Published in final edited form as:

Neuroscience. 2019 June 01; 408: 400–417. doi:10.1016/j.neuroscience.2019.04.005.

μ -opioid receptor activation directly modulates intrinsically photosensitive retinal ganglion cells

Allison M. Cleymaet^{1,2,*}, Shannon K. Gallagher^{1,*}, Ryan E. Tooker¹, Mikhail Y. Lipin¹, Jordan M. Renna^{3,#}, Puneet Sodhi⁴, Daniel Berg³, Andrew T.E. Hartwick⁴, David M. Berson³, and Jozsef Vigh¹

¹Dept. of Biomedical Sciences, Colorado State University, Ft. Collins, CO 80523

²Dept. of Biomedical Sciences, Colorado State University, Ft. Collins, CO 80523

³Dept. of Neuroscience, Brown University, Providence, RI 02912

⁴College of Optometry, Ohio State University, Columbus, OH 43210

Abstract

Intrinsically photosensitive retinal ganglion cells (ipRGCs) encode light intensity and trigger reflexive responses to changes in environmental illumination. In addition to functioning as photoreceptors, ipRGCs are post-synaptic neurons in the inner retina, and there is increasing evidence that their output can be influenced by retinal neuromodulators. Here we show that opioids can modulate light-evoked ipRGC signaling, and we demonstrate that the M1, M2 and M3 types of ipRGCs are immunoreactive for μ -opioid receptors (MORs) in both mouse and rat. In the rat retina, application of the MOR-selective agonist DAMGO attenuated light-evoked firing ipRGCs in a dose-dependent manner ($IC_{50} < 40$ nM), and this effect was reversed or prevented by co-application of the MOR-selective antagonists CTOP or CTAP. Recordings from solitary ipRGCs, enzymatically dissociated from retinas obtained from melanopsin-driven fluorescent reporter mice, confirmed that DAMGO exerts its effect directly through MORs expressed by ipRGCs. Reduced ipRGC excitability occurred via modulation of voltage-gated potassium and calcium currents. These findings suggest a potential new role for endogenous opioids in the

Correspondence should be addressed to: Jozsef Vigh, Dept. of Biomedical Sciences, Colorado State University, 1617 Campus Delivery, Ft. Collins, CO 80523, jozsef.vigh@colostate.edu.

*:equal contribution

#:current address: Dept. of Biology, University of Akron, Akron, OH 44325

Author contribution

Most experiments were performed at Colorado State University (Fort Collins, CO), except for loose patch single cell recordings of light responses that were done at Brown University (Providence, RI) and the MEA recordings from adult rat retinas that were performed at Ohio State University (Columbus, OH).

A.T.H. D.M.B. and J.V. designed the experiments. A.M.C., S.K.G., M.Y.L., R.E.T., J.V., P.S. A.T.H., J.M.R. and D.M.B. performed the experiments. A.M.C., S.K.G., J.V. and J.M.R. analyzed the data. A.T.H., D.M.B. and J.V. were involved in the interpretation of the results. A.M.C., S.K.G. drafted the paper, which was critically revised by all authors. All authors approved the final version of the manuscript. All those who qualify for authorship are listed.

Competing interest

Authors declare no competing interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

mammalian retina and identify a novel site of action—MORs on ipRGCs—through opioids might exert effects on reflexive responses to environmental light.

Keywords

opioids; retina; intrinsically photosensitive ganglion cell

Introduction

The discovery of melanopsin-containing intrinsically photosensitive retinal ganglion cells (ipRGCs) has fundamentally altered our understanding of how light influences mammalian physiology and behavior. These ganglion cells were initially identified as a third photoreceptor type that respond to environmental light cues and help synchronize circadian rhythms to external day/night cycles (Berson et al., 2002; Hattar et al., 2002). Since their discovery, intense research has broadened our understanding of morphology and function of ipRGCs. These photosensitive cells are now classified into several distinct subtypes (M1-M6 cells) that, as a group, send axons to diverse brain areas that participate in both image-forming and non-image-forming vision (Baver et al., 2008; Schmidt and Kofuji, 2009; Ecker et al., 2010; Schmidt et al., 2011; Lee and Schmidt, 2018; Quattrochi et al., 2018). In addition, ipRGCs have been implicated in light-mediated pathological processes such as light-evoked exacerbation of migraine headache (photophobia) (Nosedá et al., 2010) and altered mood and cognitive function associated with irregular light schedules (LeGates et al., 2012).

Although capable of producing light responses intrinsically (Berson et al., 2002; Hartwick et al., 2007), ipRGCs receive rod/cone-mediated light information through synapses employing fast excitatory and inhibitory transmitters (Perez-Leon et al. 2006; Wong et al. 2007; Schmidt et al. 2008). IpRGCs are also subject to neuromodulatory influences that tune their signaling to physiological needs. For example, dopamine acts through D1 receptors to directly modify ipRGC signaling (van Hook et al., 2012). Adenosine inhibits light-stimulated responses in ipRGCs via A1 receptor activation (Sodhi and Hartwick, 2014), and somatostatin has been implicated in parallel inhibition of dopaminergic amacrine cells and ipRGCs (Vuong et al., 2015). Acetylcholine stimulates ipRGC spiking even in the absence of light through a muscarinic receptor-mediated mechanism (Sodhi and Hartwick, 2016).

We have previously confirmed the expression of the endogenous opioid, β -endorphin, and its preferred receptor, the μ -opioid receptor (MOR), in the mouse retina (Gallagher et al., 2010, 2012). Specifically, we have shown that, besides dopaminergic amacrine cells, other GAD-67-expressing amacrine cells and some Brn3a-positive ganglion cells also express MORs (Gallagher et al., 2012). Here we show that the M1-M3 types of ipRGCs express MORs in both rats and mice. Further, we show that exogenously applied opioids act on MORs inhibit light-evoked ipRGC signaling in the following two ways: by delaying the onset of light-evoked firing and by reducing the duration of spiking. We propose that the delayed onset of light-evoked firing is caused by a shift in the activation of voltage-gated potassium currents (I_K) to hyperpolarized potentials, thereby elevating the current threshold

of voltage-gated sodium currents (I_{Na}) and spike initiation. We provide evidence that the MOR-mediated reduced duration of light-evoked spiking of ipRGCs results from suppression of non-inactivating voltage-gated calcium currents. These findings outline a previously unrecognized role for endogenous opioids in the mammalian retina.

Experimental procedures

Animals—Animals were handled in compliance with the Institutional Animal Care and Use Committees of Colorado State University, Ohio State University, and Brown University, and all procedures met United States Public Health Service Guidelines. Every effort was made to minimize the number of animals used and to mitigate any possible discomfort. Experiments were performed using both rat and mouse tissue. Rats were young (postnatal day 6–11) or adult (>3 months) males and females of the Sprague Dawley strain (Harlan Laboratories, Indianapolis, IN). For multielectrode array experiments on adult rat retinas, adult (>3 months) males and females of the Long-Evans strain were utilized (Charles River, Wilmington, MA). Mice were of the transgenic Tg(Opn4-EGFP)ND100Gsat/Mmucd strain, generated by the GENSAT project. These mice carry a bacterial artificial chromosome (BAC) in which the melanopsin (Opn4) promoter drives expression of enhanced green fluorescent protein (EGFP); for simplicity, they will be referred to here as *Opn4::EGFP* mice. Animals were kept on a 12 hr light:12 hr dark cycle, with lights on at 6:00 AM, and were fed standard chow and water *ad libitum*. Adult rats were anesthetized with 0.2ml sodium pentobarbital (i.p. injection) or isoflurane (inhalation) and euthanized by decapitation; postnatal day 6–11 (P6-P11) rats, and wildtype mice were anesthetized with isoflurane and euthanized via decapitation, *Opn4::EGFP* mice were euthanized with CO₂ asphyxiation or anesthetized with isoflurane and euthanized via decapitation.

Patch-clamp recording solutions—For investigation of ipRGC excitability in whole cell current-clamp experiments, a K-gluconate based internal solution was used. It contained (in mM) the following: 110 K-gluconate, 7 phosphocreatine-di(tris) salt, 10 L-ascorbic acid, 2 EGTA, 3 Mg-ATP, 0.5 Na-GTP, 20 KCl, 10 HEPES, pH 7.2 (adjusted with KOH) and osmolarity of 300 ± 5 mOsmol. For isolation of I_K in whole-cell voltage-clamp, 2 mM QX 314 was added to the above K-gluconate based internal solution to block I_{Na} with appropriate adjustments made to the solution to maintain constant osmolarity. For I_{Ca} recordings, a Cs-gluconate based internal solution was used that contained (in mM) the following: 100 Cs-gluconate, 10 phosphocreatine-di(tris) salt, 10 L-ascorbic acid, 2 EGTA, 3 Mg-ATP, 0.5 Na-GTP, 10 tetraethylammonium chloride, 0.1 CaCl₂, 10 NaCl, pH 7.2 (adjusted with CsOH) and osmolarity of 300 ± 5 mOsmol, and the extracellular solution was supplemented with 5 mM CaCl₂ (Hu et al., 2013). The standard extracellular / bathing solution was Ames' medium (US Biological), with osmolarity of 300 ± 10 mOsmol constantly gassed with 95% O₂ / 5% CO₂. [D-Ala², MePhe⁴, Gly-ol⁵]-enkephalin (DAMGO), H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP), H-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP), QX 314 and 4-Aminopyridine (4-AP) were obtained from Tocris Bioscience (Bristol, UK). Tetrodotoxin (TTX) obtained from Alomone Labs (Jerusalem, Israel). Other salts or chemicals were purchased from Sigma (St. Louis, MO).

Dissociated ipRGC preparation for loose patch recording—Eyes were enucleated and hemisected posterior to the limbus; the lens and vitreous humor were removed. Retinal neurons from *Opn4::EGFP* mice were dissociated using enzymatic digestion for 30 min at 37°C with 20 U/mL papain (Worthington, Lakewood, NJ), 1 mM L-Cysteine, B-27 (Invitrogen, Grand Island, NY), 0.5 mM GlutaMAX (Gibco, Grand Island, NY), and 0.004% DNase in Hibernate-A without calcium (BrainBits, Springfield, IL). The cells were centrifuged (3 min at 200g) then washed and gently triturated with Hibernate-A (with calcium) containing 10% (vol/vol) heat-inactivated fetal calf serum, 0.004% DNase and 0.5 mM GlutaMAX. Retinal ganglion cells (RGCs) were enriched by incubating with magnetic nanoparticles conjugated to antibodies towards the pan-RGC surface marker Thy1.2 and filtering the suspension through a 30µm Pre-Separation Filter and magnetic columns (Miltenyi Biotec, Auburn, CA). The eluted RGCs were then plated and cultured on coverslips for 18–64 hr as previously described (Van Hook et al., 2012) with culturing additives (Chen et al., 2008).

Dissociated ipRGC preparation for whole cell recording—IpRGCs were enzymatically dissociated from *Opn4::EGFP* mouse retina as previously describe (Meyer-franke et al., 1995; van Hook and Berson, 2010). In brief, eyes were enucleated and hemisected posterior to the limbus; the lens and vitreous humor were removed. Retinas were detached in dark from the retinal pigmented epithelium and incubated for 15 min at 37°C in a papain solution (10 U/ml, Worthington; Lakewood, NJ). After rinsing in a papain free solution, manual trituration was performed with a large-bore Pasteur pipette and dissociated cells were plated on poly-d-lysine/laminin coated coverslips (Corning™BioCoat™; Bedford, MA) followed by overnight incubation in MACS® NeuroMedium without L-Glutamine (Miltenyi Biotech; Auburn, CA). The medium was supplemented with MACS® NeuroBrew-21 as per the manufacturer's instructions, antibiotics (100 u/ml penicillin and 100 µg/ml streptomycin), ciliary neurotrophic factor (10 ng/ml; Sigma), brain-derived neurotrophic factor (25 ng/ml; Sigma), and forskolin (5 mM; Tocris; Ellisville, MO). Coverslips were transferred to a perfusion chamber mounted on an upright microscope (Akioskop 2 FS plus, Zeiss) and superfused at 2–5 ml/min with 300 ± 10 mOsmol bicarbonate buffered Ames' medium (US Biological; Swampscott, MA) constantly gassed with 95% O₂ / 5% CO₂. Coverslips were viewed through a 40X water immersion objective, infrared differential contrast, and an infrared CCD camera with 2.5 pre-magnification (XC-75; Sony, Japan) connected to a Camera Controller C2741–62 (Hamamatsu; Japan), which directed output to a 19" monitor (Westinghouse; Santa Fe Springs, CA). Dissociation yielded a mixture of retinal neurons from which M1 ipRGCs were identified based on their large size (~ 10 µm) and bright green fluorescence.

Multielectrode array recordings of opioid effects on ipRGC photoresponses—Retinas of P6-P10 rats were isolated from eye cups in bicarbonate buffered Ames' medium (A1372–25; US Biological, Swampscott, MA) supplemented with 0.1 mM EGTA (Sigma) and bubbled with 95% O₂ / 5% CO₂. A flat portion of the central retina not including the optic nerve head was placed with the ganglion cell layer down on a multielectrode array (60MEA200/30iR-ITO; Multi Channel Systems, Reutlingen, Germany) and was secured with nylon mesh and a wire weight. For all recordings, retinas were superfused with Ames'

medium constantly gassed with 95% O₂ /5% CO₂ at 37 °C. Synaptic inputs to ipRGCs were blocked by bath application of a cocktail of pharmacological agents in Ames' medium as previously described (Wong et al., 2007; Perez-Leighton et al., 2011). The cocktail contained 100 μM L-(+)-2-Amino-4-phosphonobutyric acid (L-AP4), 30 μM D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5) or 100 μM D-(-)-2-Amino-7-phosphonoheptanoic acid (D-AP7), 25 μM 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX), 50 μM picrotoxin, 5 μM [S-(R*,R*)]-[3-[[1-(3,4-Dichlorophenyl)ethyl]amino]-2-hydroxypropyl](cyclohexylmethyl) phosphinic acid (CGP54626), 50 μM (1,2,5,6-Tetrahydropyridin-4-yl) methylphosphinic acid (TPMPA), 10 μM strychnine, 10 μM atropine and 100 μM (+)-Tubocurarine chloride. Apart from the experiment shown in Fig 3Ai–Aiv, single doses (1 nM–10 μM) of DAMGO were bath applied with the synaptic blockers. Results were considered for further processing only if DAMGO-mediated (inhibitory) effects were reversed with 1μM–10μM of D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP, a MOR-selective antagonist). Apart from atropine and strychnine (Sigma), all pharmacological agents were obtained from Tocris (Ellisville, MO).

Full-field light stimuli were generated using a light-emitting diode (470 nm; Digikey, Thief River Falls, MN). The intensity of light pulses was set to 7.5×10^{14} photons cm⁻² s⁻¹ by a function generator (Berkley Nucleonics, CA) and calibrated by an optical power meter (Newport, model 1918-C). Retinas were dark adapted for at least one hour prior to initial light stimulation. Responses to 20 s flashes presented every 15 min were recorded, amplified, band-pass filtered between 500 Hz and 1.5 kHz, and digitized at 25 kHz using MCRack software (Multi Channel Systems). Spikes were isolated using a –4.5 standard deviation of noise threshold filter (MCRack software, MCS).

Adult rats were dark adapted for 1 h prior to enucleation, and the retinas were dissected under dim red light. Retinas were placed RGC-side down on multielectrode arrays as described above for the neonatal retinas. Prior to recordings, array-mounted retinas were stored in Hibernate-A medium plus 2% B-27 supplements (Life Technologies), and during recordings, the superfusing Ames' medium was buffered with 10 mM HEPES (pH 7.4) and constantly gassed with 100% O₂. The light stimulus (20 s duration) was generated by a blue LED source (470 nm, Colibri system, Zeiss, Germany) and delivered through a 40x objective on an upright microscope (Axio Examiner, Zeiss) in previous work (Sodhi and Hartwick, 2014). The irradiance of the 20 s light stimulus was 3.9×10^{15} photons cm⁻² s⁻¹ at 470 nm. After an initial light pulse to confirm retina viability, the array-mounted adult rat retinas were superfused with a cocktail of glutamatergic antagonists (100 μM L-AP4, 25 μM NBQX, 10 μM MK-801) to block rod/coned driven excitatory signaling.

Cluster analysis of the isolated spike data (obtained from both neonatal and adult rat retinas) was performed using Offline Sorter (Plexon Inc., Dallas, TX) in two consecutive steps (first using a T-distribution Expectation-Maximization algorithm followed by iterative K-means sorting) and then the number of spikes in 1 s bins were separated and counted using Neuroexplorer (Plexon Inc. Dallas, TX). Only cells with robust intrinsic light responses were used for further analysis; specifically, all analyzed cells produced at least twice as many spikes during the first 10 seconds of light stimulation as during the 10 seconds immediately

preceding the light stimulation (i.e., in darkness). Due to these relatively strict criteria, the cell sample was likely biased towards the selection of M1-type ipRGCs. Further information on the spike sorting and ipRGC identification criteria can be found in previous work (Sodhi and Hartwick, 2014, 2016).

Peristimulus time histograms for each channel (1 ms binwidth) were normalized to their maximum spike frequency, then pooled and averaged across channels to yield a light response for a given retina. The duration of the light response was defined as the time (in seconds) from light onset to the time when binned spike frequency fell below the prestimulus baseline. Using Graphpad Prism software, best-fit dose response curves were generated for DAMGO concentrations of 1 nM-10 μ M. We used the following two alternative output measures: the duration of light response and the average number of spikes during the 20 s light stimulus. Both measures were normalized to their control value, assessed under synaptic blocker cocktail (presented as mean $\pm$ SEM).

When testing the effect of DAMGO on ipRGC photoresponses, individual retinas were exposed to only a single concentration; concentrations across experiments ranged from 1 nM-10 μ M. Only ipRGCs showing recovery of photoresponses in the MOR antagonist CTOP were included in the analysis. We assessed the magnitude of the opioid effect from the duration of the light-evoked spike train spiking and the number of spikes fired during the light stimulation, both normalized to pre-drug control responses. Normalized data were then averaged across all recorded ipRGCs exposed to a given DAMGO dose to generate dose-response curves.

Whole cell voltage- and current-clamp recording from dissociated, solitary ipRGCs—A horizontal puller (model p-97, Sutter; Novato, CA) was used to pull patch pipettes of 5–15 M Ω from 1.5-mm-diameter, thick-walled borosilicate glass (World Precision Instruments; Sarasota, FL). The pipettes were subsequently coated with dental wax (Cavex; Netherlands) to minimize stray pipette capacitance. Whole-cell voltage- and current-clamp recordings were made from dissociated ipRGC somas using an EPC-10 USB patch-clamp amplifier and Patchmaster software (version 2.3; HEKA) at room temperature during daytime. Membrane current and voltage data were filtered at 3 kHz. Recordings with leak >50 pA at -70 mV holding potential and/or series resistance (R_s) >30 M Ω at any time during the recording were terminated and excluded from analysis. Similarly, if the leak or R_s changed more than 44% and 13%, respectively during the recording, data was not considered for further analysis (see Results for details). The holding current to set the holding potential at -70 mV at break in was determined in voltage-clamp mode and maintained via Patchmaster's "Gentle CC-switch" function in current-clamp mode. Membrane potential spikes were evoked from ipRGCs using a current-clamp ramp protocol that lasted 2 seconds and extended from -20 to 25 pA relative to the holding current injection required to introduce -70 mV membrane potential; the sampling rate was 20 kHz. Voltage-gated potassium current (I_K) was evoked by a voltage-clamp ramp protocol that lasted 2 seconds and extended from -100 to 50 mV (sampled at 5 kHz) or with 500 ms voltage-clamp steps between -120 mV and 50 mV in 5 mV increments, with a 5 second interval between each step (sampled at 50 kHz). Inactivating and non-inactivating portions of the total I_{Ca} were determined as previously described (Hu et al., 2013). In brief, ipRGCs

were held at -80 mV and subjected to a voltage-clamp step protocol consisting of 150 ms steps, from -90 mV to 30 mV in 10 mV increments, with 2 seconds between steps and a sampling rate of 50 kHz to obtain total I_{Ca} . To reveal the non-inactivating portion of I_{Ca} , cells were then held at -40 mV to apply steps from -40 mV to 30 mV in 10 mV increments, with 2 seconds between steps.

Loose patch recording of light responses of dissociated, solitary ipRGCs—

Following identification of an ipRGC by EGFP fluorescence, the cell was dark adapted for 10–30 minutes, and drugs were bath-applied 1–2 minutes prior to a 10 s light stimulus. White light stimuli (2.7×10^{15} photons $\text{cm}^{-2} \text{s}^{-1}$ at 500 nm) were generated by a 100 W tungsten-halogen lamp and blue light stimuli (10^{14} photons cm^{-2}/s at 470 nm) by a LED (Digikey, Thief River Falls, MN). To record light-evoked spiking of single ipRGCs at room temperature via loose patch, pipettes made of borosilicate glass were filled with extracellular solution.

Recording light-evoked responses from ipRGCs in whole-mount preparation—

Euthanasia of *Opn4::EGFP* mice and tissue preparation for whole-mount preparation were performed under infrared illumination. Both eyes were enucleated and retinas were detached from the retinal pigment epithelium and placed in Ames' medium gassed with 95% $\text{O}_2/5\%$ CO_2 at room temperature. A piece of retina was secured with a tissue anchor (harp) to the glass bottom of a superfusion chamber with the ganglion cell layer up. The retinas were visualized with an upright microscope (Axioskop; Zeiss) with a custom-built infrared LED (940 nm; Osram) light source through a $40\times$ water-immersion objective coupled to a $2.5\times$ magnification Optovar (Zeiss) and camera (AxioCam; Zeiss). The chamber sat in a light-tight Faraday cage and except during brief epifluorescence viewing (470 ± 20 nm) to locate EGFP-positive large, putative M1 type ipRGCs, the retina was maintained in darkness. In the presence of synaptic blocking cocktail (see *Multielectrode array recordings*) retinas were stimulated with full-field blue light (10^{14} photons $\text{cm}^{-2} \text{s}^{-1}$ at 470 nm) stimuli with an LED (Digikey, Thief River Falls, MN) positioned 3 cm above the preparation at a 30° angle. The LED voltage was controlled by the EPC-10 (HEKA Elektronik) through D/A output. Whole cell voltage- and current-clamp recordings were made from ipRGCs using an EPC-10 USB patch-clamp amplifier and Patchmaster software (version 2.3; HEKA) at room temperature as described for solitary ipRGCs above.

Data analysis—Data was analyzed off-line using IgorPro software (version 5.03; Wavemetrics), SigmaPlot (version 11; Systat Software), and Excel (Microsoft). Current-ramp-evoked spike threshold was defined as the membrane potential value at which the sharpest phase of the voltage-gated Na^+ influx-mediated depolarization started, and current threshold was defined as the injected current which correlated with the spike threshold (Hu et al., 2013). Current-clamp recordings were neither baseline-subtracted nor normalized. For I_K analysis, voltage-clamp ramp and step evoked I-V curves were leak subtracted and normalized to the peak (Tooker et al., 2013). Briefly, leak current, estimated from extrapolation of the slope of the line between -100 to -60 mV in voltage-clamp ramp experiments or the first 13 points i.e., from -120 to -60 mV in voltage-clamp step experiments, was subtracted from the raw recording to determine the actual I_K . The

normalized leak-subtracted ramp and step evoked I-V curves were then fit using SigmaPlot with the following third order sigmoidal equation:

$$I = a / (1 + \exp(-(V - V_{0.5})/b))$$

where $V_{0.5}$ is the half-activation potential, b is the slope of the voltage dependency, and a is the maximal I_K (constrained to 1 for normalized traces). I-V kinetic analysis was performed using SigmaPlot. Activation was defined as the voltage at which the resulting I_K was 5% of the peak ($V_{0.05}$) and half-activation as the voltage at which the resulting current was 50% of the peak ($V_{0.5}$). For I_{Ca} analysis, voltage-clamp step evoked I-V curves were generated from leak subtracted data (Hu et al., 2013; Tooker et al., 2013), with the first three points (−90, −80, −70 mV) used to estimate the leak current for extrapolation. The total I_{Ca} ($I_{Ca, total}$) was considered to be the I_{Ca} elicited by the step protocol applied to the cell held at −80 mV. The non-inactivating component of I_{Ca} ($I_{Ca, non-inact}$) was considered to be the I_{Ca} elicited by the step protocol applied to the cell held at −40 mV. The inactivating component of I_{Ca} ($I_{Ca, inact}$) was calculated as the difference between the total and the non-inactivating component ($I_{Ca, total} - I_{Ca, non-inact} = I_{Ca, inact}$). Liquid junction potential (LJP) was calculated as 13.05 mV for I_K and 13.1 mV for I_{Ca} recordings. All voltage-clamp recordings were a posteriori corrected for LJP.

Statistics were performed with SigmaPlot (version 11; Systat Software) and Excel (Microsoft). Paired and unpaired Student t-tests, Mann-Whitney Rank Sum tests, and one way ANOVA were used for comparisons between groups of paired traces. Data are presented as mean ± SEM and $p < 0.05$ considered significant.

Immunohistochemistry—Immunohistochemical procedures were conducted as previously described for retinal sections (Gallagher et al., 2010). In brief, animals were deeply anesthetized with isoflurane and decapitated before both eyes were enucleated. A small incision was made anterior to the ora serrata, and the whole eye was fixed at room temperature in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.35) for 15 min. The cornea and lens were removed and the eyecups left in the same fixative solution for an additional 5 min. Fixed eye cups were cryoprotected in 30% sucrose overnight, embedded in OCT (Ted Pella Inc.) and cut into 20 µm thick vertical sections. Sections were mounted on glass slides and stored frozen until immunostained. The melanopsin immunolabeling was done according to a previously described protocol (Van Hook et al., 2012); primary antibody: c26962, 1:50; Santa Cruz Biotechnology, Santa Cruz, CA). Methods for anti-MOR immunostaining (AOR-011, 1:200; Alomone Labs, Jerusalem, Israel) were also described previously (Gallagher et al., 2012). Retinal sections from *Opn4::EGFP* mice in some cases were also colabeled with an anti-GFP antibody (ab13970, 1:500; Abcam, Cambridge, MA). Fluorescent images were taken with a Zeiss LSM 800 confocal microscope (Carl Zeiss, Oberkochen, Germany). For all acquisitions, sequential scans at the different wavelengths were performed. Z-stack images through the full thickness of immunolabeled tissues were taken at 40x, with 1 µm increments between images. Brightness and contrast of images were adjusted uniformly in Photoshop CS3 (Adobe 10.1). Images were compiled and analyzed using Zeiss LSM Image Examiner software (Carl Zeiss,

Oberkochen, Germany). Subjective assessment of fluorescent signal colocalization was performed on single plane optical sections.

Results

ipRGCs express μ -opioid receptors in rat and mouse retinas

We detected immunoreactivity for μ -opioid receptors (MORs) in ipRGCs of both rats and mice. In adult rats ($n=3$), substantial anti-MOR immunolabeling marked the inner retina (Fig. 1A). The labeling pattern resembled that observed previously in mice (Gallagher et al., 2012), but in the rat retina, labeling of the inner plexiform layer (IPL) was more robust, with discernible MOR+ processes. The anti-melanopsin antibody strongly labeled ipRGCs of the M1 type, with dendrites extending into the outermost layer of the IPL (Fig. 1B, white arrow). The M2/M3 types were also identified as more weakly immunolabeled cells of the ganglion-cell layer, often with dendrites extending into the innermost layer of the IPL (Fig. 1F, white arrow). Melanopsin+ dendrites were invariably MOR immunoreactive (Fig. 1, white arrow, 28/28 dendrites from 3 animals). Melanopsin+ cell bodies were also typically labeled by the MOR antibody, although usually more weakly than the dendrites (Fig. 1: hollow arrowhead, insets).

To evaluate MOR expression in mouse ipRGCs, we used retinas from adult *Opn4::EGFP* melanopsin reporter mice, generated by the GENSAT project ($n=3$). In other melanopsin reporter mice generated by using BAC technology (Schmidt et al., 2008; Do et al., 2009), only ipRGCs of the M1, M2 and M3 types express detectable levels of the fluorescent reporter evidenced by the high coincidence of transgenic reporter protein and melanopsin immunolabeling (Lee and Schmidt, 2018); similarly, in the *Opn4::EGFP* mice, we found that 173 of 182 EGFP-expressing cells were also melanopsin-positive. M1 cells are easily distinguished from the other types by their brighter fluorescence and dendritic arborizations in the outer IPL (Fig. 2B). The pattern of MOR immunolabeling resembled that previously reported in wild type mice (Gallagher et al., 2012), with heaviest MOR immunolabeling occurring in a minority of somata in the inner nuclear and ganglion-cell layers (INL and GCL), as well as puncta and some dendritic profiles in the IPL. Nearly all EGFP+ somas of ipRGCs were strongly MOR immunopositive (173/182 cells from 3 animals; Fig. 2). The double labeled cells included M1 ipRGCs (including ‘displaced’ M1s, with somata in the INL), M2 cells (characterized by weak EGFP fluorescence and processes in the inner IPL), as well as M3 cells with bistratified dendrites occupying the same layers as M1 and M2 cells (Schmidt et al., 2011) (Fig. 2H, hollow arrowhead and white arrow, respectively). Importantly, ipRGCs dissociated enzymatically from the *Opn4::EGFP* mouse retina showed positive immunolabeling with the anti-MOR antibody, suggesting MOR expression (Fig. 2I–M).

Multielectrode array recordings reveal dose-dependent μ -opioid attenuation of light responses in ipRGCs

To test whether MOR activation affects ipRGC signaling, we recorded light-evoked spiking of ipRGCs in early postnatal rat retinas (P6–P11) on a multielectrode array (MEA). A drug cocktail blocked all major retinal neurotransmitters (GABA, glycine, acetylcholine and both

ionotropic and metabotropic glutamate receptors (Wong et al., 2007; Perez-Leighton et al., 2011; Sodhi and Hartwick 2016, see Experimental Procedures). Synaptogenesis is incomplete in rat retinas at this young age (P6–11) (Sernagor et al., 2001), further minimizing any influence of synaptic inputs on ipRGCs in these studies.

Intrinsic photoresponses of ipRGCs were clearly modulated by bath application of the MOR-specific opioid antagonist DAMGO. Figure 3Ai shows the intrinsic light responses of a representative ipRGC recorded under synaptic blockade. In control medium (top), spiking remained elevated through the full stimulus duration (20 s) and persisted for many seconds after stimulus offset. This is as expected for intrinsic responses derived from melanopsin phototransduction (Emanuel and Do, 2015). The MOR-specific agonist DAMGO shortened the duration of the light response in a dose-dependent manner (Fig. 3Ai, 3Aii; doses: 10 nM, 100 nM, 1 μ M and 10 μ M). Even the lowest dose (10 nM) significantly reduced the number of light-evoked spikes ($n=43$ cells from 3 retinas, ANOVA, $p<0.05$), and the effect appeared to saturate because increasing the concentration from 1 μ M to 10 μ M did not further reduce the number of spikes (ANOVA, $p=0.72$). Subsequent application of the MOR-selective antagonist CTOP (10 μ M) not only restored the intrinsic light responses but actually increased the number of spikes compared to the control (Fig. 3Ai, 3Aii). This increase, though slight, was significant (ANOVA, $p<0.05$). Thus, the reduction of ipRGC in response to increasing DAMGO concentrations was not due to rundown.

A second functional effect of DAMGO application was to delay the onset of light-evoked spiking in ipRGCs (Fig. 3Aiii, 3Aiv). Group data revealed no clear dose dependence of this effect (Fig. 3Aiv), and dose-response curves for individual cells were highly variable (Fig. 3Aiii). Nonetheless, for the population of ipRGCs ($n=43$ cells from 3 retinas) DAMGO significantly increased the delay to the first spike (Fig. 3Aiv). The MOR antagonist CTOP (10 μ M) reversed the opioid-induced delay to levels statistically indistinguishable from the initial control response (ANOVA, $p=0.96$).

Importantly, the robust effects on spiking of DAMGO (1 μ M; Fig. 3Ai) were abolished by simultaneous application of CTOP (10 μ M) (Fig. 3B; $n=60$ from 2 retinas). Application of CTOP alone (1 μ M and 10 μ M) did not alter the light responses of ipRGCs (Fig. 3C; $n=52$ from 3 retinas).

These results collectively suggest a dose-dependent and MOR-specific opioid modulation of ipRGC photoresponses. Because these effects may have been distorted by opioid receptor desensitization during prolonged agonist exposures (Kelly et al., 2009; Dang and Christie, 2012; Williams et al., 2013), we constructed dose-response curves for MOR-mediated inhibition of ipRGC photoresponses (see Experimental Procedures) (Fig. 3Di, Dii). The dose-response relationships revealed IC_{50} values of 23 nM for the duration index (Fig. 3Di) and 39 nM for the spike-count measure (Fig. 3Dii) with saturation occurring at ~ 1 μ M in both cases. At saturating DAMGO concentrations, the suppression of the spike-count ($\sim 70\%$) was greater than that for the decrease in response duration ($\sim 50\%$). Regardless, both outcome measures indicated that the intrinsic light response of ipRGCs in the isolated juvenile rat retina was highly sensitive to selective activation of MORs.

To determine whether similar MOR-mediated ipRGC modulation was present in more developed retinas with fully functional retinal circuit wiring, we assessed the effects of DAMGO on MEA-mounted retinas from adult (> 3 month old) rats. A saturating dose (10 μ M; see Fig. 3Di, 3Dii) of DAMGO was chosen for these experiments. IpRGCs were identified by their sustained spiking responses to a bright blue light stimulus in the presence glutamate receptor antagonists (Fig. 4A). As in previous work (Sodhi and Hartwick, 2014), the number of ipRGCs recorded per retina decreased in the adult rat retinas, as compared to neonatal, and this is likely influenced by the relatively strict criteria for ipRGC identification (see Methods) and the potential persistence of vitreal strands. DAMGO significantly reduced both peak spike rate and response duration of the light response relative to control (n=6 from N=5 retinas; Fig. 4B; p=0.03, one way repeated measures ANOVA, Holm-Sidak post-hoc) (Fig. 4C). After 40 min of drug washout, responses exhibited partial recovery (Fig. 4B, 4C), so that the light-evoked spike count was no longer significantly different from that measured for the initial control response (p=0.10). Thus, the effect of DAMGO on ipRGC light responses is not restricted to early development. This is consistent with our immunohistochemical evidence demonstrating MOR localization to ipRGC dendrites in adult rat retinas (Fig. 1).

Loose-patch recordings from dissociated ipRGCs confirm direct modulation by μ -opioid receptors

Though retinal cells other than ipRGCs do express MORs (Gallagher et al., 2012), the DAMGO effects on ipRGCs we observed occurred during blockade of fast neurotransmitters in our experiments. This suggests that the observed effects were likely due to direct action on MORs expressed by ipRGCs themselves. As a more stringent test of this interpretation, we made loose patch recordings from isolated ipRGCs, which have been shown to maintain their light sensitivity in primary culture (Hartwick et al., 2007; van Hook et al., 2012). We enzymatically dissociated retinas from *Opn4::EGFP* mice and targeted the largest and brightest EGFP+ cells (presumably corresponding to M1 ipRGCs) for loose-patch single cell recordings (Fig. 5A). Bath application of DAMGO (1 μ M) diminished light-evoked spiking in isolated ipRGCs (5/5 cells), as shown for a representative cell in Fig. 5B. In this cell, the light response partially recovered following a washout of DAMGO (“recovery”) but in most cases (3/5) no recovery was observed before deterioration of the loose patch during the long wash. It is important to note that recovery of ipRGC light responses from intact retinas recorded on the MEA, following DAMGO treatment, was not complete after tens of minutes of wash without subsequent application of a MOR antagonist, which is consistent with the recovery paradigm used in other neural preparations following DAMGO application (Pennock and Hentges, 2011; Qu et al., 2015). Furthermore, phototransduction of ipRGCs at room temperature has been found weaker than at 37 °C (Do et al., 2012) which might explain the more robust DAMGO-mediated inhibition of light responses in the loose patch experiments at room temperature compared to the results of MEA experiments that were performed at 37 °C. Nonetheless, as for the earlier MEA experiments, co-application of the MOR antagonist CTOP (1 μ M) blocked the effects of DAMGO on the light responses of solitary, cultured ipRGCs (n=3) (Fig. 5C).

MOR signaling reduces excitability of ipRGCs

The sequence of depolarizing events along with the ion channels that mediate the characteristically sluggish but sustained intrinsic light responses of melanopsin-expressing ipRGCs have not been fully identified, but evidence supports the involvement of TRP channels, voltage-gated sodium currents (I_{Na}), and voltage-gated calcium currents (I_{Ca}) (Warren et al., 2006; Hartwick et al., 2007; Xue et al., 2011). A set of voltage-gated and calcium-dependent potassium currents (I_K and $I_{K(Ca)}$, respectively) are also critical to repolarizing the membrane potential of ipRGCs after each spike (Hu et al., 2013).

To determine how MOR activation reduces light responses of ipRGCs, first we recorded melanopsin-driven light responses from ipRGCs in whole mount preparation, bathed in Ames' medium that was supplemented with the synaptic blocking cocktail in the presence of 2 mM Co^{2+} to block I_{Ca} ; the recording pipette solution contained 2 mM QX 314 to eliminate I_{Na} (Fig. 6). QX 314 at this concentration is expected to slightly inhibit I_{Ca} (Talbot and Sayer, 1996), acting in concert with Co^{2+} in these experiments. Under these conditions DAMGO (1 μ M) altered neither the light-induced inward current in voltage-clamp recordings (Fig. 6A; $V_{hold} = -60$ mV) nor the light-evoked membrane depolarization (Fig. 6B) of the same ipRGCs ($n=3$; V_m set by current injection at -60 mV). These results indicate that in ipRGCs MOR activation does not affect melanopsin-dependent phototransduction including the photocurrent flowing through TRP channels, unlike in sensory neurons where MOR activation reduces TRPV1 currents (Bao et al., 2015).

Therefore, we next tested whether MOR activation altered spiking of ipRGCs depolarized by current injections in whole mount preparations that were bathed in Ames media supplemented with the synaptic blocking cocktail. IpRGCs held at ~ -70 mV resting potential in current clamp by injecting -70 pA holding current were subjected to a depolarizing current ramp from -70 pA to -20 pA over 2 s (see Experimental Procedures). A representative recording is shown in Fig. 7Ai. In this cell, the depolarizing current ramp evoked the first spike with 0.68 ± 0.04 s delay ($n=15$ trials) in control, but the delay increased to 0.87 ± 0.03 s ($n=15$ trials) after 3 min in the presence of 1 μ M DAMGO ($p < 0.0004$; Student t-test). In other words, DAMGO increased the current threshold of action potential generation in this ipRGC (Fig. 7Aii) from 15.07 ± 0.98 pA (relative to the holding current maintaining the membrane potential at -70 mV) in control to 20.82 ± 1.08 pA ($n=15$; $p < 0.007$, Student t-test) but did not alter the membrane potential threshold (Fig. 7Aiii) for action potential generation (-54.02 ± 3.59 mV vs. -54.09 ± 3.62 mV, in control and DAMGO, respectively). Importantly, DAMGO application did not cause a significant change in the resting membrane potential measured just before the depolarizing ramp (-79.47 ± 1.14 mV vs. -76.76 ± 0.87 mV, in control and DAMGO, respectively; $p=0.07$, Student t-test). Similar results were obtained from two other intact ipRGCs in whole mount preparation. To confirm that DAMGO exerted its effect on the excitability of ipRGCs directly, via MORs expressed by ipRGCs, we turned to solitary ipRGCs enzymatically dissociated from the *Opn4::EGFP* mouse retina. In solitary, dissociated ipRGCs, as in intact retina, DAMGO (1 μ M) consistently increased the delay of the first spike evoked by a depolarizing current ramp (Fig. 7Bi) by increasing the current threshold of action potential generation (Fig. 7Bii) from 3.95 ± 1.05 pA (relative to the holding current injected to

maintain the membrane potential at -70 mV) in control to 6.02 ± 1.20 pA ($n=11$, paired Student t-test, $p < 0.004$) (Fig. 7Ci) without affecting the membrane potential threshold for spike generation (Fig. 7Cii) that was -52.06 ± 0.56 mV in control and -52.39 ± 0.66 mV in DAMGO ($n=11$; $p = 0.37$, paired Student t-test). Importantly, DAMGO did not alter the holding current injected into ipRGCs to maintain their resting V_m at -70 mV (Fig. 7Ciii), indicating that in ipRGCs DAMGO did not activate G-protein activated inward rectifier K^+ currents (GIRK) (Pennock and Hentges, 2011) that are widely distributed effectors of MOR signaling in the CNS (Williams et al., 2001, 2013).

In parallel experiments, pretreatment of solitary ipRGCs with the MOR selective antagonist CTAP ($1 \mu\text{M}$) did not alter current threshold for depolarizing current ramp-evoked action potentials (2.18 ± 0.6 pA) compared to control (3.39 ± 0.86 pA) or to that seen during the consecutive application of CTAP+DAMGO ($1 \mu\text{M}$ each) (2.37 ± 0.66 pA) ($n=5-8$, $p=0.19$, one way repeated measures ANOVA, data not shown). Similarly, the membrane potential threshold of depolarizing ramp-evoked action potential firing did not change in consecutive treatments with CTAP and CTAP+DAMGO (control: -50.28 ± 1.12 mV; CTAP: -50.32 ± 0.93 mV; CTAP + DAMGO: -50.61 ± 0.89 mV, $n=5-8$, $p=0.43$, one way repeated measures ANOVA, data not shown).

Effectors of MOR signaling in ipRGCs

The above results collectively suggested that MOR signaling altered the excitability of ipRGCs without interfering with the melanopsin-mediated signal transduction, TRP channel function, or by opening GIRK channels. Furthermore, the fact that DAMGO did not alter the membrane potential threshold for spike generation indicated that I_{Na} in ipRGCs is not modulated upon MOR activation; this is consistent with the lack of evidence for I_{Na} being an effector of MOR signaling-evoked neuronal responses.

To test whether MOR activation affects voltage-gated potassium currents (I_K) of enzymatically dissociated ipRGCs, I_K was isolated in the presence of 2 mM Co^{2+} in the bath solution to eliminate I_{Ca} and by using a recording pipette solution containing 2 mM QX 314 to eliminate I_{Na} . I_K was then evoked in voltage-clamp using both depolarizing voltage steps and depolarizing ramp protocols (see Experimental Procedures). DAMGO ($1 \mu\text{M}$) shifted activation ($V_{0.05}$) of I_K to more negative potentials regardless of the voltage-clamp protocol (i.e., sequential steps or continuous ramp). When depolarizing ramps were used, 6–10 minutes of DAMGO ($1 \mu\text{M}$) application reduced the activation threshold ($V_{0.05}$) of I_K (Fig. 8Ai) from -39.44 ± 2.85 mV in control to -51.43 ± 3.36 mV ($n=10$, $p < 0.001$, paired Student t test) as well as the half activation potential ($V_{0.5}$) from -6.14 ± 2.28 mV in control to -12.30 ± 1.93 mV in DAMGO ($n=10$, $p < 0.001$, paired Student t test) (Fig. 8Aii). The ramp evoked I_K activation steepness, defined as the slope of the sigmoidal fit to I-V curve (b), was not significantly altered by DAMGO (13.02 ± 0.95) relative to control (11.26 ± 0.67 , $n = 10$, $p = 0.101$, paired t test, data not shown). Similar results were obtained when I_K was activated by a voltage step protocol (see Experimental Procedures). Namely, the activation threshold of step-evoked I_K ($V_{0.05}$: -40.63 ± 0.92 mV) was significantly lowered by DAMGO ($V_{0.05}$: -48.51 ± 1.22 mV, $n=10$, $p < 0.001$, paired Student t test, data not shown) along with the half activation potential ($V_{0.5}$ of -8.24 ± 1.07 mV in control to -12.16 ± 1.31

mV in DAMGO, $n=10$, $p=0.003$, data not shown). We found no statistical difference between the ramp-evoked versus step-evoked I_K parameters ($V_{0.05}$, $V_{0.5}$ and b) in similar conditions (i.e. in control or in DAMGO, respectively; $p=0.04$ – 0.95 , Mann-Whitney Rank Sum test).

To make sure that the shift in I_K kinetics was due to MOR activation, we again performed a parallel series of experiments in which CTAP (5 μ M) was applied for at least 2 min prior to concurrent application of both DAMGO (1 μ M) and CTAP (5 μ M) for at least 3 min. Neither treatment with CTAP alone, nor consecutive application of CTAP+DAMGO together altered the depolarizing ramp-evoked I_K activation parameters (Fig. 8Bi) in ipRGCs ($n=8$) ($V_{0.05}$ control: -37.31 ± 2.28 mV, $V_{0.05}$ CTAP: -41.30 ± 3.27 mV, $V_{0.05}$ CTAP+DAMGO: -36.11 ± 4.71 mV, $p=0.47$; $V_{0.5}$ control: -6.57 ± 1.78 mV, $V_{0.5}$ CTAP: -5.32 ± 1.82 mV, $V_{0.5}$ CTAP+DAMGO: -6.61 ± 2.12 mV, $p=0.28$; b control: 10.74 ± 0.59 , b CTAP: 12.13 ± 0.78 , b CTAP + DAMGO: 10.01 ± 1.63 , $p = 0.39$; one way repeated measures ANOVA) (Fig. 8Bii).

In addition, we explored the possibility whether the rundown of I_K in ipRGCs could artificially cause a negative shift of the activation curve (DiFrancesco et al., 1986) although there was no appreciable loss of I_K amplitude after DAMGO application (Fig. 8Ai). To test this notion we held the dissociated ipRGCs in whole-cell voltage-clamp as long as the amplitude of I_K started to decay, up to 10 min; no significant difference was found for any of the measured I_K kinetic parameters between the first (control) trace obtained within seconds of patch break and the latest (“second”) trace without amplitude rundown ($V_{0.05}$: -39.95 ± 1.23 mV vs. -40.35 ± 1.14 mV, $p = 0.18$; $V_{0.5}$: -7.76 ± 1.25 vs. -7.59 ± 1.38 , $p = 0.74$; b : 10.38 ± 0.40 vs. 10.25 ± 1.06 mV, $p = 0.72$; for control and second traces, respectively, $n = 8$, paired Student t test, data not shown). We also considered the possibility that small uncompensated increases in inter-trace series resistance (R_s) could result in hyperpolarizing shifts of $V_{0.05}$ and $V_{0.5}$ between control and DAMGO treated traces (Armstrong and Gilly, 1992). We tested and found that the presence or absence of automatic R_s compensation up to $54.83\% \pm 2.42$ ($n=13$) did not cause a significant difference between the first, uncompensated control trace and the second, R_s compensated trace for any of the measured I_K kinetic parameters ($V_{0.05}$: -41.45 ± 1.87 mV vs. -42.17 ± 1.42 mV, $p = 0.38$; $V_{0.5}$: -10.71 ± 0.94 mV vs. -12.74 ± 1.12 mV, $p = 0.002$; b : 10.42 ± 0.43 vs. 9.91 ± 0.34 , $p = 0.15$ for control and R_s compensated traces, respectively, paired Student t test, data not shown) for the recordings falling within the range of acceptable R_s (< 30 M Ω , see Experimental Procedures). With the small, round, electronically compact soma of dissociated ipRGCs that lack processes and the gradual activation kinetics of I_K , it is likely that these small ($< 13\%$), uncompensated increases in R_s did not cause significant shifts in $V_{0.05}$ and $V_{0.5}$. Notably, a leak increase of up to 44% did not affect measured I_K kinetic parameters in these parallel experiments and these cut-offs were accordingly imposed on recordings chosen for analysis across experiments (see Experimental Procedures).

The activation properties of I_K , namely the $V_{0.05}$ of ~ -40 mV in control, suggested that the voltage-gated potassium channels expressed by ipRGCs might belong to the K_v1 or perhaps to the K_v4 family (Grissmer et al., 1994; Cox, 2005). To investigate the identity of K_v gene product(s) that might be responsible for mediating the DAMGO effect in ipRGCs, we exploited the differences in efficacy of I_K inhibition by two broad-based K^+ channel

blockers, 4-aminopyridine (4-AP) and tetraethyl ammonium (TEA). Namely, K_v4 family members are inhibited by 4-AP only at 5 mM or higher concentrations, whereas K_v1 channels are blocked by 2 mM 4-AP (Grissmer et al., 1994; Cox, 2005). We found that 2 mM 4-AP not only markedly reduced I_K in ipRGCs (Fig. 8Ci), but 4-AP prevented significant shift of the I_K activation to more negative potentials by DAMGO (1 μ M) ($V_{0.05}$ 4-AP: -37.31 ± 2.28 mV, $V_{0.05}$ 4-AP+DAMGO: -41.30 ± 3.27 mV, $n=7$, $p=0.33$) (Fig. 8Cii). The action of 4-AP in blocking the DAMGO-sensitive I_K component in ipRGCs supports the premise that K_v1 family members mediate the DAMGO-sensitive I_K component in ipRGCs. We also found that 10 mM TEA eliminated I_K in ipRGCs (data not shown). Importantly, K_v4 channels, as well as most K_v1 channels, are resistant to TEA of ~10 mM concentration (Jerng et al., 2004), except $K_v1.1$, which is inhibited by TEA with an IC_{50} of ~0.3 mM (Grissmer et al., 1994; Gutman et al., 2005). In our hands, 1 mM TEA reduced I_K in dissociated ipRGCs (Fig. 8Di) and also markedly reduced the potential of DAMGO (1 μ M) to shift the activation to hyperpolarized potentials ($V_{0.05}$ TEA: -42.60 ± 2.54 mV, $V_{0.05}$ TEA +DAMGO: -44.45 ± 2.42 mV, $n=5$, $p=0.61$) (Fig. 8Di, 8Dii). Taken together, the pharmacological and biophysical data together strongly implicate $K_v1.1$ channels as the effector of MOR signaling that mediate DAMGO effects in ipRGCs.

Next we tested if MOR activation affects voltage-gated Ca^{2+} currents (I_{Ca}) (Kieffer, 1995) in ipRGCs (Hartwick et al., 2007; Hu et al., 2013). I_{Ca} in voltage-clamped solitary ipRGCs was recorded in the presence of 5 mM extracellular Ca^{2+} (Hu et al., 2013) using cesium-based pipette solution (see Experimental Procedures). Inactivating and non-inactivating components of I_{Ca} in ipRGCs were separated according to Hu et al. (2013): Total I_{Ca} ($I_{Ca, total}$) was obtained with depolarizing steps from -80 mV holding potentials (Fig. 9A). The non-inactivating I_{Ca} ($I_{Ca, non-inact}$) component was recorded in response to depolarizing voltage-steps from the holding potential of -40 mV (Fig. 9B). Peak $I_{Ca, non-inact}$ values were subtracted from the peak values of $I_{Ca, total}$ at corresponding step potentials to calculate the inactivating portion of I_{Ca} ($I_{Ca, inact}$) in ipRGCs (Fig. 9C). These were lengthy experiments, and we often found I_{Ca} run down well before the desired 3–5 min DAMGO application following the acquisition of control data. Therefore, I_{Ca} recordings in control ($n=26$) and DAMGO ($n=6$) were not performed on the same cells. Our results show that in the presence of DAMGO (1 μ M) the current density of $I_{Ca, total}$ was significantly smaller than that in control ($p=0.01$, two way ANOVA) (Fig. 9A). Similarly, the non-inactivating component ($I_{Ca, non-inact}$) was significantly reduced in DAMGO ($p=0.01$, two way ANOVA) (Fig. 9B) but not the calculated $I_{Ca, inact}$ ($p=0.43$, two way ANOVA) (Fig. 9C). Together, these results suggest that voltage-gated Ca^{2+} channels mediating the non-inactivating component of I_{Ca} in ipRGCs are also subject to opioid modulation upon MOR activation.

Discussion

We have previously shown β -endorphin and MOR expression in the mouse retina (Gallagher et al., 2010, 2012). Here we present convergent evidence that: (1) ipRGCs in both mouse and rat retinas express MORs; (2) the activation of MORs on ipRGCs results in the suppression of light responses by (3) increasing the delay of the first light-evoked spike as well as by reducing the duration of the spike train through a (4) shift in the activation of K_v1 channels to hyperpolarized membrane potentials and (5) inhibition of the non-inactivating

component of voltage-gated I_{Ca} . In addition to being observed in both mouse and rat, the MOR-mediated effect was present in young animals (P6–10 rats) as well as in adults (rats and *Opn4::EGFP* mice). Whether opioid modulation of ipRGCs has a fully conserved role at distinct time-points during development and adulthood remains to be examined. Interestingly, the MOR mediated physiological effect was shown in rat at a developmental time-point in which ipRGCs can modulate retinal wave activity and development of the visual system (Renna et al., 2011).

MOR activation and downstream modulation of Ca_v and K_v channels

Voltage-gated calcium (Ca_v) channels are activated downstream of TRP and I_{Na} in ipRGCs during light-evoked signaling (Hartwick et al., 2007), and they are thought to contribute to sustained firing of ipRGCs that characteristically outlasts the duration of stimulation: indeed, blocking I_{Ca} resulted in reduced spiking upon light stimulation (Berson et al., 2002). MOR activation in ipRGCs caused dose-dependent reduction of the duration of light-evoked ipRGC signaling (Fig. 3Ai, 3Aii) that is consistent with the observation that the non-inactivating component of I_{Ca} in ipRGCs (Hu et al., 2013) was inhibited by DAMGO (Fig. 9). MOR activation can result in the activation of multiple downstream pathways, including G-protein-dependent and -independent ones (reviewed by Williams et al., 2013). Furthermore, some effectors are directly coupled to MORs, such as the G protein-gated inwardly rectifying potassium [GIRK, GIRK isoform (Kir3)] channels, in which case the amplitude of GIRK is proportional to the MOR activation by a given agonist (i.e. dose-dependent) (Pennock and Hentges, 2011). Similarly, many types of Ca_v channels have been shown to be inhibited directly by G proteins where, upon activation of various G protein-coupled receptors, in a dose-dependent manner the $G\beta\gamma$ dimer binds to Ca_v channels to inhibit I_{Ca} (reviewed by Proft and Weiss, 2015). It is noteworthy, however, that activation of somatic MOR in hypothalamic proopiomelanocortin (POMC) neurons leads to inhibition of I_{Ca} and activation of GIRK, with apparently distinct MOR reserves for the separate process (Fox and Hentges, 2017). In our experiments, we isolated I_K by blocking voltage-gated I_{Ca} with Co^{2+} . This pharmacological manipulation has been shown to eliminate the calcium-dependent potassium currents ($I_{K(Ca)}$) (Solessio et al., 2002), therefore the DAMGO-mediated changes of I_K in our hands could not include a potential DAMGO-mediated increase in $I_{K(Ca)}$ at ~ -50 mV. Nonetheless, the fact that MOR-mediated analgesic effects were not sensitive to $I_{K(Ca)}$ blockers such as apamin or charybdotoxin (Welch and Dunlow, 1993; Ocaña et al., 2004), suggests that a direct interaction between MOR signaling and $I_{K(Ca)}$ is unlikely.

The I_K that we identified to be modulated via MOR activation by DAMGO in ipRGCs was blocked by 1 mM TEA or by 2 mM 4-AP, making $K_v1.1$ the most plausible candidate (Grissmer et al., 1994; Cox, 2005; Gutman et al., 2005). However, it has been shown that $K_v1.1$ channels are capable of heterotetramerization *in vivo*, often with $K_v1.2$ and that TEA sensitivities as well as half activation values of these $K_v1.1$ and $K_v1.2$ heterotetramers can vary depending on both subunit composition and arrangement (Wang et al., 1993). The IC_{50} of TEA for a $K_v1.1$ homotetramer ranges from 0.47 mM to 0.67 mM, for a $K_v1.2$ homotetramer ranges from 47 mM to 50 mM, and for a $K_v1.1$ and $K_v1.2$ heterotetramer ranges from 0.8 mM to 10 mM, depending on subunit arrangement. As well, while the

activation threshold of $K_v1.1$ homotetramers has been reported near -50 mV and that of $K_v1.2$ near -40 mV, varying spatial arrangements of 2:2 $K_v1.1:K_v1.2$ heterotetramers in heterologous systems can alter measured half activation of I_K by ~ 5 mV (Al-Sabi et al. 2010; Kew and Davis, 2010). Given our pharmacological data, it seems most likely that the DAMGO sensitive channel in ipRGCs is a $K_v1.1$ and $K_v1.2$ heterotetramer. While a $K_v1.1$ homotetramer cannot be entirely ruled out based strictly upon TEA affinity, it would seem less likely given the reported activation threshold of near -50 mV (Kew and Davis, 2010).

Of particular relevance to this study, $K_v1.1$ and $K_v1.2$ have been shown to form heterotetramers *in vivo* (Wang et al., 1993; Shamotienko et al., 1997; Coleman et al., 1999), and opioid induced negative regulation of GABAergic tone of basolateral amygdala (BLA) output neurons occurs through modulation of pre-synaptic $K_v1.1$ and $K_v1.2$ channels (Finnegan et al., 2006). Dendrotoxin-K and tityustoxin-K α , purported to be $K_v1.1$ and $K_v1.2$ specific blockers, respectively, each blocked the inhibitory effects of $1 \mu\text{M}$ DAMGO on miniature inhibitory postsynaptic currents (mIPSCs) in the BLA, leading the authors to suspect that BLA $K_v1.1$ and $K_v1.2$ form heteromeric complexes. $K_v1.1$ and $K_v1.2$ are important determinants of cellular excitability (Smart et al., 1998; Glazebrook et al., 2002; Brew et al., 2003, 2007) and as such are key players in nociceptive pathways and their modulation by opioid signaling (Clark and Tempel, 1998; Galeotti et al., 1999; Finnegan et al., 2006). For example, mice with an antisense oligonucleotide on the $K_v1.1$ gene lack morphine and baclofen-induced antinociception (Galeotti et al., 1997), and $K_v1.1$ null mice have reduced morphine-induced antinociception (Clark and Tempel, 1998). In a sense, $K_v1.2$ provides for increased neuronal excitability, and $K_v1.1$ provides for negative regulation of that excitability; adjustments of the $K_v1.1:K_v1.2$ stoichiometric balance may represent a precise, real-time method for down-regulation of neuronal excitability (Brew et al., 2007). Another mechanism of $K_v1.2$ subunit containing channel modulation by opioids could involve $K_v\beta$ subunit modulation of I_K activation. Coexpression of $K_v1.5$ and $K_v\beta2.1$ in heterologous systems results in a 10 mV hyperpolarizing shift in $V_{0.05}$ without alteration of I_K amplitude as seen in our experiments (Fig. 8Ai), with phosphorylation of $K_v\beta2.1$ postulated to rapidly regulate its interaction with the α subunit (Uebele et al., 1996). $K_v\beta2$ is the predominant subunit isoform present in the brain, and it has additionally been shown to positively regulate $K_v\beta2/K_v1.2$ complex stability and $K_v1.2$ surface expression (Shi et al., 1996).

MOR activation and consequent $G_{\alpha i/o}$ signaling might be coupled to the effectors through enzymatic steps: for example, in pyramidal neurons of the lateral amygdala, activation of the PLA2/arachidonic acid/12-lipoxygenase cascade with morphine and DAMGO enhances spike frequency adaptation, which involves shifting the voltage dependence of K_v channels containing $K_v1.2$ subunits to more negative potentials by ~ 14 mV (Faber and Sah, 2004). Furthermore, extensive literature documents G protein coupled receptor - mediated changes in $K_v1.1$ and $K_v1.2$ surface expression via clathrin-dependent endocytotic mechanisms (Bosma et al. 1993; Cachero et al. 1998; Connors et al. 2008; Hattan et al. 2002; Huang et al. 1993; Nesti et al. 2004; Stirling et al. 2009; Williams et al. 2007, 2012). In addition, MOR activation results in decreased adenylyl cyclase (AC) activity and thus cAMP levels and PKA activity; to that end, $K_v1.2$ is affected by cAMP levels, with elevation of cAMP increasing $K_v1.2$ surface expression and low cAMP decreasing it. Thus, through its effects

on $K_v1.2$ surface levels, cAMP homeostasis also functions as buffer for cellular excitability (Connors et al., 2008).

Integration of opioid signaling with the retinal-ipRGC circuit

What might be the retinal circuit that leads to a rise in endogenous retinal opioid levels and what are the functional consequences of the effect of these opioids on ipRGC excitability? Similar to how MOR activation in the lateral amygdala serves to attenuate neuronal spiking in depolarizing conditions (Faber and Sah, 2004), modulation of K_v and Ca_v channels in ipRGCs by MOR activation may serve to limit ipRGC output in response to depolarizing stimuli. When might ipRGC output need to be suppressed? The biological clock is located in the suprachiasmatic nucleus in the hypothalamus, and it receives photic information through ipRGCs. As clock neurons are active during the day / light cycle and its output accordingly integrated by central sleep-regulatory systems, there would be advantages to mechanisms of ipRGC output suppression during the dark cycle that are capable of modulating the cells sensitivity to depolarizing input (Saper et al., 2005).

IpRGCs exhibit both intrinsic (melanopsin-driven) and extrinsic (synaptically-driven) light responses (Wong et al., 2007; Schmidt et al., 2011), and these responses have a powerful impact on ipRGC-mediated central processes. The intrinsic phototransduction cascade has very high gain, with ipRGCs capable of signaling single photon absorption to the brain via spiking, as a 1 mV depolarization results in a several-fold increase in the spike rate of ipRGCs. Such high efficiency signaling of ipRGCs could be achieved by the ipRGCs operating near spike threshold. Furthermore, at the organism level, only a few hundred melanopsin molecules need to undergo photoisomerization in order to trigger the pupillary light reflex (PLR) (Do et al., 2009). Selective elimination of 99% of ipRGCs does not eliminate the PLR completely (Güler et al., 2008), confirming that signaling from even a very limited number of ipRGCs has significant downstream behavioral consequence. These findings suggest that relatively small shifts in ipRGC spiking could be expected to have discernable impact on behaviors and reflexes regulated by these photoreceptors. Opioid signaling could serve to modulate the efficiency of ipRGC signaling in darkness when such high gain is both unnecessary and counter-productive. As even a slight rise in the spike threshold would decrease ipRGC light signaling by orders of magnitude, the spike threshold of ipRGCs has previously been postulated to be a regulatory point for ipRGC sensitivity (Do et al., 2009). We have shown that the spike threshold is indeed a regulatory point, although MOR activation in ipRGCs reduces ipRGC excitability not by increasing the spike threshold itself but by increasing I_K at the threshold of voltage-gated Na^+ channels, thereby delaying the Na^+ -mediated depolarization of ipRGCs.

Negative regulation of ipRGCs by opioids during darkness could serve as an effective nighttime counterpart to the known regulation by dopamine (DA) of ipRGCs during daylight (van Hook et al., 2012). DA, via D1-receptor activation, has been shown to affect light-evoked spiking in ipRGCs by both attenuating the photocurrent and depolarizing ipRGC resting membrane potentials (van Hook et al., 2012). While cAMP's effects on light evoked spiking were not directly investigated in the study by van Hook et al (2012), a subsequent study showed that elevated cAMP increased light evoked spiking via a PKA-dependent

pathway (Sodhi and Hartwick, 2014). Given that opioids are known to decrease cAMP (Kieffer, 1995), in ipRGCs DA and opioids might act to promote the transition between daytime and nighttime, respectively, as it was proposed for avian retinas (Morgan and Boelen, 1996). In support of this notion in the rabbit retina, exogenous opioids were shown to inhibit the release of dopamine (Dubocovich and Weiner, 1983). The increased number of light-evoked spikes after application of CTOP (Fig. 3Ai, 3Aii) suggests the presence of a weak inhibitory tone mediated by endogenous opioids in dark-adapted retinas (Morgan and Boelen, 1996). With our experimental paradigm, however, this effect of CTOP could instead be the result of a homeostatic sensitization of AC triggered by the long exposure to multiple concentrations of DAMGO, resulting in an overshoot of cAMP production upon the addition of a competitive MOR antagonist (Watts, 2002; Levitt et al., 2010).

The fact that CTOP alone did not increase light-evoked signaling (Fig. 3D) suggests that the CTOP-mediated increase of ipRGC light responses seen in our experiments, which were performed during the day following long DAMGO exposures (Fig. 3Ai, 3Aii), was most likely caused by MOR antagonist-induced cAMP overshoot (Watts, 2002). In the mouse retina β -endorphin, the endogenous opioid that is preferentially bound by MORs, is expressed by a subpopulation of ON and OFF cholinergic amacrine cells (Gallagher et al., 2010): the OFF types somas are located at the INL/IPL border and their processes arborize in a thin layer between sublaminae 1 and 2 of the IPL, whereas the ON types somas are displaced to the GCL and whose processes arborize between IPL sublaminae 3 and 4 (Haverkamp and Wässle, 2000). In essence, this close spatial apposition of putative β -endorphin release sites to M1 and M3 ipRGC processes that cross the inner retina might support either direct synaptic or paracrine opioid regulation of ipRGCs, whereas a paracrine opioid regulation of M2 type ipRGCs with processes running along in sublamina 5 is more likely. Although it is not known whether the expression and release of β -endorphin follows a circadian rhythmicity in the retina, it is tempting to speculate that endogenous opioid levels, akin to those of adenosine, rise at night to likewise co-regulate nighttime signals from ipRGCs to the brain. A1 adenosine receptor activation in ipRGCs, like MOR activation, decreases AC activity, cAMP levels, and PKA activity, with the consequence of decreased light evoked spiking. While not yet explicitly investigated, postulated downstream targets of A1 receptor signaling include Ca_v channels, TRPCs, and (less likely) hyperpolarization-activated cyclic nucleotide-gated channels (Sodhi and Hartwick, 2014). It would appear that opioids and adenosine are poised to work synergistically to inhibit light-evoked spiking in ipRGCs. While in the basal forebrain increases in adenosine promote sleep and increases in opioids promote insomnia (as reviewed by Nelson et al. 2009), the effects of adenosine and opioids in the spinal cord are not in opposition but are instead additive (Sawynok, 1998), and this is consistent with how they appear to function in the retina.

Acknowledgement

This research was funded by the following grants: US National Institute of Health-National Eye Institute EY020108 to JR, EY12793 and R01 EY17137 to DB, EY019051 to JV; US National Institute of Health -sponsored National Center For Advancing Translational Sciences Grant 8KL2TR000112-05 to AH; John H. Venable Memorial Scholarship to RET and CSU CVMBBS Research Council grant to JV.

Abbreviations

AC	adenylate cyclase
4-AP	4-Aminopyridine
BLA	basolateral amygdala
I_{K(Ca)}	calcium-dependent potassium currents
CGP54626	[S-(R*,R*)]-[3-[[1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl] (cyclohexylmethyl) phosphinic acid
CTOP	H-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH ₂ CTAP, H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH ₂
DAMGO	[D-Ala ² , MePhe ⁴ , Gly-ol ⁵]-enkephalin
D-AP5	D-(-)-2-Amino-5-phosphonopentanoic acid
D-AP7	D-(-)-2-Amino-7-phosphonoheptanoic acid
EGFP	enhanced green fluorescent protein
GAD	glutamic acid decarboxylase
GCL	ganglion cell layer
GIRK	G-protein-activated inwardly rectifying K ⁺ channels
IC₅₀	half-blocking concentration
V_{0.5}	half-activation potential
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
INL	inner nuclear layer
IPL	inner plexiform layer
ipRGCs	intrinsically photosensitive retinal ganglion cells
L-AP4	L-(+)-2-Amino-4-phosphonobutyric acid
LJP	liquid junction potential
V_m	membrane potential
MEA	multielectrode array
MOR	μ-opioid receptor
NBQX	2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide
ONL	outer nuclear layer

OPL	outer plexiform layer
R_s	series resistance
TPMPA	(1,2,5,6-Tetrahydropyridin-4-yl) methylphosphinic acid
TTX	tetrodotoxin
Ca_v	voltage-gated calcium channel
I_{Ca}	voltage-gated calcium current
K_v	voltage-gated potassium channel
I_K	voltage-gated potassium current
I_{Na}	voltage-gated sodium current
V_{0.05}	command voltage at which the resulting I _K was 5% of the peak

References

- Al-Sabi A, Shamotienko O, Dhochartaigh SN, Muniyappa N, Le Berre M, Shaban H, Wang J, Sack JT, Dolly JO (2010) Arrangement of Kv1 α subunits dictates sensitivity to tetraethylammonium. *J Gen Physiol* 136:273–282. [PubMed: 20805574]
- Armstrong CM, Gilly WF (1992) Access resistance and space clamp problems associated with whole-cell patch clamping. *Methods Enzymol* 207:100–122. [PubMed: 1528114]
- Bao Y, Gao Y, Yang L, Kong X, Yu J, Hou W, Hua B (2015) The mechanism of μ -opioid receptor (MOR)-TRPV1 crosstalk in TRPV1 activation involves morphine anti-nociception, tolerance and dependence. *Channels* 9:235–243. [PubMed: 26176938]
- Baver SB, Pickard GE, Sollars PJ, Pickard GE (2008) Two types of melanopsin retinal ganglion cell differentially innervate the hypothalamic suprachiasmatic nucleus and the olivary pretectal nucleus. *Eur J Neurosci* 27:1763–1770. [PubMed: 18371076]
- Berson DM, Dunn FA, Takao M (2002) Phototransduction By Retinal Ganglion Cells That Set The Circadian Clock. *Science* 295:1070–1073. [PubMed: 11834835]
- Bosma MM, Allen ML, Martin TM, Tempel BL (1993) PKA-dependent regulation of mKv1.1, a mouse Shaker-like potassium channel gene, when stably expressed in CHO cells. *J Neurosci* 13:5242–5250. [PubMed: 8254371]
- Brew HM, Gittelman JX, Silverstein RS, Hanks TD, Demas VP, Robinson LC, Robbins CA, McKee-Johnson J, Chiu SY, Messing A, Tempel BL (2007) Seizures and reduced life span in mice lacking the potassium channel subunit Kv1.2, but hypoexcitability and enlarged Kv1 currents in auditory neurons. *J Neurophysiol* 98:1501–1525. [PubMed: 17634333]
- Brew HM, Hallows JL, Tempel BL (2003) Hyperexcitability and reduced low threshold potassium currents in auditory neurons of mice lacking the channel subunit Kv1.1. *J Physiol* 548:1–20. [PubMed: 12611922]
- Cachero TG, Morielli AD, Peralta EG (1998) The Small GTP-Binding Protein RhoA Regulates a Delayed Rectifier Potassium Channel. *Cell* 93:1077–1085. [PubMed: 9635436]
- Chen Y, Stevens B, Chang J, Milbrandt J, Barres BA, Hell JW (2008) NS21: Re-defined and modified supplement B27 for neuronal cultures. *J Neurosci Methods* 171:239–247. [PubMed: 18471889]
- Clark JD, Tempel BL (1998) Hyperalgesia in mice lacking the Kv1.1 potassium channel gene. *Neurosci Lett* 251:121–124. [PubMed: 9718989]
- Coleman SK, Newcombe J, Pryke J, Dolly JO (1999) Subunit composition of Kv1 channels in human CNS. *J Neurochem* 73:849–858. [PubMed: 10428084]
- Connors EC, Ballif BA, Morielli AD (2008) Homeostatic regulation of Kv1.2 potassium channel trafficking by cyclic AMP. *J Biol Chem* 283:3445–3453. [PubMed: 18003609]

- Cox RH (2005) Molecular determinants of voltage-gated potassium currents in vascular smooth muscle. *Cell Biochem Biophys* 42:167–195. [PubMed: 15858231]
- Dang VC, Christie MJ (2012) Mechanisms of rapid opioid receptor desensitization, resensitization and tolerance in brain neurons. *Br J Pharmacol* 165:1704–1716. [PubMed: 21564086]
- DiFrancesco D, Ferroni A, Mazzati M, Tromba C (1986) Properties of the hyperpolarizing-activated current (if) in cells isolated from the rabbit sino-atrial node. *J Physiol* 377:61–88. [PubMed: 2432247]
- Do MTH, Kang SH, Xue T, Zhong H, Liao H-W, Bergles DE, Yau K-W (2009) Photon capture and signalling by melanopsin retinal ganglion cells. *Nature* 457:281–287. [PubMed: 19118382]
- Dubocovich ML, Weiner N (1983) Enkephalins modulate [3H]dopamine release from rabbit retina in vitro. *J Pharmacol Exp Ther* 224:634–639. [PubMed: 6827486]
- Ecker JL, Dumitrescu ON, Wong KY, Alam NM, Chen SK, LeGates T, Renna JM, Prusky GT, Berson DM, Hattar S (2010) Melanopsin-expressing retinal ganglion-cell photoreceptors: Cellular diversity and role in pattern vision. *Neuron* 67:49–60. [PubMed: 20624591]
- Emanuel AJ, Do MTH (2015) Melanopsin tristability for sustained and broadband phototransduction. *Neuron* 85:1043–1055. [PubMed: 25741728]
- Faber ESL, Sah P (2004) Opioids Inhibit Lateral Amygdala Pyramidal Neurons by Enhancing a Dendritic Potassium Current. *J Neurosci* 24:3031–3039. [PubMed: 15044542]
- Finnegan TF, Chen S, Pan H (2006) Opioid Receptor Activation Inhibits GABAergic Inputs to Basolateral Amygdala Neurons Through Kv1.1/1.2 Channels. *J Neurophysiol* 95:2032–2041. [PubMed: 16306173]
- Fox PD, Hentges ST (2017) Differential Desensitization Observed at Multiple Effectors of Somatic μ -Opioid Receptors Underlies Sustained Agonist-Mediated Inhibition of Proopiomelanocortin Neuron Activity. *J Neurosci Off J Soc Neurosci* 37:8667–8677.
- Galeotti N, Ghelardini C, Papucci L, Capaccioli S, Quattrone A, Morgagni VGB, Florence I-(1997) An Antisense Oligonucleotide on the Mouse Shaker-like Potassium Channel Kv1.1 Gene Prevents Antinociception Induced by Morphine and Baclofen *Br J Pharmacol* 281:941–949.
- Galeotti N, Ghelardini C, Vinci MC, Bartolini A (1999) Role of potassium channels in the antinociception induced by agonists of a 2-adrenoceptors. *Br J Pharmacol* 126:1214–1220. [PubMed: 10205011]
- Gallagher SK, Anglen JN, Mower JM, Vigh J (2012) Dopaminergic amacrine cells express opioid receptors in the mouse retina. *Vis Neurosci* 29:203–209. [PubMed: 22643230]
- Gallagher SK, Witkovsky P, Roux MJ, Low MJ, Otero-Corchon V, Hentges ST, Vigh J (2010) β -Endorphin expression in the mouse retina. *J Comp Neurol* 518:3130–3148. [PubMed: 20533364]
- Glazebrook PA, Ramirez AN, Schild JH, Shieh CC, Doan T, Wible BA, Kunze DL (2002) Potassium channels Kv1.1, Kv1.2 and Kv1.6 influence excitability of rat visceral sensory neurons. *J Physiol* 541:467–482. [PubMed: 12042352]
- Grissmer S, Nguyen N, Hanson C, Mather J, Gutman A, Karmilowicz J, Auperin D, George K (1994) Pharmacological Characterization of Five Cloned Voltage-Gated Expressed in Mammalian Cell Lines. *Mol Pharmacol* 45:1227–1234. [PubMed: 7517498]
- Güler AD, Ecker JL, Lall GS, Haq S, Altimus CM, Liao W, Barnard AR, Cahill H, Badea TC, Zhao H, Mark W, Berson DM, Lucas RJ, Yau K, Hattar S (2008) Melanopsin cells are the principal conduits for rod/cone input to non-image forming vision. *Nature* 453:102–105. [PubMed: 18432195]
- Gutman GA, Chandy KG, Grissmer S, Lazdunski M, McKinnon D, Pardo LA, Robertson GA, Rudy B, Sanguinetti MC, Stühmer W, Wang X (2005) International Union of Pharmacology. LIII. Nomenclature and molecular relationships of voltage-gated potassium channels. *Pharmacol Rev* 57:473–508. [PubMed: 16382104]
- Hartwick ATE, Bramley JR, Yu J, Stevens KT, Allen CN, Baldridge WH, Sollars PJ, Pickard GE (2007) Light-Evoked Calcium Responses of Isolated Melanopsin-Expressing Retinal Ganglion Cells. *J Neurosci* 27:13468–13480. [PubMed: 18057205]
- Hattar D, Nesti E, Cachero TG, Morielli AD (2002) Tyrosine Phosphorylation of Kv1.2 Modulates Its Interaction with the Actin-binding Protein Cortactin. *J Biol Chem* 277:38596–38606. [PubMed: 12151401]

- Hattar S, Liao HW, Takao M, Berson DM, Yau KW (2002) Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. *Science* 295:1065–1070. [PubMed: 11834834]
- Haverkamp S, Wassle H (2000) Immunocytochemical analysis of the mouse retina. *J Comp Neurol* 424:1–23. [PubMed: 10888735]
- Hu C, Hill DD, Wong KY (2013) Intrinsic physiological properties of the five types of mouse ganglion-cell photoreceptors. *J Neurophysiol* 109:1876–1889. [PubMed: 23343892]
- Huang X-Y, Morielli AD, Peralta EG (1993) Tyrosine Kinase-Dependent Suppression of a Potassium Channel by the G Protein-Coupled ml Muscarinic Acetylcholine Receptor. *Cell* 75:1145–1156. [PubMed: 8261514]
- Jerng HH, Pfaffinger PJ, Covarrubias M (2004) Molecular physiology and modulation of somatodendritic A-type potassium channels. *Mol Cell Neurosci* 27:343–369. [PubMed: 15555915]
- Kelly E, Bailey CP, Henderson G (2009) Agonist-selective mechanisms of GPCR desensitization. *Br J Pharmacol* 153:S379–S388.
- Kew JN, Davies CH eds. (2010) *Ion Channels: From Structure to Function*, 2nd ed. New York: Oxford University Press.
- Kieffer BL (1995) Recent advances in molecular recognition and signal transduction of active peptides: Receptors for opioid peptides. *Cell Mol Neurobiol* 15:615–635. [PubMed: 8719033]
- Lee SK, Schmidt TM (2018) Morphological Identification of melanopsin-Expressing Retinal Ganglion Cell Subtypes in Mice. *Methods Mol Biol* 1753:275–287. [PubMed: 29564796]
- LeGates TA, Altimus CM, Wang H, Lee H-K, Yang S, Zhao H, Kirkwood A, Weber ET, Hattar S (2012) Aberrant light directly impairs mood and learning through melanopsin-expressing neurons. *Nature* 491:594–598. [PubMed: 23151476]
- Levitt ES, Purington LC, Traynor JR (2010) Gi/o-Coupled Receptors Compete for Signaling to Adenylyl Cyclase in SH-SY5Y Cells and Reduce Opioid-Mediated cAMP Overshoot. *Mol Pharmacol* 79:461–471. [PubMed: 21098043]
- Meyer-franke A, Kaplan MR, Pfrieger FW, Barres BA (1995) Characterization of the Signaling Interactions That Promote the Survival and Growth of Developing Retinal Ganglion Cells in Culture. *J Neurosci* 15:805–819.
- Morgan IG, Boelen MK (1996) A retinal dark-light switch: A review of the evidence. *Vis Neurosci* 13:399–409. [PubMed: 8782368]
- Nelson AM, Battersby AS, Baghdoyan HA, Lydic R (2009) Opioid-induced decreases in rat brain adenosine levels are reversed by inhibiting adenosine deaminase. *Anesthesiology* 111:1327–1333. [PubMed: 19934879]
- Nesti E, Everill B, Morielli AD (2004) Endocytosis as a Mechanism for Tyrosine Kinase-dependent Suppression of a Voltage-gated Potassium Channel. *Mol Biol Cell* 15:4073–4088. [PubMed: 15215309]
- Noseda R, Kainz V, Jakubowski M, Gooley JJ, Saper CB, Digre K, Burnstein R (2010) A neural mechanism for exacerbation of headache by light. *Nat Neurosci* 13:239–245. [PubMed: 20062053]
- Ocaña M, Cendán CM, Cobos EJ, Entrena JM, Baeyens JM (2004) Potassium channels and pain: Present realities and future opportunities. *Eur J Pharmacol* 500:203–219. [PubMed: 15464034]
- Pennock RL, Hentges ST (2011) Differential Expression and Sensitivity of Presynaptic and Postsynaptic Opioid Receptors Regulating Hypothalamic Proopiomelanocortin Neurons. *J Neurosci* 31:281–288. [PubMed: 21209213]
- Perez-Leighton CE, Schmidt TM, Abramowitz J, Birnbaumer L, Kofuji P (2011) Intrinsic phototransduction persists in melanopsin-expressing ganglion cells lacking diacylglycerol-sensitive TRPC subunits. *Eur J Neurosci* 33:856–867. [PubMed: 21261756]
- Perez-Leon JA, Warren EJ, Allen CN, Robinson DW, Brown LR (2006) Synaptic inputs to retinal ganglion cells that set the circadian clock. *Eur J Neurosci* 24:1117–1123. [PubMed: 16930437]
- Proft J, Weiss N (2015) G protein regulation of neuronal calcium channels: back to the future. *Mol Pharmacol* 87:890–906.
- Qu CL, Huo FQ, Huang FS, Tang JS (2015) Activation of mu-opioid receptors in the ventrolateral orbital cortex inhibits the GABAergic miniature inhibitory postsynaptic currents in rats. *Neurosci Lett* 592:64–69. [PubMed: 25711796]

- Quattrochi LE, Stabio ME, Kim I, Ilardi MC, Fogerson PM, Leyrer ML, Berson DM (2018) The M6 cell: A small-field bistratified photosensitive retinal ganglion cell. *J Comp Neurol* 527:297–311. [PubMed: 30311650]
- Renna JM, Weng S, Berson DM (2011) Light acts through melanopsin to alter retinal waves and segregation of retinogeniculate afferents. *Nat Neurosci* 14:827–829. [PubMed: 21642974]
- Saper CB, Scammell TE, Lu J (2005) Hypothalamic regulation of sleep and circadian rhythms. *Nature* 437:1257–1263. [PubMed: 16251950]
- Sawynok J (1998) Adenosine receptor activation and nociception. *Eur J Pharmacol* 347:1–11. [PubMed: 9650842]
- Schmidt TM, Chen SK, Hattar S (2011) Intrinsically photosensitive retinal ganglion cells: Many subtypes, diverse functions. *Trends Neurosci* 34:572–580. [PubMed: 21816493]
- Schmidt TM, Kofuji P (2009) Functional and morphological differences among intrinsically photosensitive retinal ganglion cells. *J Neurosci* 29:476–482. [PubMed: 19144848]
- Schmidt TM, Taniguchi K, Kofuji P (2008) Intrinsic and extrinsic light responses in melanopsin-expressing ganglion cells during mouse development. *J Neurophysiol* 100:371–384. [PubMed: 18480363]
- Sernagor E, Eglén SJ, Wong ROL (2001) Development of retinal ganglion cell structure and function. *Prog Retin Eye Res* 20:139–174. [PubMed: 11173250]
- Shamotienko OG, Parcej DN, Dolly JO (1997) Channel Kv1 Subtypes in Synaptic Membranes from Bovine Brain. *Biochemistry* 36:8195–8201. [PubMed: 9204863]
- Shi G, Nakahira K, Hammond S, Rhodes KJ, Schechter LE, Trimmer JS (1996) Beta Subunits promote K⁺ channel surface expression through effects early in biosynthesis. *Neuron* 16:843–852. [PubMed: 8608002]
- Smart SL, Lopantsev V, Zhang CL, Robbins CA, Wang H, Chiu SY, Schwartzkroin PA, Messing A, Tempel BL (1998) Deletion of the K(v)1.1 Potassium channel causes epilepsy in mice. *Neuron* 20:809–819. [PubMed: 9581771]
- Sodhi P, Hartwick ATE (2014) Adenosine modulates light responses of rat retinal ganglion cell photoreceptors through a cAMP-mediated pathway. *J Physiol* 592:4201–4220. [PubMed: 25038240]
- Sodhi P, Hartwick ATE (2016) Muscarinic acetylcholine receptor-mediated stimulation of retinal ganglion cell photoreceptors. *Neuropharmacology* 108:305–315. [PubMed: 27055770]
- Solessio E, Vigh J, Cuenca N, Rapp K, Lasater EM (2002) Membrane properties of an unusual intrinsically oscillating, wide-field teleost retinal amacrine cell. *J Physiol* 544:831–847. [PubMed: 12411527]
- Stirling L, Williams MR, Morielli AD (2009) Dual Roles for RHOA/RHO-Kinase In the Regulated Trafficking of a Voltage-sensitive Potassium Channel. *Mol Biol Cell* 20:2991–3002. [PubMed: 19403695]
- Talbot MJ, Sayer RJ (1996) Intracellular QX-314 inhibits calcium currents in hippocampal CA1 pyramidal neurons. *J Neurophysiol* 76:2120–2124. [PubMed: 8890325]
- Tooker RE, Lipin MY, Leuranguer V, Rozsa E, Bramley JR, Harding JL, Reynolds MM, Vigh J (2013) Nitric Oxide Mediates Activity-Dependent Plasticity of Retinal Bipolar Cell Output via S-Nitrosylation. *J Neurosci* 33:19176–19193. [PubMed: 24305814]
- Uebele VN, England SK, Chaudhary A, Tamkun MM, Snyder D (1996) Functional Differences in Kv1.5 Currents Expressed in Mammalian Cell Lines Are Due to the Presence of Endogenous Kvbeta2.1 Subunits. *J Biol Chem* 271:2406–2412. [PubMed: 8576199]
- van Hook MJ, Berson DM (2010) Hyperpolarization-activated current (h) in ganglion-cell photoreceptors. *PLoS One* 5.
- van Hook MJ, Wong KY, Berson DM (2012) Dopaminergic modulation of ganglion-cell photoreceptors in rat. *Eur J Neurosci* 35:507–518. [PubMed: 22304466]
- Vuong HE, Hardi CN, Barnes S, Brecha NC (2015) Parallel Inhibition of Dopamine Amacrine Cells and Intrinsically Photosensitive Retinal Ganglion Cells in a Non-Image-Forming Visual Circuit of the Mouse Retina. *J Neurosci* 35:15955–15970. [PubMed: 26631476]

- Wang H, Kunkel DD, Martin TM, Schwartzkroin PA, Tempel BL (1993) Heteromultimeric K⁺ channels in terminal and juxtaparanodal regions of neurons. *Nature* 365:75–79. [PubMed: 8361541]
- Warren EJ, Allen CN, Brown RL, Robinson DW (2006) The light-activated signaling pathway in SCN-projecting rat retinal ganglion cells. *Eur J Neurosci* 23:2477–2487. [PubMed: 16706854]
- Watts VJ (2002) Molecular mechanisms for heterologous sensitization of adenylate cyclase. *J Pharmacol Exp Ther* 302:1–7. [PubMed: 12065693]
- Welch SP, Dunlow LD (1993) Antinociceptive activity of intrathecally administered potassium channel openers and opioid agonists: a common mechanism of action? *J Pharmacol Exp Ther* 267:390–399.
- Williams JT, Christie MJ, Manzoni O (2001) Cellular and synaptic adaptations mediating opioid dependence. *Physiol Rev* 81:299–343. [PubMed: 11152760]
- Williams JT, Ingram SL, Henderson G, Chavkin C, Zastrow M Von, Schulz S, Koch T, Evans CJ, Christie MJ (2013) Regulation of μ -Opioid Receptors: Desensitization, Phosphorylation, Internalization, and Tolerance. *Pharmacol Rev* 65:223–254. [PubMed: 23321159]
- Williams MR, Fuchs JR, Green JT, Morielli AD (2012) Cellular mechanisms and behavioral consequences of Kv1.2 regulation in the rat cerebellum. *J Neurosci* 32:9228–9237 [PubMed: 22764231]
- Williams MR, Markey JC, Doczi MA, Morielli AD (2007) An essential role for cortactin in the modulation of the potassium channel Kv1.2. *PNAS* 104:17412–17417. [PubMed: 17959782]
- Wong KY, Dunn FA, Graham DM, Berson DM (2007) Synaptic influences on rat ganglion-cell photoreceptors. *J Physiol* 582:279–296. [PubMed: 17510182]
- Xue T, Do MTH, Riccio A, Jiang Z, Hsieh J, Wang HC, Merbs SL, Welsbie DS, Yoshioka T, Weissgerber P, Stolz S, Flockerzi V, Freichel M, Simon MI, Clapham DE, Yau KW (2011) Melanopsin signalling in mammalian iris and retina. *Nature* 479:67–72. [PubMed: 22051675]

Highlights

- In the rodent retina M1-M3 types of intrinsically photosensitive ganglion cells (ipRGCs) express μ -opioid receptors (MORs).
- Light-evoked firing of ipRGCs is attenuated by the MOR-specific agonist DAMGO in a dose-dependent manner.
- MOR activation reduces ipRGC excitability by modulating I_K and reducing the amplitude of non-inactivating I_{Ca} .
- These findings suggest a potential new role for endogenous opioids in the mammalian retina.

Figure 1.

In the rat retina M1 ipRGCs are immunopositive for μ -opioid receptors (MORs). **A:** Single-plane confocal image of vertically sectioned adult rat retina showing a MOR+ dendrite (red) within the IPL (white arrow). Weaker MOR immunofluorescence is apparent in a ganglion cell body (hollow arrowhead). **B:** Immunolabeling for melanopsin (green) in the same optical section as in **A**, showing a single melanopsin+ ipRGC soma in the GCL (hollow arrowhead) and a well-labeled dendrite in the IPL (white arrow). **C:** A merged image of **A** and **B**, showing that the same cell is immunoreactive for MOR and melanopsin. Inset: expanded view of MOR+ labeling of melanopsin+ ipRGC soma marked by the box in **C** (brightness and contrast adjusted). **D:** Projected image compiled from five single-plane Z-stack confocal images of the same field of view as in **A-C** showing that the melanopsin immunopositive dendrite derives from the labeled soma; this appears to be an M1 cell based on its strong melanopsin staining and dendrites ascending into the outer IPL. Note that punctate MOR+ labeling decorates most of this dendrite. **E:** Single-plane confocal image of vertically sectioned adult rat retina showing a MOR+ dendrite (red) deep within the IPL (white arrow). **F:** melanopsin immunolabeling (green) in the same optical section as in **E**, showing a single melanopsin+ ipRGC soma in the GCL (hollow arrowhead) and a well-labeled dendrite deep in the IPL (white arrow). **G:** A merged image of **E** and **F**, showing that the melanopsin+ dendrite is immunoreactive for MOR (white arrow); based on its position at the border of IPL and GCL it originates from a putative M2 or M3 ipRGC. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; Scale bars: **D** and **G**: 20 μ m; **C inset**: 5 μ m.

Figure 2.

EGFP-expressing ipRGCs in the *Opn4*:EGFP mouse retina are immunopositive for μ opioid receptors (MORs). **A**: Single-plane optical section of the *Opn4*:EGFP mouse retina showing red MOR immunolabeling in a soma of the GCL (hollow arrowhead) and a dendritic process in the IPL (arrow). **B**: The same optical section as in **A**, but showing a green EGFP+ ipRGC soma in the GCL (hollow arrowhead) and its processes in the IPL (white arrow). **C**: A merged image of **A** and **B**, indicating colocalization of MOR immunolabeling and EGFP in the GCL (hollow arrowhead). Weak MOR immunoreactivity also marks the dendrite (white arrow), as shown more clearly in the inset in **C**, represented the area marked by the rectangle in **C**, with brightness and contrast adjusted. **D**: Projected image of the same field of view compiled from four single-plane Z-stack confocal images. **E**: Single-plane confocal image of vertically sectioned adult *Opn4*:EGFP mouse retina showing a MOR+ puncta (red) within the GCL (white arrow, hollow arrowhead). **F**: melanopsin immunolabeling (green) in the same optical section as in **E**, showing two EGFP+ somas of putative ipRGCs in the GCL. **G**: A merged image of **E** and **F**, showing that the EGFP+ somas are immunoreactive for MOR. **H**: Projected image compiled from five single-plane Z-stack confocal images of the same field of view as in **E-G** revealing that EGFP+ putative ipRGCs expressing MOR+ immunolabeling are most likely M2 (hollow arrowhead) and M3 (white arrow) types based their dendritic arborization pattern. **I-M**: DIC image (**I**) of a representative EGFP-expressing (**J**), putative ipRGC enzymatically dissociated from the *Opn4*:EGFP mouse retina. The same cell shows both anti- MOR (**K**) and melanopsin immunolabeling (**L**), evident on the

merged fluorescent image (**M**). ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; Scale bars: **D**: 20µm; **H** and **M**: 10µm.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3.

The μ -opioid-specific agonist DAMGO inhibits intrinsic light responses of ipRGCs in isolated retinas. **Ai**: Multielectrode array (MEA) recording of the light responses a representative ipRGC in response to a 20 s stimulation (3.9×10^{15} photons $\text{cm}^{-2} \text{s}^{-1}$ at 470 nm, black bar) a P10 rat retina in the presence of increasing concentrations of MOR-selective agonist DAMGO (0.01–10 μM) followed by application of MOR-selective antagonist CTOP (10 μM). Light-evoked ipRGC spiking was greatly attenuated by DAMGO in a dose-dependent manner, and rescued by consecutive application of CTOP. **Aii**:

Cumulative ipRGC light response data obtained in increasing concentrations of DAMGO (0.01–10 μ M) followed by CTOP as in Ai. Data is shown as Average $\pm$ SEM, n=43 from 3 retinas. *: p<0.05; **: p<0.001. **Aiii:** Delay of the 1st light-evoked spikes of representative ipRGCs (Cell 1–4) recorded by MEA in the presence of increasing concentrations of MOR-selective agonist DAMGO (0.01–10 μ M) followed by application of MOR-selective antagonist CTOP (10 μ M). **Aiv:** Cumulative data summarizing 1st spike delays ipRGC light response as in Aiii. DAMGO significantly increased the 1st spike delays in all instances, whereas consecutive CTOP treatment resulted in a delay close to that observed in control. Average $\pm$ SEM, n=43 from 3 retinas. *: p<0.05; **: p<0.001. **B:** Simultaneous application of DAMGO (1 μ M) and CTOP (10 μ M) did not alter light-evoked ipRGCs firing. Data is shown as Average $\pm$ SEM, n=60 from 2 retinas **C:** CTOP (1 and 10 μ M) application does not alter light-evoked ipRGCs firing. Dots and error bars representing Average $\pm$ SEM were omitted for better visibility of the lines connecting the average values; n=52 from 3 retinas. **Di:** Dose-response curve of the duration of the ipRGC light responses in DAMGO (1nM-10 μ M). Data are plotted as a percentage of light response under control conditions (synaptic blocker cocktail without DAMGO). Parenthetical numerals indicate the number of retinas studied for each DAMGO dose. Every retina was exposed to a single DAMGO concentration. Error bars represent $\pm$ SEM. **Dii:** Dose-response curve plotting the number of spikes recorded in ipRGCs during the 20 s light stimulus as a function of the applied DAMGO concentration; data normalized as in **Di**. Parenthetical numerals indicate the number of retinas studied for each DAMGO dose.

Figure 4.

DAMGO modulates spiking activity in adult rat ipRGCs. **A:** Spike rasters from an example MEA recording from an ipRGC. Sustained spiking response to bright (3.9×10^{15} photons $\text{cm}^{-2} \text{s}^{-1}$, 10 s) blue light persisted in the presence of glutamatergic antagonists, confirming ipRGC identity. Rasters of spiking activity recorded before, during and after treatment with 10 μM DAMGO illustrates inhibitory effect of this MOR agonist on ipRGC spiking. **B:** Summary of mean spike frequency (spikes per 1 s bins) and **C:** total counts of spikes fired over 80 s period (20 s light stimulation plus 60 s post-light) by light-stimulated ipRGCs ($n=6$ from $N=5$ retinas) before during and after DAMGO treatment. * $p<0.05$, one way repeated measures ANOVA, Holm-Sidak post-hoc testing.

Figure 5.

The DAMGO modulated intrinsic light responses of dissociated ipRGCs directly, by MORs expressed by ipRGCs. **A:** Direct opioid modulation of intrinsic light responses via MORs expressed by ipRGCs was revealed by loose-patch recordings of an isolated EGFP+ ipRGC dissociated from an *Opn4::EGFP* mouse retina. **B:** Representative recording showing that bath application of DAMGO (1 μ M) reversibly eliminated the light-evoked spikes of an enzymatically dissociated ipRGC. **C:** Simultaneous application of DAMGO (1 μ M) and CTOP (10 μ M) did not alter light-evoked firing of enzymatically dissociated ipRGCs.

Figure 6.

MOR agonist DAMGO did not alter the melanopsin-driven increase in light-evoked cationic conductance/depolarization in ipRGCs. Representative light responses of the same ipRGC were evoked by a 10 s light flash (10^{14} photons $\text{cm}^{-2} \text{s}^{-1}$, 470 nm, black bar) and recorded in voltage-clamp mode at -60 mV holding potential (**A**) or in current-clamp mode with resting potential set at -60 mV (**B**).

Figure 7.

MOR agonist DAMGO reduced excitability of ipRGCs. **Ai:** Representative current clamp recording from an M1 ipRGCs made in whole mount preparation in the presence of the synaptic blocking cocktail. DAMGO (1 μ M) increased delay of the 1st spike evoked by a depolarizing current ramp from -70 pA to -20 pA over 2 s, starting at 1 s. **Aii:** Replotting V_m changes shown in Ai against the injected current (relative to the holding current of -70 pA) revealed that DAMGO increased the current threshold for the 1st spike. **Aiii:** Extended timescale view of Aii shows that DAMGO did not alter the V_m threshold for spike

generation in ipRGCs. **Bi:** Representative current clamp recording from an enzymatically dissociated solitary ipRGCs showing that similar to intact cells, DAMGO (1 μ M) increased delay of the 1st spike evoked by a depolarizing current ramp. **Bii:** Plotting V_m changes against the injected current (relative to the holding current necessary to maintain V_m at -70 mV) from the same recordings as in Bi revealed that DAMGO increased the current threshold for the 1st spike. Note the smaller current values here, due to the higher input resistance of dissociated ipRGCs compared to the intact ones in situ (Aii). **Ci:** Summary graph showing that current threshold for spike generation is significantly increased by DAMGO (D) compared with control (cont). White circles represent control; gray circles represent DAMGO. $*p < 0.004$ (paired Student t test). $n=11$. **Cii:** Summary graph showing that membrane potential (V_m) threshold for spike generation was not altered by DAMGO (D) compared with control (cont). White circles represent control; gray circles represent DAMGO. $p=0.37$ (paired Student t test) $n=11$. **Ciii:** Summary graph showing that holding current necessary to maintain V_m at -70 mV was not altered by DAMGO (D) compared with control (cont). White circles represent control; gray circles represent DAMGO. $p=0.51$ (paired Student t test). $n=11$.

Figure 8.

MOR agonist DAMGO alters I_K activation in ipRGCs. **Ai:** Representative leak-subtracted current traces show that DAMGO (1 μ M) increased the voltage ramp-evoked I_K between -55 mV and -15 mV by shifting the activation to hyperpolarized potentials without increasing the overall I_K amplitude. **Aii:** Summary graph showing I_K activation ($V_{0.05}$) and half-activation ($V_{0.5}$) from Boltzmann fits in control and DAMGO (*: $p < 0.001$, paired Student t test, $n=10$). **Bi:** Representative leak-subtracted current traces show that pretreatment with MOR selective antagonist CTAP (5 μ M) or consecutive application of

CTAP (5 μ M) +DAMGO (1 μ M) did not alter voltage ramp-evoked I_K . **Bii:** Summary graph showing I_K activation ($V_{0.05}$) and half-activation ($V_{0.5}$) from Boltzmann fits in control, followed by pretreatment with CTAP and with CTAP+DAMGO ($V_{0.05}$: $p=0.47$, one way repeated measures ANOVA, $n=8$; $V_{0.5}$: $p=0.28$, one way repeated measures ANOVA, $n=8$). **Ci:** Representative leak-subtracted current traces show that I_K evoked by depolarizing voltage steps in ipRGCs was markedly reduced by 4-AP (2 mM). In presence of 4-AP, DAMGO (1 μ M) did not shift the activation of the remaining I_K . **Cii:** Same as in Ci, but traces obtained in 4-AP and 4-AP+DAMGO normalized to their peak showing no difference in their activation kinetics. **Di:** Representative leak-subtracted current traces show that I_K evoked by depolarizing voltage ramps in ipRGCs was markedly reduced by TEA (1 mM). In presence of TEA, DAMGO (1 μ M) did not shift the activation of the remaining I_K . **Dii:** Same as in Di, but traces obtained in TEA and TEA+DAMGO normalized to their peak showing no difference in their activation kinetics.

Figure 9.

MOR agonist DAMGO inhibits I_{Ca} in ipRGCs. **Ai:** DAMGO (1 μ M) inhibited the total I_{Ca} ($I_{Ca,total}$) evoked with depolarizing steps from -80 mV holding steps between -10 mV and 0 mV ($p=0.01$, two way ANOVA). **Aii:** The non-inactivating I_{Ca} ($I_{Ca,non-inact}$) component, recorded in response to depolarizing voltage-steps from the holding potential of -40 mV was also significantly reduced by DAMGO ($p=0.01$, two way ANOVA) at -10 mV and 0 mV. **Aiii:** Peak $I_{Ca,non-inact}$ values were subtracted from the peak values of $I_{Ca,total}$ at

corresponding step potentials to calculate the inactivating portion of I_{Ca} ($I_{Ca,inact}$), which was not inhibited significantly ($p=0.43$, two way ANOVA) by DAMGO.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript