Supporting Information

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SI Methods

Animals. C3H MT1$^{-/-}$ knock-out mice homozygous for the *rd1* mutation, generously donated by Drs. Reppert and Weaver (University of Massachusetts Medical School), were backcrossed with C3H/f$^{+/+}$ mice in which the *rd1* mutation has been removed to produce C3H/f$^{+/+}$MT1$^{-/-}$. The genotypes were determined according to the protocols described (1, 2).

All of the experimental procedures were carried out in accordance with Association for Assessment of Laboratory Animal Care policies and approved by the Morehouse School of Medicine Animal Care and Use Committee.

In Situ Hybridization and Immunocytochemistry (ICCH). Details about the procedures used for in situ hybridization and ICCH are described in detail in published work (3–5). For additional details, see Table S1.

Laser Capture Microdissection (LCM) of Retinal Layers and RT-PCR Analysis. Tissue preparation and RT-PCR were performed as described (6, 7).

Dark-Adapted Electroretinogram (ERG). Mice were dark adapted for at least 30 min, and anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg). The pupils were dilated with 1% tropicamide and 1% cyclopentolate HCl (Bausch and Lomb) and mice were placed on a regulated heating pad set at 37°C with feedback from the rectal temperature probe. ERGs were recorded using a silver/nylon fiber electrode placed on the cornea moisturized with 1% methylcellulose. Platinum needle electrodes placed in the cheek and tail served as the reference and ground, respectively.

A 6-step intensity series from 0.11 to 5.85 cd*s/m^2 was presented to the right eye. Flashes (0.01–1 ms) were generated using 530-nm green LEDs from Agilent Technologies. With increasing intensity, the interstimulus interval increased from 15 to 60 s. Signals were amplified (amplification gain 1,000) using a model 1700 differential amplifier from A-M Systems, filtered from 0.1 to 20,000 Hz, and recorded using a custom built digital acquisition system based on the NI-6250 M-series board from National Instruments. Responses of 3–10 flashes were averaged to generate a waveform for each flash intensity. The number of waveforms averaged varied depending on the flash intensity to get more reliable quantification (by averaging multiple responses to dim flashes) and to avoid light adaptation of the rods (by reducing the number of bright flashes). The bright flashes produce large a- and b-waves and therefore averaging of multiple responses is not necessary for reliable quantification. While developing the ERG protocol we examined the individual responses to bright flashes to ensure that no bleaching (manifested by gradual decrease in amplitudes) occurs.

Scotopic Threshold Response. Mice were injected (i.p.) with melatonin (1 mg/kg, Sigma) or sterilized PBS (10 mL/kg), and dark adapted for 1 h before recording ERGs. The pupil of the eye recorded was dilated with 1% atropine (Sigma) and 2.5% phenylephrine (Sigma) 30 min before ERG recordings. Mice were anesthetized as described above. The eye was lubricated with 0.5% carboxy methylcellulose sodium (CVS) and a contact lens type electrode (LKC Technologies model: N1530NNC) was inserted to the eye. A needle reference was inserted in other side of cheek, and the ground needle was inserted in the tail. The electrode placement and the injection of anesthetic were conducted under dim red light (<3.5 lux). Electrodes were connected to a Universal DC Amplifier (LKC Technologies model: UBA-4200) and bands were filtered from 0.3 to 500 Hz. Data were recorded and analyzed by EM for Windows (ver. 8.2.1, LKC Technologies). Core body temperature was maintained in 37 °C by a feedback temperature control system (FHC). Six series of 10 consecutive flashes delivered at 0.612-s intervals were introduced to mice in a Ganzfeld illuminator. The light intensity of flashes started at 6.60 log cd*s/m^2 and increased 10 dB between each series up to −1.60 log cd*s/m^2 (0.11 cd*s/m^2). The trace of ERG was averaged and stored to a computer for later analysis. The scotopic response threshold was calculated with a similar method to what described in Cameron et al. (8, 9).

Photopic ERG. For photopic ERG, cone associated activity was isolated by saturating rods with 63 cd*s/m^2 white LED background light. The four series of consecutive 10 white flashes (79.06 cd*s/m^2) were introduced at 2.5 min, 5 min, 10 min, and 15 min during the background light exposure. Background light was left on for 15 min while photopic ERGs records were measured (8). The traces of the ERG were averaged and stored to computer for later analysis.

The amplitude of the a-wave was measured from the prestimulus baseline to the a-wave trough. The amplitude of the b-wave was measured from the trough of the a-wave to the peak of the b-wave or, if no a-wave was present, from the baseline to the b-wave peak. Implicit times were measured from the time of stimulus presentation to the a-wave trough or the b-wave peak.

Retinal Morphology. Mice were euthanized and before eye nucleation the superior cornea was marked with a hot needle. After a 1-h fixation in 4% paraformaldehyde, the anterior segment was removed, except for the superior cornea, and the eyes were fixed overnight at 4°C. After dehydration through a graded ethanol series, eyecups were embedded in Durcopan, sectioned at 1.5 μM thickness, and stained with Toluidine blue. To assess the structure of the retina in the two genotypes we used the following morphometric measures: (*i*) Photoreceptor nuclei were counted in a 10-μm microscopic field that was centered at 300 μm above the edge of the optic nerve head. For each sample, we counted the number of photoreceptor cells in 10 different locations within each of three adjacent sections. The number of photoreceptors, INL and GCL cells were counted using the Image-Pro Plus 3.0 software. The data obtained from the different adjacent sections were combined and the mean ± SEM was calculated. Comparisons among the different genotypes were carried out using parametric ANOVA followed by a multiple comparison test, where appropriate (Sigma Stat version 3.5). (*ii*) The length of the rod outer segment (OS) was assessed by measuring the distance from the OS base to the tips of the OS layer adjacent to the RPE, using the Image-Pro Plus 3.0 software. Similarly, the length of the rod inner segment (IS) was assessed by measuring the distance from the OS base to the ONL. Morphometric measurements were made by observers who were blinded to the genotype and age of the samples. This methodology has been successfully used in previous studies that investigated age-related morphometric changes in the mouse retina (10).

1. Liu C, et al. (1997) Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock. *Neuron* 19:91–102.
2. Lem J, et al. (1992) Retinal degeneration is rescued in transgenic *rd* mice by expression of the cGMP phosphodiesterase beta subunit. *Proc Natl Acad Ssi USA* 89:4422–4426.
3. Jeon CJ, Strettoi E, Masland RH (1998) The major cell populations of the mouse retina. *J Neurosci* 18:8936–8946.
4. Damiani D, et al. (2008) Dicer inactivation leads to progressive functional and structural degeneration of the mouse retina. *J Neurosci* 28:4878–4887.
5. Gargini C, Terzibasi E, Mazzoni F, Strettoi E (2007) Retinal organization in the retinal degeneration 10 (rd10) mutant mouse: A morphological and ERG study. *J Comp Neurol* 500:222–238.
6. Liu C, Fukuhara C, Wessel JH, Iuvone PM, Tosini G (2004) Localization of *Aa-nat* mRNA in the rat retina by fluorescence in situ hybridization and laser capture microdissection. *Cell Tissue Res* 315:197–201.
7. Fukuhara C, et al. (2004) Gating of the cAMP signaling cascade and melatonin synthesis by the circadian clock in mammalian retina. *J Neurosci* 24:1803–1811.
8. Cameron MA, et al. (2008) Electroretinography of wild type and cry mutant mice reveals circadian tuning of photopic and mesopic retinal responses. *J Biol Rhythms* 23:489–501.
9. Sugawara T, Sieving PA, Iuvone PM, Bush RA (1998) The melatonin antagonist luzindole protects retinal photoreceptors from light damage in the rat. *Invest Ophthalmol Vis Sci* 39:2458–2465.
10. Li C, et al. (2001) Age-related changes in the mouse outer retina. *Optom Vis Sci* 78:425–430.

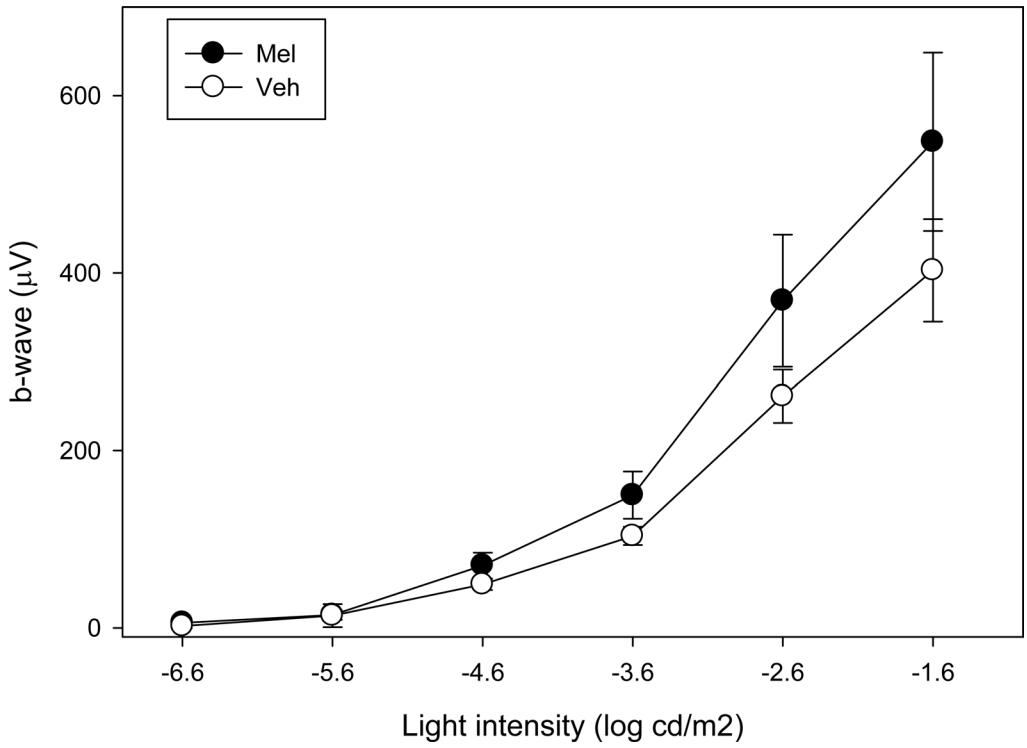

Fig. S1. Quantification of the dark-adapted response of the b-wave to flashes of light (−6.6 to −1.6 log cd*s/m^2 [0.11 5 cd*s/m^2]) recorded after 1 h of dark adaptation and intraperitoneal injection of melatonin (1 mg/kg) or vehicle in the middle of the night. Data are presented as mean ± SEM; $n = 6–7$ for each time point and genotype. In C3H/f$^{+/+}$ mice melatonin injection induced a significant reduction in the scotopic threshold response and increased the amplitude of the b-wave (two-way ANOVA, $P < 0.05$).

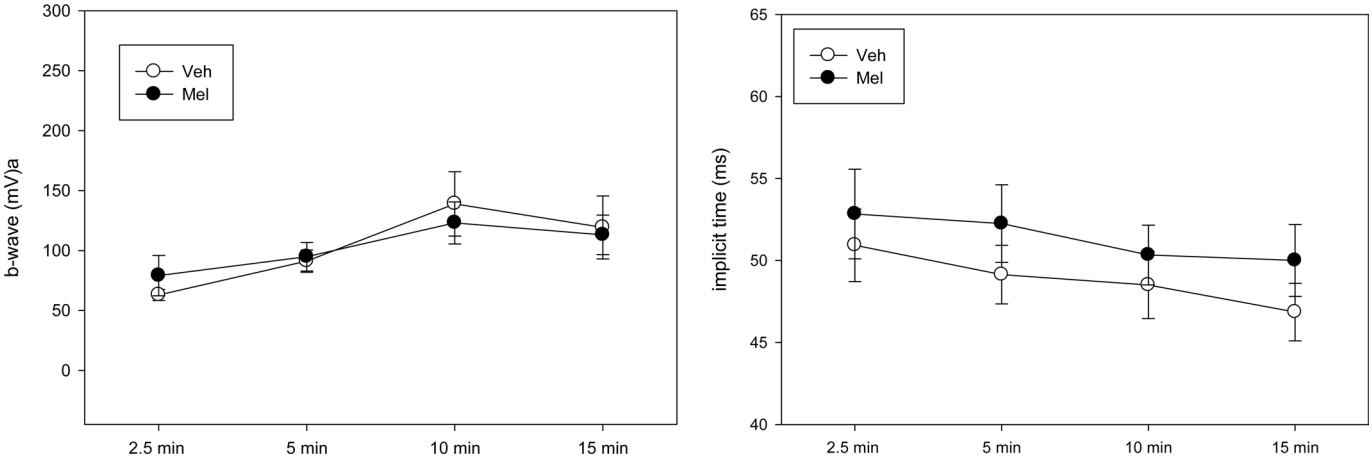

Fig. S2. Quantification of light-adapted ERG responses to flashes of light recorded after 1 h of dark adaptation and intraperitoneal injection of melatonin (1 mg/kg) in the middle of the night (ZT18). Data are presented as mean ± SEM; $n = 6–7$ for each time point and genotype. In C3H/f$^{+/+}$ mice melatonin injection did not affect the amplitude of the b-wave (two-way ANOVA, $P > 0.1$) and the implicit time (two-way ANOVA, $P > 0.1$). Mice were 3–4 months old at the time of the experiment.

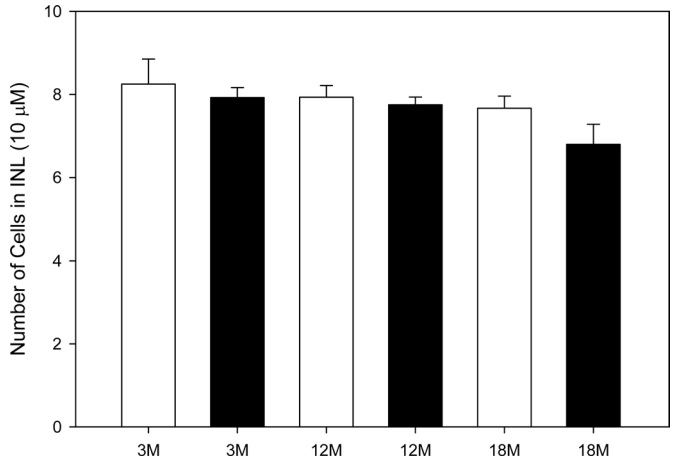

Fig. S3. Number of inner retinal neurons in C3H/f$^{+/+}$ (white bars) and C3H/f$^{+/+}$MT1$^{-/-}$ (black bars) at the three different ages. No significant differences in the number of inner retinal neurons were detected between the same genotype at three different ages (two-way ANOVA, $P > 0.14$) or between genotypes (two-way ANOVA, $P < 0.07$). Each bar, the mean ± SEM; $n = 4–6$. *, $P < 0.05$.

Table S1. Antibodies

Primary antibody	Source	Host	code	Immunogen	Recognized bands
Bassoon	Stressgen, BC, Canada	Mouse	VAM-PS003	Recombinant rat Bassoon fragment (residues 738-1035) expressed as GST fusion protein in *Escherichia coli*	A 400-kDa protein. Additional bands between 97 and 400 kDa are also identified as proteolytic degradation products of Bassoon
Calbindin D, clone CB-955	Sigma, St. Louis, MO	Mouse	C 9848	Derived from the CB-955 hybridoma produced by the fusion of mouse myeloma cells and splenocytes from BALB/c mice immunized with purified bovine kidneycalbindin-D-28K	A single 28-kDa band
Goα	Chemicon, Temecula, CA	Mouse	MAB 3073	Purified bovine brain Goα	A single protein band of 39–42 kDa
Glutamine synthetase (GS)	Chemicon, Temecula, CA	Mouse	MAB302	Gs purified from sheep brain	Single band
Kinesin II	Covance, Babco, CA	Mouse	MMS-198P	Purified via protein-A chromatography from unfertilized sea urchin egg cytosol	Epitopes on the 85- and 95- kDa subunits of Kinesin II lying outside the conserved motor domain
Neurofilament 200-kDa, cloneN52	Sigma, St. Louis, MO	Mouse	N 0142	C-terminal tail segment of enzymatically dephosphorylated pig neurofilament H-subunit	200-kDa band
Protein kinase C, clone MC5	Sigma, St. Louis, MO	Mouse	P 5704	Purified bovine brain PKC	80-kDa band
Synaptophysin	Boehringer Mannheim (Germany)	Mouse	902314	BALB/c mice were immunized with a vesicular fraction of bovine brain	
Calbindin D-28K CB38	Swant, Switzerland	Rabbit	CB38a	Recombinant rat calbindin D-28k	A single 28-KDa band
Glial fibrillary acidic protein (GFAP)	Sigma, St. Louis, MO	Rabbit	G 9269	GFAP purified from human brain	
mGlur6	Chemicon, Temecula, CA	Rabbit	RA13105	Synthetic C-terminal peptide (KKTSTMAAPPQNENAEDAK) of the rat sequence	Two bands of 96 and 200 kDa (possible dimer)
Protein kinase C (PKCα)	Sigma, St. Louis, MO	Rabbit	P 4334	A synthetic peptide (Lys-Val-Asn-Pro-Gin-Phe-Val-His-Pro-Ile-Leu-Gin-Ser-Ala-Val) conjugated to KLH	80-kDa polypeptide and the minor band at 45 kDa
Recoverin	Chemicon, Temecula, CA	Rabbit	AB5585	Recombinant human recoverin	One band of 26 kDa (manufacturer's technical information)
S-cone opsin	Chemicon, Temecula, CA	Rabbit	AB5407	Recombinant human blue opsin	
Tyrosine hydroxylase (TH)	Chemicon, Temecula, CA	Rabbit		Denatured tyrosine hydroxylase from rat pheochromocytoma	
ZO-1 (N-term)	Zymed Laboratories, Invitrogen, Canada	Rabbit	40–2300	Synthetic peptide derived from the N-terminal region of human, dog, mouse, and rat ZO-1	225-kDa ZO-1 protein