Diminished Pupillary Light Reflex at High Irradiances in Melanopsin-Knockout Mice

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In the mammalian retina, a small subset of retinal ganglion cells (RGCs) are intrinsically photosensitive, express the opsin-like protein melanopsin, and project to brain nuclei involved in non–image-forming visual functions such as pupillary light reflex and circadian photoentrainment. We report that in mice with the melanopsin gene ablated, RGCs retrograde-labeled from the suprachiasmatic nuclei were no longer intrinsically photosensitive, although their number, morphology, and projections were unchanged. These animals showed a pupillary light reflex indistinguishable from that of the wild type at low irradiances, but at high irradiances the reflex was incomplete, a pattern that suggests that the melanopsin-associated system and the classical rod/cone system are complementary in function.

It had long been assumed that rods and cones were the only intrinsically photosensitive cells in the mammalian retina. However, this assumption was questioned when mice lacking functional rods and cones were shown to retain circadian photoentrainment and the pupillary light reflex (*1*, *2*). Most recently, a small subset of rat RGCs have been shown to be directly photosensitive (*3*, *4*). These RGCs express melanopsin (*4*–*7*), an opsin-like protein (*8*), and project to retino-recipient areas of the brain responsible for non–image-forming visual functions (*4*–*6*), raising the possibility that these RGCs serve as photoreceptors for these functions. The action spectra of these RGCs are consistent with an opsin:vitamin A–based photopigment underlying the process (*2*, *3*). To determine whether melanopsin and the RGCs expressing it indeed have physiological roles, we have examined the pupillary reflex of mice lacking this gene.

Previously, we have generated mice in which the melanopsin gene (*mop*) is replaced by a tau-LacZ coding sequence (*4*). We bred these animals to homozygosity. We found that the RGCs that would normally express melanopsin are still present in $mop^{-/-}$ (*tau-LacZ*$^{+/+}$) mice, as revealed by blue X-Gal labeling (*9*) (Fig. 1A). Their morphology and number (~600 per mouse retina) are similar to those in $mop^{+/-}$ (*tau-LacZ*$^{+/-}$) mice and, by implication (*4*), wild-type animals. Their axons converge on the optic disk and still innervate the olivary pretectal nuclei (OPN) (Fig. 1B), the retino-recipient area responsible for the pupillary light reflex (*10*, *11*), and the suprachiasmatic nuclei (SCN) (Fig. 1C), the circadian pacemaker in the brain. In both structures, the spatial pattern of the innervation is similar to that previously observed in $mop^{+/-}$ animals (*4*). Thus, the absence of melanopsin does not affect the genesis, survival, or connectivity of the melanopsin-associated RGCs.

The genetic deletion does, however, eliminate the intrinsic photosensitivity of these RGCs. For wild-type and $mop^{+/-}$ mice, RGCs retrograde-labeled from the SCN and tested under synaptic blockade (*9*) were tonically depolarized by light (13 of 13 cells tested for wild type, and 3 of 3 cells tested for $mop^{+/-}$) (Fig. 1D), just as in rat (*3*, *4*). In contrast, none of 10 cells tested for $mop^{-/-}$ mice were photosensitive under the same conditions (Fig. 1E). The morphologies of recorded cells were indistinguishable across genotypes and closely resembled those of the photosensitive RGCs innervating the SCN in rat (*3*, *4*).

We compared the consensual pupillary light reflex of $mop^{+/+}$, $mop^{-/-}$, and $mop^{+/-}$

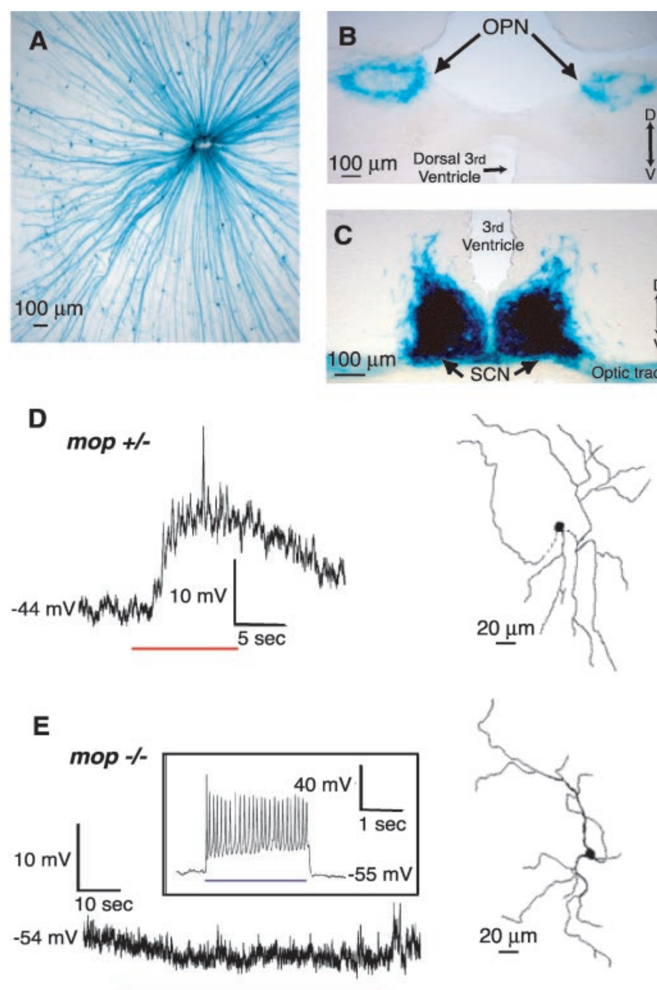

Fig. 1. RGCs that would normally express melanopsin in wild-type and $mop^{+/-}$ mice are still present in $mop^{-/-}$ mice and project to the OPN and SCN but have lost their intrinsic photosensitivity. (**A**) Flat-mount view of a $mop^{-/-}$ retina stained with X-Gal (blue labeling). (**B** and **C**) Coronal sections of $mop^{-/-}$ mouse brains showing normal innervation of the OPN and the SCN by X-Gal–labeled axons. D, dorsal; V, ventral. (**D** and **E**) Intrinsic photosensitivity of SCN-projecting RGCs of $mop^{+/-}$ (D) but not $mop^{-/-}$ (E) mice. Left: Voltage responses to light stimuli (red line) of RGCs retrograde-labeled from the SCN. Recordings were made from an isolated, flat-mount retina in the presence of 2 mM $CoCl_2$ to block synaptic transmission. Right: Camera-lucida drawings of the cells recorded. Inset in (E): Depolarization and action potentials evoked by current injection (50 pA; blue line), demonstrating functional viability of this cell.

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animals (*9*). All three genotypes showed a light-dependent pupillary constriction, but the minimal pupil area attained by dark-adapted $mop^{-/-}$ animals in 1 min of steady bright light was three times that of $mop^{+/-}$ and wild-type animals (Fig. 2, A and B). This difference was not due to an intrinsic defect in the iris sphincter of $mop^{-/-}$ animals, because parasympathetic activation by topical application of carbachol (*9*) elicited equally strong constrictions in $mop^{-/-}$ and wild-type animals (Fig. 2A). Nor was it due to an alteration in circadian entrainment (pupillary recordings in the day and night revealed a similar phenotype) or to the mixed C57 Bl6/129 genetic background of the three genotypes (littermates were used and both parental strains assessed) (*9*). Thus, the impaired response of $mop^{-/-}$ mice appeared to have resulted directly from a loss of intrinsic photosensitivity of the melanopsin-expressing RGCs.

With a stimulus of bright, steady monochromatic light, the pupil constriction in wild-type mice was greater than that in $mop^{-/-}$ animals during both the transient phase and steady state of the response (Fig. 2C, compare open and filled circles). With dimmer light (green squares), the reflex was slower, but also became indistinguishable in speed and amplitude between the two genotypes, indicating that melanopsin exerted an influence only at high irradiances. With an intense, 100-ms white flash (Fig. 2D, circles), which should give little opportunity for the rods and cones to light-adapt (*12*, *13*), the maximal constriction of the $mop^{-/-}$ pupil remained weaker than that of the wild type. This result suggests that there is probably a genuine limit to how far rods and cones can drive the mouse pupil reflex—a ceiling independent of adaptation.

The complete step irradiance–response relations (*9*, *14*) for $mop^{+/+}$ and $mop^{+/-}$ animals are similar (Fig. 3A and supporting online text). The relation for $mop^{-/-}$ mice is also similar to that of the wild type at irradiances less than $10^{11.5}$ photons cm^{-2} s^{-1}, but diverges at higher irradiances (supporting online text), approaching a minimum attainable pupil size that is larger than that of wild-type mice. Mice lacking detectable rods and cones (*rd/rd cl*) retain a pupillary light reflex, with an action spectrum (*2*) resembling that for the intrinsic photosensitivity of melanopsin-expressing RGCs in rat (*3*). The *rd/rd cl* pupil reflex

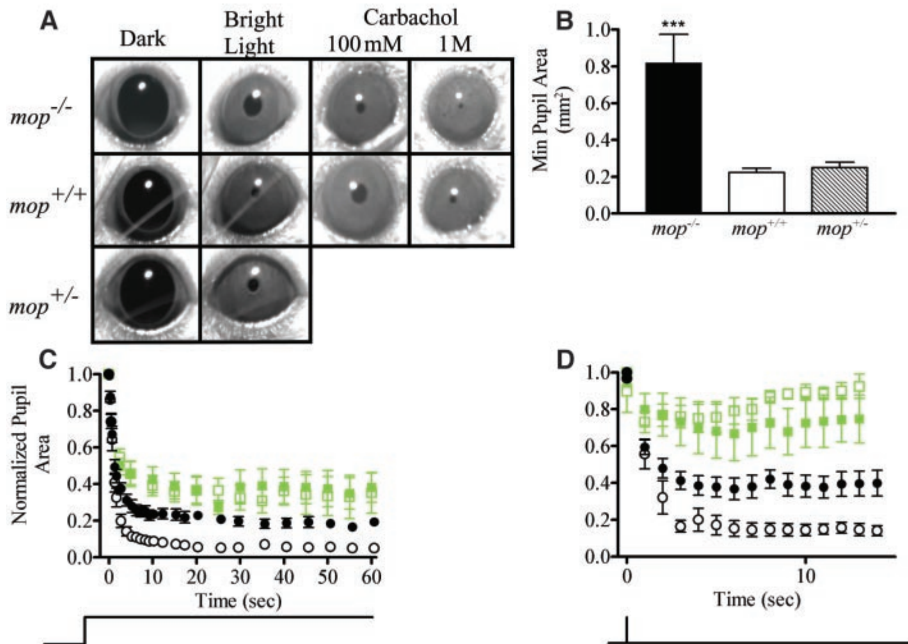

Fig. 2. Mice lacking melanopsin exhibit an incomplete pupillary light reflex at high irradiances. (**A**) Wild-type ($mop^{+/+}$), $mop^{+/-}$, and $mop^{-/-}$ mice all showed consensual pupillary constriction in response to 1 min of bright (145 $\mu W\ cm^{-2}$) monochromatic (480 nm) light, but the constriction was less in $mop^{-/-}$ than in $mop^{+/+}$ and $mop^{+/-}$ mice. A brighter light step (25 mW cm^{-2} white light) did not increase the response of $mop^{-/-}$ mice (not shown). By contrast, topical application of carbachol resulted in further pupil constriction of both $mop^{-/-}$ and $mop^{+/+}$ animals. There was no difference in dark-adapted pupil size between the three genotypes. (**B**) Collected results showing the minimum pupil areas (mean ± SEM, mm^2) attained by the three genotypes during bright-light exposure. The minimum pupil size attained by $mop^{-/-}$ mice was significantly larger than that of either $mop^{+/+}$ or $mop^{+/-}$ animals (one-way analysis of variance, $P < 0.0001$; post hoc Newman-Keuls multiple comparisons, $P < 0.001$). The responses of $mop^{+/+}$ and $mop^{+/-}$ mice were not significantly different from one another ($P > 0.05$). Data are from eight $mop^{-/-}$, eight $mop^{+/-}$, and five $mop^{+/+}$ mice. (**C**) Time courses of pupillary responses of $mop^{-/-}$ and wild-type mice to a light step at two different irradiances: 1-min light step at 0.12 $\mu W\ cm^{-2}$, 480 nm (five $mop^{+/+}$ and six $mop^{-/-}$ mice; open and filled green squares, respectively) and at 110 $\mu W\ cm^{-2}$, 480 nm (five $mop^{+/+}$ and eight $mop^{-/-}$ mice; open and filled black circles, respectively). (**D**) Same experiment as in (C), but with a brief flash of white light at two irradiances: 100-ms flash at 1.5 mW cm^{-2} (four $mop^{+/+}$ and four $mop^{-/-}$ mice; open and filled green squares, respectively) and at 25 mW cm^{-2} (four $mop^{+/+}$ and four $mop^{-/-}$ mice; open and filled black circles, respectively). The pupil area normalized with respect to the initial dark value is plotted (mean ± SEM).

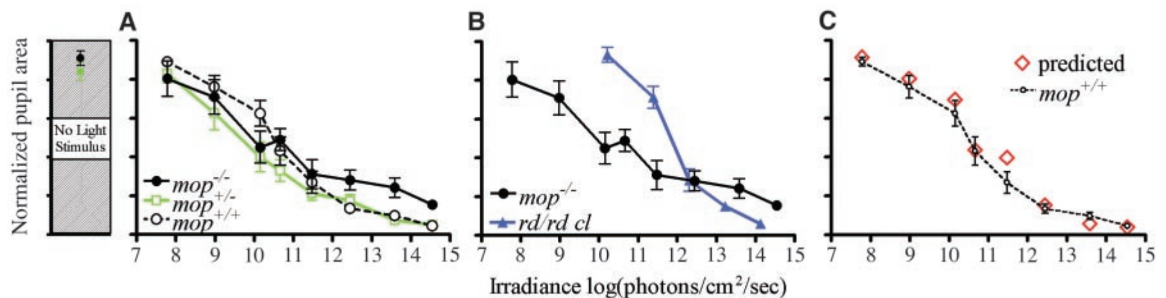

Fig. 3. Irradiance-response relations for the pupil reflex for the three genotypes. The minimum pupil area (mean ± SEM; normalized with respect to dark-adapted value) attained during exposure to 1-min 480-nm monochromatic light is plotted against irradiance (*14*). Dark bar on left represents control data, showing minimum pupil size attained during a 1-min period with the animal in place but without activation of light stimulus. (**A**) The responses of the three genotypes were similar at irradiances $\leq 10^{11.5}$ photons cm^{-2} s^{-1}, but at higher irradiances, the $mop^{-/-}$ response was impaired (supporting online text). Five to eight animals per genotype are shown at each irradiance. (**B**) Comparison of relations for $mop^{-/-}$ [same as in (A)] and *rd/rd cl* mice (four to six animals at each irradiance). (**C**) The wild-type irradiance-response relation can be well predicted by summing the relations from $mop^{-/-}$ and *rd/rd cl* mice (*15*).

shows greatly reduced sensitivity, but nonetheless reaches the same maximum response as that of the wild type at high irradiances (*2*). Thus, the *rd/rd cl* phenotype is the complement of the $mop^{-/-}$ phenotype. To test this apparent complementarity quantitatively, we measured the irradiance-response relation for the pupillary reflex of *rd/rd cl* mice (*9*) under conditions identical to those for $mop^{-/-}$ and wild-type animals (Fig. 3B) and then summed it with the relation for $mop^{-/-}$ mice (*15*). The resulting irradiance-response relation provides a good prediction of that measured from wild-type mice (Fig. 3C). This agreement suggests that the rod/cone and melanopsin systems together provide the full dynamic range of the normal pupillary reflex. We cannot rule out the existence of a third photodetection pathway, but if present, its contribution should be minor. The threshold corneal irradiance at 500 nm reported for exciting the melanopsin-positive RGCs is $\sim 10^{13}$ photons cm^{-2} s^{-1} [(*3*) and supporting online text]. By contrast, reproducible pupillary constriction in *rd/rd cl* mice (reflecting the activity of the melanopsin-associated system and of any unidentified contributing system) was observed to stimuli as dim as $10^{11.5}$ photons cm^{-2} s^{-1} at 480 nm (Fig. 3B) (*16*). In principle, any convergence of photosensitive RGCs driving the reflex would substantially lower the threshold of the reflex compared with that of individual photosensitive RGCs (supporting online text).

The retention in $mop^{-/-}$ mice of the otherwise melanopsin-expressing RGCs and of their proper central projections, combined with the loss of their intrinsic photosensitivity and the defective pupillary reflex of these animals, provides conclusive evidence that melanopsin is an indispensable component of a photoreceptive system with genuine physiological functions. The precise role of melanopsin in the RGC phototransduction process, however, remains uncertain. It may be the photopigment of the intrinsically photosensitive RGCs, or it may perform some other function critical to their photosensitivity (supporting online text).

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14. Irradiance-response relations were based on light intensities at the cornea because the dynamic nature of pupil size in these experiments precluded accurate assessment of the effective retinal irradiance. Pupil response was quantified as the minimum pupil area achieved during 1 min of light exposure, expressed as a fraction of the initial pupil area measured over a 2-s period before light exposure. In control experiments (dark bar at left in Fig. 3), the same measurements were made over a period of 1 min without activation of light stimulus. Even without a light stimulus, the pupil constricted slightly, presumably because of its high sensitivity and a very low level of stray light around the pupillometer and/or a tiny percentage of visible light from the infrared light-emitting diodes used for viewing the eye (*9*).
15. The formula for summing the irradiance-response relations from $mop^{-/-}$ and *rd/rd cl* mice is given by Response (normalized) = $\alpha(f_1 + f_2 - 2f_1f_2)/(1 - f_1f_2) + (1 - \alpha)f_2$, where f_1 and f_2 are the fractional responses, at a given irradiance, of the $mop^{-/-}$ and *rd/rd cl* mice in relation to the respective saturation levels, and α ($0 \leq \alpha \leq 1$) is the ratio of the maximum response levels attainable by $mop^{-/-}$ mice and *rd/rd cl* (same level as wild-type) mice. In our experiments, $\alpha = 0.86$. It is derived from the simple model that a component (α) of the pupil reflex can be driven by the rod/cone system and/or the melanopsin system, but the remainder ($1 - \alpha$) can only be driven by the melanopsin system. Data were interpolated where the two mouse lines did not receive identical irradiances.
16. With the λ_{max} for the action spectrum of the photosensitive RGCs at 484 nm (*3*) and that for the pupil response of *rd/rd cl* mice at 479 nm (*2*), the sensitivities of the two systems in the wavelength range of 480 to 500 nm can be compared directly without corrections because sensitivity changes little around λ_{max}.

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Supporting Online Material

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Materials and Methods

Supplementary Text

Fig. S1

References

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Regulation of Blood and Lymphatic Vascular Separation by Signaling Proteins SLP-76 and Syk

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Lymphatic vessels develop from specialized endothelial cells in preexisting blood vessels, but the molecular signals that regulate this separation are unknown. Here we identify a failure to separate emerging lymphatic vessels from blood vessels in mice lacking the hematopoietic signaling protein SLP-76 or Syk. Blood-lymphatic connections lead to embryonic hemorrhage and arteriovenous shunting. Expression of *slp-76* could not be detected in endothelial cells, and blood-filled lymphatics also arose in wild-type mice reconstituted with SLP-76–deficient bone marrow. These studies reveal a hematopoietic signaling pathway required for separation of the two major vascular networks in mammals.

Mammals have two circulatory systems, a closed blood vasculature and an open lymphatic vasculature, that operate in parallel but develop in series (*1, 2*). Although derived from venous endothelial precursors, lymphatic vessels do not communicate with blood vessels except at a single point where the thoracic duct empties into the subclavian vein (*1–3*). Recent studies have identified specific transcription factors and growth factors required to regulate the development of lymphatic vessels (*4–6*), but how emerging lymphatics remain separate from preexisting blood vessels is not known.

In mice, loss of the hematopoietic intracellular signaling proteins Syk (*7, 8*), SLP-76 (*9, 10*), or PLCγ2 (*11*) results in embryonic hemorrhage and perinatal death in addition to loss of immune receptor signaling. SLP-76–deficient mice that survive to adulthood were noted to have significant cardiac enlargement with an increase in the heart/body mass ratio of 40% compared with wild-type littermates at 12 weeks of age (Fig. 1, A and B). Analysis

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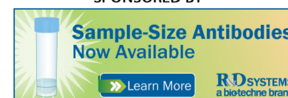

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