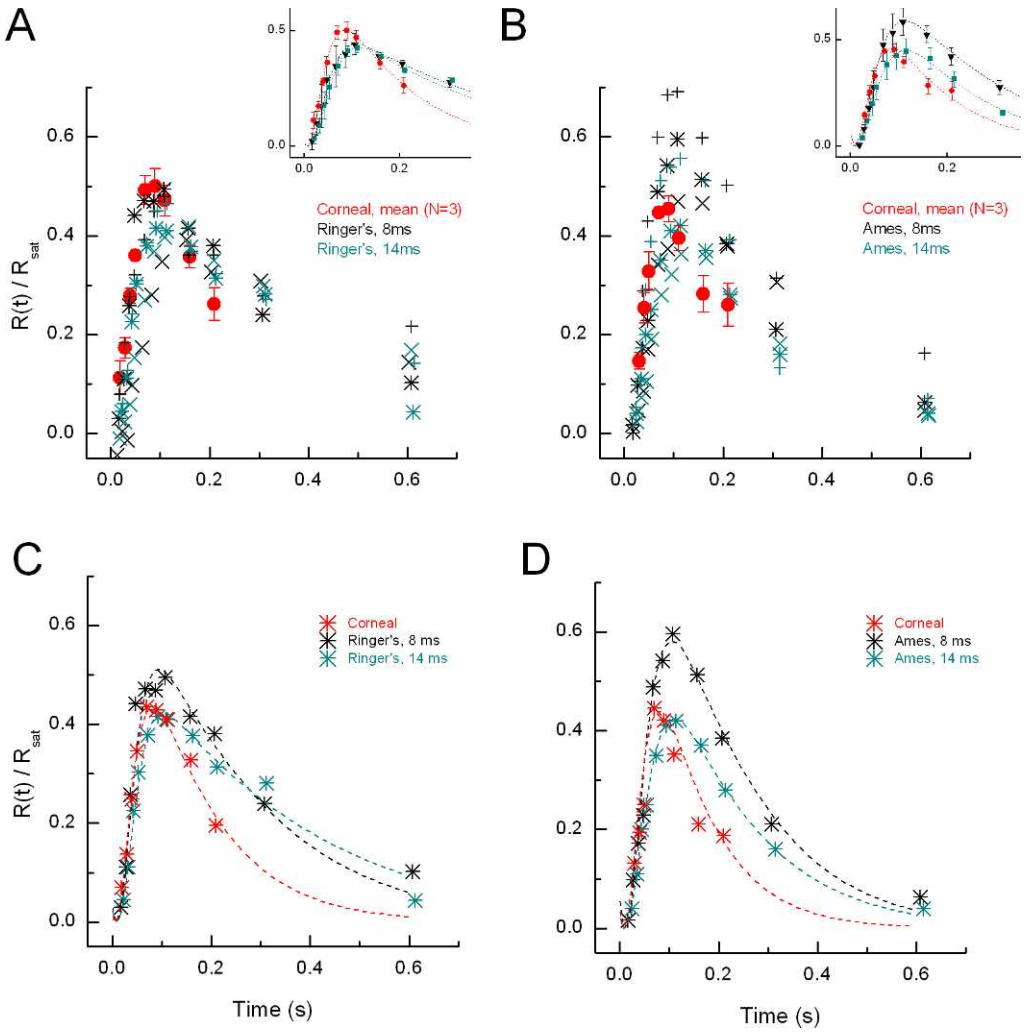

Figure 5. Comparison of the rod photocurrent response kinetics in vivo and ex vivo with paired flash method. (**A**) The *red circles* denote the derived rod photoresponse to a test flash ($\Phi_{test} = 20$ Rh*/rod), as averaged from three corneal recordings. The black symbols show individually the derived responses (Φ_{test} ranged from 20–40 Rh*/rod) from the same retinas following isolation, perfused with the Ringer's solution. The response amplitudes were determined 8 ms after the probe flash onset. The same analysis was done also at a fixed time (13–14 ms) near the probe flash response peak (*dark cyan symbols*). Despite variation among individual responses obtained with $t_{probe} = 8$ ms and $t_{probe} = 14$ ms, both the overall time course and the size of the averaged flash response values were generally similar (the inset). (**B**) An average of the corneally derived rod photoresponses from another three mice (*red circles*), compared to the responses derived ex vivo from 2 of the same retinas and the retina of the third mouse's littermate isolated on the same day, all perfused with Ames' solution. The color coding is as in (**A**). The inset again shows the mean of the transretinal responses determined at the two time points along with the averaged corneal responses. The *dotted lines* in each figure represent fits of Equation 3 to the responses for the purpose of determining the time of peak from the pointwise responses. (**C**, **D**) Individual representative experiments from (**A**) and (**B**), respectively, denoted with the same symbols as in those panels.

20%. Xylazine at 0.3 to 3 μM did not have noticeable effect on the pharmacologically isolated rod flash responses (Fig. 6C).

Both ketamine and xylazine, like many other anesthetics, are also known to have systemic metabolic effects such as inducing hyperglycemia, which in turn affects at least some ERG components.[31,32] However, doubling of glucose level into 20 mM caused no significant changes in the gain and kinetics of the rod responses in the two isolated retinas tested (Fig. 6D).

Discussion

Rod Flash Response Kinetics

The rod flash responses determined with the paired flash protocol in vivo are similar in kinetics and sensitivity to those published by other investigators,[26,33] and they were well conserved ex vivo. Yet despite similar activation efficiency of the responses, the subsaturated flash responses derived with the paired flash method peaked consistently later ex vivo.

The slowed kinetics of the transretinal rod photoresponses may originate in part from the geometry of our setup: the green stimuli arrived to the retina from its distal side. Thus the distribution of absorbed photons favored the distal part of the outer segments in the isolated retina as opposed to their proximal parts in vivo. It remains to be tested whether the light responses generated in the distal end of the mouse rod outer segments are slower than those originating in the proximal end, but it has been reported so in toad rods.[34] However, the chemical and physical environment of the retina is likely to be a more significant factor in shaping the responses known to be sensitive to perfusion composition, temperature and pH.[11,22,35,36] While trying to optimize these factors for the mouse retina, we found that besides the obvious need for

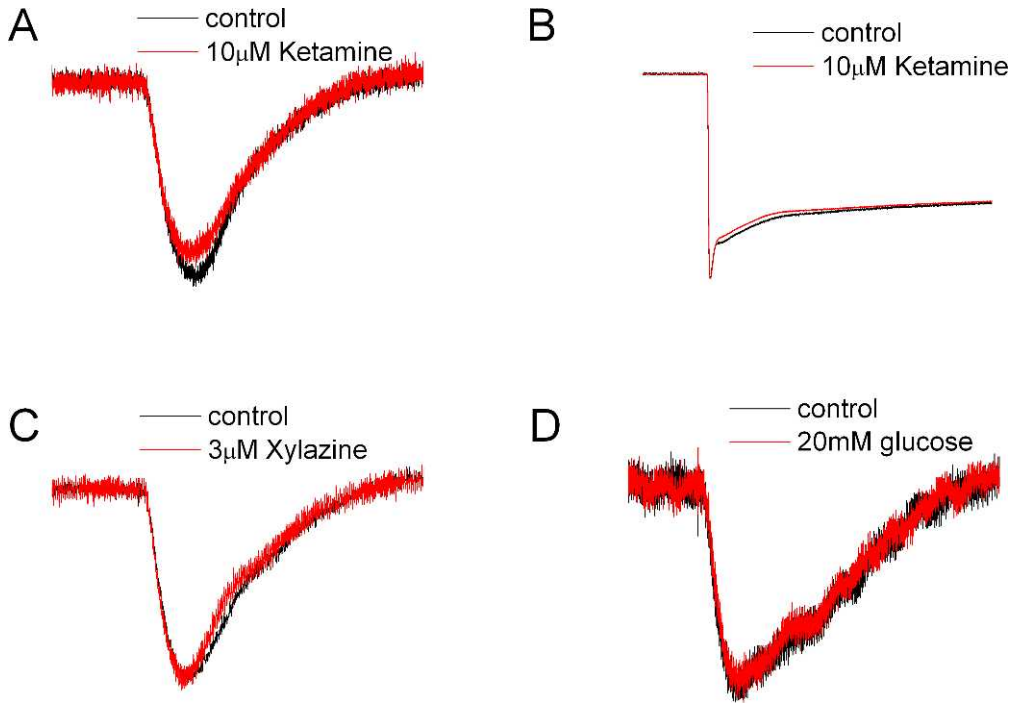

Figure 6. Effect of ketamine, xylazine, and raised glucose level on the pharmacologically isolated rod flash responses. (**A**) Linear range (<10% of the saturation level) responses to the same stimulus before (*black*) and after (*red*) administration of 10 μM ketamine HCl to the perfusion. (**B**) Responses of the same retina to a saturating stimulus of constant strength before (*black*) and after introducing ketamine HCl (*red*) in the same experiment, normalized to the a-wave peak. Neither the leading edge kinetics (a-wave) nor the transient negative "nose" are changed by ketamine. (**C**) Linear range responses of another retina before (*black*) and after (*red*) administration of 3 μM xylazine. (**D**) Increasing glucose level on the retina did not affect rod flash responses: *black trace* represents linear range response in standard Ringer's solution and *red trace* after doubling the glucose level of the perfusion to 20 mM. To better compare the response gain and kinetics, all responses in (**A**–**D**) have been fractionalized by dividing with the saturated a-wave amplitude, masking slight overall drop of amplitude following ketamine application. The responses of (**C**) and (**D**) were digitally band block filtered (FFt filter, OriginPro 8.1) with lower and upper cutoff frequencies 48 and 52 Hz, respectively.

temperature and pH regulation, a high perfusion flow rate was a necessary prerequisite for stable and near physiologic rod function. The flow rate of 4 to 5 mL perfusant per minute through the small (approximately 5 mm^3) confined space above the retina indicated complete renewal of the liquid near the retina at approximately one thousand times per minute.

The perfusant flow was applied only on the distal side of the retina, which may have affected the latency and size of the b-waves. Control experiments indicated that the inner retinal components (b–wave and oscillatory potentials) were less variable and could be better preserved and maintained in Ames medium. Yet the initiation of the b-wave was still delayed as compared to corneal ERG. The generalizability of the apparent delay in the synaptic transmission upon isolation of the retina and its dependence on experimental conditions certainly warrant further investigation.

The photoreceptor responses obtained with Ames and with our Ringer's solution showed little difference. Yet as a general observation, the pharmacologically isolated linear range responses tended to peak later in the limited subset of retinas perfused with Ames than under our standard solution, although we did not perform explicit statistical analysis. This is qualitatively in line with the differences in single cell current response kinetics reported[11] in different media, even though the ERG flash responses are less affected by the choice of solution.

The Rod Flash Responses: ERG versus the Suction Pipette

The rod flash responses determined with the electroretinogram ex vivo are still considerably faster than the respective responses recorded from the rod circulating current with the suction pipette. This may be partly attributed to the different origins of the two signals: the electroretinogram originates from the radial potential distribution of the extracellular space and might be shaped by the voltage-gated conductances of the inner segment and/or capacitive currents,[37,38] whereas the suction electrode records the current between inner and outer segment reflecting the cyclic nucleotide-gated (CNG) channel current more straightforwardly compared to the ERG. On the other hand, the transretinal recording can be argued to be less invasive to the cells, better maintaining their "natural" state. Specifically, the part of the cell drawn into a suction pipette is blocked from the perfusion flow. The solution inside the pipette also generally lacks HCO_3-buffering. Both of these conditions tend to slow down responses in our setup. Lack of efficient perfusion flow through the proximal side of the retina in our setup may also explain the slowing down of our responses compared to those published for the isolated retina by Azevedo and Rieke.[11] Settling the origins of the differences between rod photoresponses recorded with ERG and suction pipette is still a work in progress.

The Role of Pigment Epithelium in Shaping the Electroretinogram

The pigment epithelium (RPE) forms a part of the blood/retina barrier and plays multiple supportive roles in the retinal function,[39] such as maintaining the rod visual cycle via supplying the photoreceptors with fresh 11-*cis*-retinal, transporting ions, proteins, nutrients and water to and from the retina and buffering ionic and pH changes in the extracellular

space. It participates in photoreceptor renewal by engulfing shed disks, and its processes surround the viable parts of the photoreceptor outer segments. Thus isolating the retina from the eyecup and the pigment epithelium is bound to affect the retinal cells and bring changes to the ERG flash response. The main alterations considered below are (1) alteration in the extracellular ionic buffering, (2) disruption of the rod visual cycle and (3) change in the geometry of the outer retina.

Ionic Buffering in the Extracellular Environment

As the rods hyperpolarize in response to light, the potassium flux through their plasma membrane is reduced and $[K^+]_0$ falls in the vicinity of the photoreceptors. The lowered $[K^+]_0$ initiates hyperpolarization in the apical (photoreceptor side) membrane of the pigment epithelial cells.[40–42] This gives rise to the c-wave, disappearance of which from the dark adapted ERG response is a readily apparent consequence of isolating the retina from the pigment epithelium (Fig. 1).

The c-wave being absent, a highly pronounced glial component (slow PIII) is evident in the transretinal ERG responses. It is not directly observable in vivo, which may be partly due to it being masked by the c-wave of the opposite polarity. In addition, the absence of the K^+- buffering action of the RPE may lead to an amplified response in the K^+ currents of the Müller cells. Thus the pronounced slow PIII of the excised retina may partly result from detaching the retina from the pigment epithelium.

Visual Pigment Regeneration

The capacity for rhodopsin regeneration is limited in the isolated retina as detachment of the RPE disrupts the visual cycle. However, a typical pair flash experiment does not bleach adapt the rods significantly. The ~ 40 to 70,000 Rh*/rod probe stimuli, which are typically presented 15 to 30 times during the protocol, bleach 2% to 5% of the visual pigment content, much of which is regenerated from the rods' moderate *11-cis* reservoirs during the 2 to 3 minute interflash intervals.[43]

Change in the Retinal Geometry

Removal of the RPE processes that tightly enclose the rod outer segments in the intact eye affects the resistance profile of the extracellular space. Thus extracellular resistance decreases around the rod outer segments, possibly exaggerating the relative contribution of currents along inner segments in the ex vivo recordings. We are not able to give a quantitative estimate for this effect. Yet it is likely to be minor, especially as forcing the retina to the flat mounted geometry tends to have the contrary effect of reducing the extracellular space around the rod outer segments.

Anesthetics and Mydriatics

The anesthetics used for the corneal recordings can affect the obtained data in two ways: either through affecting the general metabolism of the animal or through direct action of the compounds on the retinal cells. In the present work, we tested for direct effects of 1 to 10 μM ketamine HCl on the flash responses of rods on the isolated retina. At these concentrations we observed minor acceleration of the isolated rod flash responses. This might contribute to the observed difference in the flash response kinetics between corneal recordings and transretinal ERG. The actual level of anesthetics in the retinal tissue is not known but plasma concentration of ketamine during maintained anesthesia is 5 to 20 μM in mammals[44] and it permeates the blood-brain barrier with ease.[45]

Ketamine has been reported to affect several targets in neuronal signaling. Of particular interest for photoreceptor study, it has been reported to inhibit recombinant HCN1-containing channels and neuronal hyperpolarization-activated cationic current (I_h) at clinically relevant concentrations.[44] HCN1 is also abundantly expressed in retinal rods, in which the I_h current shapes the photovoltage response, effectively band-pass filtering the information passed to the bipolar cells.[46–49] Inhibiting these channels with Cs^+ or ZD7288 also markedly shapes the pharmacologically isolated rod ERG response to strong stimuli by removing the prototypical nose-plateau behavior and increasing the plateau amplitude of the responses.[50] However, we could not observe such behavior with the ketamine concentrations that were tested, suggesting that ketamine/xylazine anesthesia does not suppress the I_h currents in mouse rod photoreceptors.

Although we could not validate each of ketamine's and xylazine's known or hypothesized molecular target separately, the minor changes in the flash responses following ketamine/xylazine application to the perfusion suggest that either none of these mechanisms shape the rod flash response or that the applied concentrations on the retina were too low to have significant modulatory effects.

The various systemic effects of the anesthetics on metabolism and subsequently on the corneal electroretinogram are intrinsically more difficult to evaluate than the direct actions of the drug on the photoreceptors. It is known that ketamine anesthesia induces hyperglycemia in human,[51] monkeys,[52] and rats.[53] Both ketamine administered alone and ketamine/xylazine combination also raise blood glucose levels in mice.[54] Hyperglycemia significantly increases the scotopic b-wave of the corneal electroretinogram in the mouse[32] and in human type 2 diabetic patients,[55] as well as in the ERG recorded from the excised, perfused eye.[56,57] Our experiments suggested that such an effect is not significant at the photoreceptor level.

According to a recent report,[58] also the generally used mydriatic atropine and phenylephrine initiate a significant augmentation in both the a- and b-waves of murine ERG when combined with the ketamine/xylazine anesthesia. The molecular route of this augmentation is not known. Further studies are also needed to find out whether this augmentation involves changes in the kinetics of the rod photoresponse or whether it arises from simple scaling of the ERG signals.

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