Cellular/Molecular

**Orexin-A Intensifies Mouse Pupillary Light Response by Modulating Intrinsically Photosensitive Retinal Ganglion Cells**

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We show for the first time that the neuropeptide orexin modulates pupillary light response, a non-image-forming visual function, in mice of either sex. Intravitreal injection of the orexin receptor (OXR) antagonist TCS1102 and orexin-A reduced and enhanced pupillary constriction in response to light, respectively. Orexin-A activated OX1Rs on M2-type intrinsically photosensitive retinal ganglion cells (M2 cells), and caused membrane depolarization of these cells by modulating inward rectifier potassium channels and nonselective cation channels, thus resulting in an increase in intrinsic excitability. The increased intrinsic excitability could account for the orexin-A-evoked increase in spontaneous discharges and light-induced spiking rates of M2 cells, leading to an intensification of pupillary constriction. Orexin-A did not alter the light response of M1 cells, which could be because of no or weak expression of OX1Rs on them, as revealed by RNAscope in situ hybridization. In sum, orexin-A is likely to decrease the pupil size of mice by influencing M2 cells, thereby improving visual performance in awake mice via enhancing the focal depth of the eye's refractive system.

**Key words:** intrinsic excitability; intrinsically photosensitive retinal ganglion cell; light response; melanopsin; orexin; pupillary light response

**Significance Statement**

This study reveals the role of the neuropeptide orexin in mouse pupillary light response, a non-image-forming visual function. Intravitreal orexin-A administration intensifies light-induced pupillary constriction via increasing the excitability of M2 intrinsically photosensitive retinal ganglion cells by activating the orexin receptor subtype OX1R. Modulation of inward rectifier potassium channels and nonselective cation channels were both involved in the ionic mechanisms underlying such intensification. Orexin could improve visual performance in awake mice by reducing the pupil size and thereby enhancing the focal depth of the eye's refractive system.

**Introduction**

Intrinsically photosensitive retinal ganglion cells (ipRGCs), containing the photopigment melanopsin, represent the third class of photoreceptors in the retina in addition to the two classical types, rods and cones (Berson et al., 2002; Hattar et al., 2002). Under physiological conditions, the responses of ipRGCs to light are driven not only by signals from photoreceptors through ON bipolar cells (extrinsic light response), but also by melanopsin (intrinsic light response) (Zhao et al., 2014). ipRGCs are commonly classified into six subtypes (M1-M6), based on their distinct dendritic branching patterns, physiological responses, and central projections (Do, 2019; Aranda and Schmidt, 2020; Sondereker et al., 2020). They are known to project to a variety of subcortical nuclei, including the hypothalamic suprachiasmatic nucleus (SCN) and the olivary pretectal nucleus (OPN), which are primarily responsible for circadian photoentrainment and the pupillary light response (PLR), respectively (Bayer et al., 2008). While the PLR is not directly involved in image-forming (pattern) visual functions, it plays an important role in non-image-forming visual functions and is principally mediated by...
ipRGCs at least in rodents, as evidenced by its complete elimination in ipRGC-ablated mice (Guler et al., 2008; Hatori et al., 2008).

The neuropeptides orexin-A and orexin-B (also known as hypocretin-1 and -2, respectively) are derived from a common precursor polypeptide (prepro-orexin) and originate from the lateral hypothalamic neurons, which project to the whole brain (de Lecea et al., 1998; Sakurai et al., 1998). These peptides regulate the sleep/wake cycle, feeding behavior, metabolism, and reproduction by activating two orexin receptors (OXRs), OX1R and OX2R (de Lecea and Sutcliffe, 2005; Matsuki and Sakurai, 2008; Sakurai et al., 2010). In the rat retina, orexins are expressed by almost all types of neurons except photoreceptors, whereas OX1Rs are expressed on dopaminergic amacrine cells (ACs) and most retinal ganglion cells (GCs), including ipRGCs (Liu et al., 2011). It has been demonstrated that orexins modify image-forming visual functions by affecting neuronal activities in both the inner and outer retina (Zheng et al., 2015; Qiao et al., 2017; G. Zhang et al., 2018). However, there are no data available concerning whether and how orexins could modulate non-image-forming visual functions. In the present work, we demonstrate that orexin-A significantly intensifies the PLR in mice by activating OX1Rs in M2 cells. We further show that this effect of orexin-A comes into play because of an increased intrinsic excitability of M2 cells following the direct activation of OX1Rs on these cells. This is the first report showing that orexins could modulate non-image-forming vision.

Materials and Methods

Animals. All animal experiments were performed in accordance with the standards of the Animal Care and Use Committee of Shanghai Medical College, Fudan University, and the National Institutes of Health's Guide for the care and use of laboratory animals. C57BL/6, Opn4-tdTomato, Opn4+/-, and rd/rd cl mice were used in the present work. In Opn4-tdTomato mice, M1-M3 ipRGCs, which are genetically labeled by the red fluorescent protein tdTomato (Do et al., 2009), could be reliably identified under the microscope used in this work. The Opn4+/- mouse (Xue et al., 2011), with melanopsin knocked out and ipRGCs labeled by tdTomato, was created by mating the melanopsin-deficient Opn4+/-cKO line (Hattar et al., 2002) with the Opn4-tdTomato line. The rd/rd cl mouse was generated by crossing rd1 with the Cone-DTA mouse, in which rods and cones progressively degenerate and are virtually absent at ~80 d postnatally (Lucas et al., 1999). Both male and female mice were used in the experiments. C57BL/6, Opn4+/-tTomato, and Opn4+/- mice were 2-4 months old, whereas rd/rd cl mice were at least 80 d old to ensure complete loss of rod/cone photoreceptors. They were housed under a 12:12 h light-dark cycle, with the light turned on at 8:00 A.M.

Electrophysiology. Before each experiment, each animal was dark-adapted for at least 2 h. All animals were overdose with 25% urethane (0.2 ml/100 g) under dim red light. Both eyes were harvested and placed in Ames’ medium (MilliporeSigma) equilibrated with carbogen. The retina was isolated from the pigment epithelium and placed in a recording chamber mounted on the stage of an upright, fixed-stage fluorescence microscope (BX51WI; Olympus). The preparation was continuously perfused with oxygenated Ames’ medium (30°C-32°C) at a rate of ~3 ml/min and kept in darkness for ~1 h before electrophysiological recordings. TdTomato-labeled ipRGCs were visualized by epifluorescence with 530–550 nm light excitation (14:45 log photons/cm²/s measured at 540 nm with a model 350 optometer (UDT Instruments)) using a rhodamine filter cube (U-MWIGi3; Olympus) through a 60× water immersion objective (numerical aperture = 0.9). After brief exposure to fluorescent excitation, the retina was maintained in darkness for 10 min before recording. Glass micropipettes with tip resistance of 5–7 MΩ were pulled from thin-walled borosilicate tubes (BF 150-110-10; Sutter Instrument) using a P-97 puller (Sutter). Cell-attached recordings were made using glass electrodes filled with a solution of 150 mM NaCl and 10 mM HEPES in voltage-clamp configuration with a holding potential of 0 mV and seal resistance >500 MΩ. For all current-clamp recordings, a K+/-based intracellular solution was used, containing the following (in mM): 120 K-glucos, 5 NaCl, 4 KCl, 10 HEPES, 2 EGTA, 4 Mg-ATP, 0.3 Na-GTP, 7 Tris-phosphocreatine, and 0.1% Lucifer yellow (LV); the pH was adjusted to 7.3 with KOH. For all voltage-clamp recordings, we used a Cs+-based intracellular solution containing the following (in mM): 120 CsMeSO₄, 5 NaCl, 4 TEA-Cl, 10 HEPES, 2 EGTA, 4 Mg-ATP, 0.3 Na-GTP, 7 Tris-phosphocreatine, and 0.1% LV; the pH was adjusted to 7.3 with CsOH. Fast capacitance was fully canceled, and cell capacitance was partially canceled using the amplifier. The holding potential was −73 mV, which was near the reversal potential of CI− for voltage clamping. The holding current was 0 pA for current clamping. Data were obtained using an Axon 700B amplifier (Molecular Devices) and a HEKA EPC10 amplifier (HEKA Elektronik). Signals were low-pass filtered at 2 kHz and sampled at 10 kHz. Series resistance was typically between 20 and 40 MΩ and was compensated by ~70%. Effects of all the pharmacological manipulations reported in this work were reversible. Data collected from cells that failed to show an evident recovery from drug treatments (at the time when the preparations had been washed out for at least 10 min) were discarded and not included for further analysis.

Light stimulation. Full-field blue light, which was emitted from an LED illuminator (X-Cite 110 LED; Excelitas Technologie) and bandpass filtered at 475 ± 15 nm with a fluorescent filter cube (U-M650; Olympus) incorporated into the microscope light path, was used to evoke light responses from ipRGCs through the 60° objective. Light intensity was adjusted by neutral density filters in a 10 position 25 mm electronic filter wheel (LB-10; Sutter) coupled to the microscope. To obtain the irradiance-response (I-R) relations in Figure 2D, stimuli were applied to two groups of cells (DMSO and TCS1102) in a series, which was monotonically ascending in intensity (each intensity applied only once), ranging from 11.52 to 15.89 log photons/cm²/s, with interstimulus intervals varied with the light intensity, being shortest for the dimmest stimuli (3 min) and longest for the highest intensities (7 min), so as to minimize the effects of light adaption while completing the experiment as quickly as possible, avoiding response rundown. In other experiments, cells were repeatedly stimulated at a single intensity, with interstimulus intervals fixed to be 7 min (for intrinsic light response, 13.84 log photons/cm²/s) or 3 min (for extrinsic light response, 9.61 log photons/cm²/s), and data collection (control, drug treatment, and washout) was not initiated until the first 1 or 2 stimulations had been applied. In such experimental paradigms, a control experiment found that the response amplitude could be very stable (<5% fluctuation of the response amplitudes) for at least 40 min in agonist/antagonist-free environments.

Identification of ipRGC subtypes. Immediately after whole-cell recording, the cell was visualized via the fluorescence of LV, which was included in the internal solution, and identified morphologically. In cell-attached patch experiments, when the recordings were finished, an electrode filled with LV-containing internal solution was sealed with the cell and the cell membrane was ruptured to allow LV to enter. Cell identity was confirmed offline by immunostaining of LV to enhance fluorescent signals, with ChAT-immunoreactive bands used as retinal laminar references. Cells with sparser dendrites exclusively stratifying in the OFF sublamina of the inner plexiform layer (IPL) were categorized as M1 cells. ON-stratified cells with wider dendritic fields and relatively dense dendrites, somewhat below the ON ChAT-labeled band, were categorized as M2 cells.

Immunohistochemical staining of retinal whole mounts. The retinas were fixed with 4% PFA in 0.1 M fresh PB for 30 min, rinsed with 0.1 M PBS, pH 7.4, and blocked for 2 h in 0.1 M PBS, pH 7.4, containing 6% donkey serum plus 1% Triton X-100. The cells were incubated with the primary antibodies for 3 d at 4°C in 0.1 M PBS containing 3% donkey serum, 1% BSA, and 1% Triton X-100. The binding sites of the primary antibodies were revealed by incubation with the fluorescent secondary
antibodies for 2 h at room temperature. A rabbit polyclonal antibody against LY (1:1000, A5750; Invitrogen), a goat polyclonal antibody against ChAT (1:1000, AB144P; Millipore), and a mouse monoclonal antibody against SM132 (1:1000, SM132R; Covance) were used to enhance LY fluorescence, to label cholinergic AChs, and to label M4 ipRGCs, respectively. The secondary antibodies were Alexa 488-conjugated donkey anti-rabbit IgG for LY, Alexa 555-conjugated donkey anti-goat IgG for ChAT, and Alexa 647-conjugated donkey anti-mouse IgG for SM132 (1:200 all from Invitrogen).

**Determination of membrane properties.** To measure input resistance ($R_m$), under current-clamp mode, ipRGCs were maintained at $-83 \pm 5$ mV with an appropriate current to eliminate spontaneous discharge. The peak voltage change ($\Delta V$) induced by injection of a 1 s hypopolarizing current with an amplitude of 35 pA ($I_{AI}$) was measured. $R_m$ was obtained using Ohm’s law: $R_m = \Delta V/\Delta I$. To measure the action potential threshold, 1 s depolarizing current, increasing in 10 pA increments, was applied to each ipRGC. The action potential threshold was defined as the membrane potential at which the upstroke of the first spike was induced by the lowest-amplitude current.

**Intravitreal microinjection and pupillometry.** All mice subjected to intravitreal injection were dark-adapted for at least 2 h and anesthetized by isoflurane using a small animal anesthesia instrument (RWD). Under dim red light, 1 µl solution containing the dual ORX antagonist TCS1102 or orexin-A was injected intravitreally into the left eyes of the mice using a Nanoject II microinjector (Drummond Scientific); an equal volume of vehicle DMSO solution for TCS1102 and saline for orexin-A was injected into the left eyes in another group as a control. The final vitreal concentration was calculated based on a mouse vitreal volume of 5.3 µl (Remtulla and Hallett, 1985). A 15 min recovery period was allowed before PLR measurements. All PLR recordings were conducted during the light period (ZT6-ZT12). Under dim red illumination, the mouse was placed in a head-and-body restrainer device with a metal bar (implanted into the skull 2 d before the recording) fixed to the device, and PLRs were recorded using a pupillometer (AZ2000; Neuroptics) in combination with Bandicam software (Bandicam). The right eye of each mouse was oriented in the direction of the infrared video camera, with the left eye subjected to the light stimulus. Pupil areas were measured both in the dark and during application of the light stimulus (20 s), and the interval between two consecutive light stimuli was 5 min. The percentage of pupil constriction was calculated as $(1 - A_{min}/A_{0}) \times 100\%$, where $A_{min}$ is the minimum pupil area during light exposure and $A_{0}$ is the pupil area immediately before light exposure. In the melanopsin-mediated PLR experiments, light of increasing intensity (11.09, 11.59, 12.09, 12.59, 13.09, 13.59, 14.09, 14.59, and 15.09 log photons/cm²/s) was presented. In the rod/cone-mediated PLR experiments, the light intensities were 8.09, 9.09, 10.09, 11.09, 11.59, and 12.09 log photons/cm²/s.

**Multiplexing of RNAscope in situ hybridization (ISH) with immunostaining.** Animals were deeply anesthetized with 25% urethane. Isolated eye cups were immersion-fixed in fresh 4% PFA in 0.1 M PB, pH 7.4, for 1 h and then cryoprotected at 4°C in successive solutions of 0.1 M PBS containing 10% (w/v), 20%, 30% of sucrose. After that, the eye cups were obtained using Ohm’s law: $R_m = \Delta V/\Delta I$. To measure the action potential threshold, 1 s depolarizing current, increasing in 10 pA increments, was applied to each ipRGC. The action potential threshold was defined as the membrane potential at which the upstroke of the first spike was induced by the lowest-amplitude current.

**Image acquisition.** Images were obtained using a fluorescent confocal microscope (Fluoview 1000, Olympus) with a 60× oil immersion objective lens. To avoid any possible reconstruction stacking artifact, images were acquired separately from each laser channel on single-layer optical sections. Photoshop CC 2019 (Adobe) was used to adjust image brightness and contrast globally.

**Materials and methods.** All chemicals were purchased from MilliporeSigma. TCS1102 and SR334867 were initially dissolved in DMSO for stock, while all other drugs were directly dissolved in ion-free water to make aliquots. All aliquots were stored at −20°C and freshly diluted to working concentrations with saline (for PLR recording) or Ames’ medium (for patch-clamp recording) on the day of the experiment.

**Experimental design and statistical analysis.** Any results reported in this study are highly reproducible, being replicated in at least 3 (typically $n > 6$) animals or cells. Control data were collected from animals/cells treated with drug vehicles. Data were analyzed using Clampfit (Molecular Devices), SigmaPlot 10.0 (Systat Software), and CorelDRAW X7 (Corel). Data are presented as mean ± SEM. For statistical analysis, Kolmogorov–Smirnov test was first used to check the normality of the data distribution, which determined whether parametric or nonparametric tests were used subsequently. Unless otherwise specified, p values represented the results of paired Student’s t test. In all cases, $p < 0.05$ was considered indicative of statistical significance.

**Results**

**Modulation by orexins of PLR in mice**

As orexin-A and -B are present in almost all retinal neurons except photoreceptors (Liu et al., 2011), it is possible that there may be a basal level of endogenous orexins in the mouse retina and retinal neurons function in an orexin-rich microenvironment. We first examined whether and how pupil constriction in response to light steps (463 nm, 20 s) were altered in WT C57BL/6 mice when retinal endogenous orexin activity was suppressed by intravitreal injection of 500 µM TCS1102, an antagonist for both ORX1R and ORX2R. In both control (treated with an equal volume of DMSO solution) and TCS1102-treated mice, pupillary constriction was increased by light steps of increasing irradiance (see the I-R curves in Fig. 1A1). However, TCS1102-treated animals showed an overall decrease in pupillary constriction, compared with control mice (two-way repeated-measures ANOVA, $F_{(1,10)} = 5.382, p < 0.05$; Sidak multiple comparison test, $p < 0.05$ at 10.09 log photons/cm²/s; $n = 10$ for control, $n = 5$ for TCS1102). Figure 1A2 shows representative images of the pupils of TCS1102-treated and control WT mice. The PLR is composed of two components, driven by activation of melanopsin and rods/cones, respectively (Hattar et al., 2003). To precisely understand whether orexins may differentially affect these two components, we next explored the effects of orexin system perturbation on the melanopsin-signal-mediated component in rd/rd rd cl mice, wherein rods/cones were degenerated, and on the rod/cone-mediated component in Opn4−/− mice, in which melanopsin was knocked out. Because of the loss of rods/cones, PLRs exhibited markedly reduced sensitivity in rd/rd rd cl mice compared with C57BL/6 mice; 10 µM TCS1102 administration significantly attenuated pupillary constriction in rd/rd rd cl mice, resulting in a clear downward scaling of the PLR I-R curve (two-way ANOVA, $F_{(1,10)} = 17.81, p < 0.001$; Sidak multiple comparison test, $p < 0.0001$ at 13.59 log photons/cm²/s, $p < 0.01$ at 12.59 and 13.09 log photons/cm²/s, $p < 0.05$ at 12.09, 14.09, and 14.59 log photons/cm²/s; $n = 10$ for control, $n = 12$ for TCS1102) (Fig. 1B1,B2). Similarly, attenuation of pupillary constriction by 10 µM TCS1102 was also observed in Opn4−/− mice (two-way repeated-measures ANOVA, $F_{(1,11)} = 8.551, p < 0.05$; Sidak multiple
The above results imply that orexins intensify pupillary constriction, thus making the pupil smaller in response to light. We found that following intravitreal injection of orexin-A at a dose close to that induces robust activation of OXRs in central neurons (15.87 μM), C57BL/6 mice showed enhanced pupillary constriction in response to light steps (Fig. 1D1), resulting in a significant upward scaling of the I-R curve relative to that of vehicle-treated control mice (two-way repeated-measures ANOVA, \( F_{(1,14)} = 10.55 \), \( p < 0.01 \); Sidak multiple comparison test, \( p < 0.01 \) at 10.09 log photons/cm²/s, \( p < 0.05 \) at 11.09 log photons/cm²/s; \( n = 7 \) for saline-treated control animals, \( n = 9 \) for orexin-A-treated animals). Figure 1D2 presents representative pupil images showing the distinct constriction amplitudes of orexin-A-treated and control mice.
We also examined whether TCS1102 and orexin-A could affect basal pupil areas measured in complete darkness for at least 10 s. Intravitreal injection of TCS1102 did not significantly alter the basal pupil size in C57BL/6, rd rd/crl, and Opn4+/− mice, nor did orexin-A change the basal pupil area in C57BL/6 mice (all p values > 0.05; Mann–Whitney U test in Fig. 1B3, unpaired t test with Welch’s correction in Fig. 1A3,C3,D3). It seems likely that orexins modulate the PLR by boosting the light-induced pupil dynamic constriction, rather than adjusting the setting of the basal level of the PLR in the dark.

Orexin-A increases the intrinsic light-evoked spiking rates of M2 cells
The PLR in mice is principally mediated by ipRGCs (Guler et al., 2008). It is known that pupillary constriction is intensified as spiking rates of ipRGCs are increased (Keenan et al., 2016; Milosavljevic et al., 2018). Because M1/M2 cells mostly project to the OPN (Baver et al., 2008) and pupillary constriction is minimized when light-induced GC spiking is blocked by TTX (Webb et al., 2013), it was reasonable to explore how the activity of M1/ M2 cells could be modulated by orexins.

We first investigated I-R relations of the “intrinsically,” melanopsin-driven light-evoked spiking rates of M2 cells with and without TCS1102 in whole-mount retinas. WT Opm4-tdTomato mice were used for this experiment, in which ipRGC somata are specifically labeled with red fluorescence protein (Do et al., 2009). We targeted cells exhibiting small somata with bright red fluorescence, characteristic of M2 and M1 cells, for recording. M2 cells can be reliably distinguished based on their distinct dendritic profiles by LY included in the recording pipettes (Fig. 2A). These cells exhibited wide dendritic fields and sparse dendrites stratifying exclusively in the ON sublamina of the IPL, somewhat below the ON ChAT-labeled band (Schmidt and Kofuji, 2009). These cells could not be M4 cells as evidenced by the absence of immunolabeling of SMI-32, a marker for M4 cells (Schmidt et al., 2014), in all 18 cells examined (a representative example is shown in Fig. 2B). They are quite different morphologically from M1 cells, which are “OFF-stratifying” cells in the retina, and from M5 cells, which are also “ON-stratifying” neurons but are characterized by small and bushy dendritic fields (Stabio et al., 2018), as well as from M3/M6 cells which possess bistratified dendrites (Schmidt and Kofuji, 2011; Quattrochi et al., 2019).

During these experiments, the retinas were perfused with a cocktail containing 50 μM L-AP4 (metabotropic glutamate receptor 6 agonist), 30 μM D-AP5 (NMDA receptor antagonist), and 40 μM DNXQ (AMP/kainate receptor antagonist) to specifically block glutamatergic transmission (Fig. 2C). Therefore, the light-induced responses to a 2 s 475 nm light step series at 11.52–15.89 log photons/cm²/s, recorded in cell-attached mode, were exclusively driven by melanopsin. In the absence of TCS1102 (control), a light step exceeding melanopsin activation threshold induced robust spike discharges in a group of M2 cells, and these spikes persisted during light exposure and then decayed slowly, lasting over for tens of seconds, as reported previously (Berson et al., 2002; Zhao et al., 2014) (Fig. 2D1, top). Administration of TCS1102 (10 μM) to another group of M2 cells led to markedly attenuated discharges (Fig. 2D1, bottom). The spiking rates, measured during a 60 s period after light onset in M2 cells, were increased as a function of light intensity in both control and TCS1102-treated M2 cells. TCS1102 administration resulted in a downward scaling of the I-R curve (two-way ANOVA, F(1,0) = 6.382, p < 0.05; Sidak multiple comparison test, p < 0.01 at 13.84, 14.81 log photons/cm²/s, p < 0.05 at 15.89 log photons/cm²/s; n = 5 for control, n = 5 for TCS1102) (Fig. 2D2). These results were similar to those observed in the melanopsin-driven PLR (Fig. 1B1), suggesting that orexins may modulate mouse PLR by changing the outputs of M2 cells.

Next, we tested the effect of orexin-A on the intrinsic light-evoked spiking rates of M2 cells in response to a 475 nm test light pulse (13.84 log photons/cm²/s, 2 s). Light-induced responses were analyzed during short (2 s) and long (60 s) periods after light onset. When 500 nM orexin-A was applied to the retina, a significant increase in the spiking rate of M2 cells was seen (Fig. 2E1). On average, the spiking rate of M2 cells in the presence of 500 nM orexin-A was increased from 7.07 ± 0.83 Hz to 11.42 ± 1.18 Hz (Fig. 2E2) and from 2.85 ± 0.53 Hz to 7.29 ± 0.80 Hz (Fig. 2E3) for short and long periods (Wilcoxon signed-rank test, p < 0.001 in both cases, n = 19).

The orexin-A-induced effect was concentration-dependent. Figure 2F1, F2 shows the dose–response relations between orexin-A and M2 cell spiking rates for short and long periods. To avoid any layering effects of different concentrations, each cell was treated with orexin-A of only one concentration, and data from four different cell groups (50, 100, 500, and 1000 nM) were pooled to generate the dose–response functions. No significant potentiation of light-induced spiking rates was seen with application of 50 nM orexin-A (105.45 ± 3.70% and 102.21 ± 7.55% of control for short and long periods, respectively, p > 0.05 in both cases, n = 3). However, the average spiking rate for the test light step was significantly potentiated by orexin-A in a dose-dependent manner for short period data when the concentration was larger than 100 nM (100 nM: 124.83 ± 10.2% of control, p < 0.05, n = 7; 500 nM: 155.00 ± 9.81% of control, p < 0.001, n = 12; 1000 nM: 178.60 ± 15.42% of control, p < 0.05, n = 3) (Fig. 2F1). Similar results were obtained for long-period data (Fig. 2F2).

To determine what subtype(s) of OXRs may mediate the orexin effect pharmacologically, we tested the effects of two distinct OXRs antagonists on the intrinsic light-evoked spiking rate of M2 cells. As shown in Figure 2G1–G3, SB334867 (5 μM), a selective antagonist for OX1R, significantly reduced the intrinsic light-evoked spiking rate of M2 cells from 18.07 ± 2.35 Hz to 14.06 ± 2.20 Hz (p < 0.001, n = 7) and from 8.70 ± 2.40 Hz to 6.42 ± 2.05 Hz (p < 0.01, n = 7), for short and long period data, respectively. In contrast, TCS OX229, a selective antagonist for OX2R, did not change the spiking rate (p > 0.05 for both short and long period data, n = 7; Fig. 2H1–H3). These results suggested a role of retinal endogenous orexins in modulating M2 cells, and raised a possibility that OX1R, but not OX2R, may be involved in the orexin effect.

This possibility was strengthened by the experiment showing that orexin-A (500 nM) failed to increase the intrinsic light-evoked spiking rate of M2 cells pretreated with SB334867. In this experiment, we first perfused the preparations with SB334867 (5 μM), which reduced the spiking rate to a steady level. In the presence of SB334867, 500 nM orexin-A no longer potentiated the spiking rates (p > 0.05 for both short and long periods data, n = 5; Fig. 2I–I3). In contrast, in the presence of TCS OX229 (5 μM), orexin-A persisted to increase intrinsic light-evoked spiking rates (16.17 ± 1.49 vs 11.63 Hz ± 1.26 Hz for short period data; 9.05 ± 1.45 Hz vs 4.63 ± 0.92 Hz for long period data; p < 0.001 in both cases, n = 12; Fig. 2I–I3).

Orexin-A enhances M2 cell excitability without changing melanopsin-based photocurrents
The next question we sought to answer is how activation of OX1Rs increases the light-evoked spiking rate. Because the
Orexin-A increases intrinsic light-evoked spiking rates of M2 cells. 

**Figure 2.** Orexin-A increases intrinsic light-evoked spiking rates of M2 cells. 

**A.** Confocal microphotographs of a whole-mount mouse retina, showing an M2 cell filled with LY (green) co-immunolabeled with ChAT (red). Side view of the same cell (below), showing that dendritic branches of the M2 cell stratify solely in the ON sublamina of the IPL. Scale bar, 40 μm. GCL, GC layer; INL, inner nuclear layer; ON, ON sublamina; OFF, OFF sublamina.

**B.** Representative microphotograph showing that SMI-32 signals were not seen in the LY-filled ipRGC. Yellow circle represents the soma of the LY-filled M2 cell. Red circles represent SMI-32-positive somata. Scale bar, 20 μm.

**C.** Schematic illustration showing how the intrinsic light responses of ipRGCs were recorded. R, Rod; C, cone; BC, bipolar cell; OPL, outer plexiform layer.

**D1.** Representative intrinsic light-evoked spiking responses of an M2 cell recorded in cell-attached mode, showing that an increase in spiking rate was reduced by TCS1102 (10 μM). Stimulation bar represents the timing of the light pulse (2 s, 475 nm flash with an intensity of 13.84 log photons/cm²/s).

**D2.** Effect of TCS1102 on I-R relations of intrinsic light-evoked spiking rates of M2 cells. TCS1102 reduced the rates evoked by light steps of three higher irradiances (13.84, 14.81, and 15.89 log photons/cm²/s). Data were obtained when the preparations were perfused with Ames’ medium containing glutamatergic blockers (L-AP4, DNQX, and D-AP5). Data obtained in the perfusion medium with DMSO instead of TCS1102 were used as control.

**E1.** Representative intrinsic light-evoked spiking responses of another M2 cell recorded in cell-attached mode, showing that an increase in spiking rate was caused by orexin-A (500 nM).

**E2, E3.** Bar charts showing that orexin-A significantly increased the average firing rates of M2 cells for short (2 s) and long (60 s) periods after light onset. 

**F1, F2.** Orexin-A dose-dependently increased the intrinsic light-evoked spiking rates of M2 cells for short (F1) and long periods (F2) after light onset. 

**G1, G2.** Representative recordings show that 5 μM SB334867, a selective OX1R antagonist, decreased the light-evoked spiking rate of an M2 cell.

**G3.** Bar charts show that SB334867 significantly decreased the average spiking rates of M2 cells for short and long periods after light onset.

**H1, H2.** Representative recordings show that TCS OX229 (5 μM), a selective OX2R antagonist, hardly changed the intrinsic light-evoked spiking rate of another M2 cell.

**H3.** Bar charts summarize the effect of TCS OX229 on the average spiking rates of M2 cells.

**I1.** The intrinsic light response of an M2 cell was no longer increased by orexin-A (500 nM) in the presence of 5 μM SB334867.

**I2, I3.** Bar charts of pooled data show that SB334867 completely blocked the orexin-A effect in M2 cells.

**J1.** Orexin-A (500 nM) persisted to increase the light-evoked spiking rate of an M2 cell during perfusion of 5 μM TCS OX229.

**J2, J3.** Bar charts of pooled data show that TCS OX229 did not abolish the orexin-A effect in M2 cells.

*p < 0.05. **p < 0.01. ***p < 0.001. n.s., not significant.
spiking rate of M2 cells depends on the amplitude of the melanopsin-based photocurrent, a measure of melanopsin phototransduction, and/or on intrinsic membrane excitability (Kowalski et al., 2016; Sonoda et al., 2018), we tested the effects of orexin-A on these two parameters.

Melanopsin-based photocurrents were recorded in whole-cell voltage-clamp mode, with bath perfusion of the cocktail for blocking rod/cone-driven synaptic inputs and TTX for silencing action currents. As shown in Figure 3A, the whole-cell photocurrent recorded from an M2 cell in the presence of 500 nM orexin-A was hardly different from the control. The average peak current collected from seven M2 cells was not affected by orexin-A [21.56 ± 2.15 pA (orexin-A) vs 22.15 ± 2.47 pA (control), p > 0.05] (Fig. 3B), suggesting no involvement of melanopsin phototransduction in the orexin-A-induced increase in spiking rates.

Spontaneous spiking rates generated in darkness may be regarded as an indicator of the excitability of M2 cells (Nelson et al., 2003). Orexin-A at 500 nM increased the M2 cell spontaneous spiking rates, both in the control (Ames’ medium) condition (8.18 ± 0.88 Hz vs 2.72 ± 0.31 Hz, p < 0.01, n = 6; Fig. 3C), and in the presence of the cocktail (6.36 ± 2.09 Hz vs 0.3 ± 0.3 Hz, p < 0.05, n = 5; Fig. 3D), implying an increase of the cell excitability. Orexin-A-induced hyperexcitability is attributed to a direct postsynaptic action of orexin-A on M2 cells, because the effect survived when glutamatergic transmission was blocked by the cocktail (Fig. 3D). Figure 3E shows how the spontaneous spiking rate changed as a function of time following orexin-A application. E, Spontaneous spiking of an M2 cell by 1 s depolarizing current of increasing strength, recorded before and during the administration of 500 nM orexin-A. F, Current versus evoked spiking rate relations. *p < 0.05. **p < 0.01. ***p < 0.001. n.s., not significant.

Orexin-A increases M2 cell excitability by depolarizing the cell membrane
To identify the mechanism underlying the increased intrinsic excitability of M2 cells caused by orexin-A, we examined the effect of orexin-A on several membrane parameters of M2 cells, which have been demonstrated to be associated with the cell excitability (Sonoda et al., 2018). Bath perfusion of orexin-A (500 nM) depolarized the M2 cell and changed the resting membrane potential from −61.5 ± 2.05 mV, which is quite comparable with those reported in several previous studies (Schmidt and Kofuji, 2010; Emanuel et al., 2017), to −56.17 ± 2.52 mV (p < 0.05, Wilcoxon signed-rank test, n = 6; Fig. 4A,E). Similar to the orexin-A-induced increase in the spontaneous spiking rate (Fig. 3C,D), the orexin-A-induced depolarization (5.33 ± 0.72 mV, n = 6) was maintained at a comparable amplitude during glutamatergic cocktail perfusion (5.0 ± 0.27 mV, Dunn’s multiple comparison test, p > 0.05, n = 17; Fig. 4B,F). Moreover, it was not changed by the addition of the chemical synapse blocker CoCl₂ (1 mM) (4.81 ± 0.27 mV, Dunn’s multiple comparison test, p > 0.05, n = 7; Fig. 4CF) or the gap junction blocker carbenoxolone (25 μM) (4.79 ± 0.52 mV, Dunn’s multiple comparison test, p > 0.05, n = 5; Fig. 4D,F). Furthermore, the orexin-A-induced depolarization was unchanged by 5 μM TCS OX229 (4.77 ± 0.43 mV, Dunn’s multiple comparison test, p > 0.05, n = 6; Fig. 4G,H), but markedly reduced by coapplication of 5 μM SB334867 (0.87 ± 0.32 mV, Dunn’s multiple comparison test, p < 0.001, n = 6; Fig. 4H,F), suggesting that such membrane depolarization is mediated by OXR1. Thus, the possibility that the orexin-A-induced depolarization was caused by the effect of orexin-A on presynaptic neurons and/or cells.
electrically coupled to M2 cells could be ruled out; rather, it is a consequence of the direct activation of OX1Rs in these cells, which in turn modifies a series of intrinsic membrane properties.

The changes in input resistance, another important membrane parameter, were assayed by measuring the voltage responses to −35 pA hyperpolarizing current steps injected in M2 cells in the absence/presence of 500 nM orexin-A. The input resistance was increased significantly by orexin-A from $195.77 \pm 15.31 \text{ M} \Omega$, a value close to previous reports (Schmidt and Kofuji, 2009), to $218.70 \pm 16.48 \text{ M} \Omega$ ($p < 0.001$, $n = 10$; Fig. 4J,K), implying the closure of certain channels and reduced leak conductance, which would in turn increase the intrinsic excitability (Kowalski et al., 2016; Sonoda et al., 2018). Orexin-A did not change the action potential threshold of M2 cells ($−57.17 \pm 1.95 \text{ mV}$ in control Ames’ medium vs $−57.50 \pm 1.95 \text{ mV}$ with orexin-A, $p > 0.05$, $n = 9$; Fig. 4L,M).

However, orexin-A depolarized M2 cells, such that the resting membrane potential of these cells became closer to the unchanged action potential threshold, indicating higher intrinsic excitability.

Inward rectifier potassium channels and nonselective cation channels (NSCCs) are both involved in orexin-A-induced depolarization

We investigated ionic mechanisms underlying the orexin-A-induced depolarization through two sets of experiments. In the first set of experiments, relations of whole-cell currents of M2 cells in response to voltage ramps from $−153$ to $27 \text{ mV}$ ($dV/dt = 90 \text{ mV/s}$) ($I-V$ relations) in the presence and absence of 500 nM orexin-A were determined (Fig. 5A). In a small fraction of M2 cells probed (3 of 17, 17.6%; Fig. 5B), orexin-A induced an inward current reversing at $−112.0 \pm 9.5 \text{ mV}$, which was close to...
the calculated equilibrium potential of K⁺ channels (~89.18 mV), suggesting a major contribution of K⁺ channels to this current. However, in a majority of them (11 of 17, 64.7%), the orexin-A-induced current reversed at ~28.9 ± 2.3 mV, which was closer to the equilibrium potential of NSCC (Fig. 5C), thus arguing for mixed ionic mechanisms involved in the orexin-A-induced excitation on M2 cells. The I-V curves of the remaining three cells (3 of 17, 17.6%) did not reverse within the entire voltage ramp range, probably because of the imperfect space clamp of M2 cells, which are reported to be gap-junction coupled to neighboring neurons (Caval-Holme et al., 2019).

The ionic basis of the effect of orexin-A was further explored using various ion channel blockers. The orexin-A-induced depolarization was significantly suppressed by 1 mM Ba²⁺, a nonselective blocker of K⁺ channels, from 5.0 ± 0.27 mV (control) to 3.17 ± 0.16 mV (n = 11) (Dunn’s multiple comparison test, p < 0.01; Fig. 5D,F,J), suggesting the involvement of K⁺ channels. The inward rectifier potassium (Kir) channel-specific blocker Tertiapin-Q (0.1 μM) also suppressed the depolarization significantly (2.44 ± 0.2 mV, p = 0.001, n = 12; Fig. 5E,J), suggesting that NSCCs may contribute to the membrane depolarization of M2 cells induced by orexin-A. Furthermore, when Ba²⁺ was coapplied with FFA (Fig. 5G,J), the orexin-A-induced depolarization was reduced to a very low level (1.33 ± 0.93 mV; Dunn’s multiple comparison test, p < 0.001, n = 6).

Although it was previously reported that sodium-calcium exchangers (NCXs) and Ca²⁺ channels were both involved in the membrane depolarization caused by orexin administration (Eriksson et al., 2001), neither the NCX blocker KB-R7943 (50 μM) nor the Ca²⁺ channel blocker NiCl₂ (1 mM; L- and T-type Ca²⁺ channels, both abundantly expressed by Gαs, are known to be blocked at this concentration) (Mukherjee et al., 2015) significantly altered the orexin-A-induced depolarization of M2 cells (5.54 ± 0.40 mV for KB-R7943, n = 16; 5.25 ± 0.35 mV for NiCl₂, n = 11; Dunn’s multiple comparison test, p > 0.05 in both cases), compared with the depolarization (5.00 ± 0.27 mV, n = 17) recorded in the control condition (in the presence of the glutamatergic blocker cocktail, but without KB-R7943 and NiCl₂) (Fig. 5H–J). Thus, it seems that NSCCs, together with Kir channels, are largely responsible for orexin-A-induced depolarization in M2 cells, whereas NCXs and Ca²⁺ channels were not associated with this action.

Orexin-A increases the extrinsic light-evoked spiking rate of M2 cells

The effect of orexin-A on “the extrinsic,” synaptically driven light responses of M2 cells was tested at 9.61 log photons/cm²/s, which is lower than the threshold for melanopsin activation (Dacey et al., 2005; Zhao et al., 2014). Following orexin-A (500 nM) administration, the extrinsic light-evoked spiking rate of M2 cells during a 3 s period after light onset, recorded under cell-attached mode in normal Ames’ medium, was increased from 12.33 ± 1.96 Hz to 19.02 ± 2.75 Hz (p < 0.01, n = 6; Fig. 6A,B). However, orexin-A had no effect on the extrinsic photocurrent of M2 cells [192.35 ± 45.92 pA (orexin-A) vs 192.82 ± 45.55 pA (control), p > 0.05, n = 8] (Fig. 6C,D). Similar experiments were performed.

**Figure 5.** Multiple ionic mechanisms underlying the orexin-A-induced depolarization in M2 cells. A, Voltage protocols for investigating the I-V relations of M2 cells. B, C, Representative I-V relation changes observed in two subsets of M2 cells tested; the orexin-A-induced current reversed in polarity near the equilibrium potential of K⁺ channels (B) and NSCCs (C), respectively. Insets, Net orexin-A-induced currents calculated by subtracting I-V curves obtained in control condition from those obtained in the presence of orexin-A. D–I, Representative current-clamp recordings showing orexin-A (500 nM)-induced depolarization of M2 cells in Ames’ solution containing the glutamatergic cocktail, along with Ba²⁺ (K⁺ channel blocker, 1 mM), Tertiapin-Q (inward rectifier K⁺ channel blocker, 0.1 μM), FFA (NSCC blocker, 0.1 μM), Ba²⁺ + FFA (G), KB-R7943 (NCX blocker, 50 μM), and NiCl₂ (Ca²⁺ channel blocker, 1 mM). J, Statistical comparison of the effects of various ion channel blockers on orexin-A-induced depolarization of M2 cells. *p < 0.05, **p < 0.01, ***p < 0.001. n.s., not significant.
Orexin-A has no effect on intrinsic and extrinsic light responses of M1 cells

It has been reported that the M1 cell is a critical subtype of ipRGCs projecting to OPN and mediates the PLR (Baver et al., 2008). Figure 7A shows an LY-filled M1 cell in the Opn4−/− tdTomato retina, which is characterized by relatively sparser dendritic branches exclusively stratifying in the OFF sublamina of the IPL. Perfusion of 500 nM orexin-A had no effect on the intrinsic light-evoked spiking rates in response to a 3 s light step (475 nm, 10.9 log photons/cm²/s) of M1 cells [16.58 ± 3.67 Hz (orexin-A) vs 19.13 ± 5.46 Hz (control) for short period, 2.87 ± 0.36 Hz (orexin-A) vs 3.12 ± 0.48 Hz (control) for long period, p > 0.05 in both cases, n = 6] (Fig. 7B–D). Even when orexin-A concentration was increased to as high as 1000 nM, the spiking rates were still not significantly changed for short period [12.63 ± 3.40 Hz (orexin-A) vs 12.75 ± 3.00 Hz (control), p > 0.05, n = 4] (Fig. 7E). Similar results were obtained when the data collection period was extended to a 60-s-long period (Fig. 7F). In addition, administration of the dual OX1R antagonist TCS1102 (10 μM) did not significantly affect the intrinsic light-evoked spiking rates of M1 cells [11.54 ± 1.06 Hz (TCS1102) vs 11.67 ± 0.55 Hz (control) for short period, 3.41 ± 0.65 Hz (TCS1102) vs 3.18 ± 0.51 Hz (control) for long period, p > 0.05 in both cases, n = 4], confirming a lack of orexergic modulation (Fig. 7G–I). Orexin-A (500 nM) also had no effect on the intrinsic photocurrent of M1 cells [136.83 ± 23.28 pA (orexin-A) vs 136.51 ± 24.16 pA (control), p > 0.05, n = 7] (Fig. 7J,K). Furthermore, orexin-A (500 nM) failed to alter the resting membrane potential of M1 cells in Ames’ medium [−54.24 ± 1.97 mV (orexin-A) vs −53.14 ± 1.68 mV (control), n = 9, p > 0.05] (Fig. 7L) or in cocktail [−63.95 ± 0.95 mV (orexin-A) vs −64.36 ± 0.71 mV (control), n = 7, p > 0.05] (Fig. 7M). The extrinsic photocurrent of M1 cells, which was recorded in the Opn4−/− mouse retina, was also not altered (data not shown). Thus, M1 cells are seemingly not involved in the orexin-A-induced intensification of the mouse PLR.

OX1R transcripts are differentially expressed in M2 and M1 ipRGCs

A possible explanation for light responses of M2, but not M1 cells, being affected by orexin-A may be that OX1Rs are differentially expressed in these two ipRGC subtypes. To address this issue, the expression levels of OX1R mRNA were compared between M2 and M1 cells by conducting RNAscope ISH for OX1R mRNA, together with immunostaining using antibodies against tdTomato and melanopsin.

First, we conducted a set of control experiments to evaluate background staining level and to ensure that the specimen for RNAscope analysis is appropriately prepared. Negative control probe to bacterial dihydrodipicolinate reductase (Dapb) revealed nearly complete absence of fluorescent punctuate staining in vertical sections prepared from Opn4−/tdTomato retinas (Fig. 8A1), a result very similar to that obtained when the OX1R probe was omitted (Fig. 8A2), suggesting no nonspecific staining. In contrast, when polymerase II subunit A (polr2a), a rigorous positive control probe widely used for low copy tissues such as the retina, was used, punctuate labeling could be clearly seen, confirming the good preservation of sample RNA (Fig. 8A3).

In Opn4−tdTomato retinas, cell bodies with immunohistochemistry enhanced tdTomato signals were targeted, and their cell-subtype identities were determined based on dendritic stratification patterns in the IPL revealed by a melanopsin antibody (UF-008), which clearly labels the entire dendritic fields of M2 and M1 cells. Whereas those with dendrites stratifying immediately adjacent to the somata and exclusively in the ON sublamina are M2 cells, those ramifying in the outermost part of the IPL are M1 cells (Berson et al., 2010). In a vast majority (14 of 15, 93.3%) of M2 cells tested, OX1R transcript puncta were colocalized with the somata (Fig. 8B1–B4). In contrast, no OX1R transcript puncta
were found in a majority (75.0%) of 16 M1 cells examined (Fig. 8C1–C4). In remaining 4 M1 cells, a trace amount of OX1R transcripts could be detected. Similar results were obtained in M1 cells of C57BL/6 mice for which RNAscope was used to label OX1R transcripts and another M1-preferred melanopsin antibody, PA1-780, was used for M1 cell labeling (X. S. Wu et al., 2019). In a comparable majority (13 of 17, 76.5%) of M1 cells examined, no OX1R transcript puncta were detected (Fig. 8D1–D3), and only a very limited number of puncta could be seen in the rest. When OX1R transcript puncta were counted and compared, it immediately became evident that M2 cells expressed much higher levels of OX1R mRNA (2.07 ± 0.36/cell) than M1 cells (UF-008-labeled M1s: 0.31 ± 0.15/cell; PA1-780-labeled M1s: 0.35 ± 0.17/cell; Dunn’s multiple comparison test, p < 0.001 in both cases; Fig. 8E1). Moreover, as a negative control, rotating the OX1R channel in the image by 180 degree eliminated the difference in puncta numbers among the three groups (UF-008-labeled M2s: 0.27 ± 0.15/cell; UF-008-labeled M1s: 0.06 ± 0.06/cell; PA1-780-labeled M1s: 0.12 ± 0.08/cell; Kruskal–Wallis test, p > 0.05; Fig. 8E2). Such differential expression of OX1R mRNA might, at least in part, account for the M2-specific modulation by orexin-A observed in this study.

Discussion
Orexin-A modulates both rod/cone-and melanopsin-mediated pupillary constriction
Modulation by orexin of the activity of retinal neurons that are responsible for image-forming visual function has been demonstrated (Zheng et al., 2015; Qiao et al., 2017; G. Zhang et al., 2018). In the outer retina, orexin-B increases the activity of rod-driven bipolar cells in rat retina by suppressing GABAergic inhibitory feedback from ACs to these cells. Such modulation improves the visual acuity and contrast sensitivity of animals in dark periods (G. Zhang et al., 2018). In the inner retina, orexin-A suppresses AMPAR-mediated currents of GCs but enhances these currents in ACs via signaling pathways driven by the activation of OX1Rs and OX2Rs, respectively (Zheng et al., 2015). Orexin-A also suppresses the light responses of dopaminergic ACs (Qiao et al., 2017). Whether and how orexins modulate non-image-forming visual function, however, are

Figure 7. Orexin-A has no effect on intrinsic light responses of M1 cells. A, Confocal microphotographs of a whole-mount mouse retina, showing that an M1 cell filled with LY (green) is communolabeled with ChAT (red). Scale bar, 40 μm. B, C, D, Bar charts show that orexin-A had no effect on the average spiking rates of M1 cells for short (C) and long periods (D) after light onset. E, F, Bar chart shows that intrinsic light-evoked spiking rates of an M1 cell recorded in cell-attached mode, before and during application of 500 nM orexin-A. G, H, Bar charts show that orexin-A had no effect on the average spiking rates of M1 cells for short (G) and long periods (H) after light onset. I, J, K, L, Bar chart shows that orexin-A had no effect on intrinsic photocurrents of M1 cells in Ames’ medium were measured in the absence and presence of 500 nM orexin-A. N.s., not significant.
Mechanisms underlying the orexin-induced increase in excitability of M2 cells

There are several reasons to believe that the orexin-A-induced effect could be because of the effect of orexin-A on light-evoked spiking rates of ipRGCs. First, PLR is controlled by the OPN (Clarke and Ikeda, 1985) to which M1 and M2 cells primarily project (Baver et al., 2008). Second, there is evidence showing that brighter light causes increased activity of M2 ipRGCs (Zhao et al., 2013). Such an increment is known to enhance pupillary constriction (Keenan et al., 2016; Milosavljevic et al., 2018), which is minimal when light-induced GC spiking is blocked by TTX (Webb et al., 2013). Third, both the effects of TCS1102 on pupillary constriction in mice and on light-evoked spiking rates of M2 cells were changed in a stimulus intensity-dependent manner, and the effects of TCS1102 on both were similar.

As in other regions of the brain (Kim et al., 2009; Mukai et al., 2009; J. Zhang et al., 2011), the increase in pupillary constriction induced by orexin-A may be attributed to an increase in the intrinsic membrane excitability of M2 cells because of the membrane depolarization. This effect of orexin seems ubiquitous and could explain why orexin excited almost all target neurons in the brain (Kukkonen et al., 2002; Sakurai, 2007), except for glucose-responsive neurons in the ventromedial hypothalamic nucleus, in which the activity was reduced by orexin (Shiraishi et al., 2000). This likely represents a direct effect on postsynaptic activity, as observed in other central neurons, because the membrane depolarization was barely affected when chemical synaptic transmission was blocked and gap junctions were uncoupled.

In different central neurons, orexin-A-induced excitatory effects may be mediated by OX1Rs (Kim et al., 2009; Ho et al., 2011; Dustrude et al., 2018), OX2Rs (Mukai et al., 2009; Yu et al., 2010), or both subtypes of OXRs (J. Zhang et al., 2011). We have shown that the increase in intrinsic light-evoked spiking rate of M2 cells by orexin-A could be reversed by the specific OX2R antagonist SB334867, but not by the specific OX1R antagonist TCS OX229, strongly suggesting the involvement of OX1Rs. Indeed, the expression of OX1R on M2 cells was demonstrated by RNAscope ISH for OX1R mRNA. As shown in Figure 8B, E, in a vast majority (93.3%) of M2 cells, OX1R transcript puncta were localized in the soma of these cells. Notably, the application of orexin-A did not affect M1 cells, indicating that these cells are not involved in the orexin-induced modulation of the PLR, although ~45% of the fibers projecting to OPN originate in M1 cells (Baver et al., 2008). Consistently, no OX1R transcript puncta were found in a majority of M1 cells (Fig. 8C–E). It should be noted that a trace amount of OX1R transcripts could be detected in a small fraction of M1 cells. Further study should be conducted to explore whether these transcripts may be eventually translated into OX1R proteins.

Similar to most of the central neurons examined to date, the increased firing rate of M2 cells induced by orexin-A was mainly because of membrane depolarization. The ionic mechanisms underlying the depolarization are closely related to the signal transduction pathways induced by activation of OX1Rs. They may be complex and cell type-dependent. A variety of processes could be involved, including inhibition of K⁺ channels, activation of NSCCs, and activation of voltage-gated Ca²⁺ channels, etc (Eriksson et al., 2001; Yang and Ferguson, 2002; J. Zhang et

Figure 8. OX1R transcript is differentially expressed on M2 and M1 cells, as revealed by RNAscope ISH in combination with immunostaining. A1–A3, Representative microphotographs showing that the negative control probe DapB detected no punctuate signals in an Opn4-tdTomato retinal section (A1), which was similar to the results obtained by omission of the OX1R probe (A2), whereas the positive control probe Pol2a detected abundant signals in the retinal sections harvested from the same animal. B1, B2, Microphotographs of an M2 cell in an Opn4-tdTomato retinal section, which was stained by antibodies recognizing tdTomato (B1) and melanopsin (UF-008, B2). B3, OX1R transcripts in the section labeled by RNAscope technique. B4, Merged and enlarged image of the boxed area in A1–A3, showing several OX1R transcript puncta in the soma of the M2 cell (arrows). C1–C4, Microphotographs of an M1 cell in another Opn4-tdTomato retinal section, which was processed with the same procedure as that shown in B1–B4. No OX1R transcript puncta were seen in the soma. D1–D3, Microphotographs of an M1 cell collected from a C57BL/6 retinal section, which was stained with a melanopsin antibody primarily probing M1 cells (PA1-780, D1), and then hybridized using RNAscope with the probe targeting OX1R mRNA (D2). D3, Merged and enlarged image showing that OX1R mRNA signals were not seen in the cell. E1, E2, Bar charts showing that significantly more OX1R mRNA puncta were localized to M2 cell somata than to M1 somata (E1), but such a difference was canceled when the OX1R channel was rotated by 180 degrees (E2), arguing against a “by chance” localization of puncta in M2s. ***p < 0.001. Scale bars: A3, C3, D2, 20 μm; C4, D3, 10 μm. n.s., not significant.
In this study, the ionic basis was first explored by examining the effects of orexin-A on I-V relations of M2 cells. It was found that more than one type of orexin-A-induced change in I-V curves was seen. Such a result was reminiscent of those reported in previous studies, in which the effects of orexin on lateral vestibular nuclear neurons (J. Zhang et al., 2011) and septo-hippocampal cholinergic neurons (M. Wu et al., 2004) were investigated, thus suggesting the contribution of multiple ion channels. In the pharmacological experiments conducted subsequently, suppression of orexin-A-induced depolarization by Ba²⁺ suggests the involvement of K⁺ channels. The involvement of Kir channels is further suggested by the reduced depolarization caused by the Kir channel blocker Tertiaripin. During depolarization, Kir channels, which control the resting membrane potential, are closed (Nishida and MacKinnon, 2002).

Therefore, orexin-A seems to increase the firing rates of M2 cells by closing Kir channels, as evidenced by the increased input resistance. K⁺ channel involvement in the effects of orexin-A is commonly seen in central neurons (Kim et al., 2009; J. Zhang et al., 2011). However, the channels that function together with K⁺ channels in central neurons could be cell type-dependent. In lateral vestibular nuclear neurons, for example, activation of Na⁺-Ca²⁺ exchangers along with closure of Kir channels mediate orexin-A-induced depolarization; but in the neurons of the nucleus accumbens shell, the orexin-A-induced depolarization is generated by activating NSCCs and suppressing K⁺ channels (Mukai et al., 2009; J. Zhang et al., 2011). NSCCs are voltage-independent channels that allow the passage of cations of different proportions (Kramer and Zucker, 1985), and they are involved in regulating the excitability of neurons in various systems (Bourque, 1989; Hiruma and Bourque, 1995; Yang and Ferguson, 2002). In this study, orexin-A was found to modify NSCCs, which induced cation influx, thereby depolarizing M2 cells.

Functional implications of orexin-induced pupillary constriction

It is generally thought that orexins are synthesized exclusively in neurons in the hypothalamus (Peyron et al., 1998; Nambu et al., 1999). Orexin activity in other regions may be because of orexinergic neurons extensively projecting to almost the whole brain (Peyron et al., 1998). However, orexins in the retina were unlikely to have been transported from the hypothalamus, and immunohistochemical assay demonstrated the presence of orexins in rat retinal neurons. Notably, orexin-A and -B were detected in ipRGCs, suggesting that the OX₁R expressed in ipRGCs may function as an autoreceptor. Irrespective of whether orexins are produced in the retina and/or the hypothalamus, a critical question is how retinal orexin release is regulated by light. We speculate that orexin release in darkness is reduced by light for the following reasons. First, in the retina, neurotransmitters are released during darkness. There is evidence suggesting that neuropeptides, such as VIP and substance P, are coreleased with certain transmitters. Second, in the rat, a nocturnal animal, the orexin-A level in the lateral hypothalamic shows a robust diurnal change, being higher during wakefulness (dark period) and lower during the sleep phase (light period) (Estabrooke et al., 2001). Therefore, the retinal orexin level might also show a similar diurnal fluctuation. Third, an effect of the dual receptor antagonist TC51102 on PLR was clearly seen in dark-adapted mouse eyes, suggesting a higher retinal orexin level in the dark. Orexins are known to promote wakefulness (Inutsuka and Yamanaka, 2013). Our results demonstrate that orexin increases pupillary constriction, which might improve the visual performance of awake mice. This is simply because, for a given optical system, a smaller aperture yields a greater focal depth.

While orexins in the brain are generally thought to be involved in regulating arousal/wakefulness (Inutsuka and Yamanaka, 2013) and higher-level functions, such as mood (Johnson et al., 2012), the present work shows that orexins also modulate pupillary constriction via a local ipRGC-mediated retinal circuit. Interestingly, ipRGCs are also known to regulate sleep/arousal via their direct projections to the ventrolateral preoptic nucleus (Hattar et al., 2006; Rupp et al., 2019) and to modulate affective responses via communication between the retina and habenular nucleus (Lazzerini Osperi et al., 2017; Fernandez et al., 2018; Huang et al., 2019). It seems likely that orexinergic modulation of these sophisticated behavioral responses occurs not only in the brain but also via a local, intraretinal pathway. ipRGC-selective modulation of OXRs is needed to evaluate this hypothesis.

References


