Protein Phosphatase 2A and Clathrin-Mediated Endocytosis Facilitate Robust Melanopsin Light Responses and Resensitization

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Purpose. Intrinsically photosensitive retinal ganglion cells (ipRGCs) that express the visual pigment melanopsin regulate non-image-forming visual tasks, such as circadian photoentrainment and pupil constriction, as well as contrast detection for image formation. Sustained ipRGC function throughout the day is, therefore, of great importance. Melanopsin is a bistable rhabdomeric-type (R-type) visual pigment, which is thought to use light to regenerate its chromophore from all-*trans*-retinal back to 11-*cis*-retinal and does not depend on constant chromophore supply to the extent required by visual pigment in rod and cone photoreceptors. Like the majority of photopigments and G-protein-coupled receptors (GPCRs), melanopsin deactivation requires C-terminal phosphorylation and subsequent β-arrestin binding. We hypothesize that melanopsin utilizes canonical GPCR resensitization mechanisms, including dephosphorylation and endocytosis, during the light, and together, they provide a mechanism for prolonged light responses.

Methods. Here, we examined expression of protein phosphatases from a variety of subfamilies by RT-PCR and immunohistochemical analyses of the mouse retina. The expression of protein phosphatase 2A (PP2A) in ipRGCs was assessed. We also examine the role of phosphatase and endocytic activity in sustaining melanopsin signaling using transiently-transfected HEK293 cells.

Results. Our analyses suggest that melanopsin-mediated light responses can be rapidly and extensively enhanced by PP2A activity. Light-activated melanopsin undergoes endocytosis in a clathrin-dependent manner. This endocytic activity enhances light responses upon repeated stimulation, implicating a role for endocytic activity in resensitization.

Conclusions. Thus, we propose that melanopsin phototransduction is maintained by utilizing canonical GPCR resensitization mechanisms rather than reliance on chromophore replenishment from supporting cells.

Keywords: melanopsin, opsin, phototransduction, phosphatase, endocytosis, dephosphorylation, clathrin

Visual pigments are specialized G-protein-coupled receptors (GPCRs) with a covalently attached chromophore ligand (typically 11-*cis*-retinal). In classical photoreceptors, such as rods and cones, the activation of a visual pigment by light initiates a phototransduction cascade, which ultimately results in image-forming vision. Non-image-forming photoreception in mammals plays several important roles. These include the regulation of circadian rhythms, sleep, and pupil size via intrinsically photosensitive retinal ganglion cells (ipRGCs) via projections to brain centers such as the suprachiasmatic nucleus and olivary pretectal nucleus.[1,2] In addition to non-image-forming tasks, a subset of ipRGCs also contributes to image formation.[3] Intrinsic light sensitivity in ipRGCs arises from the expression of melanopsin, an R-type visual pigment that exhibits greater sequence homology to *Drosophila* rhodopsin rather than rhodopsin expressed in rod photoreceptor cells.[4,5] Sequence homology and experimental evidence suggest that melanopsin signals via Gαq-mediated activation of phospholipase C.[6,7] This phototransduction mechanism is more similar to those typically found in rhabdomeric photoreceptors such as those in *Drosophila* eyes,[8,9] rather than the cyclic GMP-based phototransduction cascade utilized by visual pigments in vertebrate rods and cones. Melanopsin's deactivation utilizes a combination of deactivation mechanisms common to both visual pigments and GPCRs.[10–14] Namely, light-dependent C-terminal phosphorylation of melanopsin activates β-arrestin 1 and 2, which then bind to melanopsin and quench G-protein signaling. Unlike visual arrestins found in rod and cone photoreceptors, β-arrestin 1 and 2 contain

clathrin-binding domains[15] that can facilitate the internalization of the receptor-arrestin complex through endocytosis of clathrin-coated pits in the membrane.[16,17]

The kinetics of ipRGC light responses are sluggish, and activity can be sustained over many hours, encoding luminance throughout the day. This is illustrated by electrophysiologic recordings that have revealed sustained light responses for up to 10 hours under constant illumination.[18–20] Consequently, we hypothesize that sustained melanopsin phototransduction in ipRGCs is sustained by adaptation and resensitization mechanisms typically observed in canonical GPCR signal transduction. Specifically, we hypothesize that phosphatase activity is involved in both immediate and long-term resensitization via dephosphorylation of melanopsin's C-terminus. Since melanopsin binds β-arrestin 1 and 2, which contain clathrin-binding domains, we also hypothesize that melanopsin can undergo clathrin-mediated endocytosis and is preferentially targeted for recycling back to the plasma membrane, rather than proteolysis, to support sustained function. Previous work shows that other endocytosed GPCRs can sustain G-protein signaling while in endocytic vesicles, by existing in GPCR–G-protein–arrestin complexes.[21] Additionally, PP2A can localize to endosomes and can dephosphorylate endocytosed receptors.[22] Thus, endocytosis presents a potential mechanism for sustained melanopsin activity and receptor resensitization. Our predictions are based on the observation that melanopsin displays bi- and possibly tristable photochemistry,[19] similar to R-type opsins, and is capable of reisomerizing bleached all-*trans*-retinal back to 11-*cis*-retinal upon the absorption of a longer wavelength photon. While melanopsin's Schiff base linkage to its chromophore has been shown to be unstable in comparison to other R-type opsins,[23] the degree to which continued melanopsin signaling relies on a continuous supply of 11-*cis*-retinal from the visual cycle remains unclear. Experiments in *Rpe65* knockout mice, whose retinal pigment epithelium (RPE) does not produce 11-*cis*-retinal, exhibit a small reduction in their melanopsin-based ipRGC light response, in comparison to a near-complete ablation of rod and cone responses.[24–26] Therefore, while melanopsin retinal bistability and/or a fresh retinal supply from the RPE (or Müller glia cells) may contribute to melanopsin resensitization, additional mechanisms are likely necessary, and these alternative mechanisms remain to be elucidated.

Drosophila rhodopsin-1 has been shown to internalize following phosphorylation and binding to arrestin,[27] and abnormalities in its endocytosis have profound effects on photoreceptor health, leading ultimately to retinal degeneration.[27,28] Furthermore, normal rhodopsin internalization in *Drosophila* is dependent upon the rhodopsin-arrestin complex's interaction with the adaptor protein AP-2,[29] suggesting that clathrin-mediated endocytosis pathways are important, similar to those required for classical vertebrate GPCR signaling.[17] In contrast, mammalian rhodopsin, a ciliary-type (C-type) opsin, does not undergo endocytosis and relies heavily on retinal supplied by the visual cycle for visual pigment regeneration[30] and can only be dephosphorylated by protein phosphatase 2A (PP2A) after regeneration with fresh 11-*cis*-retinal.[31–33] Also, dephosphorylation of bovine rhodopsin after illumination with dim light is a slow process, where two phosphorylation sites, S338 and S334, are dephosphorylated after 30 and 60 minutes, respectively.[33,34] Mice expressing melanopsin with C-terminal phosphorylation sites mutated to alanine display greatly prolonged pupil constriction in response to light and faster circadian photoentrainment in response to jet lag,[14] suggesting a profound impact of melanopsin's phosphorylation state in both short-term and prolonged behavioral responses. We propose that melanopsin activity is modulated by resensitization mechanisms, specifically by PP2A activity and clathrin-mediated endocytosis. Functional analyses of the melanopsin-based light responses in transfected HEK293 cells indicate rapid modulation of light responses by PP2A and light-dependent endocytosis of melanopsin. By describing these previously unexplored facets of melanopsin signaling, we provide insight into ipRGC sustained function and open new targets for further analysis or modulation of melanopsin phototransduction.

Experimental Procedures

Plasmids for Expression of Melanopsin and PP2A

Mouse melanopsin, long isoform (NCBI accession: NM_013887.2) and human protein phosphatase 2A catalytic subunit, α isoform (NCBI accession: NM_002715.2) were inserted into the mammalian expression plasmid, PMT3,[35] through cassette synthesis.

Cell Culture and Heterologous Expression of Melanopsin and Protein Phosphatase 2A

HEK293 cells (ATCC, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) high glucose, pyruvate (ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum, and 1% (v/v) antibiotic-antimycotic (ThermoFisher Scientific) in a humidified 5% CO_2 incubator set at 37°C. Cells were passaged using trypsin-EDTA (0.25%; ThermoFisher Scientific) and seeded onto new plates or onto lysine D–covered coverslips (Corning, Corning, NY, USA) for preparation for fixation and fluorescence microscopy.

HEK293 cells were transfected with Turbofect Transfection Reagent (ThermoFisher Scientific) or polyethylenimine (PEI) (Polysciences Inc, Warrington, PA, USA) as per the manufacturer's protocol. Briefly, 3 µg DNA was diluted in 400 µL nonsupplemented DMEM, and then 9 µg Turbofect or PEI was added to the mixture. After a 20- or 15-minute incubation (for Turbofect and PEI, respectively), the transfection mixture was gently pipetted into a well of a 6-well culture dish. Transfected cells were used for experiments 24 hours after transfection.

RT-PCR of the Mouse Retina to Analyze Protein Phosphatase Expression

Fresh eyes from TRPM5-GFP mice (generously provided by Kayla Lemons and Dr. Weihong Lin) were placed in PBS, and their corneas and lens were removed to facilitate the removal of the retinas. Isolated retinas placed in microcentrifuge tubes were homogenized using a pestle in the presence of a 1 mL TRIzol Reagent (ThermoFisher Scientific). After centrifugation for 10 minutes at 13,000 × g at 4°C, the supernatant was transferred to a fresh tube, incubated at room temperature for 5 minutes, and then incubated with 250 µL

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Table. Forward and Reverse Primer Used for Phosphatase Amplification in RT-PCR of Retinal mRNA Samples

Characteristic	Primer	Expected Amplicon Size, bp
PP1α (Ppp1ca)	Forward: CTTCTGCCCCAGATCGTTTGReverse: GATGGGTTGCCCCAGGATCT	278
PP1β (Ppp1cb)	Forward: AGTGAAAATGGGAAGAGCTTReverse: CCAAATCAGTGGCAATTTTG	249
PP1γ (Ppp1cc)	Forward: GAGCATTTTGTTTTGAACCTReverse: GTGCACGCAGCCTCGATGCC	514
PP1A (Ppm1a)	Forward: GACACTGATTCTGCGTCAACReverse: GACAGTTTCCATGAGCCTGG	537
PP1G (Ppm1g)	Forward: GAAGGCCAAGAGGGACTAGTReverse: AGTGTTGCTTGAAAGTGTAC	402
PP1H (Ppm1h)	Forward: CCTCAGAGGAAGCCTTGAGTReverse: CTAAGTATTCTTGATCATCA	558
PP2Aα (Ppp2ca)	Forward: CACACGGACCAAAAGATGTGCCReverse: CAGCACCAGTCGTGCCCACTG	563
PP2Aβ (Ppp2cb)	Forward: TCATGTGACCCGGCGCACCCCAReverse: CCAGCAGGCAGGCTGTGGGCTGAC	272
PP3α (Ppp3ca)	Forward: CTGGGGGCCAGGAATTGGATTCReverse: GGTGTTTAATCACCACCCTCAC	108
PP3β (Ppp3cb)	Forward: CCCAGGGACTCTCACATCTTGGGReverse: GATGGGGGGAGTTCCACGTTAT	389
PP3γ (Ppp3cc)	Forward: TGGGTCCCAACCCTATGAGCReverse: GCGACTGAATTACAAATGCACCTC	102
PP4 (Ppp4c)	Forward: CCTCCAAAAAGCCAGTGGCCGACTReverse: CTCCACCTCTCCAGAGCAAGTCCG	162
PP5 (Ppp5c)	Forward: GGAATGATGTGAGGCTGTCAGCAGReverse: TCTTCCTATGGCAGGGTCCT	333
PP6 (Ppp6c)	Forward: TCCTCCTACCCCACCTTGGACTCGReverse: GGTTTACAGCCGGGTTGACA	284

chloroform for 3 minutes. After centrifugation for 15 minutes at 13,000 × *g* at 4°C, the top layer was transferred to a fresh tube. RNA was precipitated using 500 µL isopropanol and incubating for 10 minutes. After centrifugation (10 minutes at 11,000 × *g* at 4°C), the supernatant was removed, and the RNA pellet was washed with 1 mL 70% (v/v) ethanol diluted in 1% (w/v) Diethyl Pyrocarbonate (DEPC)-treated H_2O. After centrifugation for 5 minutes at 9000 × *g*, the supernatant was air dried and then resuspended in 1% (w/v) DEPC H_2O. RNA was stored at −80°C until use.

Isolated RNA was used to synthesize cDNA using oligo$(dT)_{17}$ primers and Superscript III Reverse Transcriptase (ThermoFisher Scientific) as per the manufacturer's protocol. The mixture was incubated at 50°C for 60 minutes. Synthesized cDNA was used to set up PCR reactions using the following gene specific primers (Table; catalytic subunits of each phosphatase were targeted, all sequences listed in 5′-3′ direction).

Immunohistochemistry of Mouse Retinas

Fresh eyes from C57BL/6J mice (eye tissue generously provided by Dr. Samer Hattar, National Institutes of Health [NIH]) were fixed in 4% Paraformaldehyde (PFA) at 4°C overnight, and then the lens and cornea were removed. The eyecups were then cryoprotected by incubating in 30% glucose at 4°C for 2 hours. The eyes were then immersed in O.C.T. Compound (VWR, Radnor, PA, USA) and frozen at –80°C prior to sectioning. The eyes were then cut into 16-µm sections on a cryostat and thaw-mounted onto glass slides. Sections were blocked with PBS containing 0.3% (v/v) Triton X-100 and 10% (v/v) normal goat serum at 4°C. The sections were then incubated with 1:200 dilutions of *N*-terminus melanopsin antibody (courtesy of Prof. Michael T. H. Do[36]) and PP2A $\alpha + \beta$ antibody (ab27267) (Abcam, Cambridge, UK). Specificity of PP2A antibody was verified through Western blot of retinal homogenate, which yielded a band at the expected molecular weight (Supplementary Fig. S4). Following primary antibody incubation, three washes were done using wash buffer containing 0.01 M Tris, 0.15 NaCl, and 0.05% (v/v) Tween-20. The sections were then incubated with Alexa Fluor–conjugated anti-mouse (Alexa 594) and anti-rabbit (Alexa 488) secondary antibodies (ThermoFisher Scientific) at 1:200 dilutions. Following the incubation, the sections were washed three times and coversliped using Fluoromount-G with DAPI (Southern Biotech, Birmingham, AL, USA).

Immunocytochemistry of Transfected HEK293 Cells

HEK293 cells were transfected with melanopsin or phosphonull melanopsin, each C-terminally appended to a 1D4 tag to facilitate immunodetection. Transfected cells were treated with trypsin-EDTA and seeded onto 12-mm BioCoat poly-D-lysine–coated coverslips (Corning). The cells were then dark-adapted overnight in a humidified CO_2 incubator. Coverslips were kept in the dark or exposed to 10, 20, or 30 minutes of white light; rinsed three times with PBS; and then immediately fixed with 4% PFA for 20 minutes at 4°C. After three rinses with PBS, coverslips were transferred to microscope slides cell-side up, and then a hydrophobic barrier was created around all coverslips using a peroxidase-antiperoxidase (PAP) pen. A droplet of Duolink Blocking Solution (Sigma-Aldrich, St. Louis, MO, USA) was placed on each coverslip and incubated 30 minutes at 37°C in a humidified microscope slide box. Then, 1D4 and clathrin (anti-clathrin heavy chain antibody, ab21679; Abcam) were diluted 1:200 in Duolink Antibody Diluent, dropped onto the coverslips, and incubated overnight at 4°C. See Supplementary Figure S3 for stain of untransfected HEK293 cells. After three washes with PBS, secondary antibodies (same antibodies as in previous section) were added to the coverslips and incubated 1 hour at 37°C. After three washes with PBS, coverslips were inverted onto new microscope slides with a droplet of Fluoromount-D with DAPI (Southern Biotech).

Confocal Microscopy

Immunolabeled retina sections and HEK293 cells were visualized using a Leica TCS SP5 confocal microscope (Leica Microsystems, Durham, NC, USA). Independent Z-stack image series were collected for each dye (DAPI, Alexa 488, Alexa 594) for both retina and HEK293 samples at 0.16-micron steps. Specifically, the Z-stack volumes were collected to capture the entirety of the HEK293 cells (Z-stack volume of ~7–10 microns) and a large portion of the retinal sections (Z-stack volume of ~50 microns). Image processing was done using the open-source software Fiji, an actively

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maintained and updatable version of ImageJ software (NIH, Bethesda, MD, USA). For HEK293 cell image processing, Z-projections of the ~3- to 5-micron portion (encompassing the middle portion of the cell) for each fluorophore were generated using the Sum Slices setting to produce a separate composite image for each dye. The retina images were processed similarly, but the entirety of the Z-stack series was used for generating Z-projection images.

Calcium Imaging of Melanopsin-Expressing HEK293 Cells

Transiently transfected HEK293 cells were seeded into a 96-well dish at a density of 10^5 cells per well, with six replicates for each individual construct tested in a given transfection. Cells were maintained in the dark overnight after seeding. The next day, the cells were incubated with Fluo-4 AM (ThermoFisher Scientific) and fresh DMEM for 1 hour, as per the manufacturer's protocol. After incubation, the media/dye mixture was replaced with Hank's balanced salt solution (HBSS; Corning) supplemented with 20 mM HEPES to allow for prolonged cell viability outside of a CO_2 incubator. Fluorescence measurements were carried out on a TECAN Infinite M200 (TECAN Trading AG, Morrisville, NC, USA) by exciting the sample with 487 nm light (light source voltage corresponds to ~588 V) and subsequently recording fluorescence emission at 516 nm at a rate of 1 Hz. For inhibition of protein phosphatase 2A, cantharidin was added to the cells during incubation with Fluo-4 AM to a final concentration of 2 µM. Various concentrations ranging from 1 to 10 µM were tested, and 2 µM did not cause any toxicity, as assayed by viable cell counts using trypan blue (data not shown). For inhibition of clathrin-mediated endocytosis, chloroquine was added to the cells during incubation with Fluo-4 AM to a final concentration of 400 µM (see Dutta and Donaldson[37] for a review on pharmacologic inhibition of endocytosis). Cell toxicity was assayed by viable cell counts using trypan blue, and no toxicity was observed (data not shown). To normalize calcium imaging data, data points for each replicate in each transfection were normalized to their own maximum fluorescence value. All normalized replicates across all transfections were then pooled together.

Statistical Analyses

Analyses of maximum calcium responses were done by pooling the nonnormalized maximum fluorescence values at all three 90-second calcium imaging cycles, across all replicates, in all the transfections analyzed. All the values were normalized to the highest value across the entire data set to quantify the maximum calcium responses as a value between 0 and 1. Statistical significance was tested using unpaired Student's *t*-tests, correcting for multiple comparisons utilizing the Bonferroni-Dunn method. Significance was denoted with *P* values of 0.05, 0.01, 0.001, and 0.001 to indicate *, **, ***, and ****, respectively.

Measurement of melanopsin deactivation rates was done by fitting the deactivation phase of all normalized data (across all replicates for every transfection), which corresponds to the portion of the calcium measurements from the peak calcium response to the end of each measurement (Supplementary Fig. S1). The data were fitted to the following exponential function:

$$y = y(0) * e^{(k*x)},$$

where $y(0)$ is the y-value at peak fluorescence, and k is the rate of exponential decay. Statistical significance between deactivation rate values (k) was tested using Student's *t*-tests, correcting for multiple comparisons utilizing the Bonferroni-Dunn method. Significance was denoted with *P* values of 0.05, 0.01, 0.001, and 0.001 to indicate *, **, ***, and ****, respectively. All analyses were done using GraphPad software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Diverse Expression of Protein Phosphatases in the Mouse Retina and Protein Phosphatase 2A Expression in IPRGCs

To assay the expression of phosphatases in the mouse retina, we synthesized cDNA from mRNA extracted from mouse retinal tissue. We then used gene-specific primers to amplify the 3′ regions of the transcripts of serine and threonine phosphatases of the protein phosphatase family. RT-PCR of retinal cDNA showed diverse expression of protein phosphatases of many subfamilies (Fig. 1), with the exception of protein phosphatase 5. From these various phosphatases, we chose to examine PP2A as a potential regulator of melanopsin resensitization due to its robust mRNA expression in the retina (Fig. 1A) and due to PP2A being reported to dephosphorylate GPCRs,[22,38,39] including rhodopsin.[39,40] To examine the localization of PP2A expression in the retina, specifically to test for its presence in melanopsin-expressing ipRGCs, we probed retinal slices with PP2A and melanopsin antibodies (Figs. 1B–E). We observed robust expression of PP2A across the entire retina (Figs. 1B–E), including photoreceptor outer segments, interneurons, and ganglion cells. Closer examination of the expression pattern reveals that PP2A colocalizes in melanopsin-expressing cells, suggesting that ipRGCs express PP2A. This raises the possibility that PP2A may mediate melanopsin resensitization in ipRGCs. Additionally, several protein phosphatases, including PP2A and one of its regulatory subunits (regulatory subunit B), were detected in a coimmunoprecipitation experiment using anti-1D4 antibodies in melanopsin-1D4 transfected HEK293 cells, when analyzed using mass spectrometry (Supplementary Table S1).

Unphosphorylated Melanopsin Displays a Higher Level of Sustained Activity in Response to Constant Light

We hypothesized that if melanopsin remains unphosphorylated and is unable to couple to signaling termination molecules such as β-arrestin and thus remains in the active state, the cell would exhibit a higher capability to sustain light responses over a long period of time. To test this hypothesis, we expressed melanopsin and phosphonull melanopsin (a mutant melanopsin with all 38 C-terminal potential phosphorylation sites mutated to alanine residues[10]) in HEK293 cells and examined intracellular calcium levels over a time frame of 1 hour. In this assay, we

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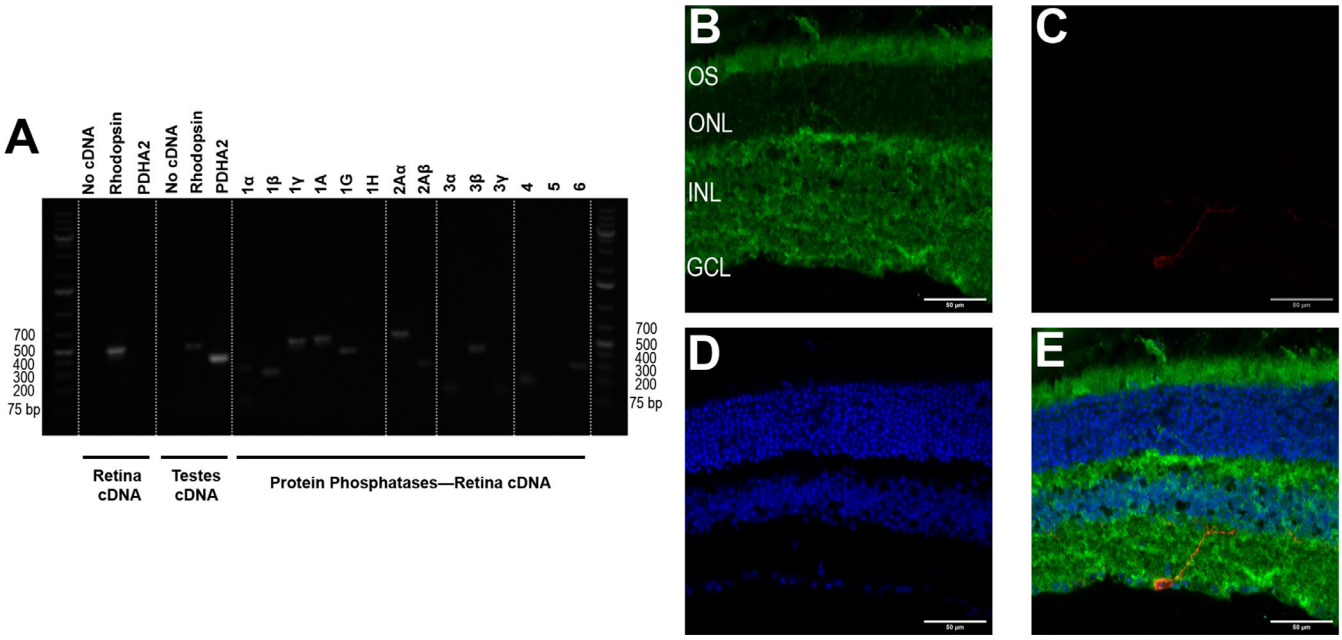

Figure 1. Diverse expression of protein phosphatases in the mouse retina, including PP2A in ipRGCs. (**A**) RT-PCR of cDNA synthesized from mouse retina homogenate. Gene-specific primers were used to amplify the 3′ and 3′UTR ends of each gene of interest. (**B–E**) Images depict immunohistochemistry of mouse retina sections using C-terminal melanopsin and protein phosphatase 2A catalytic subunit-specific antibodies. (**B**) PP2A antibody. (**C**) Melanopsin antibody. (**D**) DAPI. (**E**) Overlay. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; OS, photoreceptor outer segments. *Scale bar*: 50 microns.

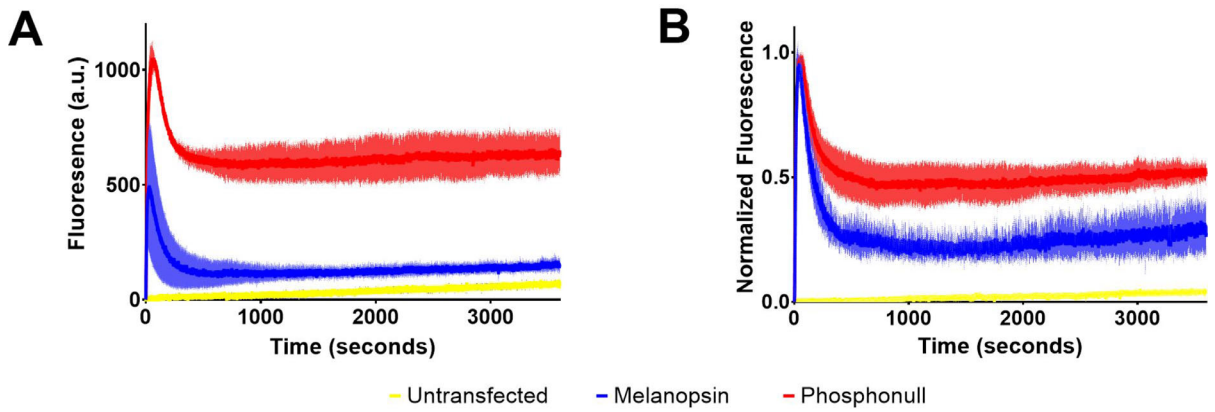

Figure 2. Permanently dephosphorylated melanopsin sustains stronger light responses than wild-type melanopsin. Representative 1-hour calcium imaging of transfected HEK293 cells expressing melanopsin or phosphonull melanopsin, a mutant in which all 38 C-terminal potential phosphorylation sites have been mutated to alanine residues. (**A**) Baseline and (**B**) normalized light responses are represented by *blue* and *red* traces for melanopsin and phosphonull, respectively. SD of data represent three replicates within one transfection; depicted as *lighter colored margins* above and below the mean. a.u., arbitrary units of fluorescence.

simulated constant light exposure by flashing melanopsin-expressing cells with light every second and simultaneously recording the cells' calcium level. We observed rapid activation for both melanopsin and phosphonull melanopsin (Fig. 2) followed by steady desensitization and then a plateau of the calcium level at a constant level for the remainder of the experiment. This plateaued portion of the calcium recording is the sustained light response. The maximum light response was higher in phosphonull melanopsin compared to wild-type melanopsin, and the light response was sustained at a considerably higher level than wild-type melanopsin throughout the entirety of the 1-hour measurement (Fig. 2A). Interestingly, this higher level of sustained response was also observed after normalizing the data, indicating that the sustained light response in phosphonull melanopsin was closer to the peak response compared to wild-type melanopsin, which possessed a sustained light response that was closer to baseline than its own maximum (Fig. 2B). This indicates that phosphonull melanopsin, which is not C-terminally phosphorylated, possesses sustained light responses at levels closer to its peak level of activity than wild-type melanopsin, further supporting the

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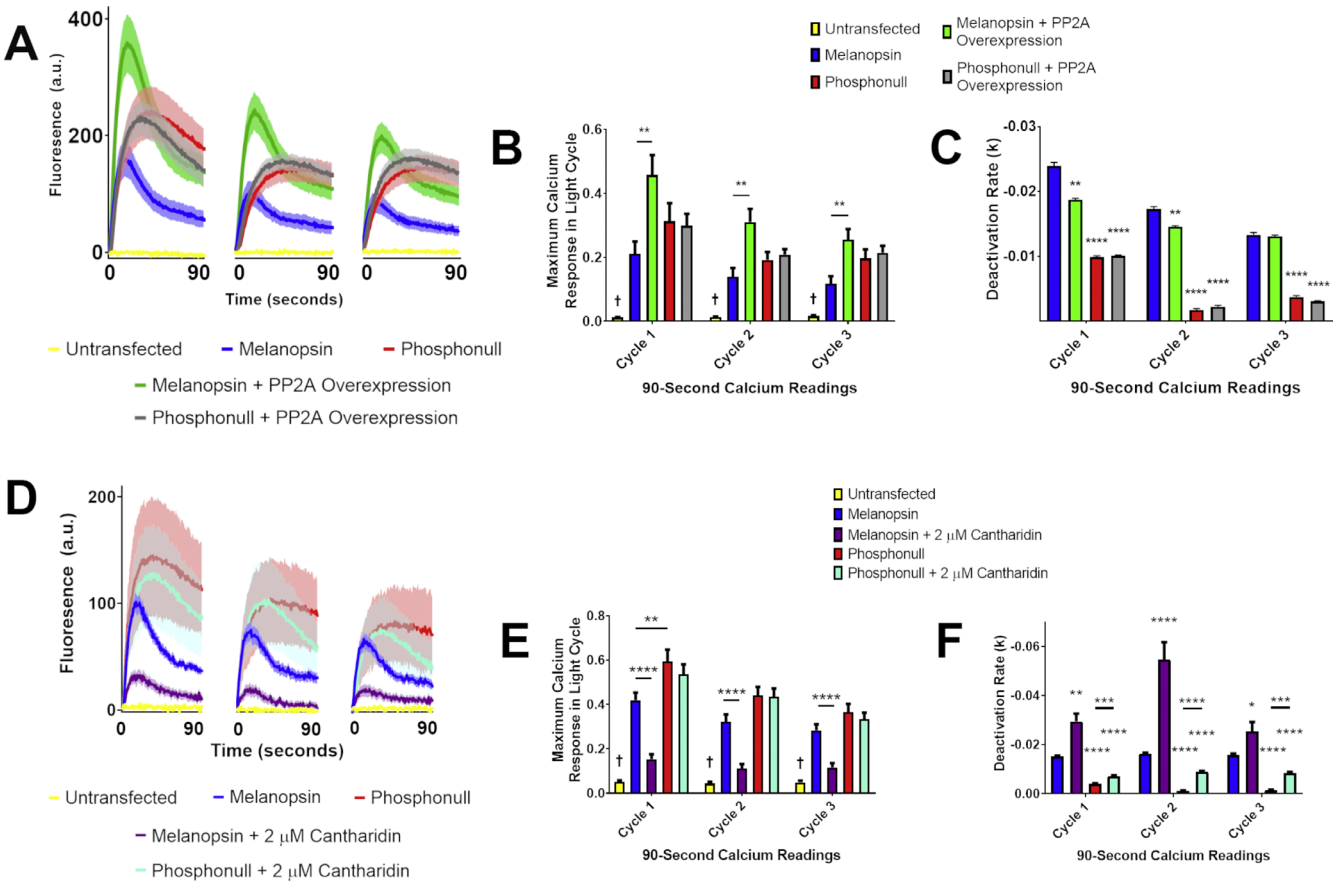

Figure 3. Analysis of protein phosphatase 2A activity on melanopsin function in transfected HEK293 cells. (**A–C**) Cotransfection of melanopsin with protein phosphatase 2A produces more robust light responses than melanopsin-only transfected HEK293 cells expressing endogenous levels of phosphatase. (**A**) Calcium imaging of melanopsin, phosphonull melanopsin, and melanopsin/phosphonull and PP2A-cotransfected HEK293 cells. Three 90-second assays were conducted with 30-minute dark incubations between rounds of calcium imaging to allow for melanopsin and cell resensitization. The x-axes on each cycle represent a duration of 90 seconds, with the gaps indicating the 30-minute dark incubation. SEM of data depicted as *lighter colored margins* above and below the mean (*dark colored line*). (**B**) Quantification of maximum light responses in each cycle. (**C**) Quantification of melanopsin deactivation rates. All rates compared to melanopsin's deactivation rate and statistical significance are indicated above the *bars* if significance is determined compared to melanopsin-only rate. (**D–F**) Chemical inhibition of endogenous protein phosphatase 2A activity reduces light response intensity in melanopsin-transfected HEK293 cells. (**D**) Calcium imaging of melanopsin and phosphonull melanopsin-transfected HEK293 cells, with or without the addition of 2 μM cantharidin, a PP2A-specific inhibitor. Three 90-second assays were done with 30-minute dark incubations between rounds of calcium imaging to allow for melanopsin and cell resensitization. The x-axes on each cycle represent a duration of 90 seconds, with the gaps indicating the 30-minute dark incubation. SEM of data depicted as *lighter colored margins* above and below the mean (*dark colored line*). (**E**) Quantification of maximum light responses in each cycle. (**F**) Quantification of melanopsin deactivation rates. All rates compared to melanopsin's deactivation rate and statistical significance are indicated above the *bars* if significance is determined compared to melanopsin-only rate. All error bars represent SEM of $n = 3$ transfections. Statistical significance tested by using Student's *t*-test. $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$. $^{****}P < 0.0001$. †Statistical significance of untransfected group (****) to all other samples.

idea that unphosphorylated melanopsin supports robust light responses.

Overexpression of Protein Phosphatase 2A Dramatically Increases the Potency of Light Responses

Previous experiments[10,13,14] and our examination of prolonged calcium signaling of melanopsin and phosphonull melanopsin (Figs. 3A–C) strongly suggest that modulation of C-terminal phosphorylation not only is critical for the kinetics of the termination of light responses but also contributes to the overall amplitude of the light responses. Due to robust expression of PP2A in the retina, we tested the effect of overexpression of PP2A in the signaling of wild-type melanopsin expressed in HEK293 cells. We cotransfected HEK293 cells with an equivalent amount of melanopsin and PP2A (PP2A catalytic subunit alpha) DNA to transiently express the visual pigment and overexpress the phosphatase. We performed calcium imaging by performing three 90-second recordings (light stimulation and calcium recording once a second) with 30-minute dark incubations between each recording to allow for cell resensitization (i.e., restoration of resting cytoplasmic calcium levels) and visual pigment resensitization, the latter through phosphatase activity. Calcium imaging of these cotransfected cells produced significantly stronger maximal light responses in cotransfected HEK293 cells compared to melanopsin-only transfected cells (Figs. 3A, 3B). Strikingly, the effect of phosphatase activity was more immediate than predicted; light responses with greater amplitude were observed in the first 90-second cycle, whereas we expected the effect of phos-

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phatase activity (i.e., light responses with greater amplitudes) to manifest after the first 30-minute dark incubation where melanopsin would resensitize. This suggests that the PP2A-mediated dephosphorylation of melanopsin is rapid and provides a powerful mechanism to modulate light responses in an immediate and sustained manner.

To test that the effects on melanopsin's light responses are specifically due to PP2A-mediated dephosphorylation of melanopsin, we performed a similar PP2A cotransfection and subsequent calcium imaging experiment using phosphonull melanopsin. Due to the mutation of C-terminal serine and threonine residues and lack of phosphorylation, the phosphonull light responses was predicted to be unaffected by the overexpression of PP2A. Calcium imaging of phosphonull and PP2A cotransfected HEK293 cells revealed light responses of similar maximal intensity in each 90-second cycle. Further analysis of the signaling kinetics of these cotransfection experiments, specifically, quantification of the deactivation phase in each 90-second cycle (deactivation phase is defined as the portion of the recording after the peak fluorescence of the data, where calcium levels decrease), suggests an effect of phosphatase activity on the deactivation rate of wild-type melanopsin (Fig. 3C). The cells where both melanopsin and PP2A were cotransfected had a slower rate of deactivation compared to cells where only melanopsin was transfected. This effect, however, was observed in the first two 90-second cycles of calcium imaging. Interestingly, while melanopsin's deactivation rate was reduced by PP2A overexpression, it did not decrease to phosphonull melanopsin's deactivation rate, which was unaffected by PP2A overexpression (Fig. 3C).

Pharmacologic Inhibition of Protein Phosphatase 2A Reduces Amplitude of Light Responses

After testing the effect of overexpression of PP2A, we sought to further test PP2A activity on melanopsin through inhibition of its catalytic activity though chemical inhibition of PP2A. Thus, we incubated melanopsin-transfected HEK293 cells with cantharidin, a potent PP2A inhibitor.[41,42] It should also be noted that cantharidin inhibits protein phosphatase 1 (PP1) activity as well, albeit at a lower capacity than the PP2A inhibition.[41] We therefore tested melanopsin and phosphonull melanopsin light responses using calcium imaging in a similar experimental paradigm as the PP2A overexpression experiments (three cycles and 30-minute dark incubation between cycles). In these experiments, cantharidin was added to the cells to a final concentration of 2 µM at the same time as the calcium dye, which was incubated for an hour prior to fluorescence measurements. Calcium imaging of melanopsin-transfected cells after cantharidin treatment resulted in a dramatic and significant decrease in the maximum light responses in each 90-second calcium recording compared to untreated melanopsin-transfected cells (Figs. 3D, 3E). Similar to the overexpression experiment, treatment with cantharidin did not affect the maximum light responses of phosphonull melanopsin-transfected cells (Figs. 3D, 3E), further implicating PP2A dephosphorylation of C-terminal serine and threonine residues in the modulation of phototransduction and light response intensity.

Cantharidin inhibition of PP2A caused an increase in the deactivation rate of wild-type melanopsin cells compared to untreated cells (Fig. 3F). This suggests inhibition of PP2A, and thus, inhibition of melanopsin dephosphorylation increases the rate of melanopsin phototransduction deactivation, presumably due to the preservation of phosphorylation modifications on melanopsin C-terminal serine and threonine residues. However, cantharidin treatment of phosphonull melanopsin-transfected cells resulted in a slight, yet significant increase in deactivation rate (Fig. 3F). We predicted that there would be no effect on deactivation kinetics in the cantharidin-treated cells compared to the nontreated phosphonull-transfected cells, similar to the maximum light responses (Fig. 3E). This result raises the possibility of functionally relevant phosphorylation on the intracellular loops, which were not mutated in the phosphonull melanopsin construct. Additionally, these non-C-terminal phosphorylations could also be regulated by a PP1-family phosphatase, and thus PP1 inhibition by cantharidin might contribute to this difference in the nontreated and cantharidin-treated phosphonull melanopsin-transfected cells. Addition of cantharidin to melanopsin-transfected cells during the first dark incubation, between the first and second 90-second cycles of calcium imaging, resulted in reduced amplitude of the light responses in cantharidin-treated cells during the second and third calcium imaging cycles, suggesting successful and rapid inhibition of PP2A activity (Supplementary Fig. S2). This further supports the idea that PP2A modulation of melanopsin light responses is a rapid process, not requiring prolonged periods of time to achieve robust light responses through receptor resensitization.

Light Exposure to Melanopsin-Expressing Cells Induces Clathrin-Mediated Endocytosis in a Phosphorylation-Dependent Manner

Melanopsin binding of β-arrestin[12] raises the possibility that melanopsin also interacts with endocytic machinery, particularly clathrin-mediated endocytosis. To test this hypothesis, we transfected HEK293 cells with wild-type melanopsin or phosphonull melanopsin (both constructs are C-terminally 1D4 tagged to facilitate immunodetection), maintained the cells overnight in the dark, and fixed the cells in the dark or after light exposure for 10, 20, or 30 minutes (Figs. 4, 5). Immunocytochemistry of these cells with 1D4- and clathrin-specific antibodies indicated localization of melanopsin primarily to the plasma membrane in both melanopsin- and phosphonull-transfected cells (Figs. 4, 5). However, in melanopsin-transfected cells, light exposure induced accumulation of cytoplasmic fluorescent puncta when probed with the 1D4 antibody, which overlapped with clathrin-positive fluorescent puncta (Fig. 4). This suggests melanopsin is trafficked into the cytoplasm via clathrin-coated endosomes after prolonged periods of light exposure. Additionally, the accumulation of melanopsin- and clathrin-positive endosomes reached a maximum after 20 minutes (Fig. 4) and decreased after 30 minutes, suggesting that after long periods of time, melanopsin is either recycled to the membrane or sent to lysosomal degradation after internalization. We propose, however, that the recycling process is utilized due to continued enrichment of melanopsin in the plasma membrane (Fig. 4).

To test if melanopsin endocytosis is dependent on C-terminal phosphorylation and β-arrestin, we analyzed the protein localization of immunolabeled phosphonull-transfected cells. Similar to melanopsin-transfected cells,

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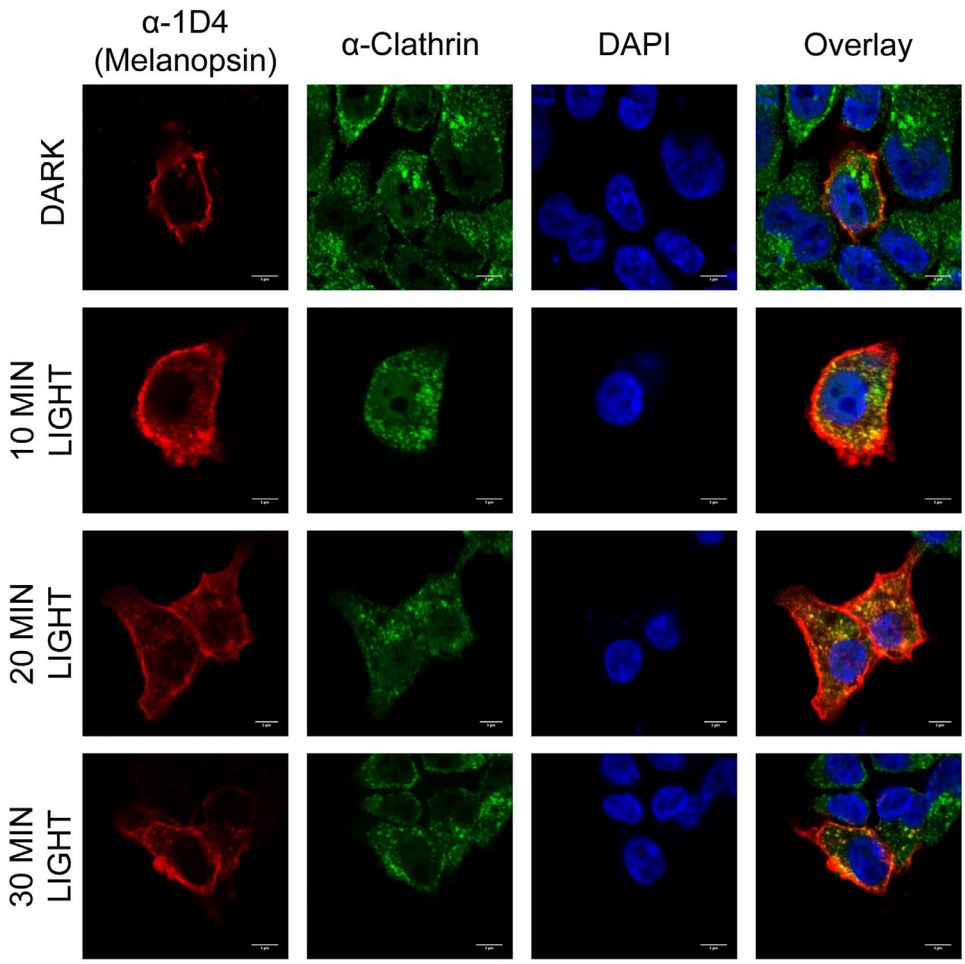

Figure 4. Localization of melanopsin in transfected HEK293 cells, after dark adaptation or prolonged exposure to light. Transfected cells were fixed after dark adaptation or exposure to various durations of white light. Fixed cells were then probed with α-1D4 and α-clathrin antibodies to visualize localization of melanopsin. Depicted are representative Z-projections (see Methods for more details) of one of $n = 4$ independent transfections. *Blue*: DAPI; *red*: melanopsin; *green*: clathrin. *Scale bar*: 5 microns.

phosphonull melanopsin was localized primarily to the cell membrane in both dark-adapted and light-exposed cells (Fig. 5). While few puncta were observed in the cytoplasm in both light and dark conditions, they did not colocalize with clathrin-positive puncta in the cells (Fig. 5), thus suggesting that the phosphonull mutant did not internalize in a light-dependent manner. These puncta instead were likely newly synthesized phosphonull melanopsin being trafficked from the cytosol to the plasma membrane. Thus, these experiments suggest that C-terminal phosphorylation and arrestin-coupling is critical for melanopsin to engage in endocytic trafficking during light exposure.

Pharmacologic Inhibition of Clathrin-Mediated Endocytosis Inhibits Melanopsin Endocytosis and Reduces Melanopsin Light Responses After Repeated Stimulation by Light

In order to test the functional role of melanopsin endocytosis through clathrin-coated endosomes, we performed calcium imaging of melanopsin-transfected HEK293 cells in the absence or presence of chloroquine, a chemical inhibitor of clathrin-coated endocytosis.[43] Specifically, chloroquine inhibits endosomal activity through the malformation of clathrin complexes at the plasma membrane[43] or through the pH alteration of acidic endosomes and lysosomes,[44,45] leading to the buildup of proteins at the plasma membrane. Similar to the previous experiments, we carried out the calcium imaging in three 90-second cycles, with a 30-minute dark incubation between cycles. Interestingly, we also observed a small increase in the amplitude of the response at the first calcium imaging cycle in the presence of chloroquine (Figs. 6A, 6B), which was also observed in phosphonull melanopsin-transfected cells (Supplementary Fig. S5), but this difference did not reach statistical significance in melanopsin-transfected cells (Fig. 6B). One possibility that may have caused this initial rise may be that the chloroquine inhibition of clathrin-mediated endocytosis may have prevented signaling molecules from internalizing upon dark adaptation, prior to the calcium measurements, thus producing the slightly increased light response. However, chloroquine treatment of melanopsin-transfected cells resulted in a robust reduction in light response amplitude after repeated light stimulation, in the third calcium imaging cycle (Figs. 6A, 6B). This is in contrast to phosphonull melanopsin-transfected cells, which did not internalize (Fig. 6B) and did not display any reduction in light

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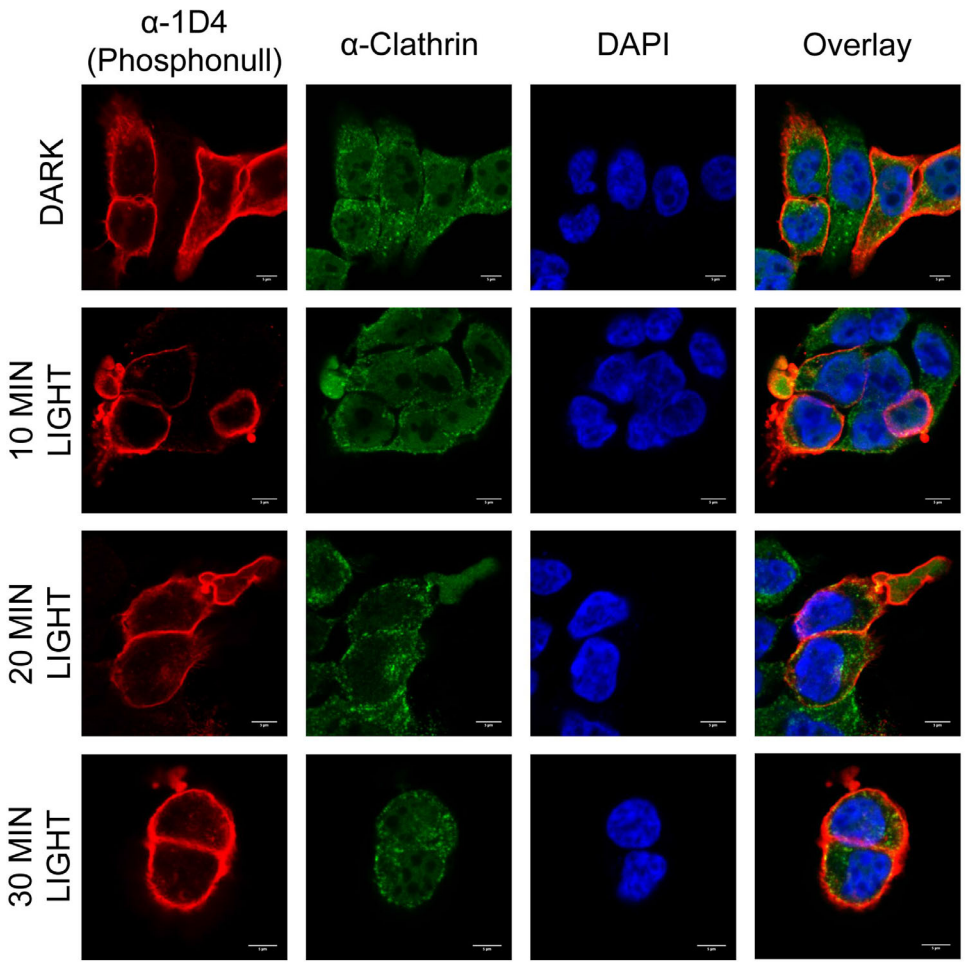

Figure 5. Localization of phosphonull melanopsin in transfected HEK293 cells, after dark adaptation or prolonged exposure to light. Transfected cells were fixed after dark adaptation or exposure to various durations of white light. Fixed cells were then probed with α-1D4 and α-clathrin antibodies to visualize localization of melanopsin. Depicted are representative Z-projections (see Methods for more details) of one of $n = 4$ independent transfections. *Blue*: DAPI; *red*: melanopsin; *green*: clathrin. *Scale bar*: 5 microns.

response amplitude after repeated stimulation (Supplementary Fig. S5). Immunocytochemical analysis of chloroquine-treated, melanopsin-transfected cells suggests that this treatment inhibits melanopsin internalization after 20 minutes of light exposure (Fig. 6C), due to lack of melanopsin puncta localized to the cytoplasm in the light-exposed cells. Furthermore, we observed altered clathrin localization in chloroquine-treated cells (Fig. 6C); specifically, there were fewer clathrin puncta in the cytoplasm compared to nontreated cells, which showed robust and plentiful cytoplasmic-localized clathrin puncta (Figs. 4, 5). These data support that chloroquine treatment of melanopsin-transfected cells is inhibiting clathrin-mediated endocytosis. After considering our previous analyses with this functional assay, we propose that melanopsin endocytosis works to efficiently maintain a constant amount of resensitized visual pigment at the membrane, which supports phototransduction during repeated or long-term stimulation.

Discussion

Analyses of melanopsin phototransduction termination have focused on mechanisms underlying its sluggish and prolonged deactivation, mediated by phosphorylation of its C-terminus. With 38 serine and threonine residues and a C-terminal domain four times longer than mouse rhodopsin, it is not surprising that this aspect of its signaling and function has been extensively studied. However, because of the rich diversity of non-image-forming responses regulated by ipRGCs and their regulation of contrast detection, which contributes to image formation,[3] we propose that additional mechanisms outside of melanopsin C-terminal phosphorylation contribute to modulating light responses in ipRGCs. Specifically, we propose that modulation of light responses by PP2A and clathrin-mediated endocytosis is important for robust light responses for rapid timescale behaviors (pupil constriction, contrast detection) and prolonged timescale behaviors (circadian photoentrainment, mood regulation). Our data support the idea that PP2A activity works rapidly, evidenced by the immediate production of robust (Figs. 3A–C) or diminished (Figs. 3D–F) light responses when overexpressing or inhibiting PP2A, respectively. Furthermore, both overexpression and inhibition of PP2A maintained their effects on the light responses after several dark incubations, suggesting that phosphatase effects on melanopsin signal transduction can be sustained and are long-lasting. We also would like to point out that while our study was conducted in a heterologous

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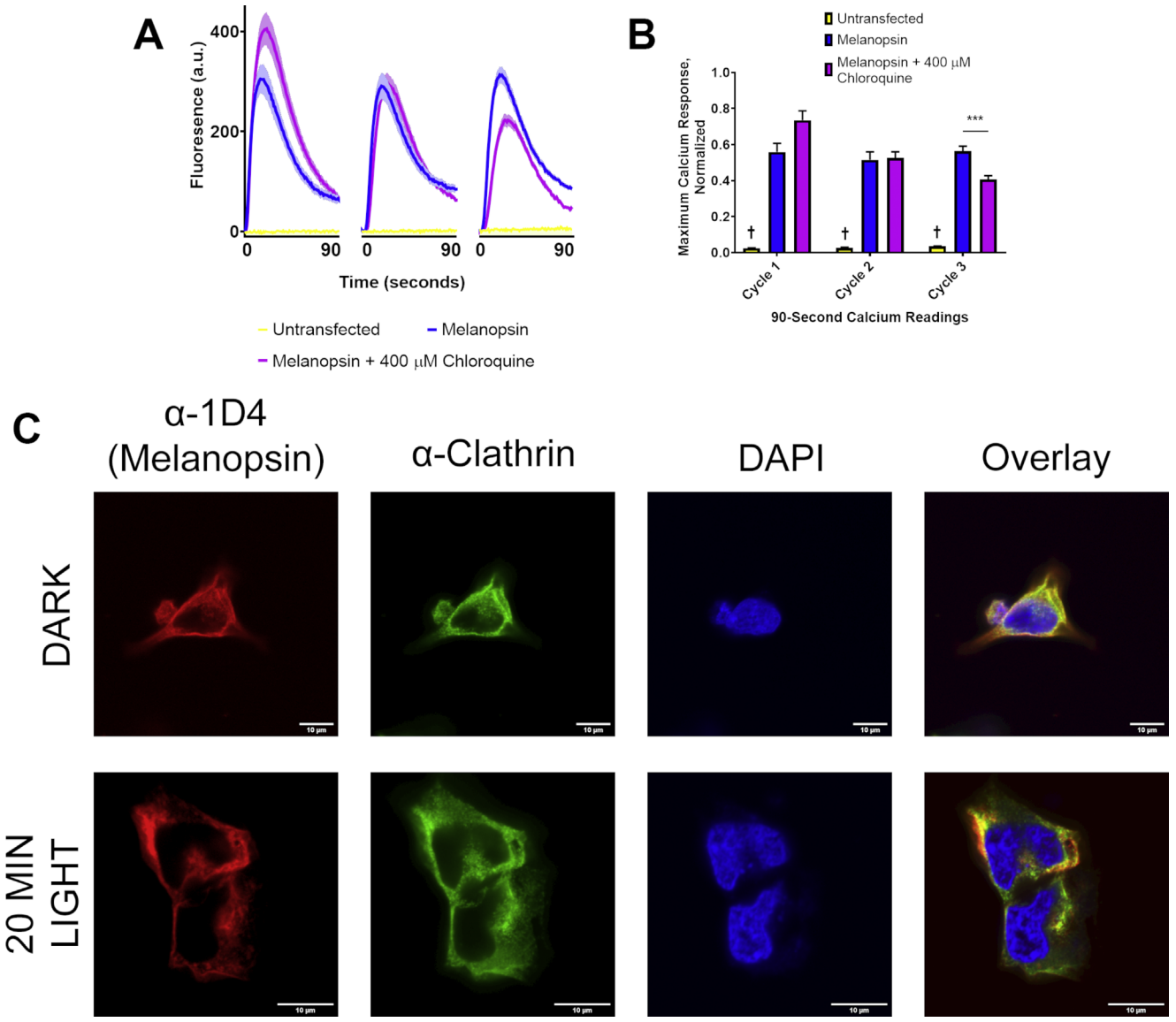

Figure 6. Pharmacologic inhibition of clathrin-mediated endocytosis using chloroquine inhibits endocytic activity in melanopsin-transfected HEK293 cells. (**A**) Calcium imaging of melanopsin-transfected cells with or without incubation with 400 μM chloroquine. Three 90-second assays were done with 30-minute dark incubations between rounds of calcium imaging to allow for melanopsin and cell resensitization. SEM of data depicted as *lighter colored margins* above and below the mean. (**B**) Quantification of maximum light responses in each cycle. All *error bars* represent SEM of $n = 3$ transfections. Statistical significance tested by using Student's *t*-test. ***$P < 0.001$. ****$P < 0.0001$ †Statistical significance of untransfected group (****) to all other samples. (**C**) Inhibition of clathrin-mediated endocytosis using chloroquine prevents endosomal localization of melanopsin after light exposure. Transfected HEK293 cells were incubated with 400 μM chloroquine for an hour prior to fixation after dark adaptation or light exposure. Fixed cells were then probed with α-1D4 and α-clathrin antibodies to visualize localization of melanopsin. *Blue*: DAPI; *red*: melanopsin; *green*: clathrin.

expression system, previous observations in HEK293 cells on melanopsin C-terminal phosphorylation[10,11] informed in vivo studies and accurately modeled melanopsin activity in murine ipRGCs expressing melanopsin C-terminal phosphorylation mutants.[13,14] Thus, we propose that the observations and conclusions derived from this study present an accurate model of phosphatase activity on melanopsin phototransduction in ipRGCs, and we put this work forward to inform future analyses on all aspects of melanopsin resensitization.

This study and previous analyses[13,14] strongly suggest that the dynamic regulation of melanopsin's phosphorylated state by kinases and PP2A is critical for melanopsin-mediated light responses, in both short-term and long-term phototransduction and ipRGC function. However, the data presented here suggest that clathrin-mediated endocytosis of melanopsin contributes to the maintenance of a constant concentration of melanopsin at the plasma membrane, which also supports melanopsin-mediated light responses during repeated light stimulation. Therefore, the dynamic modulation of C-terminal phosphorylation, or potentially the chromophore state (i.e., chromophore bi- or tristability[19]), serves to immediately regulate melanopsin-mediated light responses, while endocytosis provides a mechanism for long-term melanopsin regulation. A fascinating direction for future study is to test the effect of

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melanopsin endocytosis in ipRGC function, as the prediction would be that long-term ipRGC functions, such as circadian photoentrainment, would be greatly impacted in the absence of endocytic function. In addition, abolishment of Rh1 endocytosis leads to degeneration of the *Drosophila* retina.[25,27] Thus, it is important to test if abolishment of melanopsin endocytosis in ipRGCs leads to photoreceptor degradation, thus implicating endocytosis as an important regulator of the cell's health under constant stimulation.

We propose that these resensitization mechanisms not only provide robust transient and sustained melanopsin light responses but also help reduce ipRGC dependence on the visual cycle from the outer retina or Müller glia. In *Drosophila*, light-activated rhodopsin-1 remains associated with its chromophore and is capable of reisomerization of the all-*trans*-retinal photoproduct back to 11-*cis*-retinal.[46] Chromophore regeneration and de novo synthesis are regulated by retinol dehydrogenases *PDH* and *RDHB*, and disruption of either enzyme's activity causes photoreceptor degeneration,[47,48] suggesting the existence and importance of a visual cycle in a rhabdomeric retina. Melanopsin displays homology to rhabdomeric visual pigments in both amino acid sequence and function. Our data also suggest nonvisual cycle mechanisms of supporting robust light responses. Thus, we propose that melanopsin's functional homology to rhodopsin-1 in *Drosophila* also encompasses their resensitization mechanisms. Specifically, in mammalian ipRGCs, melanopsin-driven phototransduction is regulated by PP2A dephosphorylation and clathrin-mediated endocytosis of melanopsin. These regulatory mechanisms reduce melanopsin's dependence on constant replenishment of its chromophore. Therefore, dephosphorylation and endocytosis of melanopsin in ipRGCs may be key to regulating cellular function in a diversity of light-driven behaviors and functions.

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