Elevated Free Calcium Levels in the Subretinal Space Elevate the Absolute Dark-Adapted Threshold in Hypopigmented Mice

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Submitted 1 August 2003; accepted in final form 28 August 2003

Lavallee, C. R., J. R. Chalifoux, A. J. Moosally, and G. W. Balkema. Elevated free calcium levels in the subretinal space elevate the absolute dark-adapted threshold in hypopigmented mice. J Neurophysiol 90: 3654–3662, 2003. First published September 3, 2003; 10.1152/jn.00736.2003. Abundant evidence spanning 25 years demonstrates that hypopigmentation is associated with sensory abnormalities manifest most clearly as elevated absolute dark-adapted thresholds in hypopigmented mice. Here we show that when ocular melanin is increased in the himalayan mouse via α-melanocyte stimulating hormone (α-MSH) injections, dark-adapted thresholds drop in proportion to the change in ocular melanin. We further measured free calcium concentration with calcium-sensitive microelectrodes in both albino and black mouse retinal eyecups in living subjects. The recordings were done in anesthetized animals as the defect is not present in isolated retinas or in the superfused eye preparation. A double-barreled electrode—pCa and Vref—was used to simultaneously record the calcium concentration and the electroretinogram (ERG) at each of many depths as the electrode was driven through the retina. The position of the electrode was confirmed with ERG and 1.1’-dioctade-cyl-3,3’,3’-tetramethylindocarbocyanine perchlorate electrode tract reconstruction. Dark-adapted albinos (n = 6) had 1.4 ± 0.015 mM calcium in the subretinal space compared with 0.80 ± 0.025 mM in black mice (n = 6). The results of these experiments are consistent with the hypothesis that ocular hypopigmentation causes elevated calcium levels in the subretinal space that in turn mimic light adaptation in hypopigmented mice.

INTRODUCTION

The controversy over the relationship between ocular pigmentation and absolute dark-adapted light sensitivity has persisted for more than two decades. Previous electrophysiological experiments in hypopigmented mammals (mice, rats, rabbits) have shown elevated thresholds in the dark-adapted state that are proportional to the deficit in ocular melanin (Balkema and Dräger 1991) Animals with the least amount of ocular melanin have the most elevated thresholds. Dark-adapted thresholds in hypopigmented mice show similar threshold elevations in behavioral tests (Hayes and Balkema 1993a). We have shown that the mouse himalayan mutation, which produces a temperature-dependent expression of melanin, provides an opportunity to adjust ocular melanin within one allele in a particular strain (Balkema and MacDonald 1998). Cold-reared himalayan mice have systemic hyperpigmentation of coat and eye relative to warm-reared himalayan mutants (Balkema and MacDonald 1998). We found a 44% increase in ocular melanin in cold-reared himalayan mice compared with room-temperature-reared himalayan mice. Cold rearing did not affect ocular melanin or visual thresholds in control animals (black mice = 10^{-5.9} cd/m² and albino mice = 10^{-4.4} cd/m²). In contrast, the himalayan mice that were maintained at 4°C had thresholds of 10^{-5.7} cd/m² compared with 10^{-5.1} cd/m² for himalayan mice kept at 20°C. The thresholds of himalayan mice that were maintained in the cold were 0.6 log lower than himalayan mice maintained at room temperatures (Balkema and MacDonald 1998).

One problem with the preceding study was that all mice maintained in the cold had decreased performance times (black and albino), although only himalayan mice showed the temperature-dependent melanin expression and threshold change. In the present study, we first repeated the earlier experiment with himalayan mice except instead of cold rearing we used α-MSH to induce ocular melanin.

Logically the next step would be to measure thresholds in either superfused isolated retinas or retinal eyecups; however, the threshold reduction in hypopigmented mice is not apparent when the retinas are isolated or tested as an eyecup and superfused with normal mouse Ringer (Balkema et al. 1983; Suzuki and Pinto 1986). This suggests that the defect is due to an ionic imbalance, possibly in the photoreceptor subretinal space, or a deleterious substance that is washed out by normal Ringer (Balkema et al. 1983; Dräger 1985; Dräger and Balkema 1987; Suzuki and Pinto 1986). We tested this hypothesis by measuring both the ERG and calcium levels throughout the retina in intact albino and black mice.

METHODS

Experimental animals

All experimental procedures and animal maintenance adhered to the APS Guiding Principles in The Care And Use Of Animals. All procedures were submitted to the IUACC at Boston College under the Animal Assurance control number A3905-01. The specific protocols were reviewed, approved, and granted protocol numbers 96-09, 00-02. Albino (c/c) and himalayan mice (c/c) mice of the C57BL/6J inbred strain were compared with normal (+/+ ) mice of the same strain. Breeding stocks for the normal and the albino mutants were purchased from Jackson Laboratory, Bar Harbor, ME. Breeding stocks for the himalayan mutant were obtained from Texas A&M University as a gift from Dr. Lynn Lamoreux, Veterinary Medicine-Pathology. The mice were maintained in a 24-h light cycle (12 h/12 h).
on/off) at room temperature (20°C). The luminance during the light phase was $<10^{-1}$ cd/m$^2$.

**Hormone injections**

Alpha-melanocyte stimulating hormone (α-MSH), a 13 amino acid peptide with an N-terminus acetylation (N-SYMEHFHWGKPV), was injected intraperitoneally into the experimental group of each mutant during the testing phase of the experiment. The hormone injections consisted of 200 μg α-MSH dissolved in 50 μl saline and emulsified in 50 μl of peanut oil as an injection vehicle to time release the absorption of the α-MSH. Sham injections consisted of the above vehicle without the α-MSH. The injections were performed every other day during testing for ~9 wk. Environmental cues during injections were equalized by housing both the experimental group and the control group in the same room. Both experimental and control (sham injection) groups were exposed to the same conditions, i.e., lights and human interaction during injections.

**Experimental apparatus**

The water-maze apparatus (Hayes and Balkema 1993b) was constructed of opaque white GP acrylic plastic, which has minimal pheromone absorption (CYRO Industries, Orange, CT). The maze was hexagonal in shape with six T-shaped compartments radiating from the center. A black GP acrylic plastic background, on which a white escape ramp was attached, was inserted randomly into a compartment and appeared to the swimming mouse as a white escape ramp against a black background. Therefore when the mice were gently placed into the center of the maze, they were presented with five white compartments with no escape ramp and one dark compartment with a white escape ramp.

The water maze was continuously supplied with fresh tap water maintained at 18 ± 1°C (Hayes and Balkema 1993b). The maze was mounted onto a rotating circular table to minimize field cues and spatial learning. To further eliminate spatial or extra-maze cues, the maze was placed in an absolute lightproof room with no visual or auditory distractions.

An incandescent light source, attached to a constant voltage transformer, was mounted above the center of the maze for the initial test luminance. Dimmer test luminances were obtained by attenuating the light source with neutral density filters. The source produced a relatively uniform lighting throughout the maze. At very low light levels during testing, the animals were viewed with light intensifying night vision goggles (Moonlight Products, San Diego, CA). Light measurements were made using a calibrated photometer (United Detector Technology, Hawthorne, CA). All light measurements reported are time-averaged luminance. Dimmer test luminances were obtained by attenuating the former, was mounted above the center of the maze for the initial test luminance level was measured. An animal’s response was considered correct if it chose the escape ramp compartment first after being dropped into the water maze. The dark-adapted threshold of each animal group was determined to be that luminance at which the percent correct of choices dropped below the chance level (chance equals 1/6 or 16.7%).

**Histology**

Animals were perfused with heparinized saline followed by 4% paraformaldehyde/4% gluteraldehyde. The corneas were nicked just rostral to the ora serrata on the superior side with a fine needle dipped in India ink to create a tattoo for later orientation. The corneas were slit and placed in cold 4% paraformaldehyde/4% gluteraldehyde for 24 h on a rotator at 4°C. The tissue was dehydrated and processed for Epon embedding. The eyes were halved along the vertical meridian passing through the optic disk. Each half eye was halved once again on the horizontal meridian. The quartered portion of retina was placed with the horizontal meridian facing the front of a coin-embedding mold. After polymerization, semi-thin (1 μm) sections were cut on an ultramicrotome and stained with toluidine blue. Digital photographs were obtained with a Zeiss Standard microscope equipped with a SPOT RT camera and analyzed with National Institutes of Health-Image and Adobe Photoshop. We measured all of the layers in the retina and counted the number of outer nuclear layer (ONL) cells within a 100-μm square. We used two eyes per condition, and 10 sections were recovered from each eye within 100 μm of the horizontal meridian.

**Data analysis**

The conditions of the test resulted in six groups with eight mice per group. For the SE calculations, $n = 8$. We decreased the light intensity every day during the testing period. When the animal was in the vicinity of the threshold, we presented the same stimulus over 3 days (4 trials/day) for a total of 12 trials per test luminance. We averaged the 12 different performance times to give 1 performance time. The number of correct first choices of the target chamber was divided by the total number of trials, 12, to give the percent correct. Significance between the groups was tested via two-tailed t-test analyses. The
Retinal rhodopsin measurement

We measured the retinal rhodopsin concentration from mice that had been in a dim cycling light facility (Van Hooser et al. 2000). Six mice for each condition were dark-adapted for 12 h. They were anesthetized under infrared light with CO₂ and the eyes were removed, weighed, and minced with iris scissors under a dissecting microscope. The tissue was homogenized, sonicated on ice for 3 min with an ultrasonic homogenizer 4710 series (Cole-Parmer, Chicago, IL), incubated at 37°C for 3 h, sonicated on ice for 1 min, and allowed to settle for 12 h. The absorbance of the solution was measured at 500 nM against an albino blank in a dual beam Shimadzu 160 spectrophotometer. We generated a standard melanin curve by extracting four albino eyes as in the preceding text and adding known amounts of synthetic melanin (Sigma).

Retinal recording apparatus

The experimental apparatus (Fig. 1) was constructed so that the reference barrel of the double-barreled calcium ion selective microelectrode (Diamond General Development, Ann Arbor, MI) would serve as the primary electrode for ERG recordings. The microelectrode was ~2 in long and had a total tip diameter of ~10 μm, 5 μm for the ion selective barrel and 5 μm for the reference barrel. Each barrel was made of 1.0-mm-thick glass capillary. The reference solution was 0.9% NaCl, and the electrode had a response slope of ~24–28 mV per decade of calcium.

The microelectrode was placed in a custom microelectrode holder (Diamond General Development) and connected to a high-impedance electrometer (Markson Science) reading millivolts. The reference barrel of the microelectrode was connected to the A channel of a differential amplifier (DAM-50; WPI, Sarasota, FL). Channel B was connected to an Ag/AgCl reference electrode placed under the skin at the dorsal extent of the skull. The amplifier was DC coupled with an amplification of 1,000 with a high-pass cutoff of 1 kHz. The output of the amplifier was recorded on a FM DC data recorder (AR Vetter, Rebersburg, PA), displayed on an oscilloscope (Hitachi V-665), and monitored on an audio monitor (Technics). The output of the DAM 50 was applied to a MacLab/4S analog to digital converter (ADInstru, Milford, MA) coupled to a Power Macintosh 8100/80 for real-time data analysis and stimulator synchronization. The MacLab unit drove a Grass PS22 photic stimulator.

In vivo eye-cup preparation

The mouse was tranquilized (Taractan 20 mg/kg IM) and maintained at 37°C. Anesthesia was induced with pentobarbital (60 mg/kg sc). The animal was placed in a head restraint, and a Ag/AgCl reference electrode wire was inserted under the skin at the dorsal extent of the skull. A local anesthetic was applied to the animal’s cornea (Tetracaine), which was then punctured with an eye blade, illuminated with dim red light, and excised just rostral to the ora serrata. The lens was gently removed.

The mouse and head restraint were placed into a faraday cage with the microelectrode apparatus and the mouse was grounded. Humidified gas (79% N₂, 21% O₂, 100% humidity) was blown over the exposed retina, but it was not superfused. The microelectrode tip was advanced to within ~1 mm of the retina surface under microscopic control and viewed with dim red light (>800 nM). The microelectrode was then manually advanced until it made contact with the surface of the retina.

Retinal depth marking

To detect the location of the retinal surface (ganglion cell layer) and the retinal pigment epithelium layer, an electrode tract (Fig. 2) was labeled by using a saturated solution of 1,1'-dioctadecyl-3', 3', 3'-tetramethylindocarbocyanine perchlorate (DiI).

After positioning the electrode at the surface of the retina, the microelectrode was withdrawn to a known and workable distance from the retina. A saturated solution of DiI (Molecular Probes, Eugene, OR) was applied to the tip of the electrode. The electrode was then inserted to a depth of 600 μm, allowed to sit for 15 min, and then withdrawn. The mouse was then perfused with saline followed by Karnovsky’s reagent made of 2%/2% buffered paraformaldehyde/glutaraldehyde in phosphate buffer. The eyecup was removed, embedded in OCT, frozen, and sectioned on a cryostat. The tissue sections were viewed under a Zeiss microscope with a rhodamine-iso-thio-cyanate (RITC) filter set. The reconstructed electrode tract was used to detect the endpoints of the retina [retinal pigment epithelium (RPE) and ganglion cell layer] and coordinate the extreme changes in ERG and calcium reading to the tract.

Calcium profile recordings

The calcium ion selective microelectrodes were calibrated before and after each trial by using calcium standard buffers with a pCa range from 1–7 (CALBUF-1; WPI). The calcium recordings were taken in 100 μm steps from a depth of zero, which was chosen as the point at which the calcium recording changed abruptly at the air-retina bound-

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**Fig. 1.** The diagram of the recording apparatus. The calcium barrel (A) and the reference barrel (B) of the electrodes were connected to the electrometer for calcium measurements and then to the computer (C). The reference barrel (B) of the electrode and the Ag/AgCl reference cell (F) were connected to the data recorder (D) and oscilloscope (E) for ERG measurements. The reference barrel of the electrode acted as a reference cell for the calcium recordings and the primary electrode for the ERG recordings.
The microelectrode was positioned perpendicular to the retina for the calcium and ERG recordings and was driven into the retina in 100-μm increments from 0 to 600 μm, while a reading was taken at each step. The electrode was then withdrawn by 100 μm increments from 600 μm, while readings were taken as before. The trace produced from the reference barrel of the calcium microelectrode against the Ag/AgCl wire under the scalp was also used as an EKG to monitor the health of the mouse during the experiment. The entire series from 0 to 600 μm and 600 to 0 μm was repeated three to five times at different penetration sites and averaged together for each mouse. After the data were collected for the calcium profile, a normal and an intensity response electroretinogram series were recorded for each point in the depth series at 100-μm increments. We used the ERG recording both as a measure of the electrode depth (surface of the retina set to 200 μm and subretinal space marked as a change in the a-wave) within the retina and as an indicator of the state of the retina. If the electrode was driven significantly beyond 600 μm, we observed an abrupt change in [Ca] marking the choroid.

**Bleaching experiments**

After a normal calcium profile was attained as described previously (series were repeated only 2 times for these sets of experiments), the retina was bleached with a bright light for 5 min. After bleaching, the series of recording from 0 to 600 μm was repeated twice and averaged.

**RESULTS**

Six groups of mice were trained in a water-maze screening apparatus until each group of mice had leveled off to their maximum performance level (no difference between groups *P* > 0.05). All mice were able to reach the escape ramp within 3–5 s.

The luminance level was decreased by 1.0 or 0.3 ND/day until the animals performed at chance (16.7%, Fig. 3).
We found that the albino performance latencies increased at brighter luminance levels than the himalayan, which in turn increased at brighter luminance levels than wild-type mice. The himalayan α-MSH tended to the wild-type pigmented and the himalayan control tended to albino results.

We also recorded the percent correct first choice or the threshold from all groups. The albinos had the most elevated thresholds; the black mice had the lowest thresholds; with the himalayan-α-MSH tending to the black mouse threshold and the himalayan-control tending to the albino mouse threshold (Fig. 4.)

The albino thresholds were $6.61 \times 10^{-6}$ ($10^{-5.18}$) cd/m$^2$ for sham injection and $5.13 \times 10^{-6}$ ($10^{-5.29}$) cd/m$^2$ for the $\alpha$-MSH-injected mice (Fig. 4). There was no significant difference seen in the thresholds of the $\alpha$-MSH-treated albinos and the sham albinos. There was no significant difference seen in the thresholds between experimental wild-type mice [$4.28 \times 10^{-7}$ ($10^{-6.57}$) cd/m$^2$] and wild-type sham injection black mice [$7.76 \times 10^{-7}$ ($10^{-6.11}$) cd/m$^2$; Fig. 4]. The sham injection himalayan group had a dark-adapted threshold of $3.61 \times 10^{-6}$ ($10^{-5.42}$) cd/m$^2$; whereas the $\alpha$-MSH-treated himalayan group exhibited a lower dark-adapted threshold of $1.30 \times 10^{-6}$ ($10^{-5.85}$) cd/m$^2$ (Fig. 4; $P < 0.0106$). These results indicate that the $\alpha$-MSH-treated himalayan mice had a 0.42 log unit lower threshold compared with their controls.

After the dark-adapted thresholds of each animal group were determined, all but six eyes (1 from each group) were removed to measure the concentration of ocular melanin (microgram of melanin per milligram of eye for each mutant). The ocular melanin concentrations of both the experimental and control albinos were determined to be 0.280 μg/mg. The experimental himalayan group had an ocular melanin concentration of 0.940 μg/mg, whereas the control himalayan group had a concentration of 0.790 μg/mg ($P < 0.03$). This result shows a 19% increase in ocular melanin of the $\alpha$-MSH-treated himalayan group compared with the controls. The experimental wild-type group had an ocular melanin concentration of 11.3 μg/mg and the wild-type controls showed a concentration of 11.1 μg/mg.

In a separate series of experiments, the ocular rhodopsin concentration from each group of mice was measured by the difference spectrum (dark adapted vs. bleached) and we found small differences between groups (+/+ 570 pm/eye ± 20 pm; c$^b$ 460 pm/eye ± 35 pm/eye; c$^{ab}$ 450 pm/eye ± 50 pm/eye).

The remaining six eyes, one taken from each group, were removed to examine the retina histologically. The plastic sections were stained with Toluidine Blue (Fig. 5). There were no differences found between the control mice (black and albino) and himalayan mice in the overall morphology of the retinas. Furthermore, there were no differences found between the $\alpha$-MSH-treated animals and the controls in overall morphology.

We next measured free calcium levels throughout the retina, particularly the subretinal space, during a perpendicular penetration with a calcium-sensitive microelectrode in intact albino and black mice.

We used a double-barreled electrode that allowed simultaneous recordings of the intra-retinal ERG and the calcium concentration. The a-wave of the ERG shows an increase in amplitude when the electrode enters the subretinal space. The ERGs at various depths in the retina (Fig. 6A) and the intensity response series of ERGs (Fig. 6B) from the mice are consistent with a healthy, viable eyecup preparation.

Calcium measurements from black control mice (C57BL/6J +/+ ) ranged from 0.37 mM at the surface of the retina [ganglion cells (GC), 200 μm] to 0.80 mM in the subretinal space (Fig. 7).

Continuing into the retinal pigment epithelium layer, we found that the extra-cellular calcium concentration decreased from the subretinal space value (0.80 mM) to 0.67 mM in the region of the RPE (Fig. 7). As the electrode tip was retracted, the calcium changes were reversed with a theoretical chance level. The error bars indicate SEM.

![FIG. 4. Threshold measurement. The percentage of correct choices made by the test mouse vs. the luminance of the target ramp. If a mouse made an initial correct choice, it was scored as correct. If a mouse made an incorrect 1st choice (swimming into a chamber without the target ramp), it was scored as incorrect (even if the animal eventually found the target ramp). Chronologically the experiment proceeded from right (end of training luminance) to left. - - -, the theoretical chance level. The error bars indicate SEM.](http://jn.physiology.org/)

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C. R. LAVALLEE, J. R. CHALIFOUX, A. J. MOOSALLY, AND G. W. BALKEMA

J Neurophysiol • VOL 90 • DECEMBER 2003 • www.jn.org
We repeated these measurements in albino mice (C57BL/c2J/c2J) and found that the overall calcium level was higher throughout the retina, ranging from 1.48 mM at the surface, remaining fairly constant through the retina to 1.39 mM in the subretinal space and dropping to 1.18 in the extra-cellular space in the retinal pigment epithelium (Fig. 7). Similar to the black control mice, we found that the extra-cellular calcium concentration in the retinal pigment epithelium was lower than in the subretinal space.

We also measured extra-cellular calcium level profiles from albino and black mice before and after a 5-min bleach. We found after a bright bleaching adaptation extra-cellular calcium levels fell an average of 53% for the albino mice and 45% for the pigmented animals.

**FIG. 5.** Semi-thin plastic sections stained with Toluidine Blue from pigmented, albino, and himalayan mice. Animals were given either α-melanocyte stimulating hormone (α-MSH) injections (bottom) or control (no injection, top). Scale indicates 20 μm.

**FIG. 6.** Electroretinographic recordings from control mice. A: a series of ERGs of a wild-type control mouse at various depths in the retina. The 7 ERG traces were taken from 0 to 600 μm at 100-μm intervals. B: an intensity response series of a wild-type mouse. The ERG recordings were taken over a 4.5 log range of intensities starting from the least bright and ending at unattenuated light. The readings were taken at 4.5, 3.9, 3.3, 2.7, 2.1, 1.5, and 0.9 log attenuation as well as unattenuated flashes. Both ERG series show the mice and retinae to be healthy and normal.

**FIG. 7.** The calcium profiles for albino (○) and control black mice (□) are shown for various depths in the retina. The initial depths (μm) from the arbitrary 0 point of the electrode (shown on the x axis) were adjusted after electrode tract analysis and labeled according to the retinal layers: RPE, retinal pigment epithelium; SR, subretinal space; GC, ganglion cells. The calcium levels for the albino mice were higher over the entire retina than the black control mice. Both groups had similar profiles, but the calcium concentration in the black mice peaked in the subretinal space.

**FIG. 8.** Calcium profiles for albino and black mice before and after a 5-min bleach.
DISCUSSION

Overview

The purpose of this study was to confirm that ocular melanin affects visual threshold and to test the hypothesis that subretinal space calcium levels are elevated in hypopigmented mice. Elevated thresholds are proportional to the degree of ocular hypopigmentation as shown in a collection of hypopigmented mouse mutants with ocular pigmentation ranging from 0, 10, 11, 19, and 26% of normal (Balkema and Drager 1991). Because the threshold elevation is present in many different hypopigmentation mutants, representing mutations of different genes and different biochemical perturbations, the threshold effect is most likely to be a consequence of the reduction in melanin pigment itself. Sanderson et al. LaVail et al. and Mangini et al. independently reached the same conclusion in their studies of two other defects in hypopigmentation mutants that affect optic nerve crossing and eye movements (LaVail et al. 1978; Mangini et al. 1985; Sanderson et al. 1974).

In the past, we have shown that the himalayan mutation, which exhibits a temperature dependent expression of melanin, allows us to adjust the ocular melanin within a mouse. Cold-reared himalayan animals have systemic hyperpigmentation of coat and eye relative to a warm-reared himalayan mutant (Balkema and MacDonald 1998). We found that himalayan mice that were kept in the cold exhibited a 44% increase in ocular melanin compared with himalayan mice kept at room temperature. Cold rearing did not affect ocular melanin or visual thresholds in control animals (Balkema and MacDonald 1998).

The himalayan mouse allowed us to directly manipulate the ocular melanin concentration and the animal’s absolute dark-adapted threshold (Balkema and MacDonald 1998). However, cold rearing partially confounded the performance times in these previous experiments by causing all mice to perform better (although, the percent correct still gave an accurate measure of each animal’s threshold). We wondered if we could adjust the himalayan’s ocular melanin with α-MSH and observe the corresponding change in absolute dark-adapted threshold.

α-MSH increases visual sensitivity in the c\textsuperscript{h} mouse

α-MSH is a hormone that is secreted from the pars intermedia of the pituitary gland and regulates the control of pigment organelle movement and synthesis. It was established from experiments on both normal and malignant melanocytes (Fritsch and Varga 1976; Fuller and Viskochil 1979; Varga et al. 1976) that α-MSH stimulates melanogenesis by binding to a membrane receptor at the cell surface, activating adenylate cyclase, and therefore increases the intracellular levels of cyclic AMP (cAMP). This increase in cAMP concentration ultimately results in the activation of the tyrosinase enzyme and the synthesis of melanin. Further studies showed that tyrosinase activity and the rate of melanin production is stimulated by adding α-MSH (Weatherhead and Logan 1981).

Tyrosinase is the primary enzyme responsible for melanin synthesis. It is an enzyme that catalyzes the hydroxylation of tyrosine to produce dopa and the oxidation of dopa to produce dopaquinone. Dopa at low concentrations is a noncompetitive activator of tyrosine hydroxylation and melanin formation (Hearing and Ekel 1976). It was also shown that an increase in cAMP levels activates a cAMP-dependent protein kinase, which activates the tyrosinase enzyme (Korner and Pawelek 1977). The activation involves the removal of an inhibitor of tyrosinase or the addition of an activator (Hearing and Ekel 1976). A phosphoprotein phosphatase is also present, distinct from the inhibitor, which seems to antagonize the kinase-mediated reaction (Korner and Pawelek 1977).

In our studies, himalayan mice treated with α-MSH developed darker coats and ~20% more ocular pigmentation. We found that this increase in ocular pigmentation resulted in a 0.42 log threshold reduction. No significant change in threshold was found in either the black or albino controls. There were also no significant differences between α-MSH-treated or untreated performance times of black or albino control mice. When we examined the retinas from all the mice, we found no evidence of light damage or other gross changes in the outer segment length or the number of nuclei in the ONL. Thus we have successfully caused an elevation in ocular melanin with a resulting reduction in the absolute dark-adapted threshold and no changes in control performance times.

Light damage does not cause elevated thresholds in c\textsuperscript{2J} or c\textsuperscript{h}

We do not think that elevated dark-adapted thresholds can be explained by the light damage demonstrated in hypopigmented animals in other studies (Noell 1980; Noell et al. 1966). The albino animals that we used had the c\textsuperscript{2J} mutation on the C57BL/6J strain, which has been shown to be relatively resistant to light damage (LaVail et al. 1987). Furthermore, the mice were housed at low ambient luminance (<10\textsuperscript{−1} cd/m\textsuperscript{2}) with a 12 h/12 h light/dark cycle. These conditions are not associated with significant light damage. No significant light-damage was detected in the mice reared in this facility (Balkema 1988; Hayes and Balkema 1993a).

A previous study artificially produced light damage in both normal and albino mice by exposing them to constant light (350 cd/m\textsuperscript{2}). After 21 days, the retinae from the albino animals displayed massive light damage. The ONL was reduced by 50% and the outer-segment layer was completely disorganized. These albino animals had a marginal elevation in threshold measuring 0.3 log units compared with their albino controls (Hayes and Balkema 1993a). There was no significant difference between their absolute dark-adapted thresholds compared with controls. It is important to note that we do not maintain the animals in constant light (350 cd/m\textsuperscript{2}) but rather in dim (<10\textsuperscript{−1} cd/m\textsuperscript{2}) cycling light. Light damage is not evident in c\textsuperscript{2J} animals reared in dim cycling light.

Ionic imbalance in the subretinal space may contribute to threshold elevation

The threshold effect originates in the retina, as it is present in recordings from retinal ganglion cells, but requires the presence of retinal pigment epithelium, as it is no longer detectable in isolated retina preparations (Balkema et al. 1983; Suzuki and Pinto 1986). These results are consistent with the idea that the subretinal space has an abnormal ionic environment in the hypopigmented animals.

Dräger in 1985 reported that the RPE melanin bound calcium in direct proportion to the level of hypopigmentation of

\[ J \text{Neurophysiol} \cdot \text{VOL 90} \cdot \text{DECEMBER 2003} \cdot \text{www.jn.org} \]
individual mouse strains; thus black mice bind the highest amount of calcium, albino the least with other hypopigmented animals falling in between these two extremes (Dräger 1985). She further suggested that the calcium buffering capacity of melanin could be the common factor in many different neurological diseases (Dräger 1985). To test this hypothesis that an abnormal ionic environment might explain the elevated thresholds found in hypopigmented animals, we measured the subretinal space free calcium in both black and albino mice (Balkema and Dräger 1991; Balkema and MacDonald 1998; Dräger 1985; Dräger and Balkema 1987). The present series of experiments required the development of an anesthetized animal eyecup that was not superfused but maintained with the animal’s natural circulation and supplemented with humidified oxygen.

Subretinal space calcium elevation mimics light adaptation and may explain the threshold elevation

We measured the free calcium concentration profile across the retina in both albino and black control mice. The depth was determined by the absolute depth from the surface of the retina, the waveform of the ERG (Penn and Hagins 1969), and confirmed by examining electrode tract reconstructions (Fig. 2). We found that the calcium levels were higher in the albino mice, 1.39 mM in the subretinal space, compared with 0.80 mM in the subretinal space of the normally pigmented control mice. A comparison of calcium levels in albino retinas and pigmented controls, shows that calcium levels were most elevated at the surface of the retina, 400% of control, reduced to 174% in the subretinal space, and was 180% of control values in the RPE. The absolute values in the pigmented animals ranged from 0.371 mM at the surface, to 0.80 mM in the subretinal space, to 0.66 mM within the RPE. In the albino animals, we found 1.48 mM calcium at the surface, 1.39 mM in the subretinal space, and only in the RPE did the value drop to 1.18 mM.

We hypothesize that albino mice are missing the buffering action of melanin within the RPE and thus the subretinal space calcium concentration is elevated. There are many reports of elevated calcium concentrations mimicking light adaptation (Fain et al. 2001; Koch and Stryer 1988; Matthews et al. 1988). Therefore the hypopigmented animals may serve as a natural model of elevated light adaptation, with the degree of light adaptation dependent on the level of hypopigmentation (Balkema 1988; Balkema and Drager 1991). One would predict that in an animal that was both hypopigmented and had diffusional abnormalities of the RPE cells, the threshold elevation would be more profound than in albinos. Measurement of visual thresholds in the pearl mutant supports this hypothesis; the pearl mutant phenotype has both hypopigmentation and significantly lower number of basal RPE enrolling and also has a threshold that is ~0.5 log units more elevated (less sensitive than albino) (Balkema and Drager 1991; Balkema et al. 1981; Williams et al. 1985).

In a separate series of experiments, we recorded calcium profiles from both black and albino mice before and after a bright 5-min bleach. Again the albino calcium profile was higher than normally pigmented animals. Initially we found it surprising that the calcium profile dropped for both albinos and pigmented animals after a bright bleach; however, this is consistent with what other workers have found (Dmitriev et al. 1999; Gallemore et al. 1994; Livsey et al. 1990; Loeffler and Mangini 1998; Mangini et al. 1997; N. Mangini, personal communication; N. Mangini, K. Loeffler, and B. Kennedy, unpublished data). The dark-adapted albino subretinal space calcium concentration (SRS [Ca]) was 1.8 mM compared with the bleached albino SRS [Ca] 0.75 mM or a difference of ~1.05 mM [Ca]. The black mouse pre bleach SRS [Ca] was 1.21 mM compared with the bleached 0.64 mM or a difference of 0.57 mM [Ca].

Initially it might appear contradictory to argue that the hypopigmented animal has an elevated calcium level that mimics light adaptation when light adaptation actually drops the extra cellular calcium level. However, these are two separate processes. The calcium levels in the subretinal space are elevated in the hypopigmented animals. The elevated subretinal space calcium in turn increases the calcium current through the Na/Ca channel, which tricks the photoreceptor into being more light adapted than it should be under a given luminance—exactly what we find experimentally (Gallemore et al. 1994; Livsey et al. 1990; Loeffler and Mangini 1998; Mangini et al. 1997; N. Mangini, personal communication; N. Mangini, K. Loeffler, and B. Kennedy, unpublished results).

Another explanation for the threshold and subretinal space calcium elevation might be that albino and himalayan mice have less retinal rhodopsin than black control mice. We found progressive, though nonsignificant, differences in ocular rhodopsin in black (570 pm/eye ± 20 pm), himalayan (460 pm/eye ± 35 pm/eye), and albino (450 pm/eye ± 50 pm/eye) mice. Williams et al. (1998) have described a phenomenon called photostasis, where ocular rhodopsin concentration is dependent on rhodopsin saturation, thus rhodopsin expression is upregulated when it is less bleached (under dim, ambient light). They suggest that reduced melanin levels and the resulting light scatter in hypopigmented the animal downregulates outer-segment rhodopsin. We do not think that photostasis directly explains our results; photostasis is not appreciable in black mice at the low cycling light levels in which our mice are maintained (Battelle and LaVail 1978; Penn and Williams 1986). Given, however, that hypopigmented animals have elevated subretinal calcium levels and that elevated calcium may mimic light adaptation (Fain et al. 2001), there may be a disproportionate effect of photostasis on rhodopsin levels in hypopigmented animals (however, see King-Smith et al. 1996). This question requires further study of visual thresholds and ocular rhodopsin levels at different ambient lighting levels in hypopigmented animals.

We thank Drs. M. P. Ogren and L.H. Pinto for reading the manuscript.

DISCLOSURES

This work was supported by IBN-9983297, the Boston College Graduate School, and Boston College research funds.

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