Nonuniform Distribution and Spectral Tuning of Photosensitive Retinal Ganglion Cells of the Mouse Retina

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Summary

Melanopsin-expressing photosensitive retinal ganglion cells (pRGCs) represent a third class of retinal photoreceptor [1–3]. These cells are intrinsically photosensitive, but also receive inputs from rod and cone photoreceptors [4–7], acting as the primary sensory conduit mediating non-image-forming responses to light [8–11]. Multiple subtypes of pRGC have been described in the mouse retina with characteristic morphologies and functional properties, and which perform distinct physiological roles [12–15]. Here, we examine the levels of melanopsin expression and distribution of pRGC subtypes across the mouse retina, identifying a previously unreported anatomical and functional specialization of the melanopsin system. Our results show a dorsal-ventral gradient in the expression of melanopsin and the distribution of pRGCs, which, combined with dorsal-ventral gradients in ultraviolet-sensitive and medium-wavelength-sensitive cone opsin expression, produce dramatic variations in the ratio of cone opsins and pRGCs across the retina. Using c-fos expression as a marker of light activation in vivo [16–18], we show that the responses of pRGCs are spectrally tuned by gradients in cone opsin expression depending on their location in the retina. These data illustrate the importance of classical photoreceptors in providing spectral tuning of pRGC light responses and have important implications for the complexity of non-image-forming responses to light.

Results and Discussion

The Melanopsin System Is Spatially Specialized in the Mouse Retina

Immunostaining with a highly sensitive melanopsin antibody revealed a nonuniform pattern of staining across the wild-type mouse retina (Figure 1 and Figure S1 available online). The total number of melanopsin-immunoreactive cells identified was typically 1,600–1,800 cells per retina, consistent with previous estimates of total photosensitive retinal ganglion cell (pRGC) numbers detected with melanopsin antibodies [19]. These include brightly stained M1-type cells with processes located in the OFF layer of the inner plexiform layer (IPL) and also weakly stained M2-type cells with processes located in ON layer of the IPL. However, the density of melanopsin-positive cells and the extent of dendritic processes are both significantly higher in the dorsal retina compared to the ventral retina (Figure 1). In the dorsal retina, both M1 and M2 cells are clearly visible, with extensive and highly overlapping dendritic networks observed in both the OFF and ON layers of the IPL. By contrast, the ventral retina is more sparsely populated and dominated by intensely stained M1-type cells (Figure S1). There was little noticeable variation in the pattern of staining observed between the temporal and nasal regions. The orientation of retinae was confirmed by double labeling for ultraviolet-sensitive (UVS) cone opsin (see below).

A similar pattern of melanopsin staining was observed for all retina examined (>50), including retina from multiple strains of wild-type mice (C57/Bl6 and C3H/He), degenerate rd/rd cl retina lacking outer retina photoreceptors, Opn4<sup>−/−</sup> mice lacking melanopsin, and also retinae collected throughout postnatal development (Figure S1). Collectively, these observations suggest that this patterning of pRGC distribution is a fundamental property of the mouse retina.

Distribution of pRGC Subtypes

The distribution of specific pRGC subtypes was determined by cell density and nearest-neighbor analysis (Figures 2 and S2). Both approaches show a significant increase in density of M1 cells and M2 cells in the dorsal retina compared to the ventral retina. There are, however, subtle differences in the distribution of these cell types. The highest density of M1 cells is observed in the upper dorsal retina, whereas the highest density of M2 cells is observed in the middorsal retina. No significant differences in density of either M1 or M2 cells were observed between the temporal and nasal regions (for detailed nearest-neighbor analysis, see Figure S2). The dorsal-ventral distribution of M1-type pRGCs was further confirmed by analysis of retina from Opn4<sup>−/−</sup> (tau-LacZ<sup>+/+</sup>) mice (Figure S2) that selectively report M1-type pRGCs [20]. Due to the rarity of bis- ratified M3 cells [19], no attempt was made to characterize the distribution of these cells.

In addition to M1–M3-type cells, there also exist at least two other subtypes of pRGC, M4- and M5-type cells, in which the levels of melanopsin expression are too low to be reliably detected with melanopsin antibodies [19], but can be detected using Opn4.Cre-based reporter mice [21]. Here, we have used the same Opn4.Cre mouse with an EYFP reporter to determine the distribution of M1–M5-type pRGCs. For a full description of this model and a discussion of potential limitations, see Figure S2. In contrast to the distribution of M1 and M2 cells, EYFP-expressing cells were found to be uniformly distributed across the retina (n = 3 retina, t test, p = 0.22) (Figures 2 and S2). Double labeling with melanopsin and EYFP antibodies again confirmed the dorsal-ventral distribution of melanopsin-immunoreactive M1 and M2 cells (n = 3 retina, t test, p = 4.1 × 10<sup>−5</sup>). Interestingly, levels of melanopsin and EYFP co-expression varied in dorsal-ventral manner, with significantly lower densities of melanopsin-negative EYFP-positive M4–M5 cells detected in the dorsal retina (n = 3 retina, t test, p = 0.002) (Figure 2). Collectively, our data indicate that melanopsin-expressing cells as a whole are uniformly distributed...
across the mouse retina, but the distribution of specific subtypes of pRGCs varies. Both M1- and M2-type cells show higher densities in the dorsal retina, whereas melanopsin-negative EYFP-positive M4–M5 cells are more numerous in the ventral retina.

Cone Opsin Gradients and Spectral Tuning of pRGC Light Responses

In addition to the dorsal-ventral gradients in melanopsin expression and distribution of pRGC subtypes that we have described, there also exist significant dorsal-ventral gradients in the expression of cone opsins within M cones (95% of all cones) of the mouse retina [22–25]. Levels of medium-wavelength-sensitive (MWS) opsin are highest in the dorsal retina, whereas levels of ultraviolet-sensitive (UVS) opsin are highest in the ventral retina. The rarer S cones (5% of cones) express only UVS opsin and show no such gradients in expression (Figure S3). When combined, these gradients result in dramatic variations in the ratio of cone opsins and pRGC subtypes present across the retina (Figure 3). Recent studies have confirmed that the changeable ratio of UVS and MWS opsin leads to a spectral tuning of light responses recorded from bipolar cells and ganglion cells of the mouse retina [26–27].

Using the expression of c-fos as a marker of cellular light activation in vivo [16–18], we confirm the spectral tuning of retinal ganglion cells by cone opsin gradients and demonstrate that the responses of pRGCs are spectrally tuned depending on their location in the retina (Figures 4 and S4).

Spectral Tuning of M1-Type pRGCs

In wild-type mice, white light pulses resulted in a near saturating activation of M1-type cells in both the dorsal and ventral retina (dorsal, 96.7% ± 1.4% and ventral, 92.8% ± 3.7%, n = 3 retina, t test, p = 0.31) (Figure 4). Robust responses to white light were also observed for M1 cells in the rd/rd cl retina (0.49 mm² each) collected from the dorsal and ventral retina (t test, p = 0.03). The direction of this gradient is again consistent with an excitatory input from M cones and the gradient of MWS cone opsin expression. We could also expect a significant contribution to M1 responses to white light from rod-based input [11, 28–31], yet it is likely this is reduced under these intensities of light [32].

In contrast to white light, a significant gradient of responses was observed for M1-type pRGCs in wild-type retina following UV light stimuli, with increased numbers of responsive cells detected in the ventral retina compared to the dorsal retina (dorsal, 56.0% ± 5.4% and ventral, 71.9% ± 6.8%, n = 3 retina, t test, p = 0.03) (Figure 4). The direction of this gradient is again consistent with an excitatory input from M cones and the gradient of UVS opsin. However, despite the more striking gradient in expression of UVS opsin compared to MWS opsin, the gradient of UV light responses observed for M1 cells in Opn4<sup>-/-</sup> mice were less obvious than those observed in responses to white light (dorsal, 47.4% ± 4.3% and ventral, 48.5% ± 4.1%, n = 3 retina, t test, p = 0.004). Overall, the pattern of c-fos expression detected in M1 pRGCs from Opn4<sup>-/-</sup> mice receiving white light pulses is consistent with a significant excitatory input from M cones and the gradient of MWS cone opsin expression. The percentage of M1-type cells responding to UV light was significantly higher in the rd/rd cl retina compared to wild-type retina (WT versus rd/rd cl t test, dorsal p = 0.03, ventral p = 0.21). Thus, it would seem that M1-type pRGCs show significantly greater responses to UV light in the absence of outer retina photoreceptors. White light activates M cones, rods, and
melanopsin, whereas UV light is most efficiently absorbed by UVS opsin (expressed in M cones and S cones) (Figure S4). It is therefore possible that S cones provide additional inhibitory signals to M1 cells under UV illumination. This conclusion is supported by recent data showing that both M cones and S cones provide excitatory inputs to pRGCs, and that S cones also mediate a marked OFF inhibition of M1 pRGC-driven responses [30].

**Spectral Tuning of M2-Type pRGCs**

In the wild-type retina, M2-type pRGCs show significant gradients in c-fos expression following both white light and UV light exposure. Following white light stimuli, greater numbers of responsive M2 cells are detected in the dorsal retina, (dorsal, 84.4% ± 1.7% and ventral, 56.9% ± 6.5%, n = 3 retina, t test, p = 0.0032), whereas following UV light stimuli higher numbers of responsive M2 cells are detected in the ventral retina (dorsal, 50.8% ± 8.4% and ventral, 75.0% ± 9.3%, n = 3 retina, t test, p = 0.038) (Figure 4). The pattern of these responses is consistent with a significant excitatory input from M cones and the opposing gradients of MWS and UVS cone opsin expression. No such gradients were observed for M2 cells in the rd/rd cl retina lacking rod and cone photoreceptors (Figure 4). For both white light and UV light stimuli, the number of responsive M2 cells was higher in the wild-type retina compared to the rd/rd cl retina, indicating an additive effect of melanopsin and outer retina-driven signals. Gradients of light-induced c-fos expression were also observed for EYFP cells in retina from Opo4.Cre. EYFP mice following UV light (n = 3 retina, t test, p = 0.0016) and to a lesser extent white light pulses (n = 3 retina, t test, p = 0.14) (Figure S4). Collectively our data indicate that gradients in cone opsin expression influence the responses of M4–M5-type cells, consistent with recent studies of M4-type cells [13].

**Differential Spectral Tuning of pRGC Subtypes and Non-Image-Forming Responses to Light**

Overall, we show that the gradients of cone opsin expression within M cones of the mouse retina lead to the spectral tuning of pRGC light responses depending on their location in the retina. However, given the high levels of endogenous photosensitivity observed for M1-type cells, it seems probable that under physiological conditions the gradients in cone opsin expression may do more to spectrally tune the responses of M2-type cells, and M4–M5 cells, than M1-type cells. These
conclusions are consistent with the current understanding of the different pRGC subtypes. The photoresponses of M1 cells are driven primarily by melanopsin phototransduction, whereas M2 cells, and also M4–M5 cells, exhibit lower levels of endogenous photosensitivity and rely upon greater excitatory inputs from the outer retina [6, 7, 11, 15, 21, 33, 34]. Specific subclasses of pRGCs innervate specific areas of the brain [12, 21, 35–37] and participate in different non-image-forming response to light [12, 13]. Based on our data, it seems highly likely that different classes of pRGCs and therefore different non-image-forming responses, are spectrally tuned to different wavelengths of light. Given their higher density in the dorsal retina it is likely that the majority of M2 cells show a green light bias, driven by the high levels of MWS cone opsin expression ($\lambda_{\text{max}}$ 508 nm). By contrast, EYFP-positive melanopsin-negative M4–M5 cells are more numerous in the ventral retina and likely receive predominantly UVS-driven inputs ($\lambda_{\text{max}}$ 360 nm). Responses from M1-type pRGCs are likely to be closer to the peak sensitivity of melanopsin ($\lambda_{\text{max}}$ 479 nm) [38].

Conclusions
In summary, our results show a previously unreported anatomical and functional specialization of the murine melanopsin system. This study provides the first evidence for a differential distribution of pRGC subtypes and illustrates the importance of classical photoreceptor input in providing spectral tuning of pRGC light responses. These data have clear implications for the complexity of sensory irradiance detection and the study of different non-image-forming responses to light.

Supplemental Information
Supplemental Information includes Supplemental Results and Discussion, Supplemental Experimental Procedures, and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.07.010.

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Figure 4. The Responses of M1- and M2-Type pRGCs Are Spectrally Tuned by Gradients in Cone Opsin Expression Present within M Cones of the Mouse Retina

(A and B) Images showing the levels of c-fos expression (red) detected within pRGCs (green) located in the dorsal and ventral regions of wild-type C57/Bl6 mice, degenerate rd/rd cl mice lacking rod and cone photoreceptors, and Opn4−/− mice lacking melanopsin expression, following white light (A) and UV light pulses (B).

(C and D) Graphs showing the percentage of M1- and M2-type pRGCs showing detectable levels of c-fos expression following white light pulses (C) and UV light pulses (D). The responses of M1- and M2-type pRGCs are spectrally tuned by gradients in MWS and UVS cone opsin expression present within M cones of the mouse retina. Spectral tuning of M1-type pRGCs is evident from Opn4−/− mice receiving white light pulses (t test, p = 0.004) and wild-type mice receiving UV light pulses (t test, p = 0.0032) and UV light pulses (t test, p = 0.038). In all cases, the direction of enhanced responses is consistent with significant excitatory input from M cones and the opposing gradients of MWS and UVS cone opsin expression. No significant gradients in c-fos expression were observed in the rd/rd cl retina for either M1-type pRGCs or M2-type pRGCs following either white light or UV light pulses. Interestingly, for UV light pulses, the percentage of responsive M1 cells and the intensity of c-fos staining were lower in the wild-type retina compared to rd/rd cl retina (t test, dorsal p = 0.03, ventral p = 0.21). Analysis is based on manual counting from n = 8–10 nonoverlapping regions (0.25 mm²) in the dorsal and ventral regions of each retina; values shown are the mean of n = 3–4 retina per group. Identification of M2 cells is not permitted using Opn4−/− mice [20]. Orientation of retina from Opn4−/− and wild-type mice was performed by colabeling with UVS cone opsin (shown for Opn4−/− only). Orientation of rd/rd cl retina was performed based on the patterning of melanopsin expression. Data are shown as mean ± SEM.

See also Figure S4 for analysis of light-induced c-fos expression in EYFP cells from the retina of Opn4.Cre+/− EYFP+/+ mice.

References

1. Hankins, M.W., Peirson, S.N., and Foster, R.G. (2008). Melanopsin: an exciting photopigment. Trends Neurosci. 31, 27–36.
2. Do, M.T., and Yau, K.W. (2010). Intrinsically photosensitive retinal ganglion cells. Physiol. Rev. 90, 1547–1581.
3. Hughes, S., Hankins, M.W., Foster, R.G., and Peirson, S.N. (2012). Melanopsin phototransduction: slowly emerging from the dark. Prog. Brain Res. 199, 19–40.
4. Belenky, M.A., Smeraski, C.A., Provencio, I., Sollars, P.J., and Pickard, G.E. (2003). Melanopsin retinal ganglion cells receive bipolar and amacrine cell synapses. J. Comp. Neurol. 460, 380–393.
5. Viney, T.J., Balint, K., Hillier, D., Siegert, S., Boldogkói, Z., Enquist, L.W., Meister, M., Cepko, C.L., and Roska, B. (2007). Local retinal circuits of melanopsin-containing ganglion cells identified by transsynaptic viral tracing. Curr. Biol. 17, 981–988.

6. Wong, K.Y., Dunn, F.A., Graham, D.M., and Berson, D.M. (2007). Synaptic influences on rat ganglion-cell photoreceptors. J. Physiol. 582, 279–296.

7. Schmidt, T.M., and Kofuji, P. (2010). Differential cone pathway influence on intrinsically photosensitive retinal ganglion cell subtypes. J. Neurosci. 30, 16262–16271.

8. Altimus, C.M., Güler, A.D., Villa, K.L., McNeill, D.S., Legates, T.A., and Hattar, S. (2008). Rods-cones and melanopsin detect light and dark to modulate sleep independent of image formation. Proc. Natl. Acad. Sci. USA 105, 19998–20003.

9. Güler, A.D., Ecker, J.L., Lall, G.S., Haq, S., Altimus, C.M., Liao, H.W., Barnard, A.R., Caihill, H., Badea, T.C., Zhao, H., et al. (2008). Melanopsin cells are the principal conduits for rod-cone input to non-image-forming vision. Nature 453, 102–105.

16. Semo, S., Daniela, L., Peirson, S., Butler, J., and Foster, R.G. (2003). Development of melanopsin-based irradiance sensitivity of cone-mediated responses in mouse retinal ganglion cells. J. Neurosci. 31, 7670–7681.

17. Lucas, R.J., Lall, G.S., Allen, A.E., and Brown, T.M. (2012). How rod, cone, and melanopsin photoreceptors come together to enlighten the mammalian circadian clock. Prog. Brain Res. 199, 1–18.

18. Berson, D.M., Castrucci, A.M., and Provencio, I. (2010). Melanopsin-expressing retinal ganglion-cell photoreceptors: many subtypes, diverse functions. Trends Neurosci. 34, 359–374.

19. Hattar, S., Liao, H.W., Takao, M., Berson, D.M., and Yau, K.W. (2002). Melanopsin-positive intrinsically photosensitive retinal ganglion cells: from form to function. J. Neurosci. 31, 16094–16101.

20. Farhangfar, F., Kage, K., Krzystolik, M.G., Lyass, L.A., and Robbins, J.T. (2000). The murine cone photoreceptor: a single cone type expresses both S and M opsins with retinal spatial patterning. Neuron 27, 513–523.

21. Barnard, A.R., Cahill, H., Badea, T.C., Zhao, H., et al. (2008). Two different visual pigments in one retinal cone cell. Neuron 12, 1159–1168.

22. Applebury, M.L., Antoch, M.P., Baxter, L.C., Chun, L.L., Falk, J.D., Farhangfar, F., Kage, K., Krzystolik, M.G., Lyass, L.A., and Robbins, J.T. (2000). The murine cone photoreceptor: a single cone type expresses both S and M opsins with retinal spatial patterning. Neuron 27, 513–523.

23. McNeill, D.S., Sheely, C.J., Ecker, J.L., Badea, T.C., Morhardt, D., Guido, W., and Hattar, S. (2011). Development of melanopsin-based irradiance detecting circuitry. Neurod. Dev. 6, 8.

24. McNeill, D.S., Sheely, C.J., Ecker, J.L., Badea, T.C., Morhardt, D., Guido, W., and Hattar, S. (2011). Development of melanopsin-based irradiance detecting circuitry. Neurod. Dev. 6, 8.

25. Nikonor, S.S., Khodolenko, R., Lem, J., and Pugh, E.N., Jr. (2000). Physiological features of the S- and M-cone photoreceptors of wild-type mice from single-cell recordings. J. Gen. Physiol. 127, 359–374.