Orexin-A Suppresses Signal Transmission to Dopaminergic Amacrine Cells From Outer and Inner Retinal Photoreceptors

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Purpose. The neuropeptides orexin-A and orexin-B are widely expressed in the vertebrate retina; however, their role in visual function is unclear. This study investigates whether and how orexins modulate signal transmission to dopaminergic amacrine cells (DACs) from both outer retinal photoreceptors (rods and cones) and inner retinal photoreceptors (melanopsin-expressing intrinsically photosensitive retinal ganglion cells [ipRGCs]).

Methods. A whole-cell voltage-clamp technique was used to record light-induced responses from genetically labeled DACs in flat-mount mouse retinas. Rod and cone signaling to DACs was confirmed pharmacologically (in wild-type retinas), whereas retrograde melanopsin signaling to DACs was isolated either pharmacologically (in wild-type retinas) or by genetic deletion of rod and cone function (in transgenic mice).

Results. Orexin-A attenuated rod/cone-mediated light responses in the majority of DACs and inhibited all DACs that exhibited melanopsin-based light responses, suggesting that exogenous orexin suppresses signal transmission from rods, cones, and ipRGCs to DACs. In addition, orexin receptor 1 antagonist SB334867 and orexin receptor 2 antagonist TCS OX229 enhanced melanopsin-based DAC responses, indicating that endogenous orexins inhibit signal transmission from ipRGCs to DACs. We further found that orexin-A inhibits melanopsin-based DAC responses via orexin receptors on DACs, whereas orexin-A may modulate signal transmission from rods and cones to DACs through activation of orexin receptors on DACs and their upstream neurons.

Conclusions. Our results suggest that orexins could influence visual function via the dopaminergic system in the mammalian retina.

Keywords: orexin, dopamine, melanopsin, amacrine cell, ipRGC, retina

Orexin-A and -B (also known as hypocretin-1 and -2) are hypothalamic neuropeptides that regulate feeding behavior, reward processes, and the sleep–wake cycle.[1–4] However, orexins are also widely expressed in human and mammalian retinas.[5,6] For instance, bipolar cells (which transmit signals from rod and cone photoreceptors to amacrine and ganglion cells) contain orexin-A and -B. Amacrine cells, which provide feedback inhibition to bipolar cells and feedforward inhibition to ganglion cells, also contain orexins. Orexin-A and -B are also expressed in ganglion cells, the output neurons of the retina, which innervate the visual centers of the brain. Despite the wide distribution of orexins throughout the retina, the role of orexins in visual function is largely unknown.[7,8]

The orexins activate two orphan G-protein–coupled orexin receptors, type 1 (OX_1R) and 2 (OX_2R). OX_1R exhibits an order-of-magnitude greater affinity for orexin-A than for orexin-B, whereas OX_2R binds orexin-A and -B with similar affinity.[9,10] In human and mammalian retinas, OX_1R immunoreactivity has been observed in ganglion cells and amacrine cells; however, no OX_2R immunostaining was obtained (but OX_2R mRNA and protein expressions were detected in the rat retina by RT-PCR and Western blot, respectively).[5,6] One subpopulation of amacrine cells called dopaminergic amacrine cells (DACs) express OX_1R in the rat retina.[6] DACs are the primary source for dopamine release within the retina,[11] which plays critical roles in visual function by modulating retinal circuits and synchronizing the retinal circadian clock.[11–13] However, it is unknown if orexins modulate DAC activity through OX_1R, thus influencing visual function.

DACs receive glutamatergic synaptic input from bipolar cells, which are driven by outer retinal photoreceptors (rods and cones).[14–20] In addition, a growing body of evidence has demonstrated that DACs also receive glutamatergic synaptic input from inner retinal photoreceptors,[15,16,21–24] a small population of ganglion cells that respond directly to light via the photopigment melanopsin (*Opn4*).[25,26] These intrinsically photosensitive retinal ganglion cells (ipRGCs) are classified into five subtypes (M1–M5).[27,28] M1 ipRGCs make putative synaptic contact with DACs, forming a retrograde signaling pathway.[23] Notably, M1 ipRGCs, like most retinal ganglion cells, contain orexins and express OX_1R.[6]

In the present study, we explore whether orexin modulates signal transmission from outer retinal photoreceptors (rods and cones) through the conventional neural pathway and inner retinal photoreceptors (ipRGCs) through the retrograde signaling pathway. First, we examined the effect of orexin-A on rod/cone-mediated DAC light responses as well as on melanopsin-based DAC responses. Second, we determined whether endogenous orexins regulate DAC activity by using OX_1R- and OX_2R-specific antagonists. Third, we determined whether intracellular dialysis of the G-protein inhibitor GDP-β-S blocks orexin-induced modulation of DAC activity. Finally, we examined the effect of orexin-A on the melanopsin-mediated activity of M1 ipRGCs.

Materials and Methods

Adult male and female mice were used in all experiments. The animals were housed on a 12:12-hour light-dark cycle, with lights on at 07.30 hours. Food and water were available ad libitum. All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee at Oakland University and Fudan University.

Three lines of transgenic mice were used for the present study. The first line was a wild-type mouse in which DACs were genetically labeled with red fluorescent protein (RFP) under the control of the promoter for the rate-limiting enzyme for dopamine synthesis, *tyrosine hydroxylase* (*TH*) (referred to as wild-type *TH*::RFP mice).[29] In the second *TH*::RFP mouse line, cone photoreceptor-specific cyclic nucleotide channel *Cnga3* and rod-specific G-protein transducin α-subunit *Gnat1* were deleted (*Opn4*-only *TH*::RFP mice).[29–32] The wild-type and *Opn4*-only *TH*::RFP mice had a mixed C57BL/6J and BL6/129 background. We also used a wild-type mouse line (C57BL/6J background), in which ipRGCs were genetically labeled using the fluorescent protein, tdTomato, under the control of the *opn4* promoter (*opn4*-tdTomato mice).[33] In total, we used 5 *opn4*-tdTomato mice, 12 *Opn4*-only *TH*::RFP mice, and 35 wild-type *TH*::RFP mice for the present study.

All electrophysiologic experiments were conducted during the day (11 AM to 5 PM) to avoid a circadian effect. We performed whole-cell voltage-clamp recordings of RFP-labeled DACs and cell-attached recordings of tdTomato-labeled ipRGCs using a flat-mount retina preparation. Retina dissection, electrophysiologic recording, infrared differential interference contrast (IR-DIC) and fluorescence imaging, and light stimulation were performed as described previously.[14,20,34,35] Briefly, the retina was dissected under dim red light and was then placed with the photoreceptor side down in a recording chamber mounted on the stage of an upright conventional fluorescence microscope. Oxygenated extracellular medium continuously perfused the recording chamber, which was kept in darkness for approximately 1 hour prior to recording. RFP-labeled DACs or tdTomato-labeled ipRGCs were visualized by fluorescence using a rhodamine filter set and the identified cells and glass electrodes were visualized using IR-DIC optics. The glass electrode for whole-cell recording was filled with an intracellular solution containing (in mM) 120 Cs-methane sulfonate, 5 EGTA, 10 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), 5 CsCl, 5 NaCl, 0.5 $CaCl_2$, 4 Na-ATP, 0.3 Na-GTP, and 5 lidocaine n-ethyl-chloride (QX-314).[20] QX-314 was used to block intrinsic Na^+ channel-mediated action potentials in DACs, thus highlighting extrinsic light-induced inward currents in the cells and improving the space clamp quality of the voltage-clamp. Whole-cell currents from DACs were amplified with an Axopatch 200B amplifier and acquired using a Digidata 1550A digitizer (Molecular Devices, Sunnyvale, CA, USA).[20] For cell-attached recordings, the glass electrode was filled with a solution containing 150 mM NaCl and 10 mM HEPES.[34] Cell-attached activity was recorded from ipRGCs using an HEKA patch-clamp amplifier and data acquisition system (HEKA, Lambrecht, Germany).[34,35] A 3-second duration light pulse with a peak wavelength of 470 nm was delivered to the retina either through the objective lens (for some DAC recordings and all ipRGC recordings)[20] or via the microscope condenser (for some DAC recordings).[14]

A nonselective G-protein inhibitor, GDP-β-S (Sigma-Aldrich, St. Louis, MO, USA), was used intracellularly to block the action of G-protein–coupled receptors. All other pharmacological agents were obtained from Tocris Bioscience (Ellisville, MO, USA). These included orexin receptor 1 antagonist SB334867, orexin receptor 2 antagonist TCS OX229, nonspecific orexin receptor antagonist TCS 1102, mGluR6 agonist L-2-amino-4-phosphonobutyric acid (L-AP4), *N*-methyl-D-aspartate (NMDA) receptor antagonist D-(−)-2-amino-5-phosphonopentanoic acid (D-AP5), and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate (KA) receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX). Drugs were stored in frozen stock solutions and dissolved in intracellular or extracellular solution before experiments. SB334867 and TCS 1102 were first dissolved in dimethyl sulfoxide and then diluted to working concentration in extracellular solution.

We performed 10 groups of experiments. Four to 10 cells were recorded for each experimental group. The cells in each experimental group were collected using retinas from at least three mice to reduce interindividual variability. Electrophysiologic data were analyzed offline using the Clampfit 10.4 (Molecular Devices) and SigmaPlot 12.0 (Systat Software, Erkrath, Germany) software packages. The light-induced peak current amplitude of each DAC was measured before and during drug application. For each cell, the peak current amplitude during drug application was normalized by dividing it by the peak current amplitude before drug application. In addition, the number of spike events in M1 ipRGCs was counted using the Event Detection feature in Clampfit. The average firing rate (Hz) was found by dividing the number of events by the length of the observation interval. Light-induced firing rates of each M1 ipRGC were measured before and during drug application. A paired *t*-test was used to identify significant differences before and during drug application for each experimental group. $P < 0.05$ was considered to be statistically significant.

Results

As described above, only OX_1R has been detected by immunofluorescence in human and mammalian retinas.[5,6] Given that OX_1R has a greater affinity for orexin-A than orexin-B,[9,10] we used orexin-A to determine the effect of orexins on the retinal dopaminergic system. Light-induced excitatory postsynaptic currents (EPSCs) from RFP-labeled DACs were recorded in flat-mount retinas using a whole-cell voltage-clamp technique. Previous studies using C57BL/6J background wild-type mice have reported that in the majority of DACs (~80%), light-induced EPSCs were completely blocked by L-AP4,[14,15] an agonist of mGluR6 receptors that selectively blocks the ON pathway of the retina.[36] This suggests that these cells receive input solely from rod and cone photoreceptors. In the present study, we used mixed C57BL/129 background wild-type *TH*::RFP mice and found that L-AP4 completely blocked light-induced EPSCs in ~50% of the recorded DACs. Figure 1A shows a representative cell. This cell was clamped at −70 mV and exhibited an inward current at light onset (ON response) that decayed back to the baseline at light cessation (left trace).

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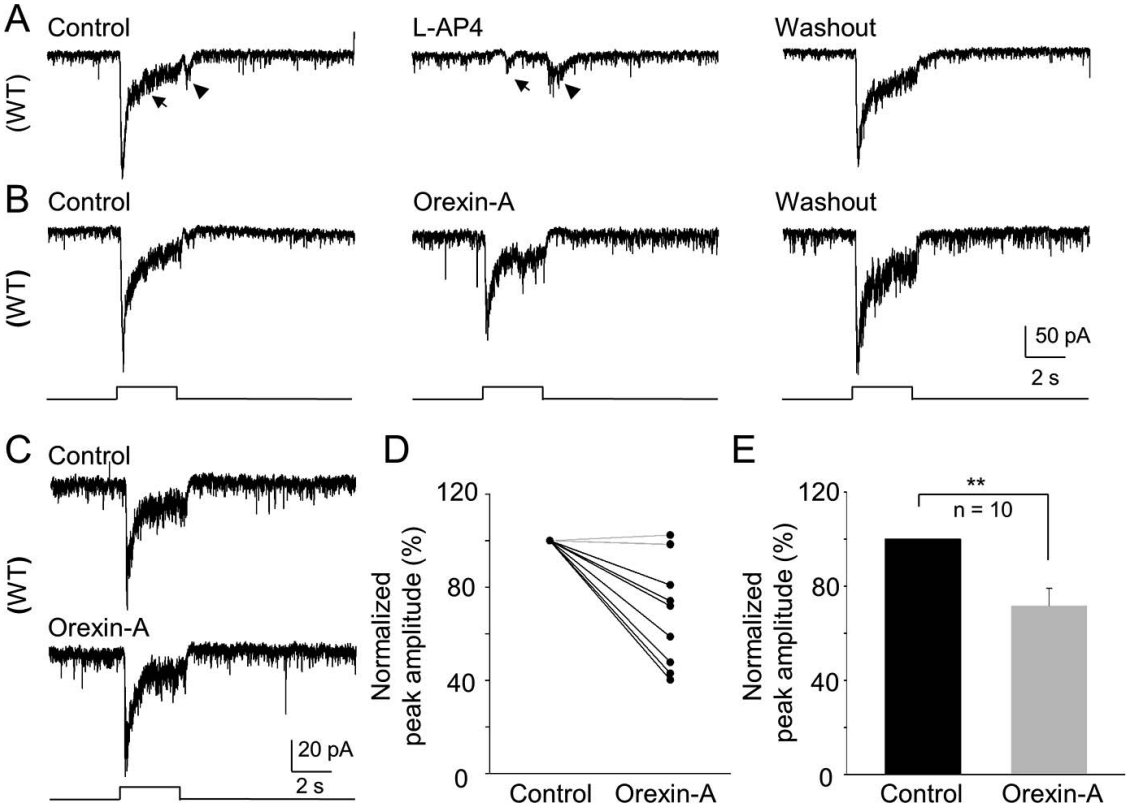

Figure 1. Orexin-A reduces rod/cone-mediated light responses in the majority of DACs in wild-type retinas. Whole-cell voltage-clamp recordings were made of RFP-labeled DACs in flat-mount retinas of wild-type mice. Light-induced EPSCs of DACs in **A**–**C** were completely blocked by 50 μM L-AP4, suggesting that these cells receive input solely from rod and cone photoreceptors. An example is illustrated in **A**; *arrows* and *arrowheads* indicate a delayed ON response and an OFF response, respectively. Upon washout of L-AP4, 500 nM orexin-A was applied to the cells shown in **B** and **C**. Orexin-A reduced the peak amplitude of the DAC EPSC in **B** but not in **C**. Stimulation bar shows the timing of light pulse (3-second, 470-nm flash with an intensity of 4.3×10^{13} photons·s^{-1}·cm^{-2}). Summarized data in **D** show the peak amplitude of the EPSC of each DAC recorded before and after application of orexin-A. Of 10 cells tested, 7 cells were inhibited by orexin-A (black lines), whereas 3 cells had no response to orexin-A (gray lines) (**D**). Average normalized data from the 10 cells in **D** indicates that the peak current amplitude was significantly reduced by orexin-A (**E**). $^{**}P < 0.005$.

In the presence of 50 μM L-AP4, the initial EPSC was completely suppressed (middle trace). This suppression was fully reversed on washout (right trace). Figure 1B illustrates one such L-AP4–sensitive cell. We found that 500 nM orexin-A reduced the peak current amplitude from 172 (left trace) to 124 pA (middle trace). This decrease was reversed on washout (right trace). However, the cell shown in Figure 1C had no response to the same concentration of orexin-A. We tested 10 L-AP4–sensitive cells and found 7 of them were substantially suppressed by orexin-A, whereas the 3 other cells showed no response to orexin-A (Fig. 1D; changes within ±10% were considered as having no effect). Although the results are inconsistent, average data show that the peak current amplitude was significantly reduced from 50.9 ± 15.1 to 35.0 ± 10.7 pA (or 71.6 ± 7.5% of control, $P < 0.01$; Fig. 1E). It is worth noting that in the presence of L-AP4, a delayed ON response (arrows) and an OFF response (arrowheads) became more evident (Fig. 1A, middle trace), as we have previously reported.[20] Because these responses are inhibitory currents,[20] we did not test whether they are modulated by orexin-A.

The remaining ∼50% of DACs recorded in C57BL/129 background wild-type *TH*::RFP mice exhibited an initial inward current, which decayed slowly and persisted for several seconds following light cessation (Fig. 2A). These dynamic characteristics suggest that these cells receive input from rods and cones, as well as ipRGCs.[15,21] To confirm this, we applied 50 μM L-AP4, which only partially blocked the light responses of these cells (Fig. 2B). We tested the effect of 500 nM orexin-A on nine such DACs and found that the light-induced EPSCs were suppressed in every cell (Figs. 2A, 2C). On average, orexin-A reduced the peak amplitude of DAC light-induced inward currents from 34.45 ± 8.3 to 19.4 ± 3.9 pA (or 61.6 ± 5.1% of control, $P < 0.001$; $n = 9$; Fig. 2D).

To isolate melanopsin-based responses in DACs, we generated a *TH*::RFP mouse line without rod or cone function. Using these *opn4*-only *TH*::RFP mice, we found that L-AP4 had no effect on the light-induced EPSCs of DACs (data not shown), suggesting that these responses are mediated exclusively by melanopsin. As expected, orexin-A (500 nM) reduced the peak amplitude of a DAC light-induced EPSC from 26 to 16 pA (Fig. 3A). Similar results were obtained from four other cells. Average data demonstrate that orexin-A reduced melanopsin-based DAC responses from 33.5 ± 7.7 to 14.0 ± 3.1 pA (or 45.0 ± 8.4% of control, $P < 0.01$; $n = 5$; Fig. 3C). To rule out the possibility that genetically removing rod and cone function alters the neural pathway to DACs, we repeated this experiment in wild-type DACs in the presence of L-AP4. As stated above, L-AP4 pharmacologically blocks excitatory rod and cone inputs in wild-type *TH*::RFP retinas, so any remaining excitatory response must be mediated exclusively by melanopsin. We found that orexin-A also suppressed L-AP4-resistant EPSCs in four of four wild-type DACs (Fig. 3B). On average, orexin-A reduced L-AP4–resistant DAC responses from 42.5 ± 16.6 to 33.4 ± 15.4 pA (or 72.6 ± 5.3% of control, $P < 0.05$; n

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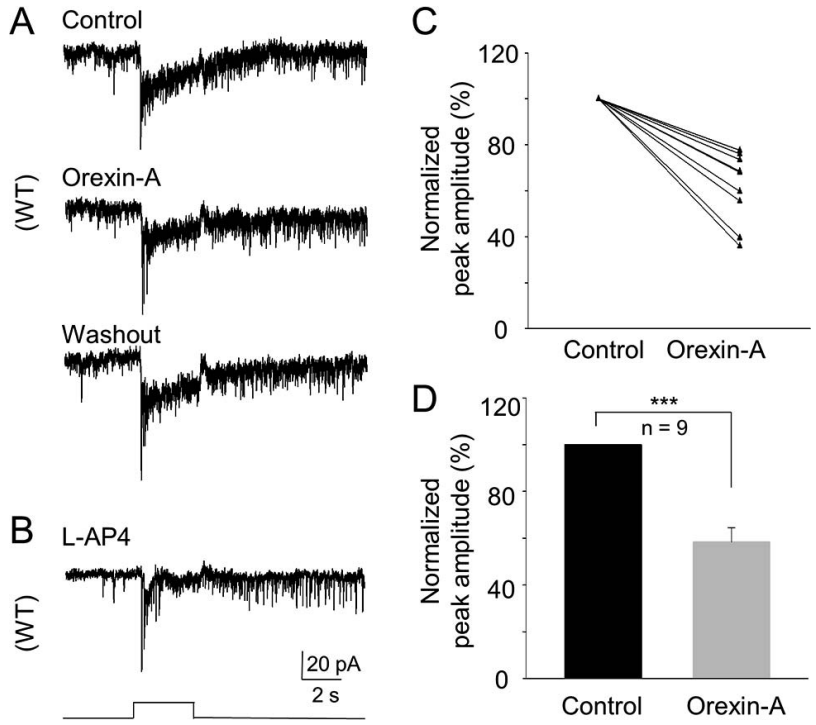

Figure 2. Orexin-A suppresses DAC light responses evoked by inputs from rods, cones, and melanopsin in wild-type retinas. Light-induced EPSCs of a DAC (**A**) exhibited slow decay kinetics following light cessation (*top trace*), suggesting that this cell receives inputs from melanopsin-expressing ipRGCs, as well as rods and cones. This was confirmed by applying L-AP4, which reduced the light response of the cell in **B**. 500 nM orexin-A reduced the peak amplitude of the light-induced EPSC (*middle trace* in **A**); this inhibition was reversed on washout (*bottom trace* in **A**). Stimulation bar shows the timing of light pulse (3-second, 470-nm flash with an intensity of 4.3×10^{13} photons·s^{-1}·cm^{-2}). Summarized data in **C** show the peak amplitude of the EPSC of each DAC recorded before and after application of orexin-A**.** Similar results were observed in all nine cells tested. Average normalized data in **D** indicate that orexin-A significantly inhibited this subclass of DACs. $^{***}P < 0.001$.

= 4; Fig. 3D). When 10 μM TCS 1102, a nonspecific orexin receptor antagonist,[8,37] was applied, additional orexin-A failed to suppress the L-AP4–resistant EPSCs of DACs (97.2 ± 1.1% of control, $P > 0.05$, $n = 5$; Fig. 3E), suggesting that orexin-mediated suppression is specifically mediated by orexin receptors. Together, these data suggest that orexin-A suppresses signal transmission from ipRGCs to DACs via activation of orexin receptors.

Because immunohistochemical studies have demonstrated that orexins are expressed throughout the retina,[6] we hypothesized that endogenous orexins also suppress signal transmission to DACs. To test this hypothesis, we first examined the effect of SB334867, a selective OX_1R antagonist,[38] on melanopsin-based DAC responses in *Opn4*-only *TH*::RFP retinas. It was found that 5 μM SB334867 significantly increased the peak amplitude of the responses (133.0 ± 6.5% of control, $P < 0.01$, $n = 5$; Figs. 4A, 4B). We then examined the effect of TCS OX229, an OX_2R antagonist,[39] on melanopsin-based DAC responses. It was found that TCS OX229 (20 μM) also significantly increased the peak amplitude of melanopsin-based DAC responses (127.0 ± 8.1% of control, $P < 0.05$, $n = 4$; Figs. 4C, 4D). These results suggest that endogenous orexins attenuate retrograde signaling to DACs in the retina.

The inhibition of glutamatergic signal transmission to DACs by exogenous orexin-A and endogenous orexins could occur on upstream presynaptic neurons or on DACs themselves. Because orexin receptors are G-protein–coupled receptors,[40] the action of orexins can be blocked by G-protein inhibitors.[7,8] To determine whether orexins act via G-protein–coupled orexin receptors expressed by DACs, we added 3 mM GDP-β-S (a nonhydrolyzable G-protein inhibitor) into the pipette solution. If dialysis of GDP-β-S into DACs blocks orexin-A inhibition, it would suggest that orexin receptors on DACs are involved in mediating the inhibition. To test this, we performed two sets of experiments in wild-type *TH*::RFP mice, as described in Figure 5A. In the first set of experiments (Fig. 5A, left), we tested the effect of orexin-A on L-AP4–resistant DAC responses (melanopsin-based responses) with 3 mM GDP-β-S in the pipette solution. After a whole-cell recording was made on a DAC, we waited 10 minutes to allow GDP-β-S to diffuse throughout the cell. We then applied orexin-A extracellularly and found that it failed to suppress the L-AP4–resistant light responses (35.4 ± 5.5 vs. 35.0 ± 5.4 pA, $P > 0.05$, $n = 7$; Figs. 5B, 5C). Compared with the results shown in Figure 3, this result suggests that orexin-A likely activates G-protein–coupled orexin receptors expressed on DACs, which in turn suppress synaptic input from ipRGCs to DACs.

Does the intracellular dialysis of GDP-β-S in DACs also block orexin-A suppression on synaptic input from rods and cones to DACs? To address this question, we performed a second set of experiments (Fig. 5A, right) to determine whether orexin-A suppresses rod/cone-mediated DAC responses through G-protein–coupled orexin receptors. Again using 3 mM GDP-β-S in the pipette solution, we first applied L-AP4 (as described in Fig. 1) to confirm whether the cell only received input from rods and cones. If L-AP4 completely blocked the DAC light response, we washed out L-AP4 and tested whether orexin-A had an effect on the cell's light response. Although average data show that orexin-A had no significant effect on the light responses (64.0 ± 13.4 vs. 61.9 ± 10.5 pA, $P > 0.05$, $n = 6$), we observed two distinct effects mediated by orexin-A. One group of cells showed an increased peak amplitude in the

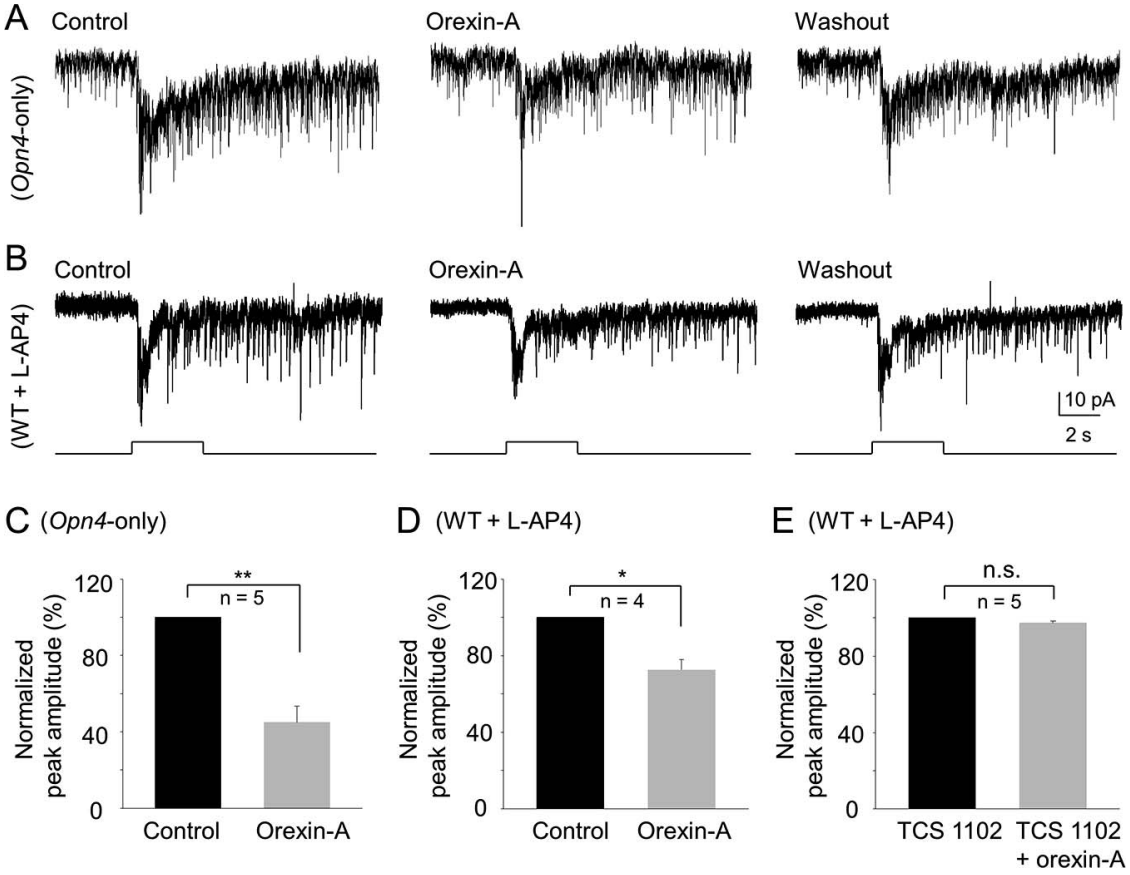

Figure 3. Orexin-A suppresses melanopsin-based DAC responses. Melanopsin-based DAC responses were isolated by either genetically removing rod and cone function in *opn4*-only *TH*::RFP mice (**A**) or by blocking rod/cone input with L-AP4 in wild-type *TH*::RFP mice (**B**). Orexin-A reduced the peak amplitude of a melanopsin-based response in a DAC recorded in an *opn4*-only *TH*::RFP retina (**A**) and in a DAC recorded in wild-type retina in the presence of L-AP4 (**B**). Stimulation bar shows the timing of light pulse (3-second, 470-nm flash with an intensity of 2.89×10^{12} photons·s^{-1}·cm^{-2}). Average data in **C** were collected from five DACs in *opn4*-only *TH*::RFP retinas. Average data in **D** were collected from four DACs from wild-type *TH*::RFP retinas in the presence of L-AP4. These data show that orexin-A significantly suppressed melanopsin-based DAC responses. Average data in **E** show that TCS 1102 completely blocked the orexin-A–mediated suppression of melanopsin-based DAC responses in wild-type *TH*::RFP retinas in the presence of L-AP4 ($P > 0.05$, $n = 5$). $^{*}P < 0.01$, $^{**}P < 0.005$, $^{n.s.}P > 0.05$.

presence of orexin-A (123.4 ± 7.1% of control, $n = 3$; Fig. 5D, gray lines), whereas the other group showed a reduced peak amplitude (82.7 ± 3.2% of control, $n = 3$; Fig. 5D, black lines). These results are considered in the discussion below.

Finally, we examined whether orexin inhibition of DAC light-induced activity is due to the action of orexin on ipRGCs. Our previous study has suggested that signal transmission from ipRGCs to DACs is likely mediated by the action potentials of M1 ipRGCs.[23] Therefore, we determined whether orexin-A had an effect on melanopsin-mediated action potentials in M1 ipRGCs using *opn4*-tdTomato mice. Action potentials of ipRGCs were recorded using a cell-attached extracellular recording method as this technique (compared with whole-cell recording) does not influence the composition of the cell cytoplasm. We also applied a cocktail of synaptic blockers (50 μM L-AP4, 30 μM D-AP5, and 40 μM DNQX) to block any excitatory inputs from rods and cones to ipRGCs (Fig. 6A). Because this cocktail almost completely eliminated ipRGC spontaneous activity, we did not subtract spontaneous activity from the light-induced action potentials. Due to depolarization-induced blockage of action potentials at high light intensities, a low stimulation intensity (8×10^{10} photons/cm^2·s) was used to evoke ipRGC light-induced action potentials. Furthermore, tdTomato labeled more than one type of ipRGCs in the *opn4*-tdTomato mouse retina.[33] To identify tdTomato-labeled M1 ipRGCs, after a cell-attached recording had been executed, a new glass electrode filled with Lucifer yellow (0.1%) was introduced into the same cell to reveal its entire morphology. Cells with dendrites stratifying exclusively in the off sublamina of the inner plexiform layer were considered M1 ipRGCs.[27]

Figure 6B depicts the response of an M1 ipRGC to a 3-second, 470-nm light pulse. The cell exhibited a robust increase in the number of action potentials at light onset. This increase persisted during light stimulation and slowly decayed, lasting over 10 seconds after stimulus cessation (Fig. 6B, top trace). This pronounced poststimulus persistence was consistent with previous publications.[15,21,23] When 500 μM orexin-A was applied to the retina, no apparent change in the number of light-induced action potentials was observed (Fig. 6B, bottom trace). We compared the frequency of action potentials during 3-second light stimulation in the absence of orexin-A with the frequency in the presence of orexin-A and found no significant change (20.2 ± 2.1 vs. 20.1 ± 1.2 Hz, $P > 0.05$, $n = 5$; Fig. 6C). The same comparison was made for the frequency of action potentials in a 10-second period after light onset. Again, no significant difference was found (11.3 ± 0.9 vs. 11.8 ± 0.8 Hz, $P > 0.05$, $n = 5$; Fig. 6D). These results suggest that orexin-A does not alter melanopsin-mediated responses of M1 ipRGCs.

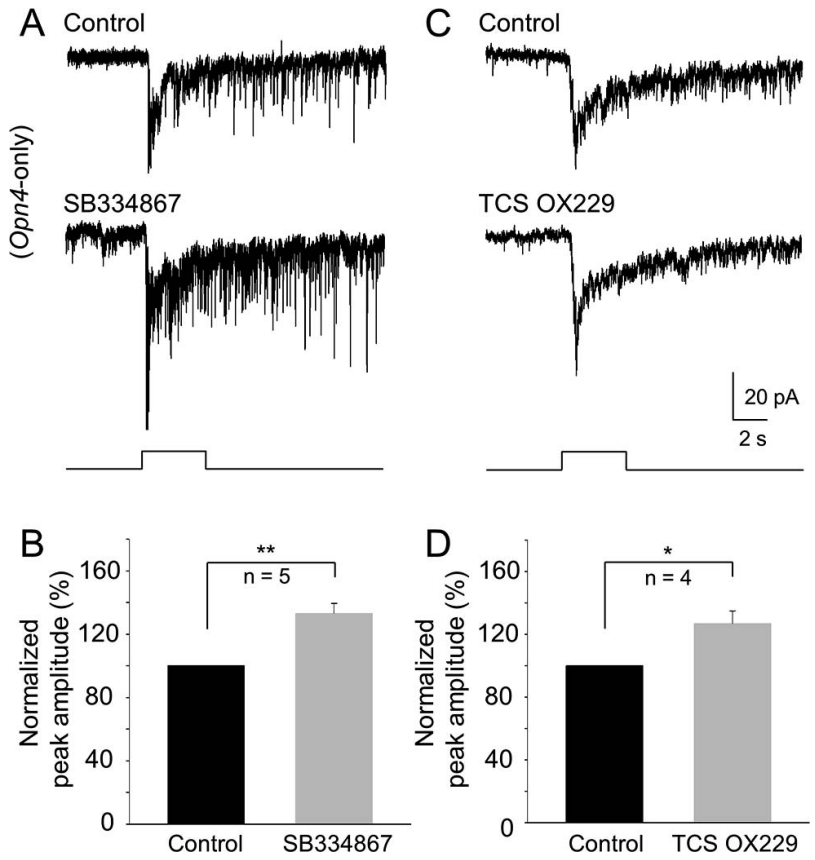

Figure 4. OX_1R and OX_2R antagonists enhance melanopsin-based DAC responses. Melanopsin-based DAC responses were recorded in *opn4*-only *TH*::RFP retinas. *Traces* in **A** show that 5 μM SB334867, a selective OX_1R antagonist, increased the peak amplitude of the melanopsin-based response of a DAC. Average data from five DACs in **B** show that the increase in the presence of SB334867 is significant ($P < 0.005$). In addition, 20 μM TCS OX229, a selective OX_2R antagonist, had a similar potentiating effect on melanopsin-based responses. A typical recording is shown in **C**, and average data are illustrated in **D** ($P < 0.01$, $n = 4$). Stimulation bar shows the timing of light pulse (3-second, 470-nm flash with an intensity of 2.89×10^{12} photons·s^{-1}·cm^{-2}).

Discussion

In the present study, we demonstrated that exogenous orexin-A and endogenous orexins suppress retrograde signaling from ipRGCs to DACs, possibly through activation of G-protein–coupled orexin receptors on DACs. We also showed that orexin-A suppresses rod and cone inputs to the majority of DACs; however, this suppression appears to be mediated by orexin receptors on DACs and their upstream neurons. Overall, the present study suggests that orexins may influence retinal function via the dopaminergic system.

To date, DACs are the only known retinal neurons that receive glutamatergic inputs simultaneously from outer retinal photoreceptors (rods and cones) and inner retinal photoreceptors (ipRGCs).[14–19,21–23] Our results reveal that orexin-A significantly inhibited all DACs that exhibited melanopsin-based light responses. This inhibition was completely blocked by a nonspecific orexin receptor antagonist, suggesting that orexin-A mediates its effects by acting on orexin receptors. In addition, the OX_1R antagonist SB334867 enhanced melanopsin-based signaling to DACs, indicating that endogenous orexins attenuate retrograde signaling from ipRGCs to DACs. This conclusion is supported by previous immunohistochemical studies demonstrating that OX_1R is expressed in most retinal ganglion cells and DACs.[5,6] We also found that the OX_2R antagonist TCS OX229 potentiated retrograde signaling from ipRGCs to DACs. However, the concentration of TCS OX229 required to achieve this potentiation is far higher than the IC_{50} (the concentration of a drug required to achieve 50% of inhibition) of this antagonist for the OX_2R (40 nM).[39] Although we believe that TCS OX229 enhances melanopsin-based DAC responses via blockade of OX_2R, it is possible that TCS OX229-induced potentiation also occurs via blockade of OX_1R. Further investigations are needed to clarify this issue. Regardless, our results clearly suggest that exogenous and endogenous orexins downregulate intraretinal retrograde signaling via activation of OX_1R and possibly OX_2R. In conjunction with a recent study showing that the neuropeptide somatostatin suppressed signal transmission between ipRGCs and DACs,[41] our results suggest that neuropeptides are likely to play an important role in modulating retrograde signaling in the retina.

OX_1R is a G-protein–coupled receptor and has been reported to be expressed in several types of retinal neurons, including DACs and ipRGCs.[6] Therefore, the site of orexin-mediated suppression could be DACs, ipRGCs, or both. Our data show that orexin-A–induced inhibition was abolished when a G-protein inhibitor was dialyzed into DACs (Figs. 5B, 5C). This result strongly suggests that DACs are likely to be the site of orexin-mediated suppression. This conclusion is also supported by our data on ipRGCs (Fig. 6). Our latest studies have shown that M1 ipRGCs are likely presynaptic to DACs (through their axon collaterals) and that the action potentials of M1 ipRGCs are the major driving source for DACs.[23] However, we found that orexin-A had no detectable effect on the frequency of light-induced action potentials in M1 ipRGCs (Fig. 6), indicating that orexin-A is not likely to act on M1

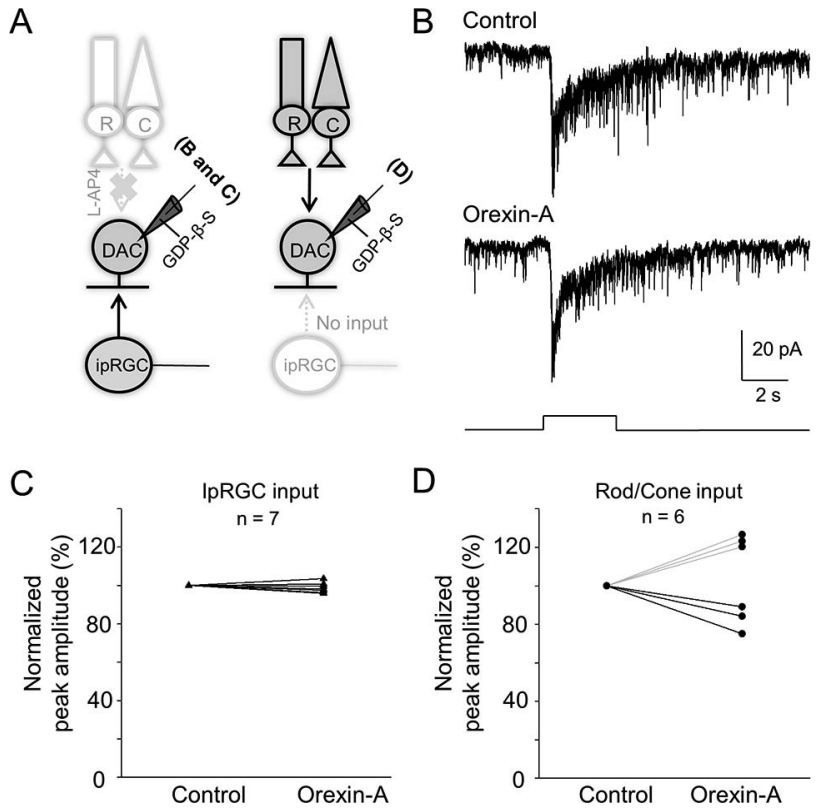

Figure 5. Effects of GDP-β-S on orexin-A affections for ipRGC- or rod/cone-mediated DAC responses in wild-type *TH*::RFP mice. Two schematic diagrams in **A** show the experimental protocols for **B** and **C** (*left diagram*) and for **D** (*right diagram*); 3 mM GDP-β-S, a G-protein inhibitor, was added to the recording pipette solution. Intracellular dialysis of GDP-β-S into DACs blocked orexin-A–mediated suppression of melanopsin-based responses, which were isolated by blocking rod and cone inputs with L-AP4 (**B**). Stimulation bar shows the timing of light pulse (3-second, 470-nm flash with an intensity of 4.3×10^{13} photons·s^{-1}·cm^{-2}). Summarized data in **C** show the melanopsin-based response of each DAC recorded before and after application of orexin-A. GDP-β-S blocked the orexin-A–induced inhibition of melanopsin-based responses in all seven cells tested (**C**). DACs in **D** receive input only from rods and cones (verified by application of LAP-4). In the presence of GDP-β-S, of six cells tested, three cells were inhibited by orexin-A (*black lines*), whereas three cells were potentiated by orexin-A (*gray lines*).

ipRGCs, thereby suppressing melanopsin-based DAC responses.

In contrast, orexin-A suppressed rod/cone-mediated responses in 70% of DACs, whereas the remaining DACs showed no response to orexin-A (Fig. 1D). This result suggests that orexin-A acts on other sites in the pathways by which rods and cones signal to DACs and not just on DACs themselves. When DAC orexin receptors were blocked by an intracellular G-protein inhibitor, we found that orexin-A was still able to suppress rod/cone-mediated responses in 50% of DACs (Fig. 5D). Apparently, this suppression occurs on neurons that are presynaptic to DACs. Orexin-A thus suppresses the activity of these DACs through both presynaptic and postsynaptic inhibition. Accordingly, these DACs would be classified with the 70% of DACs in which we observed orexin-mediated inhibition (Fig. 1D). Interestingly, in the presence of an intracellular G-protein inhibitor, we observed that orexin-A enhanced rod/cone-mediated responses in 50% of DACs (Fig. 5D), suggesting that in some cases orexin-A increases presynaptic glutamatergic transmission to DACs. This presynaptic potentiation by orexin-A would act against the postsynaptic inhibition mediated by DAC orexin receptors, resulting in one of three outcomes. First, if the presynaptic potentiation is weaker than the postsynaptic inhibition, the overall effect would be inhibitory, and these DACs would display an orexin-A-mediated inhibition (Fig. 1D). Second, if the presynaptic potentiation is roughly equal to the postsynaptic inhibition, the potentiation and inhibition would cancel each other out, resulting in an apparent unresponsiveness to orexin-A. This would account for the 30% of DACs we observed that showed no response to orexin-A (Fig. 1D). Finally, the presynaptic potentiation could be stronger than the postsynaptic inhibition. However, this may be very rare (or nonexistent), as we did not observe any DACs that showed enhanced rod/cone-mediated responses in the presence of orexin-A (Fig. 1D).

The present study does not attempt to determine which types of upstream neurons are inhibited or excited by orexin-A, as multiple neurons and neural pathways are involved in signal transmission to DACs. Rods signal to DACs through the primary rod pathway (rod → rod bipolar cell → AII amacrine → cone bipolar cell → DAC), the secondary rod pathway (rod → cone → cone bipolar cell → DAC), and the tertiary pathway (rod → cone bipolar cell → DAC).[15,16] In addition, cones can excite DACs through ON cone bipolar cells directly or indirectly via ipRGCs.[14,17–20] Cones can also produce ON and OFF inhibitory responses on DACs through distinct OFF bipolar cells and inhibitory amacrine cells.[15,20] The effects of orexins on each type of cell involved in these pathways deserve a thorough investigation in the future.

Orexin-A and -B are expressed in all classes of postreceptoral neurons including bipolar cells, DACs, and ipRGCs.[6] Therefore, orexins could corelease with glutamate from bipolar cells and ipRGCs onto DACs as both compounds are colocalized in these cells. Orexin-A and -B could also be released from DACs onto their autoreceptors.[6] The regulation of retinal orexin release by light and the biological clock is

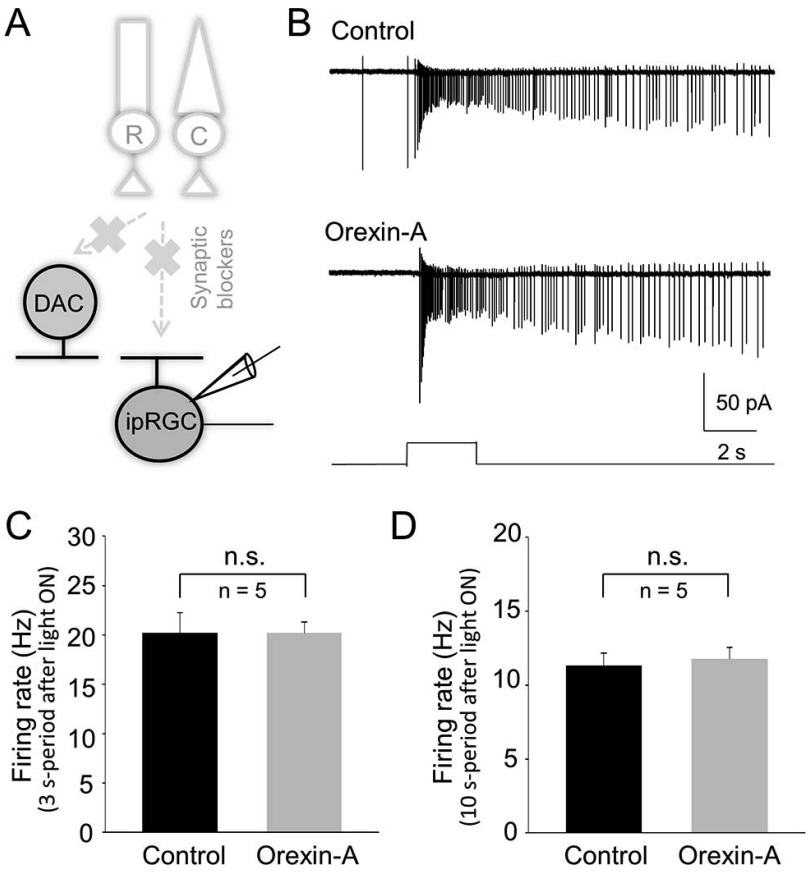

Figure 6. Orexin-A has no effect on melanopsin-mediated action potentials of M1 ipRGCs. Cell-attached extracellular recordings were made from tdTomato-labeled ipRGCs in flat-mount retinas of *opn4*-tdTomato mice. A schematic diagram in **A** shows the experimental protocol. Light-induced action potentials of ipRGCs were recorded in the presence of a cocktail of synaptic blockers (50 μM L-AP4, 30 μM D-AP5, and 40 μM DNQX). These blockers eliminated any excitatory inputs from rods and cones to ipRGCs. A representative recording from an M1 ipRGC in **B** (*top trace*) shows an increased number of action potentials at light onset. The number of action potentials decreased after cessation of the light stimulus, but activity continued for more than 10 seconds. Application of 500 nM orexin-A did not change the number of light-induced action potentials (*bottom trace* in **B**). Stimulation bar shows the timing of light pulse (3-second, 470-nm flash with an intensity of 8×10^{10} photons/cm^2·s). Bar charts in **C** and **D** show that orexin-A had no effects on average firing rates of M1 ipRGCs during 3-second (**C**) and 10-s periods after light onset (**D**), respectively.

unknown; however, in the brain, the suprachiasmatic nucleus (a primary circadian pacemaker) controls the daily rhythm of orexin-A release, with more orexin being released at night than during the day.[42,43] If this is also the case in the retina, orexin levels would be expected to be higher at night. These elevated levels of orexin could suppress dopamine release. Indeed, the suppression of dopamine release by endogenous orexins was observed in the prefrontal cortex during the dark phase.[44] During the daytime, however, the orexin-mediated inhibition of retinal dopamine could be relieved, which may result in higher levels of dopamine secretion. It is well known that increased levels of retinal dopamine play a critical role in the light adaptation of the visual system.[11,12] Therefore, we speculate that the orexinergic system in the retina could influence visual function by regulating the levels of retinal dopamine. This is consistent with the modulatory effects of orexins on the central brain dopaminergic system, as exemplified in several studies dealing with motivated behavior, reward processes, and restraint stress–induced cocaine relapses.[45–47]

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