Differential Expression of Brn3 Transcription Factors in Intrinsically Photosensitive Retinal Ganglion Cells in Mouse

Varsha Jain, Ethiraj Ravindran, and Narender K. Dhingra*
National Brain Research Centre, Manesar (Haryana) 122050, India

ABSTRACT
Several subtypes of melanopsin-expressing, intrinsically photosensitive retinal ganglion cells (ipRGCs) have been reported. The M1 type of ipRGCs exhibit distinct properties compared with the remaining (non-M1) cells. They differ not only in their soma size and dendritic arbor, but also in their physiological properties, projection patterns, and functions. However, it is not known how these differences arise. We tested the hypothesis that M1 and non-M1 cells express Brn3 transcription factors differentially. The Brn3 family of class IV POU-domain transcription factors (Brn3a, Brn3b, and Brn3c) is involved in the regulation of differentiation, dendritic stratification, and axonal projection of retinal ganglion cells during development. By using double immunofluorescence for Brn3 transcription factors and melanopsin, and with elaborate morphometric analyses, we show in mouse retina that neither Brn3a nor Brn3c are expressed in ipRGCs. However, Brn3b is expressed in a subset of ipRGCs, particularly those with larger somas and lower melanopsin levels, suggesting that Brn3b is expressed preferentially in the non-M1 cells. By using dendritic stratification to distinguish M1 from non-M1 cells, we found that whereas nearly all non-M1 cells expressed Brn3b, a vast majority of the M1 cells were negative for Brn3b. Interestingly, in the small proportion of the M1 cells that did express Brn3b, the expression level of Brn3b was significantly lower than in the non-M1 cells. These results provide insights about how expression of specific molecules in a ganglion cell could be linked to its role in visual function. J. Comp. Neurol. 520:742–755, 2012.

INDEXING TERMS: melanopsin; Brn3a; Brn3b; Brn3c

A subset of retinal ganglion cells (RGCs) expresses the photopigment melanopsin, and is intrinsically photosensitive (ipRGCs; Provencio et al., 2000, 2002; Berson et al., 2002). These cells are involved in a range of visual functions. At least five subtypes of ipRGCs have been reported, denoted M1–M5; they differ in their morphological and physiological characteristics (Hattar et al., 2002, Viney et al., 2007; Baver et al., 2008, Schmidt et al., 2008, Schmidt and Kofuji, 2009, 2010, 2011; Pires et al., 2009; Berson et al., 2010; Ecker et al., 2010). Specifically, the properties of M1 cells are strikingly different from those of the non-M1 cells. For example, M1 cells stratify in the Off sublayer of the inner plexiform layer (IPL), whereas non-M1 cells stratify in the On sublayer (M3 cells stratify in both). Similarly, M1 cells have a smaller soma and a simpler dendritic arbor, but higher melanopsin levels, and therefore higher sensitivity and faster response kinetics.

Until recently, ipRGCs were considered to mediate only non-image-forming visual functions, such as circadian photoentrainment and pupillary reflex (Gooley et al., 2001; Berson et al., 2002; Hannibal et al., 2002; Hattar et al., 2002; Panda et al., 2002; Hatori et al., 2008). However, the non-M1 types of ipRGCs have been shown recently to additionally mediate image-forming vision (Ecker et al., 2010). Consistent with this, M1 and non-M1 cells exhibit distinct patterns of projection to various brain areas (Ecker et al., 2010). For example, the suprachiasmatic nucleus (SCN) and intergeniculate leaflet...
TABLE 1.
Primary Antibodies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Immunogen</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brn3a(^1)</td>
<td>Raised against 1–109 N-terminal amino acids</td>
<td>Santa Cruz Biotechnology; mouse monoclonal, #sc-8429</td>
<td>1:250</td>
</tr>
<tr>
<td></td>
<td>of mouse Brn3a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brn3b(^1)</td>
<td>Raised against 397–410 C-terminal amino acids</td>
<td>Santa Cruz Biotechnology; goat polyclonal, #sc-6026</td>
<td>1:250</td>
</tr>
<tr>
<td></td>
<td>(QRQKQRMKYSAGI) of human Brn3b(^2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brn3c</td>
<td>Raised against a GST-tagged partial recombinant</td>
<td>Santa Cruz Biotechnology; mouse monoclonal, #sc-81980</td>
<td>1:50</td>
</tr>
<tr>
<td></td>
<td>protein containing amino acids 100–191 of human</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Melanopsin</td>
<td>Raised against a synthetic peptide consisting</td>
<td>Advanced Targeting Systems; rabbit polyclonal, #UP006</td>
<td>1:7,500 (flatmount)</td>
</tr>
<tr>
<td></td>
<td>of 1–15 N-terminal amino acids (MDSPGSQVLSLTLQ)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>of mouse melanopsin(^3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Already included in the primary antibody database of the Journal of Comparative Neurology.
\(^2\)Xiang et al., 1993.
\(^3\)Provencio et al., 2000.

(IGL) are innervated predominantly by the M1 cells, whereas the superior colliculus and dorsal lateral geniculo-nucleus receive their melanopsin cell input primarily from the non-M1 cells. However, it is not known how these differences between M1 and non-M1 cells are generated.

One possibility is that these differences originate from differential expression of specific molecules during development. The Brn3 family of class IV POU-domain transcrip-tion factors, (Brn3a, Brn3b, and Brn3c) is known to regulate the differentiation, axonal pathfinding, and dendritic stratification of RGCs (Xiang et al., 1993; Gan et al., 1996, 1999; Erkman et al., 2000; Wang et al., 2000, 2002; Badea et al., 2009). In mouse retina, Brn3b is the first Brn3 transcription factor to appear during development and is critically required for the survival and differentiation of RGCs and in guiding their axonal projections (Gan et al., 1996, 1999; Erkman et al., 1996, 2000; Wang et al., 2000, 2002; Badea et al., 2009). Brn3a plays a role in regulating patterns of dendritic stratification of RGCs (Badea et al., 2009). Brn3c is expressed in fewer RGCs and is involved in RGC differentiation and axonal outgrowth (Wang et al., 2002). We asked here whether Brn3 transcription factors are expressed differentially in M1 and non-M1 cells.

A part of this work was published as an abstract (Jain et al., 2010).

MATERIALS AND METHODS
Animals and tissue preparation

All experiments reported here were approved by the Institutional Animal Ethics Committee of the National Brain Research Centre, India. Adult mice (C57BL/6, 8–12 weeks old, male) were maintained on a 12-hour light/dark cycle with an average ambient daylight of approximately 200 lux (measured with an IL1400 photometer, International Light Technologies, Peabody, MA). Nine retinas from nine animals were used to quantify the number of cells in the ganglion cell layer (GCL), including the number of RGCs and the number of Brn3a-, Brn3b-, Brn3c- or melanopsin-expressing cells. To average the potential daily oscillation in the number of immunodetectable melanopsin cells (González-Menéndez et al., 2009), we sacrificed one animal at Zeitgeber time (ZT) = 3 (ZT = 0 represents onset time of the day cycle), another at ZT = 23, and seven animals between ZT = 4 and ZT = 12.

Eyes were removed after cervical dislocation, and a small incision was made near and perpendicular to limbus. Eyes were then immersed in 4% paraformaldehyde (PFA; pH 7.4) at 4°C for 10 minutes and hemisected, followed by fixation of the posterior eyecup in 4% PFA for 45–60 minutes at 4°C. To prepare a whole-mount, the retina was isolated from the posterior eyecup, incised radially, and flattened on a membrane filter. The membrane filter with the attached flattened retina was used for further processing. To prepare retinal sections, the posterior eyecup was immersed in 30% sucrose for 1–2 hours at 4°C and sectioned at 30- or 50-μm thickness on a cryostat (model CM3050S; Leica Microsystems, Wetzlar, Germany). The sections were collected on 3-amino-propyl, triethoxysilane (APES)-coated glass slides.

Characterization of primary antibodies

The primary antibodies against Brn3a and Brn3c were mouse monoclonal, whereas the Brn3b antibody was goat polyclonal (Table 1; Santa Cruz Biotechnology, Santa Cruz, CA; Wagner et al., 2002; Elshatory et al., 2007; Poche et al., 2008; Kawano et al., 2008). The Brn3a and Brn3c antibodies recognize in Western blot 47- and 37-kDa bands, respectively (manufacturer’s specifications). The specificity of the Brn3b antibody was confirmed by preadsorption with a Brn3b blocking peptide (Santa Cruz Biotechnology; #sc-6026P [not illustrated]). The Brn3b blocking peptide sequence consisted of amino acids 397–410, as determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and liquid
chromatography mass spectrometry (Poche et al., 2008). Furthermore, by using double immunofluorescence for Brn3a and Brn3b, and for Brn3b and Brn3c, we found that these antibodies identified distinct subsets of RGCs, implying absence of any cross-reactivity among each other (Fig. 1). The melanopsin antibody was rabbit polyclonal (UFO06; Advanced Targeting Systems, San Diego, CA), which was raised against 15 N-terminal amino acids of the mouse melanopsin, and has been extensively used previously (Provencio et al., 2002; Hattar et al., 2002; Baver et al., 2008; Dumitrescu et al., 2009; Berson et al., 2010). The specificity of this antibody has been verified by the lack of immunoreactivity in melanopsin knockout mouse (Panda et al., 2002; Berson et al., 2010). See Table 1 for details on immunogens and dilutions for the antibodies used here.

**Immunohistochemistry**

Double immunofluorescence was carried out for melanopsin and Brn3a/Brn3b/Brn3c. The retinal flat-mounts or sections were incubated for 1 hour in a cocktail solution to block nonspecific staining. For melanopsin and Brn3b/Brn3c, the cocktail included 10% normal donkey serum, 3% bovine serum albumin (BSA), and 0.3% Triton X-100 in PBS (pH 7.4), and for melanopsin and Brn3a, the cocktail included 10% normal horse serum, 3% normal donkey serum, 3% BSA, and 0.3% Triton X-100 in PBS (pH 7.4).

The whole-mounts were incubated in a mixture of primary antibodies against melanopsin (diluted 1:7,500) and Brn3a (1:250)/Brn3b (1:250)/Brn3c (1:50) for 3 days, and sections were incubated for 2 days (melanopsin antibody was diluted 1:10,000 here). In each experiment, a sample was used as negative control in which the primary antibody was omitted. After washing in PBS for 5× 5 minutes, the samples were incubated in the appropriate secondary antibody (donkey anti-rabbit conjugated with Alexa Fluor 488 [1:500] for melanopsin, donkey anti-goat conjugated with Alexa Fluor 594 [1:500] for Brn3b, donkey anti-mouse conjugated with Alexa Fluor 594 [1:500] for Brn3c [all from Molecular Probes, Eugene, OR], and horse anti-mouse conjugated with Texas Red [1:250] for Brn3a [Vector, Burlingame, CA]) for 1 hour at room temperature. The samples were washed again in PBS for 5× 5 minutes and mounted by using Vectorshield containing 4′,6-diamidino-2-phenylindole (DAPI; Vector). DAPI binds to double-stranded DNA and labels the nuclei of all cells, with blue fluorescence.

**Morphometric measurements and analyses**

The images of immunostained retinal flat-mounts and sections were captured on an epifluorescent microscope (Axiolmager.Z1; Carl Zeiss, Göttingen, Germany) by using a CCD camera (AxioCam MRm) or a confocal microscope (LSM 510 Meta, Carl Zeiss). The Axiolmager microscope was equipped with an ApoTome grid projection system that enabled us to capture images at higher contrast and enhanced optical resolution in the z-axis. Briefly, the grid was projected on to the image by the reflected light. The image of the grid was shifted in three defined phases by using a scanner in ApoTome. This reduced the background in the image and produced a stronger signal. Later, these grid images were offset against one another to get an optical section without the grid (manufacturer’s specifications).

For the whole-mounts that were used for cell counting (see Table 2), and for measurement of staining intensity and soma size of melanopsin cells (see Figs. 3, 4), several 1-μm-thick optical sections in the z-axis were captured to cover the somas of all labeled cells in the GCL, which were stacked offline to generate a single image by using ImageJ software (NIH). Contiguous frames were imaged to cover the entire retina (except for Brn3c, for which we used retinal pieces; Table 2), by using specific landmarks in each frame, such that all labeled cells were imaged and none was imaged more than once. The cells labeled for DAPI, melanopsin, Brn3a, Brn3b, or Brn3c were counted in all the frames by using ImageJ. The intensity of melanopsin-positive cells was estimated by marking their soma boundary and measuring the mean gray level after background subtraction in 8-bit grayscale images. The perimeter of the soma boundary was used to calculate soma diameter, assuming circular shape. Because both Brn3 and melanopsin expression levels vary among cells, the images were enhanced digitally in Adobe Photoshop (San Jose, CA) to reveal the cells with very low expression levels. However, the quantitative intensity measurements shown in Figure 3 were made before any image enhancement.

For tracking the dendrites of melanopsin cells, six retinal pieces were double-labeled for melanopsin and Brn3b. Several 1-μm-thick serial optical sections were captured in the z-axis on the confocal or epifluorescent ApoTome microscope to cover the GCL and the IPL. Only the melanopsin cells in which dendrites were clearly visible traversing through the IPL for 100 μm or more, with their cell somas typically located near the center of the frame, were selected. The optical sections were visually examined dendrite by dendrite for each selected cell to track its dendrites through the depth of the IPL, paying special attention to whether any of the dendrites traversed vertically to stratify in the outer IPL. For these cells, we then determined whether they were bistriated. This enabled us to differentiate between the M1 and M3 cells. The remaining cells were identified after dendritic tracking as other non-M1 cells.
Figure 1. Specificity of Brn3 antibodies in mouse retina. 

A: Representative field of a retinal whole-mount showing RGCs immunoreactive for Brn3a (green) and Brn3b (red) (blue: DAPI). Some cells expressed both Brn3a and Brn3b (double arrow), and others expressed only Brn3a (single arrow) or only Brn3b (arrowhead). This implied that the Brn3a and Brn3b antibodies were specific and did not cross-react. 

B: Same field as in A, showing only Brn3a labeling. Note the absence of labeling in the cell expressing only Brn3b (arrowhead). 

C: Same field as in A, showing only Brn3b labeling. Note the absence of labeling in the cell expressing only Brn3a (single arrow). 

D: Representative field showing RGCs immunoreactive for Brn3b (red) and Brn3c (green) (blue: DAPI). Some cells expressed both Brn3b and Brn3c (double arrow), and others expressed only Brn3b (arrowhead) or only Brn3c (single arrow). This implied that the Brn3b and Brn3c antibodies were specific and did not cross-react. 

E: Same field as in D, showing only Brn3c labeling. Note the absence of labeling in the cell expressing only Brn3b (arrowhead). 

F: Same field as in D, showing only Brn3b labeling. Note the absence of labeling in the cell expressing only Brn3c (single arrow). Scale bar = 20 µm in F (applies to A–F). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
For more than one-fourth of the selected cells, we also made 3D reconstructions of their dendrites, by using Neuromantic software (Darren Myatt, www.reading.ac.uk/neuromantic; Freed and Liang, 2010; see Fig. 7). Here, the dendrites were tracked by moving through all optical sections one by one and were drawn from the best focused optical section. In addition, for more than one-third of the M1 cells (a different subset from the one analyzed with Neuromantic), we generated 3D images from the optical sections by using the Volume Viewer plug-in in ImageJ (http://rsbweb.nih.gov/ij/plugins/volume-viewer.html). The optical sections were opened in Volume J display mode, and the intensity threshold and other parameters were adjusted to get a clear view of the dendritic tree. The 3D reconstructions or images were rotated freely in all axes to get the best view of the dendrites stratifying in the IPL (see Fig. 7).

The Brn3b staining intensity in the ipRGCs was measured by using a previously described method with slight modifications (M. Bongard, http://naranja.umh.es/C24/atg/tutorials/VGIV-MeasuringCellsImageJ.pdf; see Fig. 8). Briefly, several 1-μm optical sections of the GCL containing Brn3b signals were merged by using ImageJ. After background subtraction, the image contrast was enhanced, which allowed detection of even the very lightly labeled Brn3b cells. The contrast enhancement was kept constant for all the frames. The image was thresholded incrementally when necessary, and all the labeled Brn3b cells were selected using the Region of Interest and Wand tools of ImageJ. The intensity of Brn3b staining was measured for each selected cell and was also visualized by using the Interactive 3D Surface Plot plug-in (see Fig. 8).

### RESULTS

**Estimates of RGCs expressing melanopsin or Brn3 transcription factors**

In six whole-mount retinas, we counted the number of DAPI-positive cells, as well as the numbers of Brn3a-, Brn3b- and/or melanopsin-immunoreactive cells in the GCL (Table 2, Fig. 2). Images were taken from contiguous areas to cover nearly the entire retina (average area covered: 10.5 mm²). Brn3c cells were counted in three flat-mounted retinal pieces, and the cell numbers were normalized to the average retinal area in our samples (Table 2). We found 6,097 ± 689 (mean ± SD) DAPI-positive cells per mm² in the GCL. Considering that approximately 44% of the cells in the mouse GCL are ganglion cells (Pang and Wu, 2011), the computed density of RGCs in these samples was 2,683 ± 303 per mm². In these samples, we found a total of 113 ± 21 cells per mm² that were immunoreactive for melanopsin, which was 4.1% of all the DAPI-positive cells.

### TABLE 2.

**Number and Density (per mm²; in parentheses) of Cells in the Ganglion Cell Layer in Whole-Mount Retinas**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Area covered (mm²)</th>
<th>DAPI-positive cells</th>
<th>Retinal ganglion cells</th>
<th>Brn3a cells</th>
<th>Brn3b cells</th>
<th>Brn3c cells</th>
<th>Melanopsin cells</th>
<th>Melanopsin cells that expressed Brn3b</th>
<th>% Melanopsin cells that expressed Brn3b</th>
<th>% Brn3b cells that expressed melanopsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retina-1</td>
<td>11.22</td>
<td>77,404 (6,899)</td>
<td>34,058 (3,035)</td>
<td>20,037 (1,786)</td>
<td>NE</td>
<td>NE</td>
<td>1,105 (98)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Retina-2</td>
<td>10.43</td>
<td>67,854 (6,506)</td>
<td>29,856 (2,863)</td>
<td>21,270 (2,039)</td>
<td>NE</td>
<td>NE</td>
<td>1,052 (101)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Retina-3</td>
<td>9.06</td>
<td>52,558 (5,801)</td>
<td>23,126 (2,553)</td>
<td>NE</td>
<td>11,178 (1,234)</td>
<td>NE</td>
<td>807 (89)</td>
<td>395 (44)</td>
<td>49</td>
<td>3.5</td>
</tr>
<tr>
<td>Retina-4</td>
<td>10.98</td>
<td>60,146 (5,478)</td>
<td>28,464 (2,410)</td>
<td>NE</td>
<td>17,947 (1,634)</td>
<td>NE</td>
<td>971 (88)</td>
<td>740 (67)</td>
<td>76</td>
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<tr>
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<td>9.25</td>
<td>46,894 (5,068)</td>
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<td>NE</td>
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<tr>
<td>Retina-6</td>
<td>11.89</td>
<td>74,701 (6,283)</td>
<td>32,868 (2,764)</td>
<td>NE</td>
<td>17,067 (1,493)</td>
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<td>1,745 (166)</td>
<td>1,138 (107)</td>
<td>67</td>
<td>6.7</td>
</tr>
<tr>
<td>Retina-7</td>
<td>1.93</td>
<td>62,343 (5,954)</td>
<td>27,428 (2,560)</td>
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<td>6,418 (613)</td>
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<td>1,313 (125)</td>
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<td>NA</td>
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<tr>
<td>Retina-8</td>
<td>3.77</td>
<td>59,451 (5,678)</td>
<td>26,158 (2,498)</td>
<td>NE</td>
<td>11,600 (1,108)</td>
<td>NE</td>
<td>1,425 (136)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Retina-9</td>
<td>3.02</td>
<td>75,453 (7,207)</td>
<td>33,199 (3,171)</td>
<td>NE</td>
<td>14,259 (1,362)</td>
<td>NE</td>
<td>1,290 (123)</td>
<td>NA</td>
<td>NA</td>
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</tbody>
</table>

**Table 2 (continued)**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Area covered (mm²)</th>
<th>DAPI-positive cells</th>
<th>Retinal ganglion cells</th>
<th>Brn3a cells</th>
<th>Brn3b cells</th>
<th>Brn3c cells</th>
<th>Melanopsin cells</th>
<th>Melanopsin cells that expressed Brn3b</th>
<th>% Melanopsin cells that expressed Brn3b</th>
<th>% Brn3b cells that expressed melanopsin</th>
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<tr>
<td>Retina-10</td>
<td>4.77</td>
<td>64,088 (6,097)</td>
<td>28,198 (2,683)</td>
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<td>NE</td>
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<td>65</td>
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</tr>
<tr>
<td>Retina-11</td>
<td>3.07</td>
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<td>24,002 (2,203)</td>
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<td>11,500 (1,040)</td>
<td>NE</td>
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<td>670 (63)</td>
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<td>66,279 (6,283)</td>
<td>33,154 (3,171)</td>
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<td>17,067 (1,493)</td>
<td>NE</td>
<td>1,745 (166)</td>
<td>1,148 (110)</td>
<td>67</td>
<td>6.7</td>
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<td>11,600 (1,108)</td>
<td>NE</td>
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<td>NE</td>
<td>1,290 (123)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

**Abbreviations:** NE, not examined; NA, not applicable (Brn3a and Brn3c cells did not express melanopsin).

1Based on Pang and Wu (2011).

2Retinal pieces, absolute cell numbers normalized to average retinal area (10.47 mm²) of whole-mount retinas (retina-1 to retina-6).
Figure 2. Differential expression of Brn3 transcription factors in melanopsin cells. A: Representative field of a whole-mount retina immunolabeled for Brn3a (red) and melanopsin (green) (blue: DAPI). None of the Brn3a-positive cells in two whole-mount retinas expressed melanopsin (Table 2). None of the melanopsin cells labeled either brightly (arrowheads) or faintly (arrows) expressed Brn3a. B: Same field as in A, with melanopsin signal removed from the image, to show that all the melanopsin cells in A are negative for Brn3a. C: Representative field of a retinal whole-mount immunoreactive for Brn3b (red) and melanopsin (green). Some of the melanopsin cells expressed Brn3b (arrows). Interestingly, nearly all of these cells were labeled faintly for melanopsin, whereas the cells that were brightly labeled for melanopsin did not express Brn3b (arrowheads). D: Same field as in C, with melanopsin signal removed from the image to show that the brightly labeled melanopsin cells in C are negative for Brn3b (arrowheads). E: Representative field of a flat-mount retinal piece immunolabeled for Brn3c (red) and melanopsin (green). None of the Brn3c-positive cells in three flat-mount retinal pieces expressed melanopsin (Table 2). None of the melanopsin cells labeled either brightly (arrowheads) or faintly (arrows) expressed Brn3c. F: Same field as in E, with melanopsin signal removed from the image, to show that the all the melanopsin cells in E are negative for Brn3c. Scale bar = 20 μm in F (applies to A–F). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
represented approximately 4% of RGCs. In addition, 1,912 ± 179 cells per mm² (71% of RGCs) expressed Brn3a (n = 2 retinas), 1,396 ± 180 cells per mm² (52% of RGCs) expressed Brn3b (n = 4 retinas), and 1,028 ± 381 cells per mm² (38% of RGCs) expressed Brn3c (n = 3 retinal pieces; Table 2).

A subset of melanopsin cells expressed Brn3b, but none expressed Brn3a or Brn3c

Double immunofluorescence for melanopsin and Brn3a/Brn3c revealed that none of the melanopsin cells expressed either Brn3a or Brn3c (Table 2, Fig. 2). However, a subset of the melanopsin cells expressed Brn3b (Fig. 2, Table 2). The number of these cells that co-expressed melanopsin and Brn3b (71 ± 22 per mm²) corresponded to approximately 3% of all RGCs, 5% of Brn3b cells, and 65% of melanopsin cells (Table 2). Interestingly, the melanopsin cells that co-expressed Brn3b were typically labeled faintly for melanopsin (Fig. 2C,D; arrows), whereas the ones that did not express Brn3b were labeled brightly (Fig. 2C,D; arrowheads). Because M1 cells express much higher levels of melanopsin than the other subtypes (Schmidt and Kofuji, 2009; Berson et al., 2010; Ecker et al., 2010), this observation indicated that the cells that expressed Brn3b were non-M1 cells, whereas those that did not express Brn3b were M1 cells. We investigated this further by classifying the melanopsin cells into M1 and non-M1 types based on three objective criteria: level of melanopsin expression, soma size, and dendritic stratification.

Brn3b expressed preferentially in cells with lower levels of melanopsin

The M1 cells are more light-sensitive than the non-M1 cells, likely because they express much higher levels of melanopsin (Schmidt and Kofuji, 2009; Ecker et al., 2010). We measured the intensity of the melanopsin antibody staining as an indicator of the level of the protein expression, and used these measurements to broadly differentiate between M1 and non-M1 cells. The frequency histogram of melanopsin levels for all cells showed a dominant peak at a low intensity (Fig. 3A; mel+; gray line), of melanopsin cells that expressed Brn3b (mel+/brn3b+; black line, filled circles), and of melanopsin cells that did not express Brn3b (mel+/brn3b−; black line, empty circles) as a function of melanopsin staining intensity (n = 4 retinas). The intensity was measured from 8-bit grayscale images after background subtraction. The arrow points to a small hump in the gray line, possibly indicating that the melanopsin cell population included at least two partially overlapping distributions. The inset shows the same data more closely, only for higher melanopsin intensities. B: Proportion of melanopsin cells that expressed Brn3b as a function of melanopsin staining intensity. The proportion reduced nearly monotonically from 100% at lowest intensity values to zero at highest intensity values.
melanopsin level range must be M1 and non-M1 cells respectively. Third, in the intermediate intensity range the proportion of cells that expressed both Brn3b and melanopsin decreased nearly monotonically with the intensity of melanopsin staining (Fig. 3A,B). Together, these results suggested that the melanopsin cells with smallest somas (diameter ≤10.2 μm), likely M1 cells, never expressed Brn3b, those with the largest somas (≥32.1 μm), likely non-M1 cells, always expressed Brn3b (Fig. 4A,B). In the intermediate range, the proportion of melanopsin cells that expressed Brn3b increased gradually with the soma size (Fig. 4B). The clear distinction in terms of Brn3b expression between the melanopsin cells with extreme soma sizes and the consistent trend in the midrange further supported the view that non-M1 cells, but not M1 cells, expressed Brn3b.

Brn3b expressed preferentially in non-M1 cells

In the absence of a known marker, dendritic stratification is currently the most reliable criterion to distinguish between M1 and non-M1 cells. The M1 cells stratify in the outermost stratum of IPL whereas non-M1 cells stratify in the inner third of IPL, including the bistratified M3 cells, which stratify in both outer and inner IPL (Hattar et al., 2006; Viney et al., 2007; Baver et al., 2008; Schmidt et al., 2008; Schmidt and Kofuji, 2009, 2010, 2011; Berson et al., 2010). We employed both retinal sections and flat-mounts to study dendritic stratification patterns of melanopsin cells.

The 30- or 50-μm-thick retinal sections were immunostained for Brn3b and melanopsin, and we analyzed the melanopsin cells where it was possible to track their dendrites in the IPL. A total of 480 cells was selected from 48 sections (eight sections each from six retinas). Out of these, 446 cells were in the GCL and 34 in the inner nuclear layer (INL; see below). The tracking was done by using 1-μm-thick serial optical sections for each cell. We found in these samples that in the GCL approximately 97% (262 of 271) of the cells that stratified in the inner IPL (M2 type), 82% (28 of 34) of the bistratified cells (M3 type), but only 14% (20 of 141) of the cells that stratified in the outer IPL (M1 type) expressed Brn3b (Fig. 5A–C).

Although dendritic stratification can distinguish among M1, M2, and M3 cells, it is not always reliable because of the difficulty of precisely tracking a cell’s dendrites in the IPL, and because some dendrites are invariably cut in a vertical section. It is therefore possible that some of the cells were incorrectly identified as a specific melanopsin cell type, which might explain why a few cells identified as M1 type expressed Brn3b, or why a few cells identified as non-M1 type did not express Brn3b. To explore this, we
applied additional criteria of soma size and melanopsin expression levels to the cells identified based on their dendritic stratification in the retinal sections, and compared within each type (M1, M2, and M3) the Brn3b-positive and Brn3b-negative cells. For the M1 and M2 cells, the Brn3b-positive cells had significantly larger soma diameters (M1: $16.6 \pm 2.6 \mu m$, $n = 20$; M2: $16.7 \pm 3 \mu m$, $n = 262$) than the Brn3b-negative cells (M1: $14.7 \pm 2.3 \mu m$, $n = 119$; M2: $13.6 \pm 2.2 \mu m$, $n = 9$) ($P < 0.01$; unpaired, two-tailed t-test), but there was no significant difference between the soma diameters of Brn3b-positive ($15.2 \pm 3.0 \mu m$) and Brn3b-negative M3 cells ($16.3 \pm 2.2 \mu m$) (Fig. 5D, left). Similarly, for the M2 and M3 cells, the Brn3b-positive cells had lower melanopsin expression levels (M2: $56 \pm 23$, $n = 262$; M3: $83 \pm 45$, $n = 29$) than the Brn3b-negative cells (M2: $73 \pm 31$, $n = 9$; M3: $105 \pm 53$, $n = 6$) (Fig. 5D, right), but there was no difference in the M1 group. These results raised the possibility that some of the cells showing contrasting Brn3b expression patterns within each melanopsin cell subtype may have been incorrectly identified.

Approximately 5–10% of the melanopsin cells are present in the INL (Hattar et al., 2002; Berson et al., 2010). In our samples of retinal sections, approximately 7% of all melanopsin cells (34 of 480) were present in the INL. Out of these, 85% (29 of 34) were M1 cells because they stratified in the outer IPL and they were labeled brightly (mean intensity, 123; see Fig. 3 for comparison), whereas the remaining 15% (5 of 34) were M2 cells because they stratified in the inner IPL and they were stained faintly (mean intensity, 54; Fig. 6). We did not find any displaced bistratified (M3) melanopsin cell in our samples. Similar to our observations for the GCL, we found in the INL that all M2 cells, but only 9 out of 29 M1 cells expressed Brn3b (Fig. 6).

Because it was possible that some of the melanopsin cells may have been misidentified (Fig. 5D), we investigated further in flattened retinas, in which a cell’s complete dendritic tree is preserved. In the flattened retinas

Figure 5. Expression of Brn3b by melanopsin cells in retinal sections. A: Double immunofluorescence labeling for Brn3b (red) and melanopsin (green) in a 50-μm-thick retinal section shows a brightly labeled M1 cell (arrowhead) that stratified in the outer IPL, and a faintly labeled M2 cell (arrow) that stratified in the inner IPL (blue: DAPI). INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. B: Same frame as in A, with melanopsin signal removed from the image, showing that the M2 cell (arrow), but not the M1 cell (arrowhead) expressed Brn3b. C: An M3 cell (green) that stratified both in outer IPL (arrows) and inner IPL (arrowheads) expressed Brn3b (red). D: The M1, M2, and M3 cells, identified based on their stratification patterns in the IPL, were further segregated on the basis of Brn3b expression. Within each melanopsin cell subtype, the Brn3b-positive and Brn3b-negative cells were compared for their soma diameter and melanopsin expression levels. Left: The Brn3b-positive M1 or M2 cells had significantly larger soma diameters than their Brn3b-negative counterparts. *, $P < 0.05$. Right: The Brn3b-positive M2 and M3 cells had lower melanopsin expression levels than their Brn3b-negative counterparts, although the difference was not statistically significant, perhaps because the number of cells in each of these subgroups was relatively small (see Results). Scale bar = 20 μm in C (applies to A–C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
double-labeled for melanopsin and Brn3b, we analyzed the dendritic stratification of several hundred melanopsin cells (n = 6 retinal pieces). To rule out experimenter’s bias, we removed digitally the Brn3b labeling from the images before analyzing stratification patterns of melanopsin cells. Only the cells in which the dendrites were clearly visible for 100 μm or more were selected. The dendrites were tracked by visually examining them through serial optical sections. The dendrites of a large number of these melanopsin cells were also analyzed by generating 3D reconstructions, by using Neuromantic or ImageJ software (see Materials and Methods). A total of 679 melanopsin cells was selected and analyzed. We found that nearly all non-M1 cells (335 of 336 M2 and 27 of 27 M3), but only approximately 9% of the M1 cells (28 of 316) expressed Brn3b (Fig. 7). These results suggested that even though some cells may have been misidentified in the retinal sections, at least a small proportion of M1 cells expressed Brn3b. Overall, approximately 77% of all melanopsin cells (1,173 of 1,528) expressed Brn3b in these samples. These estimates were slightly higher than in the samples shown in Table 2, likely because the images here were digitally enhanced to reveal even the cells that were labeled very lightly for Brn3b.

**Level of Brn3b expression in M1 cells was consistently and considerably lower than in non-M1 cells**

The expression levels of each Brn3 transcription factor vary among RGCs (Xiang et al., 1995; Fig. 1). However, the functional significance of this variability is not known.
Because a small proportion (9–14%) of the M1 cells expressed Brn3b, we asked whether the level of Brn3b expression in these cells was different from that in the non-M1 cells. By using ImageJ, we measured the intensity of Brn3b staining in 8-bit grayscale images that were digitally enhanced to reveal the very lightly labeled Brn3b cells (see Materials and Methods). Only the frames in which both M1 and non-M1 cells were present were analyzed. We found that the expression levels of Brn3b were consistently and significantly lower in the M1 cells (11.9 ± 7.7; mean ± SD; n = 27) than in the non-M1 cells (68.9 ± 28.6; n = 99; unpaired, two-tailed t-test, P << 0.0001) in the GCL (Fig. 8). Similar patterns were observed for the M1 and M2 cells in the INL (not illustrated).

**DISCUSSION**

By using multiple criteria to identify melanopsin cell subtypes, we asked whether they express Brn3 transcription factors differentially. We found that the melanopsin cells expressed neither Brn3a nor Brn3c, but a subset of the melanopsin cells expressed Brn3b. By using 3D reconstruction and dendritic tracking of melanopsin cells in retinal flat-mounts, we found that Brn3b was expressed overwhelmingly in the cells that stratified in the inner third of the IPL, whereas it was absent in a large majority of cells that stratified in the outer IPL, implying that Brn3b was expressed preferentially by non-M1 type of ipRGCs. This was consistent with our finding that Brn3b was expressed preferentially in the melanopsin cells with larger somas and lower melanopsin levels. That a small proportion (9–14%) of the M1 cells expressed Brn3b could come from our misidentification of some of the M3 cells as M1 cells. The M3 cells comprise a heterogeneous population with considerable variability in their dendritic stratification patterns (Schmidt and Kofuji, 2011). For example, some M3 cells stratify primarily in the outer IPL whereas others stratify primarily in the inner IPL (Schmidt and Kofuji, 2011), which could result in their identification as M1 and M2 cells, respectively. Including some M3 cells as M2 cells would not affect our results because they were both grouped together as non-M1 cells, but including them in the M1 group could be responsible for our finding of a few “M1” cells expressing Brn3b.

However, our finding that the level of Brn3b expression in these few M1 cells was significantly lower than in the non-M1 cells suggests that they were not misidentified, and that the M1 cells may comprise two subgroups: Brn3b-positive and Brn3b-negative, which may regulate different aspects of the non-image-forming vision. It is possible that the Brn3b-expressing M1 cells are the

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**Figure 8.** Brn3b expression levels in M1 cells were significantly lower than in non-M1 cells. **A,B:** Two representative fields from different flat-mount retinas double-labeled for melanopsin (green) and Brn3b (magenta), each showing an M1 cell (arrowhead) and M2 cell (arrow) both positive for Brn3b. The melanopsin cells were identified as M1 or M2 based on their dendritic stratification patterns (not shown). **C,D:** Same fields as in A and B, with melanopsin signal removed to reveal the Brn3b signal in the M1 and M2 cells. The Brn3b signal in the M2 cells (arrows) is considerably higher than in the M1 cells (arrowheads). **E,F:** Images in C and D were converted to 3D surface plots to show the staining intensity of Brn3b cells as grayscale values in the z-axis. Each peak represents a single cell. Note the difference in Brn3b signals between M1 (arrowheads) and M2 (arrows) cells. The Brn3b signal for most of the M1 cells fell in the range shown in these two examples. **G:** Frequency distribution of Brn3b staining intensity in M1 and non-M1 cells. The Brn3b expression levels in the M1 cells (black) were distinctly lower than in the non-M1 (gray) cells. Scale bar = 10 μm in A (applies to A–D). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
atypical variants that have been reported to be physiologically similar to M2 cells (Schmidt and Kofuji, 2010).

After this article was submitted, and while it was in revision, an article appeared showing that Brn3b-positive and Brn3b-negative ipRGCs have distinct projection patterns (Chen et al., 2011). These authors further showed that selective ablation of Brn3b-positive ipRGCs severely impaired pupillary reflex but not circadian photoentrainment, implying that Brn3b-negative M1 cells are responsible for photoentrainment whereas pupillary reflex is predominantly regulated by Brn3b-positive ipRGCs. However, it is not clear whether these Brn3b-positive cells are M1 or non-M1, or both. We report here that all non-M1 cells and some M1 cells express Brn3b, but the level of Brn3b expression in the non-M1 cells is remarkably higher than in the M1 cells (Fig. 8G). It is possible that these two types of Brn3b-positive ipRGCs mediate different functions.

Currently there are no known molecular markers to distinguish between M1 and non-M1 types of ipRGCs. An antibody against the C-terminal of melanopsin has been claimed to label only M1 cells (Baver et al., 2008). However, we found in our samples that this antibody also stained some non-M1 cells (not shown). Similarly, a transgenic mouse (Opn4<sup>tlacZ</sup>) has been claimed to have only M1 cells labeled (Hattar et al., 2002, 2006). However, because the labeled melanopsin cells in this mouse are detected by the C-terminal antibody, it was possible that even this mouse has some non-M1 cells labeled. In fact, a recent report demonstrated the presence of M2 cells in EGFP-Opn4<sup>+/−</sup> mouse (Schmidt and Kofuji, 2010). Even M3 cells may be labeled in the Opn4<sup>tlacZ</sup> mouse (Table 1 in Schmidt et al., 2011). Considering that the β-galactosidase reporter in the Opn4<sup>tlacZ</sup> mouse is linked to an axonal protein, tau, it is possible that the low expression of the reporter in the dendrites of non-M1 cells makes them undetectable in the retina. Replacing β-galactosidase with enhanced green fluorescent protein (EGFP), for example, might have revealed the dendrites of even the M2 cells (Schmidt and Kofuji, 2010). This would also explain why Baver et al. (2008) found 56% of the melanopsin cells labeled in the Opn4<sup>tlacZ</sup> mouse retina, which is inconsistent with our observation and that of Berson et al. (2010) that only 30–35% of the melanopsin cells are of the M1 type. Our finding that Brn3b is expressed preferentially in the non-M1 type of ipRGCs should allow one to distinguish these cells from the M1 cells.

**Estimates of Brn3-expressing and melanopsin-expressing cells**

The cell density in the GCL of C57BL/6J mouse in this study (6,097 cells per mm<sup>2</sup>) was lower than previously reported (Jeon et al., 1998; Pang and Wu, 2011). The sampling area in the previous reports was much smaller than in the present work, in which we sampled nearly the entire retina (mean area covered per retina > 10 mm<sup>2</sup>). Sampling a larger area should minimize the sampling bias, although the differences could also have come from genetic and environmental factors, or tissue shrinkage from fixation (Williams et al., 1996; Jeon et al., 1998). However, this should not affect our proportional estimates for Brn3 cells or melanopsin cells counted in the same samples.

We found that approximately 4% of RGCs in the GCL were immunoreactive for melanopsin, which is similar to previous estimates (Berson et al., 2010; Ecker et al., 2010). We also found that approximately 70% of the RGCs in the GCL expressed Brn3a, 50% expressed Brn3b, and 40% expressed Brn3c (Table 2), implying that a significant number of RGCs express more than one type of Brn3 transcription factors, which is consistent with earlier reports (Xiang et al., 1995; Badea and Nathans, 2011). However, unlike in Xiang et al. (1995), who reported that Brn3a and Brn3b are expressed in similar number of RGCs, we find considerably higher numbers of Brn3a cells.

**Role of Brn3- and melanopsin-expressing RGCs in vision**

Genetic manipulations to delete or label RGCs that express specific Brn3 transcription factors have demonstrated that these cells include a variety of RGC classes (Xiang et al., 1995; Lin et al., 2004; Badea and Nathans, 2011), presumably involved in a variety of visual functions. One possibility is that Brn3 transcription factors are expressed specifically in RGCs that mediate image-forming vision. Quina et al. (2005), using the Brn3a<sup>tlacZ</sup> transgene, showed that Brn3a cells project to thalamic and collicular targets, but not to the accessory visual areas. A later report (Badea et al., 2009) that used the Brn3a<sup>AP</sup> transgene, confirmed that Brn3a fibers do not project to the SCN or IGL, but suggested that they may have minor projections to the olivary pretectal nucleus and accessory optic nuclei, which are involved in accessory visual functions (Trejo and Cicerone 1984; Simpson, 1984; Clarke and Ikeda, 1985). Here, we show that not only the Brn3a cells but also Brn3c cells did not express melanopsin, supporting the view that these cells are involved exclusively in the image-forming vision.

Approximately 95% of the Brn3b-expressing cells also did not express melanopsin (Table 2). The remaining 5% that did express melanopsin were predominantly the non-M1 type of ipRGCs. Interestingly, both Brn3b cells and non-M1 cells have been separately shown to project to both image-forming and non-image-forming areas,
and that they are involved in both types of visual functions (Badea et al., 2009; Ecker et al., 2010). Together, these findings provide evidence that a small proportion of RGCs that express both Brn3b and melanopsin are involved in both image- and non-image-forming visual functions. We propose that image-forming and non-image-forming channels are characterized respectively by their expression of Brn3 transcription factors or melanopsin, and that the cells that express both Brn3b and melanopsin provide a platform for cross-talk between the two visual channels.

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