Chlorophyll derivatives as visual pigments for super vision in the red

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The primary event in vision is light-initiated activation of visual pigments. All visual pigments consist of the protein opsin bound to 11-*cis*-retinal and are responsible for initiating the transformation of light into an electrical signal. In a mouse model, we show that derivatives of chlorophyll can act as visual pigments initiating the transformation of light into an electrical signal and thus change the primary event in vision to initial activation of a chlorophyll derivative. Electroretinographic b-wave amplitudes recorded in response to red and blue light were two-fold greater in mice administered chlorin e_6, which accumulated in photoreceptor outer segments.

Introduction

The primary event in vision is light initiated activation of visual pigments. All visual pigments consist of the protein opsin bound to 11-*cis*-retinal and are responsible for initiating the transformation of light into an electrical signal, which travels down the optic nerve to the brain. In humans three visual pigments, used primarily for daytime color (photopic) vision, reside in cone cells and sense blue (450 nm), green (530 nm) and red (560 nm) light. One visual pigment is found in rod cells whose use is relegated to night-time (scotopic) vision and responds primarily to green (500 nm) light.[1] As a result of less light scattering, dim light vision in the red region of the spectra would impart a large biological advantage, especially in conditions such as of haze, fog and underwater,[2] with a strong Rayleigh type $1/\lambda^{-4}$ dependence.[3] Scattering of light at 400 nm is 9.4 times greater than at 700 nm for equal incident intensity. However, as the absorption by rhodopsin, the opsin–retinal complex responsible for night vision in most mammals, is minute above 600 nm, the pigment is not believed to sense red light. Red light vision is thus limited to red cones. Red cones, however, are about 100 times less sensitive than rhodopsin in detecting light due to their relative rarity, higher rate of thermal isomerization[4] and the higher reversibility of the red pigment complex *vs.* apoprotein and 11-*cis*-retinal,[5] making them inefficient in night-vision. How might one enhance red light night-time vision?

In the compound eye of the fly (*Musca*, *Calliphora*, *Drosophila*) high UV sensitivity arises from the photostable pigment 3-hydroxyretinol that acts as a sensitizer for rhodopsin. According to this model the photostable UV-absorbing pigment absorbs light quanta and transfers the energy to the blue-absorbing visual pigment.[6] Similar energy transfer has been observed between retinol and rhodopsin in *Simuliid males* flies.[7] Energy transfer from the carotenoid salinixanthin to bacteriorhodopsin has been observed in the eubacterium *Salinibacter ruber*.[8]

Based on the observations that a photostable derivative of chlorophyll is isolated with the rhodopsin of the deep-sea dragonfish (*Stomiidae*) and that this visual pigment is bleached with long wavelength light absorbed primarily by the photostable chlorophyll derivative, it has been suggested that the fish has evolved to use the chlorophyll derivative as a sensitizer to see red light.[9,10] We have shown that in the presence of various porphyrins the bleaching of bovine rhodopsin in response to red light is also enhanced leading us to conclude that vision enhancement by an unbleachable chlorophyll derivative might therefore be a general phenomenon in vertebrate photoreception.[11] We further showed that living rods extracted from a salamander accumulate an exogenous chlorophyll derivative that rendered them as sensitive to red light as they were to green.[12] Recent observations of the *Erenna* siphonophore, a relative of the jellyfish, suggest red light vision may be widespread in the deep-sea[13] despite the fact that no red-sensitive visual pigments have ever been isolated from deep-sea fish.[2,14–16] Given the above data coupled with the observations that porphyrins of virus architectures are actively transported into mammalian cells[17] we hypothesized that porphyrins may be utilized to effectively enhance mammalian red light vision. In order to investigate this hypothesis, we studied whether an intravenously injected chlorophyll derivative accumulates in the eyes of mice and increases the response to red or blue light.

Experimental

Animals

BALB/C albino mice (Charles River Breeding Laboratories), which weighed 25–35 g, were used throughout these experiments. All animals were maintained on a 12 h light–dark schedule in a temperature and humidity-controlled environment. All protocols were approved by the Institutional Animal Care and Use Committee of Columbia University and complied with guidelines set forth by The Association for Research in Vision and Ophthalmology.

Chlorin e_6 localization

Mice ($n = 4$) were intravenously injected with 2 mg kg^{-1} of chlorin e_6 (Frontier Scientific, Logan, Utah, in a solution of phosphate buffered saline, adjusted to pH 7.4 with $NaHCO_3$). After 1 h, the mice were sacrificed, the whole eyes were enucleated, the anterior chambers including the lenses and vitreous were removed and the remaining eyecups were homogenized in phosphate

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buffered saline (1 ml; pH 7.0). The solution was extracted with a chloroform : MeOH (3 : 1) solution (1 ml). The organic phase was concentrated under a stream of argon to 0.5 ml and used directly for fluorescence spectroscopy measurements (excitation 400 nm).

Mice ($n = 3$) were intravenously injected as described. One, two and three hours after injection the eyes along with eyes from control (non-injected) mice were dissected and the fresh retinas were prepared as whole mounts and viewed using an automated Axioplan II fluorescence microscope (Carl Zeiss, Inc.) with an AxioCam HRc digital camera and Axiovision 4.3 software (Carl Zeiss, Inc.) (fluorescence conditions: excitation 400 nm; emission >640 nm).

In other experiments, whole eyes were fixed using a published procedure with slight modification.[18] Briefly, eyes were removed and placed in a 25 ml glass vial containing a 20 ml solution of NaCl (124 mM), KCl (5 mM), $MgSO_4$ (2 mM), $NaHCO_3$ (22 mM), glucose (10 mM), $CaCl_2$ (2 mM), and NaH_2PO_4 (1.25 mM). This mixture along with a 200 ml water load (in a glass beaker) was placed in a domestic microwave and heated for 45 s at power lever 5 (temperature reached 70 °C). The samples were allowed to cool to room temperature, rinsed once with the above solution, embedded (in OCT) and frozen with liquid nitrogen for cryostat sectioning, and subsequent fluorescence microscopy.

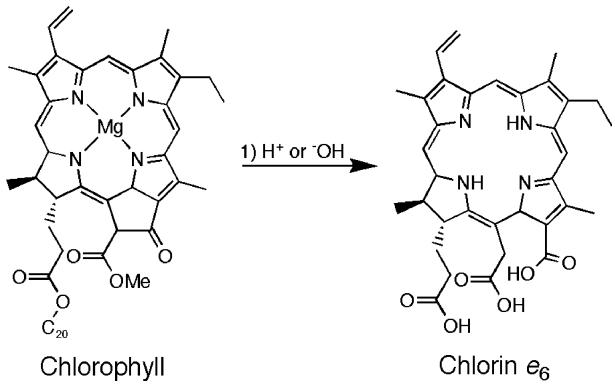
ERG recordings

Seven mice ($n = 7$) were used as control mice (non-injected) and ten mice ($n = 10$; five of which were from the control group after recording their normal, non-injected responses), were given chlorin e_6 prior to ERG recordings. After dark-adaptation overnight, mice were intravenously injected with 2 mg kg^{-1} of chlorin e_6 (in a solution of phosphate buffered saline, adjusted to pH 7.4 with $NaHCO_3$) and ERG recordings were measured 1–3 h after injection. All procedures were carried out under red safelight light (Bright Lab Jr, Delta 1/CPM, Inc., Dallas).

All ERG recordings were performed using published procedures with modification as indicated below.[19–21] Pupils were dilated with phenylephrine hydrochloride (2.5%) and cyclopentolate hydrochloride (0.5%) applied topically to cornea. After 10 min, mice were placed on a heating pad to maintain body temperature at 37 °C and anaesthetized with an intraperitoneal injection of a mixture of ketamine (about 80 mg kg^{-1}) and xylazine (about 5 mg kg^{-1}) and corneal hydration was maintained by topical application of methyl cellulose (1 drop; ~50 mg; topical to cornea, Methocel; Dow Chemical Co., Zürich, Switzerland). In addition to general anaesthesia, corneal anaesthesia was achieved with tetracaine hydrochloride (0.5%; Bausch and Lomb) and mice were situated for ERG recordings. The reference electrode (tungsten) was incorporated into an eyelid speculum and placed under the upper and lower eyelids (in contact with the sclera) of the right eye. The active electrode was a platinum–iridium wire, formed in a loop and placed on the right cornea below the pupil. Electrical contact between the cornea and electrode was achieved with a drop of methyl cellulose. The ground electrode was clamped onto the tail. To maintain body temperature at 37 °C, the mouse rested on homeothermic blanket connected to a control unit (Harvard Apparatus). The light stimulus was delivered from a desk-top ganzfeld stimulator (Color Dome, Diagnosys LLC, Littleton, MA) and responses were recorded on the Espion console. LED output wavelengths were measured with a CCD-spectrometer (Ocean Optics Inc., Dunedin, FL). Recordings lasted about 20 min, blue or amber LED flashes (5-flashes) preceded red flashes (17-flashes; Xenon lamp with <640 nm cut off filter) and a 20–30 s adaptation time was allowed between flashes. Recordings were repeated in each mouse up to three times.

Results

For our studies we selected the water-soluble chlorophyll derivative chlorin e_6 (Scheme 1), which is used as a food colorant, a dietary supplement[22] and in cancer therapy.[23] Commercially, chlorins are prepared from acid or base treatment and/or transmetalation of chlorophyll *a* according to Scheme 1.[24–26]



Scheme 1

In order to determine whether the water-soluble chlorophyll derivative chlorin e_6 could penetrate the mouse blood-retina barrier and localize in mammalian photoreceptor cells that contain the visual pigments, mice ($n = 4$) were intravenously injected with a solution of chlorin e_6. One hour after administration the eyes were removed and fluorescence spectroscopy was performed on a chloroform : MeOH (3 : 1) extract of the posterior eyecup tissue, the results of which are shown in Fig. 1. Along with a weak emission band centered at 450 nm, which was assigned to native eye pigments,[27] a strong emission band centered at 675 nm was

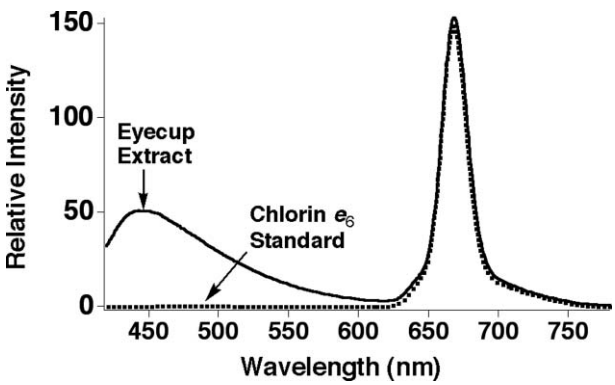


Fig. 1 Solid line: fluorescence spectrum of a chloroform : MeOH (3 : 1) extract of eyecups dissected form mice ($n = 4$) 1 h after i.v. administration of chlorin e_6 (2 mg kg^{-1}); 400 nm excitation. Dotted line: fluorescence spectrum of a chloroform : MeOH (3 : 1) solution of chlorin e_6; 400 nm excitation.

observed. This 675 nm band was assigned to chlorin e_6, since in a standard solution of chlorin e_6 prepared in the same solvent, (Fig. 1; dotted line) a strong fluorescence band centered at 675 nm was also observed. This 675 nm band was not observed in eyecup extracts of non-injected mice (data not shown).

A second group of mice ($n = 3$) was administered chlorin e_6. One, two and three hours after injection the eyes were dissected and the whole retinas mounted and viewed under a fluorescence microscope. At all three times, red fluorescence (>640 nm) was observed in the retinas of injected mice (data not shown) but not in retinas of control mice. These observations indicated that intravenously injected chlorin reaches the retina and remains three hours post administration. These observations are in accord with similar experiments in rabbit models.[28]

In order to determine whether the red fluorescence was localized within the retina, a third group of mice ($n = 2$) was administered chlorin e_6. One and two hours after injection, the eyes were enucleated, cross-sectioned and viewed under a fluorescence microscope. Fig. 2A shows a microscopic image of a cross section of a retina. The pigment epithelium (PE) and outer segment layer (OS), inner segment (IS), outer nuclear layer (ONL) and the ganglion cell layer (GCL) are labeled. Fig. 2B shows the same cross section under fluorescence conditions (ext. 400 nm band pass filter; em. < 640 mn cut off filter). Red fluorescence was localized in the outer and inner segment layers and the pigment epithelium layer. Very little red fluorescence was found in the other layers of the retina, such as the ONL and GCL layers. This suggests preferential accumulation of chlorin near the visual pigments.

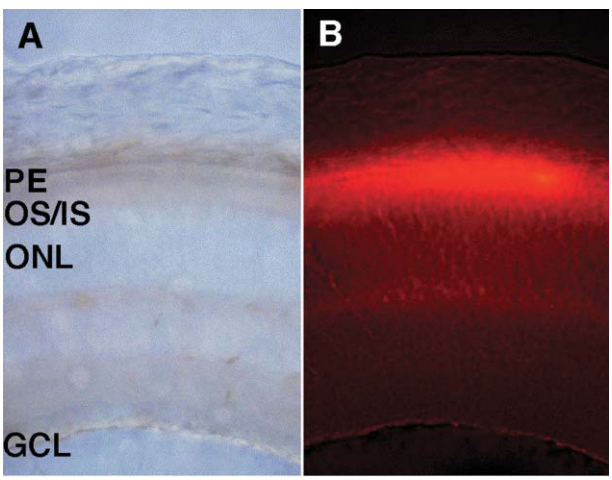


Fig. 2 Microscope images of a cross section of a retina dissected from a mouse 1 h after i.v. administration of chlorin e_6(2 mg kg^{-1}). (A) PE: pigment epithelium, OS: outer segment layer, IS: inner segment layer, ONL: outer nuclear layer, GCL: ganglion cell layer. (B) Under fluorescence conditions (ext. 400 nm band pass filter; em. <650 nm cut off filter) accumulation of red fluorescence is seen.

The retinal response to red (>640 nm) light was subsequently measured by ERG recordings. Typical ERG curves are shown in Fig. 3. For both injected and control mice, at low red light stimulus the ERG showed a slow and positive b-wave. As the light intensity increased, the b-wave increased in amplitude. With yet brighter stimuli, the negative a-wave appeared. With further increase in the flash intensity, both the a-wave and the b-wave increased in amplitude and oscillatory potentials were seen on the rising phase of the b-wave.

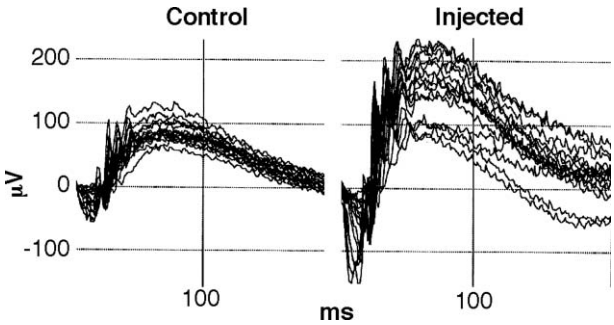


Fig. 3 Typical ERG curves for mice exposed to red (<650 nm cut off filter) light for a control, left plot, and a chlorin e_6 administered, right plot, mouse for light intensities spanning 1.3–2.85 relative log units.

Fig. 4 shows mean b-wave amplitudes generated in response to a single red light stimulus for injected and non-injected mice. Chlorin administered mice showed an almost two-fold increase in b-wave amplitude when compared to control mice. Similar plots where obtained for fourteen other red light stimuli with light intensities spanning 1.3–2.85 relative log units. For all intensities studied, the chlorin administered mice showed an almost two-fold increase in b-wave amplitudes.

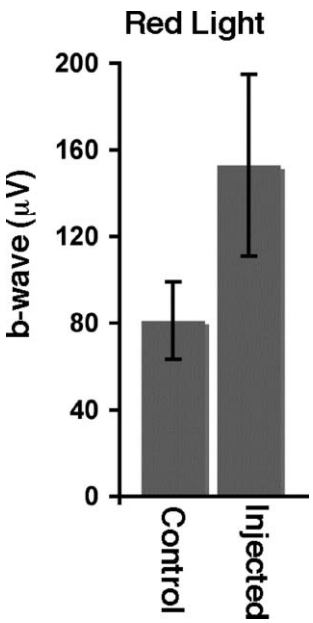


Fig. 4 ERG-b wave amplitudes (Ganzfeld ERG equipped with a <640 nm cut off filter) in response to a single flash of red light for control mice ($n = 7$) and mice administered chlorin e_6 ($n = 10$). Data are presented as mean ± standard deviation. The difference in b-wave amplitudes was statistically significant for control and injected mice. Student's t-test: $p = 0.0001$.

Chlorin e_6 strongly absorbs light centered at both 400 and 665 nm, corresponding to the Soret and Q-bands, respectively. Thus in addition to red light (>640 nm), the retinal response to blue light (456 nm ± 30 nm; 0.01 cd s m^{-2}) was measured for injected and non-injected mice. As shown in Fig. 5, the injected mice ($n = 10$) exhibited an almost two-fold increase

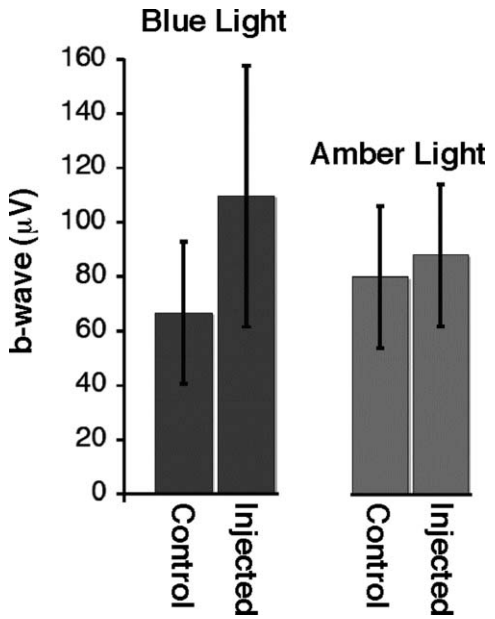


Fig. 5 ERG b-wave amplitudes plotted against blue (456 ± 30 nm) and amber (589 ± 20 nm) LED flashes at (0.01 cd s m^{-2}) for control (blue: $n =$ 10; amber: $n = 4$) and chlorin injected (blue: $n = 10$: amber: $n = 4$) mice. Data are presented as mean ± standard deviation.

in b-wave amplitudes as compared to control mice in response to blue light, although more scatter was observed compared to responses elicited from the red light flashes. On the other hand, the absorbance of chlorin e_6 to amber centered light, 589 ± 20 nm, is minimal; no increase in retinal activity to amber light (0.01 cd s m^{-2}) was observed by ERG measurements in chlorin-administered mice ($n = 4$), as shown in Fig. 5.

Discussion

We observe that mice intravenously injected with a solution of 2 mg kg^{-1} of chlorin e_6 preferentially accumulate the chromophore in the retina outer segment. It has been observed that humans given chlorin supplements (12 mg d^{-1}; orally) accumulate steady-state plasma concentrations of up to 2 mg ml^{-1}.[29] The similar chlorophyll derivative, phylloerythrin, has been detected in the blood[30] and urine[31,32] in many mammals including humans on vegetable diets.

The photoreceptor cells of the mouse retina are predominantly (98%) rods and thus maximally absorb in the blue–green region of the spectrum (500 nm).[33] The remaining cone cells have peak sensitivities at 360 (UV-pigment) and 509–512 nm (M-pigment).[34] Following protocols widely used in clinical and experimental analysis of retinal function, we plotted b-wave amplitudes generated in response to red, blue and amber light for chlorin e_6-injected and non-injected mice. The b-wave arises largely from the polarization of bipolar cells and can be used as a downstream measure of photoreceptor function. In the presence of chlorin an almost two-fold increase in neural impulse was generated in the retina in response to red and blue light, but not amber light. The stimulus wavelengths generating the increased visual response correspond to the absorption spectrum of chlorin e_6. Since systemically injected chlorin was also localized to retina, we conclude that the increase in visual sensitivity is a result of light absorption by chlorin.

In summary, the above data along with our past research shows that derivatives of chlorophyll can act as visual pigments initiating the transformation of light into an electrical signal. In doing so the primary event in vision is changed from initial activation of the protein opsin to initial activation of a chlorophyll derivative. This mechanism is shown to enhance vision in a mouse model and perhaps could also do so in humans.

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