Systems/Circuits

Acute Suppressive and Long-Term Phase Modulation Actions of Orexin on the Mammalian Circadian Clock

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Circadian and homeostatic neural circuits organize the temporal architecture of physiology and behavior, but knowledge of their interactions is imperfect. For example, neurons containing the neuropeptide orexin homeostatically control arousal and appetitive states, while neurons in the suprachiasmatic nuclei (SCN) function as the brain’s master circadian clock. The SCN regulates orexin neurons so that they are much more active during the circadian night than the circadian day, but it is unclear whether the orexin neurons reciprocally regulate the SCN clock. Here we show both orexinergic innervation and expression of genes encoding orexin receptors (OX1, OX2) in the mouse SCN, with OX2 being upregulated at dusk. Remarkably, we find through in vitro physiological recordings that orexin predominantly suppresses mouse SCN Period1 (Per1)-EGFP-expressing clock cells. The mechanisms underpinning these suppressions vary across the circadian cycle, from presynaptic modulation of inhibitory GABAergic signaling during the day to directly activating leak K+ currents at night. Orexin also augments the SCN clock-resetting effects of neuropeptide Y (NPY), another neurochemical correlate of arousal, and potentiates NPY’s inhibition of SCN Per1-EGFP cells. These results build on emerging literature that challenge the widely held view that orexin signaling is exclusively excitatory and suggest new mechanisms for avoiding conflicts between circadian clock signals and homeostatic cues in the brain.

Key words: arousal; circadian; electrophysiology; GABA; orexin A; suprachiasmatic

Introduction

Temporal organization of behavioral and metabolic states arises from the integrated activities of circadian and homeostatic neural networks. For example, key circuits involved in setting patterns of sleep and arousal include orexin/hypocretin (orexin A and B) neurons in the tuberal hypothalamus, and circadian clock neurons of the suprachiasmatic nuclei (SCN). Within the SCN, the molecular circadian clock (of which the Period1–2 genes are key components) drives daily rhythms in electrical activity, with rhythmic SCN neurons being maximally active during the circadian day and minimally active across the circadian night (Ikeda, 2004; Brown and Piggins, 2007). Such pronounced day–night variation in a neuronal state allows the SCN to communicate temporal information to the rest of the brain and body. In nocturnal rodents, SCN output signals directly and/or indirectly suppress neural centers controlling motor activity and arousal during the day (Li et al., 2012). Indeed, the activity of the orexin neurons whose activation and suppression plays critical roles in the timing of arousal and sleep states (Chemelli et al., 1999; Tsunematsu et al., 2011) is under SCN circadian control (Zhang et al., 2004). In rats and mice, orexin neuronal activity (Estabrooke et al., 2001; Marston et al., 2008) and orexin release are most prevalent throughout their behaviorally active circadian night (Deboer et al., 2004).

Intriguingly the phase of the SCN circadian clock is also sensitive to feedback from arousal stimuli, particularly during the circadian day (Mistlberger and Antle, 2011; Hughes and Piggins, 2012). Such stimuli can recruit neuropeptide Y (NPY)-containing neurons of the intergeniculate leaflet (IGL), which project to the SCN via the geniculohypothalamic tract (GHT; Harrington, 1997). NPY released from the GHT suppresses SCN neuronal activity (Gribkoff et al., 1998), inhibits clock gene expression in the SCN (Maywood et al., 2002), and in doing so shifts the phase and output of the clock. During the day, orexin neurons are also activated by such arousal-promoting stimuli (Estabrooke et al., 2001; Marston et al., 2008; Webb et al., 2008). Given that orexin-synthesizing neurons project widely throughout the brain (Peyron et al., 1998; Nambu et al., 1999), including structures of the neural circadian system (Bäckberg et al., 2002), orexin released during states of arousal may also influence SCN cellular activity. However, this raises an interesting paradox because activation of brain orexin receptors is generally considered excit-
atory (Kukkonen, 2013) and yet SCN neuronal activity is low across the circadian night when orexin excitatory activity and release in nocturnal rodents are maximal.

Here, we describe a variant that unlike its typical excitatory actions in the brain, orexin A (OXA) acts within the SCN to primarily suppress cellular activity, and that the mechanisms underlying neuronal suppression differ across the circadian cycle. In addition, we demonstrate that OXA application does not itself shift the phase of the clockwork, but instead orexin can enhance the actions of NPY on SCN clock cells. These results indicate that the output of the master circadian pacemaker is modulated by homeostatic circuits involved in arousal and energy balance in a manner that allows acute adaptation to changes in the internal and external environments.

Materials and Methods

Animals

For calcium imaging, electrophysiological, PCR, and immunohistochemical investigations, this study used male and female mice [8 weeks to 6 months old] hemizygous for the Per1-EGFP (Camden Instruments) and transferred to sterile tissue culture dishes (Corning) in cold HBSS. Using a dissecting microscope, the SCN were identified and microdissected before excised unilateral SCN nuclei were cultured on interface-style cell culture inserts (PICM0RG50; Millipore) in standard 35 mm plastic-based culture dishes (Corning). One millimeter of sterile culture medium (DMEM (D-2902; Sigma)) supplemented with 3.5 g/L glucose (Sigma), 0.035% sodium bicarbonate (Sigma), 10 mM HEPES buffer (Sigma), 25 µg/mL penicillin-streptomycin (Gibco), B27 (Invitrogen) and 0.1 mM luciferin (Promega) in autoclaved Milli-Q water was used per culture. Dishes were sealed with a glass coverslip using autoclaved high-vacuum grease (Dow Corning) and transferred directly to light-tight incubators (Galaxy R+; RS Biotech) equipped with photomultiplier tube assemblies (HR259/R7518P; Hamamatsu) for photon acquisition (integrated for 299 s every 3 min). The incubators were maintained at 37°C.

Whole-cell recordings

Targeted recordings (current clamp or voltage clamp) were made from Per1-EGFP-positive (henceforth referred to as Per1-EGFP) neurons, visually identified using a Leica epifluorescent microscope (DMLFS; Leica Microsystems) equipped with a Leica camera and capturing software (DFC 350 FX and Application Suite; Leica Microsystems), as previously described (Belle et al., 2009; Scott et al., 2010). Final gigahm seal and cell membrane rupture were done under infrared video-enhanced differential interference contrast microscopy. For current-clamp recordings an Axoclamp 2A amplifier (Molecular Devices) in bridge mode was used (Belle et al., 2009). Patch pipette electrodes (7–10 MΩ) were fashioned from thick-walled borosilicate glass capillaries (Harvard Apparatus) using a two-stage vertical micropipette puller (PB-7; Narishige). Unless otherwise stated, electrodes were filled with an internal solution containing the following (in mM): 120 K-glucuronate, 2.5 MgCl2, 2 K-ATP, 0.5 Na-GTP, 10 HEPES, and 0.5 EGTA, pH adjusted to 7.3 with KOH, measured osmolarity 295–300 mMol/kg). In some recordings, K-glucuronate was substituted for equimolar KCl. Input resistance (Rinput) was estimated by a series of hyperpolarizing current pulses (~10 to ~30 pA for 500 ms) Signals were sampled at 30 kHz, and stored and analyzed on a personal computer using Spike2 software (Cambridge Electronic Design, CED). All data acquisition and step protocols were generated through a micro 1401 mkII interface (CED).

Synaptic current measurement.

Synchronous current activity was measured with an Axopatch 200B amplifier (Molecular Devices). To record miniature and spontaneous postsynaptic currents (mPSCs and sPSCs, respectively), patch pipettes (4–6 MΩ) were filled with an internal solution that was identical to the one used in current-clamp recordings, except for 120 mM K-glucuronate and 20 KCl mM. In SCN neurons, this concentration of Cl– in the intracellular pipette solution causes the IPSCs to reverse between ~40 to ~50 mV (data not shown; but see Itri and Colwell, 2003). This positive shift in Cl– reversal potential causes both GABA and glutamate PSCs to appear as inward currents when membrane voltage is clamped at ~70 mV. To discriminate between excitatory and inhibitory PSCs, a mixture of receptor antagonists (n-2-amino-5-phosphonopentanoate, AP-5; 6-cyano-7-nitroquinoxaline-2,3-dione, CNQX; and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzof[1]quinolizine-7-sulfonamide, NBQX) for the glutamate receptors and/or for the GABA receptor (gabazine) were used. All recording protocols were similar to established methods for measuring PSCs in SCN neurons (Itri and Colwell, 2003). Whole-cell leak K+ current measurement. Slow voltage ramps from 0 to ~120 mV, at a rate of 20 mV/s, were performed using patch pipettes (3–5 MΩ) filled with an internal solution that was identical to the one used in previous current-clamp recordings above. These ramps were sufficiently slow to allow background leak currents to achieve steady-state conductance at each potential (Liu et al., 2002; Jackson et al., 2004). Pipette and cell capacitive transients were compensated. Membrane series resistance was ~20 MΩ and was compensated (70–80%). Seal resistance was typically ~10 GΩ, and access resistance ~20 MΩ. Recordings in which the series resistance changed by >15% were not included for analysis. Signals for voltage-clamp recordings were acquired using pClamp 10.2 and analyzed off-line using Clampfit 10.2 (Molecular Devices). All data acqui-
sition and ramp protocols under voltage-clamp conditions were generated through a Digidata 1440A interface using the pClamp software (Molecular Devices).

**Calcium imaging**

SCN brain slices (200 μm thick) were prepared from 2- to 6-month-old Per1-EGFP mice (n = 18), as described above. Multiple SCN neurons maintained in these hypothalamic slices were loaded with the leakage-resistant calcium-sensitive dye fura-2 AM (LeakRes fura-2; TETFlabs; 1 μM stock made on the day of the experiment in 20% Pluronic F-127 in dimethylsulfoxide (DMSO); Molecular Probes/Invitrogen) for recording of intracellular calcium responses simultaneously in all loaded cells (Fig. 2A). Methods for calcium loading were modified from previous work on SCN and other hypothalamic neurons (Irwin and Allen, 2007, 2010; Yuste et al., 2011). Here, hypothalamic slices containing the mid-rostralcaudal section of the SCN were incubated with LeakRes fura-2 AM (10 μM) in well gassed (95% O2 and 5% CO2) aCSF at 37°C for 20 min, followed by 1 h incubation at room temperature. During incubation, the gaseous composition immediately above the aCSF in which the SCN slices were kept was maintained at 95% O2 and 5% CO2. Following incubation, slices were washed in gassed aCSF for 1.5 h in a holding chamber to allow for fura-2 AM de-esterification. The SCN brain slices were then transferred to a recording chamber and maintained in a continuous flow of aCSF (1–2 ml/min). The composition of the aCSF used for calcium imaging was similar to the recording solution used for electrophysiology, except phenol red was omitted to avoid any optical distortion of the fluorescent images. Ratiometric intracellular calcium ([Ca2+]i) ratio levels were measured from pairs of background-subtracted intensity images (14 bit, 1.24 × 1.24 mega pixels) captured every 2 s at 510 nm by a cooled Rolera EM-C2 camera (QImaging) from excitation wavelengths at 365 nm (isosbestic point for fura-2) and 385 nm. Here, a rise or drop in ([Ca2+]i) ratio levels caused an increase or decrease in F365/F385 wavelengths, respectively. The sensitivity of this camera system allows minimal light exposure of SCN neurons at these excitation wavelengths, which significantly prolongs recording time with minimal photo-bleaching. All calcium imaging was done on an upright Olympus microscope using a water-immersion UV objective (UPLPlanFL N 20 X/0.5 Olympus). Florence was excited by 365 and 385 nm Opto-Light-Emitting Diodes (OptoLED; Cairn Research; Briston et al., 2011), controlled by the acquisition software OptoFluor (Cairn Research and Molecular Devices). Image capture and analysis were done in OptoFluor (Cairn Research). Acquisition time, camera setup, and LED intensities were adjusted to minimize photo-bleaching while maintaining excellent signal-to-noise ratio. Intracellular calcium response (increase, decrease, or no effect) to OXA in the presence/absence of various antagonists was defined by a ratio change ≥2× SDs from the mean of 10 baseline calcium ratio measurements prior to OXA application (Irwin and Allen, 2010). The magnitude of calcium response was determined as the maximal response after 3 min of OXA application.

**Drugs and analysis**

Stock solutions for an orexin receptor 1 (OX1R) antagonist (SB 334867), quinidine, and GABA receptor antagonist (CGP55845) were prepared by dissolving in DMSO: final concentration of DMSO in extracellular solutions did not exceed 0.001%. OXA, tetrodotoxin (TTX), AP-5, CNQX, NBQX, quinine, and gabazine (Tocris Bioscience) were dissolved in aCSF and NPY (Tocris Bioscience) in purified water. 1,2-Bis(2-aminophenoxy) ethane-N,N,N’,N”-tetraacetic acid (BAPTA) and all other drugs were purchased from Tocris Bioscience.

Unless specified, all drugs were bath applied in aCSF. Where necessary, a brief focal application of drugs was delivered to the recorded neurons with a quartz puffer pipette micro-manifold (QMM) fitted to a computer-controlled pressurized drug delivery system (ALA Instruments). The QMM comprised of 12 individual microcapillaries converging into a single tip, which had a diameter of 250 μm, and was positioned in-line with the recorded neuron at a close distance to the SCN (Fig. 2). Under these configurations, drugs were delivered only to the SCN.

During current-clamp recordings OXA was bath applied for 3 min, and the cell’s activity and/or resting membrane potential (RMP) were allowed to return to, or closely to, predrug baseline levels before any subsequent drug treatments. The effects of drug treatment on spontaneous firing rate (SFR), RMP, sPSCs, or mPSCs were sampled in gap-free acquisition mode. Responses to OXA were defined by a change in RMP that was greater than ±2 mV. The duration (min) of OXA-induced change in RMP was measured by the time taken for the cell’s RMP value to depart from the predrug baseline level to the time it returned to, or close to, the baseline values. The amplitude (mV) was measured by calculating 1 min averages of the RMP at predrug baseline level and at the maximum steady-state change in RMP during OXA application. The amplitude was obtained by subtracting these two values. Recording aCSF (drug-free or containing drugs) solution was applied to SCN slices by gravity-feed bath perfusion. Dose-dependent responses of SCN neurons to 40, 60, and 80 nM OXA were performed using the puffer pipette manifold, positioned close to the SCN (see above).

The mediating effects of OXA on NPY’s inhibitory actions on SCN Per1-EGFP cells were tested by comparing the amplitude and duration of membrane hyperpolarization to NPY in the presence and absence of bath application of OXA (Fig. 8). First, the response of each cell to two consecutive brief focal applications of NPY (40 nM) was tested (Fig. 8A) using the puffer pipette manifold placed close to the SCN. Following the recovery of the neuron’s RMP to baseline values, OXA was bath applied. A positive steady-state current injection was used to counteract any hyperpolarizing effect of OXA by returning the RMP of the cells to pre-OXA baseline levels. Then in the presence of OXA, the cell’s response to NPY was again tested (Fig. 8). Because SCN cells showed heterogeneity in their sensitivity to NPY, the duration of NPY application varied from cell to cell, but to ensure comparability of the responses the duration/volume of its repeated application to each neuron was identical, and this was accurately timed through the micro 1401 mKII interface by the Spike2 software.

Groups of cells were statistically compared using ANOVA with post hoc comparison (Sidak where repeated measures were required) or paired/unpaired t test (SPSS version 16 and 20). For these tests, p < 0.05 was considered significant. All numerical data both in text and in graphs represent mean ± SEM. Analysis of PSC frequency and amplitude were conducted off-line by template-based sorting using Clampfit 10.2 (Molecular Devices). Synaptic events were detected with an amplitude threshold of 10 pA. The Kolmogorov–Smirnov (K–S) test was used for statistical comparisons of amplitude and interevent interval distributions of PSCs. Cumulative probability distributions were considered significantly different with a p value <0.01 (K–S test). No overt gender differences were found in any of the electrophysiological or calcium imaging parameters measured, so all data obtained from the male and female mice were appropriately pooled.

**SCN slice culture**

On the fourth day after cull/culture preparation, SCN slice cultures were treated with OXA (80 nM), NPY (50 nM or 200 nM), or both at circadian time. On the fourth day after cull/culture preparation, SCN slice cultures were treated with OXA (80 nM), NPY (50 nM or 200 nM), or both at circadian time (CT) 5 (middle of the day) or CT17 (middle of the night). Treatment times were calculated as projections from the light–dark cycle under which mice were housed before cull, and drugs were delivered as a 1 μl 1000× concentrate microdrop directly into the existing culture medium. No two unilateral SCN cultures from the same slice were treated with the same drug. Raw luminometry data were processed by subtracting a 24 h running mean to remove long-term trends (baseline subtracted) then smoothed with a 3 h running average (Hughes et al., 2011). To assess phase shifts, a period for each individual tissue explant was calculated as peak–peak duration averaged across three cycles, discounting the first 24 h of data. This period was used to calculate a projected peak phase for the cycle after drug treatments, and phase shifts were measured as the time difference between this projected phase and actual peak phase for that cycle. Statistically significant differences between groups were determined using t test for 200 ns data and ANOVA with a priori pairwise comparisons for all other comparisons; p < 0.05 is required for significance.

**Immunofluorescence**

Six adult Per1-EGFP mice (three males and three females) were terminally anesthetized with pentobarbital (80 mg/kg) and they were perfused

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transcardially with 25 ml of a balanced Kreb’s solution, followed by 25–50 ml of ice-cold 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Animals were perfuse fixed at ZT8–12 to maximize EGFP signal in the SCN. Prepro-orexin knock-out mouse brains (University of Lausanne) were fixed using similar protocols to those outlined. The brains were removed, post-fixed in the same fixative buffer for 4 h, and stored for 2 d in 30% sucrose at 4°C. The tissues were then flash-frozen in cooled isopentane and cut (40 μm) on a freezing sledge microtome (Bright Instruments). After washing in 0.1 M PBS, the 40-μm-thick brain sections were incubated in Triton X-100 (0.1% in PBS) for 20 min, and nonspecific protein binding blocked with normal donkey serum (5% in PBS containing 0.05% Triton X-100) for 30 min. Sections were incubated for 48 h at 4°C in anti-orexin A (sc-8070; 1:1000; Santa Cruz Biotechnology). To test for antisera specificity, controls were performed on SCN brain sections prepared from prepro-orexin knock-out mice or by incubating Per1-EGFP SCN sections in a working dilution of the orexin A primary antisera that had been pre-adsorbed with 5 μM orexin A peptide (Tocris Bioscience) overnight. In other control Per1-EGFP SCN sections, anti-orexin A antisera was omitted from the incubating solution. Following a 2 h wash. Sections were then incubated overnight in donkey anti-goat Alexa 568 secondary antibody (A-11057; Invitrogen 1:800). Although the EGFP protein was readily detectable, for more detailed anatomical analysis EGFP signal was further enhanced with a rabbit anti-GFP antibody (Invitrogen, 1:1000), followed by an overnight incubation in donkey anti-rabbit Alexa 488 secondary antibody (A-21206; Invitrogen 1:800). All primary antibodies were diluted in PBS containing 0.5% blocking serum and 0.05% Triton X-100. Secondary antibodies were diluted in PBS alone. Following further washes, sections were mounted in Vectashield anti-fade mounting medium (Vector Laboratories). Stacks of digital images were captured in series with a Leica SP5 inverted laser scanning confocal microscope (Leica Microsystems). For detailed visual inspections of putative synaptic contacts on Per1-EGFP cells by OXA-immunoreactive (OXA-ir) fibers, 3D projections of stacked confocal images (60 images per stack taken at 63× magnification) were made using Leica LAS AF software (Leica Microsystems).

**SCN quantitative PCR for OX1, OX2, and Per2 genes** Quantitative real-time PCR (qRT-PCR) for OX1, OX2, and Per2 genes was performed on SCN tissue punches collected from Per1-EGFP mice at CT0 (n = 3) and CT12 (n = 3). Animals were kept in constant darkness to eliminate light/dark time cues 1 d before tissue collection, and maintained in the dark during exsanguination and brain dissection. SCN slices were cut using a vibratome (Camden Instruments) followed by micropunch dissection of the nuclei and collection into RNAlater (Sigma). Total RNA isolated from the SCN (RNaseasy micro kit; Qiagen) were reverse transcribed (RNA-to-cDNA Reagents; Life Technologies) for analysis by qRT-PCR. Gene analysis was performed using SYBR Green technology (Life Technologies), and measured using a StepOnePlus Real Time PCR system (Life Technologies). OX1 and OX2 transcripts were amplified by QuantiTect primer assay (Qiagen). The following primers to mouse Per2 were used (5’ to 3’: Sense:GCCTTCAGACTCAGTGAGCAGA; Antisense:TTTGTGTGCGTCAGTTTGG). 18S was used as an internal control (5’ to 3’: Sense: ACGGAAGG GCCACACCCAGGA; Antisense:CACACCACCCACGAAATG). Data were analyzed using the ΔΔCT method and are expressed relative to target gene expression at CT0 (p < 0.05, n = 6, Student’s t test). Data shown are expressed as mean ± SEM. OX, optic chiasm; 3V, third ventricle.

**Results**

**Per1-EGFP neurons in the SCN are surrounded by OXA fibers** Previous neuroanatomical studies using light microscopy report that immunostaining for orexin is sparse to absent within the rodent SCN ( Peyron et al., 1998; Mcgranaghan and Piggins, 2001; Nixon and Smale, 2005). To determine the extent to which orexin neurons innervate the mouse SCN and identify neurons potentially innervated by OXA-containing fibers, we used immunofluorescence and confocal microscopy and visualized OXA immunoreactivity in the hypothalamus of Per1-EGFP mice. OXA-ir fibers were present throughout the anterior hypothalamus. These fibers were readily visualized along the dorsal and lateral boundaries of the SCN (Fig. 1A). To determine how the innervation of the SCN by orexin cells mapped onto Per1-EGFP neurons, we used anti-GFP (GFP-ir) to further enhance visualization of Per1-
EGFP neurons in fixed SCN brain sections and coimmunolabeled for OXA. The distribution of Per1-EGFP neurons at the mid-cortical level of the SCN (Fig. 1A) was as previously described (Kuhlman et al., 2003; Hughes et al., 2008). At this plane of the SCN, OXA-ir processes innervated many sites containing Per1-EGFP-positive and -negative (unidentified) cells. Detailed visual inspection of 3D rotated projections of confocal image stacks (comprised of 60 optical image slices, of 0.3 μm thickness each) at higher magnification (63×) showed varicose OXA-ir axonal boutons converged and appeared to delineate the soma of these cells (Fig. 1d1–e3). In contrast, OXA-ir staining was absent in SCN brain sections from preproorexin knock-out mice (Fig. 1B), and in brain sections from Per1-EGFP mice incubated overnight with either preadsorbed OXA antiserum (Fig. 1C), or with omission of OXA antiserum (Fig. 1D). Figure 1, E and F, shows the nonenhanced Per1-EGFP expression of Figure 1, C and D, respectively. These data provide neuroanatomical support for orexin release in the vicinity of Per1-EGFP neurons and other cells in the SCN.

OXA, and OX2 receptor mRNAs are expressed in the mouse SCN

To determine whether orexin receptors are also expressed in the SCN, we performed qPCR on mouse (n = 6) SCN brain tissue to measure the levels of mRNA transcript encoding for OX1, and OX2 receptors at circadian dawn and dusk. Our results indicate that both OX1 and OX2 mRNA are expressed within the mouse SCN, and that OX2 (t4 = 4.33, p < 0.05) but not OX1 exhibited a similar profile to the core clock gene Per2 (t4 = 4.29, p < 0.05), with a significant increase in expression at dusk (CT12) relative to dawn (CT0; t test; p < 0.05; Fig. 1G).

OXA indirectly and directly suppresses intracellular Ca2+ levels in SCN neurons

To investigate if and how OXA influences SCN cellular activity, we next determined the impact of OXA on the intracellular cytosolic calcium [Ca2+]i levels in SCN neurons. To do this we loaded SCN brain slices with leakage-resistant fura-2 AM and imaged the mean [Ca2+]i ratio (F365/F385 wavelengths) of multiple SCN cells before and during OXA application across the projected day (ZT4–10) and night (ZT16–22; Fig. 2A1–A3). Figure 2A1 illustrates how multiple SCN cells can show robust increases in intracellular Ca2+ following brief focal application of NMDA. Consistent with previous work (Colwell, 2000; Ikeda et al., 2003; Irwin and Allen, 2010), the baseline cytosolic calcium levels in all SCN cells measured were significantly higher during the middle of the day (ZT4–10, n = 369) than at the middle of the night (ZT16–22; n = 333; t645.40 = 13.3, p < 0.0001; Fig. 2B). Overall, bath application of 80 nM OXA caused significant changes (decrease or increase) in baseline cytosolic calcium levels in ~67% of these SCN cells during the day (249 of 369 cells tested over ZT4–10), and ~40% of neurons at night (133 of 333 cells tested over ZT16–22) with remaining cells unaltered by OXA application. Of the

Figure 2. OXA mainly suppresses intracellular Ca2+ levels in SCN neurons. A, Simultaneous [Ca2+]i measurements in multiple SCN neurons in a hypothalamic slice, with (1) recording setup with pressurized puffer pipette (P) used for brief (5 s) focal application of NMDA; (2) SCN neurons (40 ×) loaded with LeakRes fura-2 AM (taken from region in (1) marked with a white box); (3) enlarged single LeakRes fura-2 AM loaded SCN neuron; (4) representative SCN neurons showing transient [Ca2+]i increase in response to a focal application of NMDA (200 μM, 5 s; duration black bar at top) in the late day (ZT10). B, Overall summary of measured baseline [Ca2+]i level in SCN cells showing significantly higher [Ca2+]i value during the day (ZT4–10) than at night (ZT16–22). During the day, OXA (80 nM) significantly suppressed (C, D; n = 172/249) or elevated (E; n = 77/249) the baseline [Ca2+]i ratio in 69 and 31% of responsive SCN neurons, respectively. At night, OXA decreased (F) the baseline [Ca2+]i ratio in 97% (n = 129/133) of responsive SCN neurons while increasing this ratio in only 3% (n = 4/133) of cells (data not shown). This nighttime effect of OXA on cytosolic calcium levels in SCN neurons was insensitive to coapplication of gabazine and CBX (K, GABAA, and GABAB receptor antagonists, respectively, but was abolished when OXA receptor antagonist SB33487 was coapplied with OXA (G). In contrast, in the day, gabazine abolished OXA’s suppression of cytosolic calcium levels in SCN cells (H), but not its induced elevation (J) of the ratio. G–I, In the presence of gabazine and SB33487 the cells showed an acute increase in their cytosolic calcium levels in response to a 200 μM focal application (S) of NMDA. L, Summary of OXA effects on [Ca2+]i level in SCN cells over the day and at night. All data shown are expressed as mean ± SEM; *p < 0.05; ***p < 0.0001. Number of cells measured is shown in brackets. OX, optic chiasm; 3V, third ventricle; P, tip of the pressurized puffer pipette manifold.
responsive cells tested during the day, most (172/249 cells; 69%) significantly reduced cytosolic calcium levels to OXA application ($t(171) = 8.32, p < 0.0001$; Fig. 2C, D, L), with the remaining cells (77/249) showing significant elevation in intracellular calcium levels to OXA ($t(76) = 9.97, p < 0.0001$; Fig. 2E, F, L). At night, the predominant response to OXA (80 nM) was a significant reduction in the cytosolic calcium level (129 of 133 OXA responsive cells; 97%; $t_{(128)} = 12.34, p < 0.0001$; Fig. 2F, K, L), with only a minority of cells (4/133) showing significant elevation in $[\text{Ca}^{2+}]_{i}$ ($t(3) = -4.59, p < 0.05$; Fig. 2L). Hence, there is a significant time of day effect on the suppressive (day: 47% of cells tested; night: 39%) and activational (day: 21% of all cells tested; night: 3%) actions of OXA on the mean cytosolic calcium levels in SCN neurons. Pretreatment with an orexin receptor antagonist SB334867 (10 $\mu$m) prevented cells from changing their intracellular calcium levels to subsequent treatment with OXA (regardless of time of day), but did not block cytosolic calcium responses to focal application of the glutamatergic agonist NMDA (200 $\mu$m for 5 s; Fig. 2G, J). Therefore, actions of OXA on intracellular calcium of SCN cells require activation of orexin receptors.

Intriguingly, most investigations of OXA on cytosolic calcium in neurons report that OXA elevates intracellular Ca$^{2+}$, an outcome interpreted as a consequence of OXA depolarizing neurons (Kohlmeier et al., 2008). Many SCN cells are GABAergic (Moore and Spre, 1993; Bujs et al., 1994; Castel and Morris, 2000) and functional GABA$_{A}$ and GABA$_{B}$ receptors are present in the SCN (Strecker et al., 1997; Itri and Colwell, 2003; Moldavan et al., 2006; Irwin and Allen, 2009). To determine whether the suppressive actions of OXA involve inhibitory GABA signaling, OXA-responsive cells were retested for the responses to this neuropeptide in the presence of gabazine and CGP55845, antagonists of GABA$_{A}$ and GABA$_{B}$ receptors, respectively. During the day, blockade of GABAergic signaling abolished all of OXA’s suppression of intracellular calcium in SCN cells ($n = 90$ cells tested). Figure 2H shows a cell that reduced intracellular Ca$^{2+}$ to OXA, but not when OXA was coapplied with gabazine. Such treatment with gabazine had no effect on the subset of cells that elevated cytosolic calcium levels in response to OXA (Fig. 2J). Subsequent focal application of NMDA (200 $\mu$m for 5 s) caused a robust acute rise in cytosolic calcium levels in all daytime SCN cells treated with gabazine (Fig. 2H). In contrast to this reliance on GABA signaling through GABA$_{A}$ receptors (GABA-GABA$_{A}$ receptor signaling) during the day, the suppression of intracellular calcium in SCN cells by OXA at nighttime was not altered when pretreated with 20 $\mu$m gabazine ($n = 42$; Fig. 2K). Since GABA$_{B}$ receptors are also present in the SCN region (Francois-Bellan et al., 2008; Gribkoff et al., 2003), we also determined whether blockade of GABA-GABA$_{B}$ receptor signaling influenced OXA’s nighttime actions. Pretreatment with the GABA$_{B}$ receptor antagonist CGP55845 (10 $\mu$m) did not affect subsequent responses to OXA ($n = 48$) (Fig. 2K). This indicates that OXA’s suppressive actions on cytosolic calcium levels of SCN cells require functional GABA$_{A}$ receptor signaling during the day, while at night neither GABA$_{A}$ nor GABA$_{B}$ receptor signaling is required for OXA’s actions. Further, these data demonstrate that the elevations in intracellular calcium evoked by OXA are unaffected by blockade of GABAergic mechanisms.

In summary, the cytosolic calcium experiments establish that OXA acts principally to suppress rather than elevate levels of intracellular calcium, thereby indicating that OXA is unlikely to depolarize the majority of SCN cells, or that if it does, then this occurs via mechanisms that do not increase intracellular calcium. Since intracellular Ca$^{2+}$ levels are associated with the intrinsic electrical state of SCN neurons (Diekmann et al., 2013), these results suggest that OXA should mostly suppress SCN neuronal activity.

**Day–night differences in the actions of OXA on SCN neurons**

To determine whether SCN neurons responsive to OXA contain clock genes and to evaluate how this neuropeptide alters the intrinsic electrical properties of SCN neurons, we used epifluorescence and performed targeted whole-cell current-clamp recordings from identified Per1-EGFP neurons in SCN brain slices ($n = 162$) in SCN brain slices ($n = 55$). We tested how bath application of OXA (80 nM for 3 min to achieve a steady-state concentration in the bath) altered the bioelectric activities [RMP, $R_{in}$, and SFR of action potentials (APs)] of Per1-EGFP neurons. Since the electrical activity of SCN neurons varies across the circadian cycle (Brown and Piggins, 2007; Colwell, 2011) and can influence how these cells respond to neurochemicals (Piggins et al., 1994), we recorded and compared OXA’s action across a 10 h epoch of the projected day (ZT2–12) and 6 h epoch of the projected night (ZT16–22). Consistent with previous reports (Kuhlman et al., 2003; Belle et al., 2009; Scott et al., 2010), SCN Per1-EGFP neurons were more active during the day than the night. Significant day–night differences were seen in mean RMP ($-40.3 \pm 0.9$ mV vs $-49.1 \pm 1$ mV; $t_{(155.62)} = 13.78, p < 0.0001$) and $R_{in}$ ($1.9 \pm 0.1$ G$\Omega$ vs $1.5 \pm 0.03$ G$\Omega$; $t_{(102.43)} = 6.97, p < 0.001$). With the exception of the middle to late projected day (ZT6–9), when some Per1-EGFP neurons stop generating APs by depolarization blockade (Belle et al., 2009; Scott et al., 2010), daytime Per1-EGFP cells showed elevated SFR compared with the low to moderate frequencies recorded at night ($2.9 \pm 0.3$ Hz vs $1.7 \pm 0.2$ Hz; $t_{(83.46)} = 4.3, p < 0.001$).

Across the projected circadian cycle, the majority of Per1-EGFP SCN neurons tested responded to OXA (119/162; 73%), with inhibitory hyperpolarizing actions predominating (100/119 responsive cells; 84%; Fig. 3A, C, E). Further, OXA-induced hyperpolarizations were significantly longer in duration at night than those elicited during the day ($54.8 \pm 4.4$ min vs $14.2 \pm 1.5$ min; $t_{(72.13)} = -13.43, p < 0.0001$). In the remaining 16% of responsive Per1-EGFP cells (19/119), OXA caused excitation with associated significant increase in AP discharge during the day ($t_{(18)} = -11.99, p < 0.0001$; Fig. 3G, I), but not at night. This tendency for OXA to suppress excitability of SCN neurons was also seen in our previous in vitro extracellular recordings (Brown et al., 2008), and in preliminary investigations we have also observed this in vivo extracellular recordings (data not shown). Independent of the polarity of the response or time of day, OXA-responsive Per1-EGFP SCN cells did not respond to this neuropeptide when tested in the presence of the OXA receptor antagonist SB334867 (10 $\mu$m; Fig. 3B; day and F; night, for example); thus indicating the dependence of OXA responses in the SCN on orexin receptor activation.

**Mechanisms of OXA signaling in SCN Per1-EGFP neurons in vitro**

Hyperpolarizations evoked by OXA during the day were not associated with changes in $R_{in}$ ($1.9 \pm 0.1$ G$\Omega$ vs $1.8 \pm 0.2$ G$\Omega$; paired $t$ test, $p > 0.05$), whereas at night, $R_{in}$ was significantly reduced ($1.6 \pm 0.1$ G$\Omega$ vs $0.8 \pm 0.1$ G$\Omega$; $t_{(29)} = 17.12, p < 0.001$, paired $t$ test). This indicates day–night differences in the potential contribution of K$^{+}$ conductances, and presynaptic and postsynaptic mechanisms to the hyperpolarizing actions of OXA. To evaluate contributions of presynaptic and postsynaptic mechanisms to these actions of OXA, cellular responses of orexin-
responsive neurons were retested to this neuropeptide in the presence of TTX-containing aCSF. TTX concentrations (0.5–1 μM), that block most voltage-dependent sodium channel conductances and hence AP-dependent synaptic communication, abolished inhibitory responses to OXA during the day (8 of 8 responsive cells; Fig. 3C,D), but not at night (0 of 8 responsive cells; Fig. 3E). Indeed, during the day when TTX was continuously bath applied before OXA treatment to a different cohort of neurons, none of these cells tested hyperpolarized to OXA (0 of 7 cells tested; data not shown). This indicates that during the day, OXA recruits presynaptic mechanisms to indirectly suppress neuronal activity in the SCN, whereas at night, it acts directly to hyperpolarize the cells.

Similar to the direct hyperpolarizing actions of OXA at night, daytime SCN neurons depolarized by OXA given in control aCSF also depolarized to OXA when AP-dependent communication was blocked by pretreatment with TTX (Fig. 3G,H). These findings establish a profound day–night difference in the actions of OXA in the SCN and suggest that stimulation of orexin receptors during the day activates indirect inhibitory neurotransmission mechanisms, whereas at night this stimulation of orexin receptors directly inhibits SCN neuronal activity.

To investigate whether OXA applied directly to the SCN would also inhibit Per1-EGFP neurons, we focally applied 40, 60, and 80 nm OXA to daytime recorded Per1-EGFP cells, by a pressurized puffer pipette placed close to the SCN (Fig. 2A1). This permits three consecutive brief pulses (35 s duration each) of OXA to be quickly delivered to the SCN, allowing shorter recovery time for the cells between each OXA dose than with bath application (Fig. 4A). As shown in Figure 4, A and B, focal application of OXA causes suppression of RMP and SFR of SCN cells (n = 4) in a dose-related manner (RMP amplitude: F(2,6) = 54.92, p < 0.001; and duration: F(2,6) = 467.21, p < 0.001; SFR: F(2,6) = 8.22, p < 0.05). Analysis from four Per1-EGFP neurons tested with these doses of OXA showed that the suppressive action of OXA (lowering of RMP and SFR) was not different at 40 and 60 nm doses (p > 0.05). In contrast, 80 nm OXA produced suppression in baseline RMP (p < 0.001) and SFR (p < 0.05) that was significantly larger than effects seen with 40 nm OXA. Focal applications of aCSF alone had no effect on membrane excitability in these cells (data not shown).

The direct depolarizing actions of OXA on central neurons in many brain sites are well described (Kukkonen and Akerman, 2005; van den Pol and Acuna-Goycolea, 2005; Eriksson et al., 2010), whereas its inhibitory effects are less frequently reported and poorly understood. Therefore, we subsequently focused on the mechanisms of OXA-induced hyperpolarization.

GABAergic signaling is differentially recruited from day to night

As revealed above, the TTX sensitivity of OXA-induced hyperpolarization of Per1-EGFP neurons during the day indicates an
underlying presynaptic mechanism. In line with the OXA-induced suppression of intracellular calcium, the inhibitory daytime actions of OXA on Per1-EGFP neurons were blocked (16 of 16 OXAreceptor-sensitive cells) by coapplying OXA with the GABA_A receptor antagonist gabazine (20 μM; Fig. 4D). In contrast, at night, neither coapplication of gabazine (20 μM; 0 of 8 responsive cells) nor the GABA_B receptor antagonist, CGP55845A (10 μM; 0 of 7 responsive cells), prevented OXA’s robust hyperpolarizing effect on Per1-EGFP neurons (Fig. 4E).

To establish whether daytime application of OXA-evoked GABA release within the SCN (as opposed to extrascN areas), gabazine (20 μM) was focally applied in the SCN (20 s with a puffer pipette; diameter 250 μm) in the presence of bath-applied OXA. As shown in Figure 4F, brief blockade of the GABA_A receptor by gabazine (n = 6) in the SCN abolished the hyperpolarizing action of OXA on Per1-EGFP cells (6 of 6 cells). Together, these findings confirm that during the day, functional GABA_A receptors are necessary for OXA to inhibit and alter the intrinsic state of SCN Per1-EGFP neurons, whereas at night, neither GABA_A nor GABA-GABA_B receptor signaling is necessary for the hyperpolarizing actions of OXA.

These results demonstrate that OXA engages neural networks intrinsic to the SCN in a dose- and time-dependent manner to suppress Per1-EGFP-expressing SCN clock cells.

OXA increases sPSCs and mPSCs in SCN neurons during the day

We next assessed the effects of OXA on the frequency and amplitude of sPSCs and mPSCs in voltage-clamp recordings of Per1-EGFP neurons. Measurement of sPSCs and mPSCs frequency assesses AP-dependent and -independent synaptic vesicle release, respectively, and allows us to determine whether OXA can also act directly on presynaptic terminals to cause the release of neurotransmitters in the absence of presynaptic APs (quantal release; recorded as mPSCs). Bath application of OXA produced an increase in the frequency and amplitude of sPSCs in ~65% of Per1-EGFP cells (25/39 cells recorded; Fig. 5A,b2,b3,C,D). In these responsive Per1-EGFP cells OXA significantly increased the mean sPSC frequency from 2.2 ± 0.2 Hz (basal) to 6.4 ± 0.8 Hz (F_124 = −11.25, p < 0.001; paired t test) and the amplitude from 45.6 ± 2.1 pA (basal) to 76.2 ± 9.4 pA (F_124 = −8.77, p < 0.05; paired t test; Fig. 5A). Pretreatment with the orexin receptor antagonist SB334867 abolished these actions of OXA (Fig. 5E,F), indicating that they are mediated by activation of orexin receptors.

Under our intracellular recording conditions (20 mK Cl- filled pipettes) Cl− currents have a reversal potential of approximately −43 mV, and thus both GABA_A and AMPA/NMDA currents appear as inward currents at the holding potential that we used (−70 mV) (Itri and Colwell, 2003). Therefore, we used receptor-selective antagonists to investigate whether the OXA-induced sPSCs were GABAergic or glutamatergic. In all OXAreceptor-sensitive Per1-EGFP cells tested, OXA-induced sPSCs were insensitive to bath application of the glutamatergic receptor antagonists, AP5, CNQX, and NBQX (0 of 10 cells; data not shown). In contrast, these OXA-sensitive spontaneous events were completely abolished by bath application of gabazine (20 μM for 5 min; 10 of 10 cells; Fig. 5b3), indicating that they were GABA_A receptor-mediated IPSCs. The rise time to peak of these spontaneous IPSCs was 2.9 ± 0.3 ms and the time constant decay was 14.7 ± 0.5 ms, values that are consistent with previous observations using similar recording conditions to study GABA_A receptor-mediated spontaneous IPSCs in SCN neurons (Itri and Colwell, 2003; Itri et al., 2004).

Bath application of TTX (0.5 μM) significantly reduced or abolished the sPSCs in OXAreactive Per1-EGFP neurons (9 of 9 cells; Fig. 5A,b5; now measured as mPSCs). However, as shown in Figure 5, A, b6, and b7, in the presence of TTX OXA significantly reduced the frequency of mPSCs in the cells (F_110 = −4.8; p < 0.01). These OXA-induced mPSCs were abolished by gabazine (20 μM; tested in four cells), demonstrating that they were GABAergic in nature (Fig. 5A,b8), indicating that they are mediated by activation of orexin receptors.
OXA hyperpolarizes SCN cells at night by opening leak K⁺ channels

At night, OXA-induced hyperpolarization persists even when AP-dependent synaptic communication and GABA-GABAR receptor signaling are impaired (with TTX and gabazine, respectively), indicating that presynaptic mechanisms do not overtly contribute to these nocturnal inhibitory effects of OXA. To establish whether Cl⁻ was involved in OXA’s hyperpolarizing postsynaptic actions at night, we substituted gluconate for equimolar K⁺; currents are responsible for this effect of orexin. OXA-induced hyperpolarization was also unaffected by fixing intracellular Ca²⁺ or Ca²⁺/Mg²⁺ extracellular solution. Under this condition, the OXA-induced current was activated by leak K⁺ channels, which in Perl-EFP cells can show increased conductance at night (Pitts et al., 2006), are unlikely to be involved in OXA’s nocturnal actions.

Previously, OXA has been shown to increase excitability of thalamic cells by suppressing the activity of leak K⁺ conductance (Govindaih and Cox, 2006). Considering the robust and long-lasting hyperpolarizing effect we observe during nighttime administration of OXA, and the very slow onset, and postsynaptic nature of this response, we tested whether OXA was activating background leak K⁺ channels to hyperpolarize SCN neurons at this time. We first investigated this in current-clamp recordings to see if the blockade of leak K⁺ channels prevented OXA’s inhibitory action. We challenged OXA-responsive neurons with quinine or quindine (500 μM), two broad-spectrum leak K⁺ channel inhibitors (Lotshaw, 2007). Both, quinine (RMP: \( F_{12,10} = 25.55, p < 0.001; \) \( R_{\text{input}}: F_{12,10} = 17.47, p < 0.01; n = 6; \) Figure 6D,E) and quinidine (RMP: \( F_{12,6} = 50.41, p < 0.001; \) \( R_{\text{input}}: F_{12,6} = 25.06, p < 0.001, n = 4; \) data not shown) reversed OXA-induced hyperpolarization, returning the RMP (post hoc tests for quinine: control vs OXA, \( p < 0.01; \) OXA vs OXA + quinine, \( p < 0.01; \) quinidine: control vs OXA, \( p < 0.01; \) OXA vs OXA + quinidine, \( p < 0.05 \) and \( R_{\text{input}} \) (post hoc tests for quinine: control vs OXA, \( p < 0.01; \) OXA vs OXA + quinine, \( p < 0.01; \) quinidine: control vs OXA, \( p < 0.05; \) OXA vs OXA + quinidine, \( p < 0.05 \) values to near baseline levels, causing some neurons to fire APs (Fig. 6D,E). This is consistent with the idea that leak K⁺ channels are important for OXA’s hyperpolarizing effects at night.

To define the nature of this leak K⁺ current, we performed subsequent experiments in voltage-clamp recordings using slow ramps of voltage in the range from 0 to −120 mV (see Materials and Methods; Fig. 6F). In the presence of TTX (1–2 μM), OXA (80 nM) caused a change in the current–voltage relationship (Fig. 6F). The net current triggered by OXA (obtained by subtracting baseline I−V from OXA I−V; Fig. 6G), had a reversal potential near −93 mV (expected \( E_R = −102 \) mV), was almost linear, and was potentially inhibited both by quinine (\( n = 11; \) Fig. 6G) and quinidine (\( n = 5; \) data not shown). Together, these observations implicate an OXA-activated current flowing through leak K⁺ channels to cause membrane inhibition. At night, Perl-EFP neurons typically rest, on average, approximately −47 mV (Belle et al., 2009). At this voltage range (approximately −47 mV), OXA significantly increased \( I_{(10)} = 12.46; p < 0.001, \) paired t-test) the hyperpolarizing background leak K⁺ conductance by ~40% in

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**Figure 6.** OXA causes direct membrane hyperpolarization and suppression of Per1-EGFP neuronal activity at night. A–C, Bath application of OXA hyperpolarized Perl-EGFP neurons, and in all cells this hyperpolarization was significantly larger in duration (>50 min) at this circadian phase than during the day. A, B, OXA-induced suppression of Perl-EGFP cells persisted when (A) intracellular K⁺-gluconate was substituted with equimolar KCl solution; (B) intracellular Ca²⁺ solution was clamped at <90 mM with BAPTA-Ca²⁺ mixture, preventing Ca²⁺ mobilization from intracellular stores; and (C) Ca²⁺ influx was prevented with low Ca²⁺/high Mg²⁺ extracellular solution. B, Bath application of quinine prevented OXA-hyperpolarizing effect. E, Summary of results obtained from six current-clamp cells where OXA-induced membrane hyperpolarization and accompanied reduction in \( R_{\text{input}} \) were blocked when OXA was coapplied with quinine. F, I–V relationship during baseline (black trace) and in the presence of OXA (gray trace) obtained by slowly ramping the membrane voltage (see Materials and Methods and inset). G, Net OXA current obtained by subtracting OXA I–V from control I–V (black trace), with quinine potently inhibiting this OXA-induced current (gray trace). **p < 0.01. Data are expressed as mean ± SEM. Number of cells measured is shown in parentheses.
responsive neurons (estimated by the size of the quinine-sensitive current). Therefore, activation of leak K⁺ channels forms part of the mechanism by which OXA causes prolonged suppression in electrical activity of Per1-EGFP neurons at night.

**OXA modulates the acute and phase-resetting actions of NPY on SCN neurons**

Previous research has implicated inhibitory signaling as conveying arousal-promoting (or nonphotic) time cue information to the SCN (Smith et al., 1989; Cutler et al., 1998; Gribkoff et al., 1998). An important source of nonphotic input to the SCN is the GHT (Harrington, 1997) with NPY being an important signaling molecule in this pathway. During the day, NPY potently inhibits SCN neuronal activity, suppresses Per1 expression, and resets the SCN clock (Medanic and Gillette, 1993; Shibata and Moore, 1993; van den Pol et al., 1996; Fukuhara et al., 2001; Maywood et al., 2002). Since the predominant action of OXA on Per1-EGFP SCN neurons is also suppression, we next asked whether daytime application of OXA phase advances the SCN molecular clock. We therefore assessed the phase of PER2::LUC rhythms in SCN brain slice cultures treated with OXA (80 nm), NPY (50 nm or 200 nm), or both in the middle of the circadian day (CT5). NPY at 200 nm evoked large (~6 h) phase advances in the timing of peak PER2::LUC expression, which were significantly different to the effects of control protocols (t(6) = 2.89, p < 0.05; Fig. 7A). Low-dose NPY (50 nm) or OXA (80 nm) alone did not alter the timing of peak PER2::LUC (net shift relative to controls of ~0.9 h and approximately ~0.2 h, respectively; both p > 0.05). However, coinoculation of OXA (80 nm) and NPY (50 nm) induced phase advances (~2 h) that were significantly larger than those measured with NPY alone (F(1,50) = 4.1, p < 0.05) or in controls (F(1,50) = 10.1, p < 0.01; Fig. 7B,C). This indicates that OXA signaling in the SCN enhances the phase-shifting capacity of NPY on core molecular clock rhythms. Further, this shows that although OXA on its own modulates the electrical activity and intracellular Ca²⁺ of the majority of SCN neurons tested over the day, this neuron peptide does not itself evoke measurable phase shifts of the SCN.

Nonphotic stimuli have much less prominent phase-shifting effects during the subjective night, and to test whether these resetting actions of OXA and NPY also show phase dependence, we assessed shifts in PER2::LUC expression following treatments with OXA (80 nm), NPY (50 nm), or OXA and NPY given at the middle of the projected night (CT17). Neither neuropeptide alone nor in combination produced phase advances; indeed, only nonsignificant small phase delays were observed (Fig. 7B). This indicates that although OXA has long-lasting suppressive effects on electrical activity and intracellular Ca²⁺ of SCN neurons at night, these acute effects do not detectably influence the phase of the SCN molecular clock during the circadian night. Collectively, these data demonstrate that during the subjective day, OXA significantly augments the phase-resetting actions of a subthreshold dose of NPY, while at night, neither neuropeptide alone or in combination can significantly reset the SCN clock.

Since OXA enhanced the daytime resetting actions of NPY, we next investigated whether OXA influences the inhibitory actions of NPY on SCN neurons. We performed targeted current-clamp recordings from SCN Per1-EGFP cells in the day and compared their responses to NPY (two 40 nm brief applications by puffer pipette) both alone and in the presence of bath-applied OXA (80 nm). To allow for appropriate comparison between NPY's actions alone and in the presence of bath-applied OXA, the following recording and drug treatment procedures were used (Fig. 8A). First, the cell's response to two consecutive brief focal applications of NPY (40 nm) was tested, allowing for full recovery of the cell's RMP between NPY applications. OXA was then bath applied until the cell's response to this neuropeptide reached maximal value and plateaued. Next, before the cell's response to NPY was resteted in the presence of OXA, the RMP of the neuron was returned to its initial baseline value (before OXA application) by manually passing a steady-state current (e.g., indicated by a +ve current in Fig. 8A). This ensures that most of the membrane's ionic conditions are restored to baseline values, allowing for appropriate comparisons with previous NPY applications (when OXA was not present). This steady-state current injection was applied in conjunction with the puffer application of NPY and removed only following the full recovery of the cells to NPY (Fig. 8A). Electrical recordings from SCN Per1-EGFP cells that showed sensitivity both to OXA and NPY (15 of 34 neurons tested) revealed that the suppressive actions of NPY (40 nm) on these cells were significantly larger in the presence of OXA (80 nm; n = 15; Fig. 8A,B). Both the amplitude (NPY = Δ3.9 ± 0.5 mV; NPY + OXA = Δ8.5 ± 1.3 mV; t(14) = 3.34, p < 0.01) and duration (NPY = Δ1.3 ± 0.2 s; NPY + OXA = Δ1.3 ± 2.7 s; t(14) = 4.33, p < 0.01) of NPY-induced membrane hyperpolarization significantly increased in the presence of OXA compared with NPY alone (Fig. 8A,B). Similar observations were seen in 5 of the 19 cells that showed no overt responsiveness to OXA (data not shown).

We next investigated if GABA-GABAA receptor signaling is recruited by OXA to potentiate these acute actions of NPY on
SCN neurons. To do this, we first tested the cell’s response to NPY alone, then to NPY and OXA, as described above. But in addition, to see if GABA_A receptor blockade prevents OXA’s facilitation of NPY’s suppressive action, we also tested the effect of NPY in the presence of bath application of gabazine and OXA. Indeed, as shown in Figure 8, A and B (n = 5 cells), blockade of GABA_A receptors with gabazine abolished the potentiating effect of OXA on NPY’s inhibitory action on Per1-EGFP neurons (hyperpolarization amplitude: NPY alone vs NPY + OXA vs NPY + OXA + Gabazine, F(2,6) = 34.93, p < 0.001; duration: NPY alone vs NPY + OXA vs NPY + OXA + Gabazine, F(2,6) = 40.38, p < 0.001; see Fig. 8B for illustration of group mean comparisons). Together, these results demonstrate that in the SCN OXA acts via GABA_GABA_A receptor signaling to facilitate the phase-shifting and electrical silencing capacity of NPY.

Discussion

Here we show a new atypical influence of orexin signaling over SCN clock cell activity and timekeeping. Physiological investigations in vitro demonstrate clear day–night differences in the polarity of SCN neuronal responses to orexin. Across the projected circadian cycle, most orexin-responsive SCN neurons respond to orexin by suppressing baseline intracellular calcium levels ([Ca^{2+}]_i) and neuronal activity. Actions of this neuropeptide are prevented by blockade of orexin receptors, but local inhibitory circuits within the SCN are differentially recruited by orexin from day to night. During the day, orexin presynaptically modulates inhibitory GABA-GABA_A receptor signaling, while at night, orexin’s suppressive actions do not require recruitment of either GABA-GABA_A or GABA-GABA_B receptor signaling mechanisms. Instead, these nighttime inhibitions to orexin arise through direct postsynaptic activation of leak K^+ currents and hyperpolarization of SCN neurons. Functionally, orexin enhanced the phase-advancing actions of NPY, another neuropeptide implicated in conveying arousal information to the SCN. This modulation of peptidergic signaling is observable at the single neuron level where orexin potentiates inhibitory effects of NPY during the day. Thus, orexin in the SCN is mostly inhibitory and functions like other neural signals implicated in relaying arousal information to the SCN clock.

Direct orexin signaling in the brain is commonly considered excitatory (Eriksen et al., 2010) and can elicit depolarization, increased AP firing, and elevations in [Ca^{2+}]_i. However, in the SCN, these actions of orexin were rarely observed, especially at night. Even during the day, direct excitatory actions of orexin on [Ca^{2+}]_i and Per1-EGFP neuronal activity were observed in only a minority of OXA-responsive cells, whereas most OXA-responsive Per1-EGFP neurons in the SCN were indirectly inhibited by OXA. Since the majority of SCN neurons are GABAergic (Antle and Silver, 2005), this indicates a possible pathway through which orexin excitation of some SCN neurons increases inhibitory GABAergic tone within the SCN overall.

In other parts of the hypothalamus and brainstem, orexin excites local GABAergic interneurons to presynaptically regulate GABAergic transmission (van den Pol et al., 1998; Liu et al., 2002; Erikssson et al., 2004; Ma et al., 2007). In our daytime SCN recordings, focal application of gabazine into the SCN blocked orexin’s suppression of the Per1-EGFP neurons, thereby showing that orexin can increase the frequency of GABA-mediated IPSCs, presumably by increasing GABA release from local SCN neurons. Indeed, across the projected day, we found that orexin evoked increases in GABA_A receptor-mediated inhibitory events both in an impulse-dependent and independent manner with gabazine preventing orexin from reducing [Ca^{2+}]_i. These observations are consistent with rat SCN recordings, where orexin increased GABAergic neurotransmission in unidentified SCN neurons (Klisch et al., 2009). In addition, we found that OXA increased the amplitude of these GABAergic IPSCs, an observation consistent with findings of orexin’s actions in the nucleus ambiguus (Dergacheva et al., 2011). This indicates that during the day, OXA
ensures release of GABA in the SCN, triggering robust GABA–GABAA receptor–mediated suppression of SCN electrical activity and [Ca^{2+}]_{i} (Fig. 9).

In stark contrast, orexin’s inhibitory actions at night are independent of GABAergic signaling, and instead arise through orexin acting directly to activate background leak K^{+} currents (Figs. 6, 9). These leak channels are important for determining and maintaining resting membrane potential and excitability in cells (Mathie, 2007), and expression of their gene transcripts (Lein et al., 2007) and their physiological activity (Wang et al., 2012) are reported in the SCN. Here we show that activation of leak K^{+} currents by orexin at night produces robust inhibition of electrical activity in SCN neurons. To our knowledge, this is the first description of a direct hyperpolarizing action of orexin in the brain. Importantly, orexin-mediated inhibition of the SCN during the night (when orexin neuronal activation and signaling are maximal; Deboer et al., 2004; Zhang et al., 2004) may therefore serve to reinforce the intrinsic rhythm in SCN electrical activity (Fig. 9). This helps to explain the paradox that orexin neuronal activity and orexin release are high when SCN neuronal activity, intracellular calcium levels, and Per gene expression are low.

Elsewhere in the brain, orexin depolarizes neurons by inhibiting K^{+} conductances, including leak K^{+} currents (Ivanov and Aston-Jones, 2000; Govindaiah and Cox, 2006), activation of nonspecific cation channels (Liu et al., 2002; Yang and Ferguson, 2003), or by activating the Na^{+}/Ca^{2+} exchanger (Burdakov et al., 2003). One possible explanation for the apparent novelty of our findings is that most interrogations of orexin’s actions are performed during the day on tissue from nocturnal rodents. Another study of the nocturnal actions of orexin found that in preoptic and anterior hypothalamic GnRH neurons, orexin profoundly suppresses electrical activity in these cells at night (Gaskins and Moenter, 2012). Hence, clear day–night differences in the responses and mechanisms of orexin’s actions in the CNS may be widespread.

The precise contribution of OX_{1} and OX_{2} receptors to these complex actions of orexin in the SCN remains to be fully elucidated. The observation that OX_{1} expression is elevated at dusk, in line with Per2 expression, suggests that the molecular clock could increase orexin receptor availability at night. Orexin receptors are promiscuous and can couple both to excitatory and/or inhibitory G-proteins (Karteris and Randeva, 2003). Since transcripts for both receptor types are expressed in the SCN, the balance between inhibitory and excitatory actions of OXA could be dictated by clock-regulated changes in the G-proteins to which the native orexin receptors in the SCN are coupled and/or by the differential expression of OX_{1} and OX_{2} (Scammell and Winrow, 2011).

During the day, the circadian rhythms in the SCN clock and in behavior can be reset by so-called nonphotic/arousal-promoting stimuli (Mistlberger and Antle, 2011; Hughes and Piggins, 2012). NPY conveys nonphotic information to the SCN and it is notable in this study that OXA enhanced the inhibitory actions of NPY on single Per1-EGFP SCN cells as well as augmented its phase-shifting effects on the SCN molecular clock during the day. Intriguingly, arousal-promoting stimuli that reset circadian rhythms in behavior activate lateral hypothalamic orexin neurons (Marston et al., 2008; Webb et al., 2008). Orexin neurons innervate the IGL (Peyron et al., 1998; McGranaghan and Piggins, 2001; Mintz et al., 2001; Nixon and Smale, 2005), the source of the NPY innervation of the SCN, and orexin modulates the activity of IGL cells in vitro (Pekala et al., 2011). Nonphotic resetting is associated with suppression of SCN cellular activity and of clock gene expression (Maywood et al., 2002) including Per1 (Hamada et al., 2004). Collectively, our results and those of others now implicate orexin signaling in the integration of nonphotic information within the SCN.

This suggests that the indirect daytime inhibitory actions of orexin play a more prominent role in regulating clock phase than its direct excitatory actions at this time. Further, as orexin is inhibitory at night and as neither orexin nor other inhibitory stimuli appear to reset the SCN clock at this time (Brown and Piggins, 2007), this shows that the orexin’s potential roles in the SCN vary from day to night. Indeed, the suppressive actions of orexin at night could act as feedback from aroused behavioral states to reinforce the molecular clock’s inhibition of SCN neuronal activity (Fig. 9). This suppressive orexinergic signal may be integral to the Zeitnehmer cues involved with SCN clock entrainment processes (Hughes and Piggins, 2012; Roenneberg et al., 2013).

Despite the ability of orexin to inhibit daytime electrical activity within the SCN, this neuropeptide (on its own) was ineffective at shifting the circadian clockwork. This is an important observation, in that it indicates that orexin signaling is capable of alleviating SCN inhibition of behavioral activity in an acute manner,
which does not have lasting impact on circadian rhythmicity overall. This is especially important in a natural setting where animals must respond to short-term alterations in the environment, yet do not want to uncouple overall behavioral rhythmicity (SCN clock controlled) from prevailing light–dark cycles. This type of paradigm is well studied, for example, during daytime restricted feeding of nocturnal rodents (Mistlberger and Antle, 2011). It is noteworthy that mice deficient in orexin signaling have impaired circadian control of wake and some sleep states (Mochizuki et al., 2004) and have reduced anticipatory responses to restricted feeding (Akiyama et al., 2004).

In summary, our findings establish that orexin signaling in the SCN is mostly inhibitory and relies on intrinsic circadian clock-controlled membrane properties and neurochemistry of SCN cells to produce its effects on SCN [Ca2+]i and electrical activity. Here, we also show that orexin acts in the SCN to modulate the way the master circadian clock responds to other key arousal inputs. These findings support the view that in vertebrates, orexin signaling plays pivotal roles in the timing of transitions in brain and behavioral states (Mochizuki et al., 2004; Elbaz et al., 2012).

Since the neural circadian system is extensive (Sakhi et al., 2013) and because orexin neurons innervate many components of this circadian timing circuitry (Pepron et al., 1998), it is important to understand how orexin signaling integrates with circadian time cues to influence neuronal activity in these areas. A key implication is that the mechanisms of orexin’s actions in the SCN switch from predominantly GABA-mediated presynaptic actions during the day to robust direct postsynaptic effects at night.

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