Supporting Information

Walker *et al.* 10.1073/pnas.0711397105

SI Text

Quantification of Melanopsin in a Single Mouse Retina. Protein standards were made from urea-stripped bovine rod outer segments (ROS) (1). Preparation of ROS protein standard was done under dim red light. ROS was solubilized in 1% dodecyl maltoside (DM) by rotating at 4°C for 1 h. The ROS sample was centrifuged at 12,000 × *g* at 4°C for 10 min. The absorbance of rhodopsin was measured by using a Hitachi model U-3300 dual path spectrophotometer. The absorbance at 498 nm ($\varepsilon = 40{,}600$) was used to calculate the concentration of rhodopsin. A serial dilution of ROS was used for immunoblot quantification of expressed melanopsin protein standard (0.8, 0.4, 0.2, 0.1, and 0.05 μM).

The expressed melanopsin protein standard was made from HEK293 cells expressing the mouse melanopsin protein appended with the 1D4 epitope tag (last 15 aa of bovine rhodopsin) (2). Melanopsin expressing cells were solubilized in 1% DM by vortexing at 4°C for 1 h. After solubilization, the suspension was centrifuged at 12,000 × *g* at 4°C for 10 min. The supernatant was removed and centrifuged at 30,000 × *g* at 4°C for 30 min. The supernatant was removed and a serial dilution of the expressed melanopsin supernatant was used for immunoblot analysis.

All retinas were taken from WT C57/B6 mice. Mice were enucleated and the retinas were dissected from the eyecup. Each retina was homogenized in 100 μl of 1% DM and vortexed at 4°C for 1 h. After vortexing, the volume was brought up to 1 ml by adding 900 μl of PBS and centrifuged at 12,000 × *g* at 4°C for 10 min. The supernatant was removed and centrifuged at 30,000 × *g* at 4°C for 30 min. The supernatant was removed and used for immunoblotting.

For each immunoblot, nitrocellulose filter membrane was fixed in a dot blot apparatus. Each well was washed with 300 μl of PBS. After washing the filter membrane, 300 μl of PBS was added to each well followed by 50 μl of sample supernatant. The filter membrane was then washed two times with 300 μl of PBS.

Immunoblots were analyzed by using a protocol adapted from the Western blotting protocol of Burnette (3). Rhodopsin and expressed melanopsin were detected by using an anti-1D4 antibody (1:10,000 dilution) and an anti-mouse IgG secondary antibody conjugated to alkaline phosphatase (Promega). Expressed melanopsin and mouse retinal melanopsin were detected by using an anti-melanopsin antibody (raised against the first 15 aa of mouse melanopsin, 1:10,000 dilution) and an anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase (Promega). Fluorescent alkaline phosphatase substrate (Attophos; Promega) was used to detect antibody binding, and blots were visualized on the Storm 860 phosphorimaging system (Molecular Dynamics).

Affinity-Column Purification of Endogenous Mouse Melanopsin. Mouse retinas (10 retinas) were dissected from the eyecup under room light. The retinas were then homogenized and solubilized in 1% DM in PBS for 1 h. The solubilized retinas were centrifuged for 20 min (14,000 × *g*) at 4°C. Melanopsin was purified from the supernatant by immunoaffinity column chromatography. Anti-melanopsin antibody raised in rabbit against a peptide derived from the first 15 amino acids of mouse melanopsin (N-Term Mel) was coupled to cyanogen bromide activated Sepharose 4B column (GE Healthcare). The solubilized retina supernatant was incubated with the affinity column for 1 h. The melanopsin column was washed with 8 volumes of 0.1% DM in PBS. Finally, melanopsin was eluted from the affinity column using 400 μl of 50 μM N-Term Mel peptide in column wash buffer (0.1% DM in PBS).

G Protein Activation Assay. To determine the ability of melanopsin to activate heterotrimeric G proteins, we used an *in vitro* assay with transducin (G_t) isolated from bovine rod outer segments (4). To determine melanopsin's light-dependent activity, G_t and [^{35}S] GTPγS were added to dark-adapted mouse melanopsin in PBS with 1% digitonin. At different time intervals, 10-μl aliquots were removed from the reaction mixture and filtered through nitrocellulose, which retains transducin-bound [^{35}S]GTPγS, and washed with PBS using a vacuum manifold system. Samples were irradiated with an Ealing 250 W xenon arc lamp. Neutral density filters were used to control light intensity, and interference filters (10-nm bandwidth) were used to select the wavelength of excitation light.

1. Rim J, Oprian DD (1995) Constitutive activation of opsin: Interaction of mutants with rhodopsin kinase and arrestin. *Biochemistry* 34:11938–11945.
2. Newman LA, *et al.* (2003) Melanopsin forms a functional short-wavelength photopigment. *Biochemistry* 42:12734–12738.
3. Burnette WN (1981) Western blotting: Electrophoretic transfer of proteins from sodium dodecyl sulfate–polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* 112:195–203.
4. Zhukovsky EA, Robinson PR, Oprian DD (1991) Transducin activation by rhodopsin without a covalent bond to the 11-*cis*-retinal chromophore. *Science* 251:558–560.

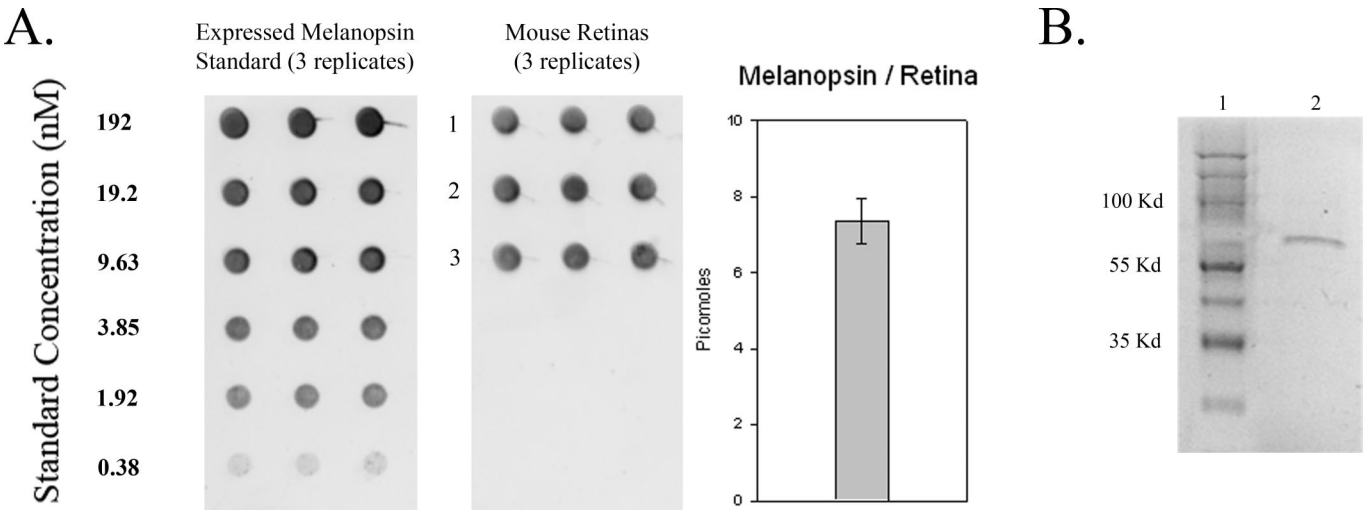

Fig. S1. Quantification of melanopsin protein in a single mouse retina and purification of endogenous mouse melanopsin from retinas. (*A*) Quantification of melanopsin protein in a single mouse retina. The melanopsin protein standards are mouse melanopsin appended with a 1D4 epitope tag solubilized in 1% dodecyl-maltoside (DM). Each tested retina was taken from an individual mouse, and all of the mice came from the same genetic background (C57/B6) ($n = 3$). Each retina was solubilized 1% DM and diluted into a 1 ml solution of 0.1% DM, and three replicates of each retina were spotted onto nitrocellulose. After immuno-staining, fluorescence was detected by using the Storm 860 phosphorimaging system (Molecular Dynamics). Immuno-detection of melanopsin indicates that there are 7.3 $\pm$ 0.5 (SEM) pmoles of melanopsin in a single retina. (*B*) Purification of endogenous mouse melanopsin from retinas. Enodogenous mouse melanopsin was affinity column-purified from 10 mouse retinas. The mouse retinas were homogenized and then solubilized in 1% DM for 1 h. The sample was separated on a SDS/PAGE gel and silver staining was used to visualize protein. Lane 1, protein molecular weight ladder. Lane 2, affinity column-purified mouse melanopsin.

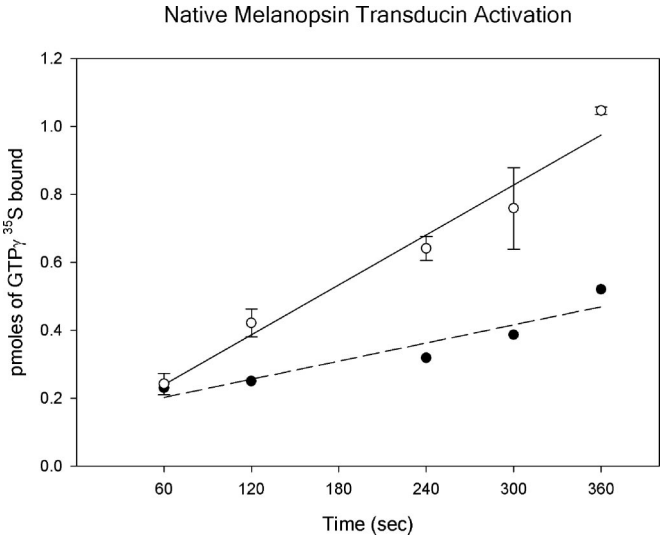

Fig. S2. Light-dependent activation of bovine rod transducin by melanopsin. In each reaction we added melanopsin solubilized in 1% digitonin and 9.4 μM bovine rod transducin. Bovine rod transducin was purified from rod outer segments (described in *Experimental Procedures*). ●, Activation of rod transducin by melanopsin in the dark. ○, Melanopsin light-dependent activation of transducin. Samples were irradiated with 1 min of 480-nm monochromatic light.