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Electrophysiological actions of orexins on rat suprachiasmatic neurons *in vitro*

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ABSTRACT

The study of neural arousal mechanisms has been greatly aided by the discovery of the orexin peptides (orexin A and orexin B), the subsequent identification of the neurons that synthesize these peptides, their projections in the brain, and the distribution of orexin receptors in the central nervous system. Orexin neuron activation is partly controlled by circadian signals generated in the brain's main circadian pacemaker, the suprachiasmatic nuclei (SCN). The SCN clock is in turn reset by arousal-promoting stimuli and, intriguingly, orexin fibers and receptor expression are detected in the SCN region. It is unclear, however, if orexin can alter SCN neuronal activity. Here using a coronal brain slice preparation, we found that orexin A and orexin B (0.1–1 μM) elicited significant changes in the extracellularly recorded firing rate and firing pattern in ~80% of rat SCN cells tested; the most common response was suppression of firing rate. Co-application of orexin A with a cocktail of ionotropic GABA and glutamate receptor antagonists did not alter the actions of this peptide on firing rate, but did change some its effects on firing pattern. We conclude that orexins can alter SCN neurophysiology and may influence the transmission of information through the SCN to other CNS regions.

The mammalian circadian clock of the hypothalamic suprachiasmatic nuclei (SCN) is reset daily by specific environmental cues (Zeitgebers) relayed via specialized neural pathways. Zeitgebers are distinguished in part by their sensory modality and are classified broadly as photic and non-photic. Photic cues involve light activation of the retina, while most non-photic stimuli are associated with the induction of locomotor activity and/or the promotion of arousal [23]. While the neural mechanisms underlying the transmission of photic cues to the SCN are well established, those of arousal are less clear. The orexin/hypocretin neuropeptides (referred to in this paper as orexins) play key roles in the promotion of arousal state [27]. Orexins occur as two forms, orexin A (OXA) and orexin B (OXB), and are made only by cells in the tuberal hypothalamus [7,8,24,26]; these neurons give rise to extensive projections throughout the brain with fibers immunoreactive (-ir) for OXA localized in the peri-SCN region [8,20,21].

There are two types of G-protein coupled receptors for orexins, OX-1R and OX-2R, both of which are widely distributed in the mammalian brain, with OX-1R-ir present in the SCN [1]. Collectively, these neuroanatomical studies raise the possibility that orexins communicate arousal state information to influence SCN cellular physiology, however, the effects of these peptides on SCN neuronal activity are currently unknown. To address this, we determined the actions of OXA and OXB on extracellularly recorded rat SCN neurons *in vitro*.

Adult male Wistar rats (150–350 g; Charles River, Margate, UK) were housed under a 12 h:12 h light–dark cycle at an ambient temperature of 22 ± 1 °C. Zeitgeber time (ZT) 0 was defined as lights on and ZT12 as lights off. Food and water were available *ad libitium*. Animals were maintained under these conditions for at least 2 weeks prior to experimental procedures, which were carried out in accordance with the UK Animals Scientific Procedures Act (1986).

Slices were prepared during the early to late projected day (ZT1-11). Rats were anaesthetized with halothane (AstraZeneca, Macclesfield, UK) followed by cervical dislocation and decapitation. The brain was removed and 500 μm thick coronal sections containing the SCN were cut using a Vibroslicer (Campden Instruments Ltd., Leicester, UK). The slices were trimmed to remove the cortex, leaving only a 'mini-slice' containing the hypothalamus, and immediately transferred to a submerged slice chamber at 37 °C. The slices were perfused (~1.5 ml/min) with standard artificial cerebrospinal fluid (aCSF; pH 7.35) of composition (in mM): NaCl 124; KCl 3.3; KH_2PO_4 1.2; $CaCl_2$ 2.5; $MgSO_4$ 1.0; $NaHCO_3$ 25.5; D-glucose 10; and allowed to equilibrate for >1 h prior to electrophysiological recording. Where appropriate, drugs were applied via the perfusion line, dissolved in aCSF. OXA was obtained from Bachem (St. Helens, UK) and OXB from Peptide Institute Inc. (Osaka, Japan). All other drugs and reagents were obtained from Sigma (Poole, UK).

Extracellular recordings were obtained from the SCN using either 2 M NaCl-filled glass microelectrodes or aCSF-filled suction electrodes [3,6] which were visually guided onto the

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transilluminated SCN slice with the aid of a stereozoom dissection microscope. Neural signals were amplified (×20 K), filtered (300 Hz to 3 kHz) using the NeuroLog system (Digitimer, Welwyn Garden City, UK) and recorded on a PC using a micro1401 and Spike2 software (Cambridge Electronic Design, Cambridge, UK). For microelectrode recordings single-unit activity was discriminated online and recorded as events. For suction electrode recordings, the multi-unit signal was digitized as a continuous waveform (50 kHz) and single-unit activity was discriminated offline using principle components-based spike sorting. The two recording methods produced essentially identical results so data obtained using the two methods were pooled.

Cells were considered responders if the firing rate changed by >20% in the 5 min period following drug perfusion, compared to the value for the 5 min period immediately preceding treatment [4]. We also examined spike patterns by calculating the coefficient of variation (CV) of interspike intervals and Fano factor (FF) [17]. For the latter, spikes were counted in bins of varying sizes (10 ms to 10 s), with the FF for that time window (T) equal to variance in spike counts divided by the mean. When FF = 1 the spike pattern approximates to a Poisson process (i.e. occurrence of a spike is independent of the arrival times of previous spikes). FF < 1 indicates regularity in the spike pattern while values >1 indicate bursting behavior. When visualized on a log–log plot a linear relationship between T and FF (with slope = α) indicates the spike pattern follows a power law relationship $FF \propto T^{\alpha}$. This scaling exponent, α, is a measure of self-similarity (scale invariance) in the data, with values >0.5 indicating positive correlations exist in the spike pattern across the whole range of timescales. We calculated α by linear regression of FF profiles for $T > 3$ and <9 s.

We recorded the activity of 251 spontaneously discharging SCN neurons for a duration of 20–185 min (mean ± S.D.: 66 ± 37 min). Initially, we examined the firing patterns of 184 of these cells for which we had obtained baseline data before the slice had been treated with any test compounds. Other researchers have differentiated SCN neuronal activity [5] using analysis of basic membrane properties [25] or interspike intervals and entropy [2]. Based on our analysis of mean firing rate (MFR), CV and α, we identified three basic firing patterns of SCN neurons (Fig. 1), which we describe as regular, irregular, or bursting. Regular cells had high MFR, low CV and FF which dipped below 1 over short timescales and usually exhibited power law-type behavior over longer timescales. In contrast, bursting cells did not show this regularity at short timescales but consistently displayed power law behavior over longer timescales and overall had lower MFR and high CV. Irregular cells had the lowest MFR, intermediate CV values, and FF profiles that stayed close to 1 regardless of the timescale.

We derived average FF profiles for the three different firing patterns and classified the spiking activity of test cells to one of these types, based on the minimal root mean square deviation, before and

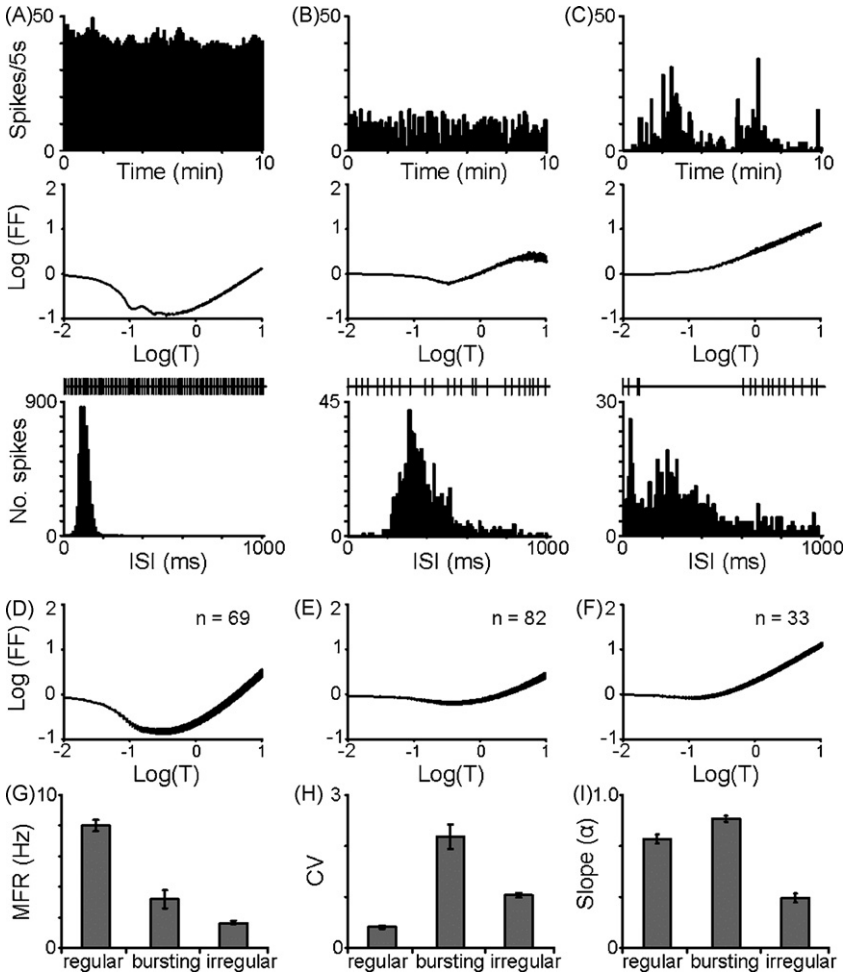

Fig. 1. SCN neurons exhibit three different types of firing pattern. Example cells classified as regular firing (A), irregular (B), and bursting (C). In (A–C), top panels show example firing rate histograms, middle panels show log Fano factor (FF) vs. log time window (T: s), bottom panels show interspike interval (ISI) distributions (raster plots above illustrate 10 s of representative spike pattern). Panels (D–F) show mean (±S.E.M.) FF profiles for all cells classified as regular, irregular or bursting, respectively. Panels (G–I) show mean (±S.E.M.) firing rate (MFR), coefficient of variation (CV) and α, respectively, for the three cell types.

after drug additions. We also compared firing patterns before and after drug treatments irrespective of their classification using the values of CV and α. Unless otherwise specified, parameters were compared by paired t-test.

We tested 185 SCN neurons for responses to OXA (0.01–1.0 μM) across the projected day to mid-projected night (ZT2-18). We did not observe any difference in responsiveness between cells tested during the projected day ($n = 140$) and projected night ($n = 45$), so data were pooled for subsequent analysis. In total, 88 cells (48%) showed suppression of firing rate, 57 (31%) were activated, and the remainder showed no change in firing rate to OXA treatment. Suppressions and activations exhibited similar dose dependency, with maximal effects observed at 0.3 μM. Activations typically lasted ~9 min, with an increase of baseline firing rate of 313 ± 77% (Fig. 2A and C). Suppressions to OXA (0.3 μM) were 6–30 min in duration, with a reduction from baseline firing rate of 56 ± 2% (Fig. 2B and D). We subsequently examined the firing patterns of these cells before and after the addition of OXA. Interestingly, OXA-responses involved changes in the spiking patterns of SCN neurons, and the type of response could be partly predicted based on the firing pattern prior to drug addition (Fig. 3). Most OXA-activated cells expressed irregular firing patterns but became more 'bursty' during responses (Fig. 3A, B and G) such that the FF scaling exponent significantly increased ($\alpha = 0.30 \pm 0.04$ vs. 0.40 ± 0.04, $P < 0.05$). OXA-suppressed cells were more likely to exhibit regular patterns which became more irregular during drug treatment (Fig. 3C, D and G), such that the CV increased significantly (Fig. 3H, $P < 0.01$). Most cells that did not respond to OXA also exhibited regular firing patterns but these were maintained during treatment with the peptide (Fig. 3E–H). Of note, most of the cells recorded during the projected night expressed irregular firing patterns before orexin

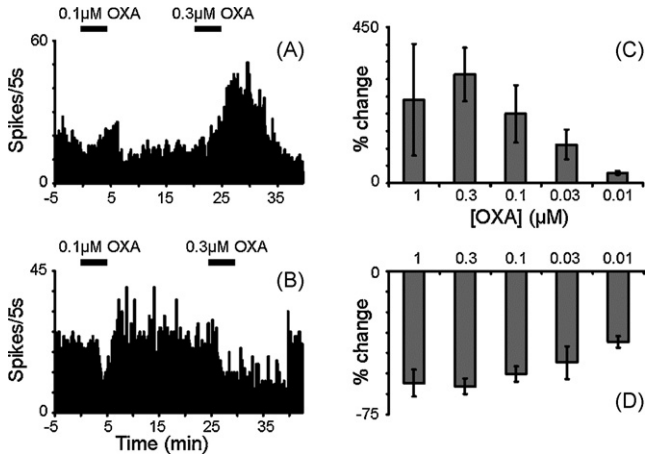

Fig. 2. OXA dose dependently alters SCN neuronal activity. Integrated firing rate histograms demonstrating the activational (A) or suppressive (B) effects of OXA treatment on the rate of discharge of SCN neurons. The filled bars indicate the timing of OXA bath application. Panels (C) and (D) represent the mean (±S.E.M.) change in firing rate 5–10 min after application of varying doses of OXA, relative to predrug values.

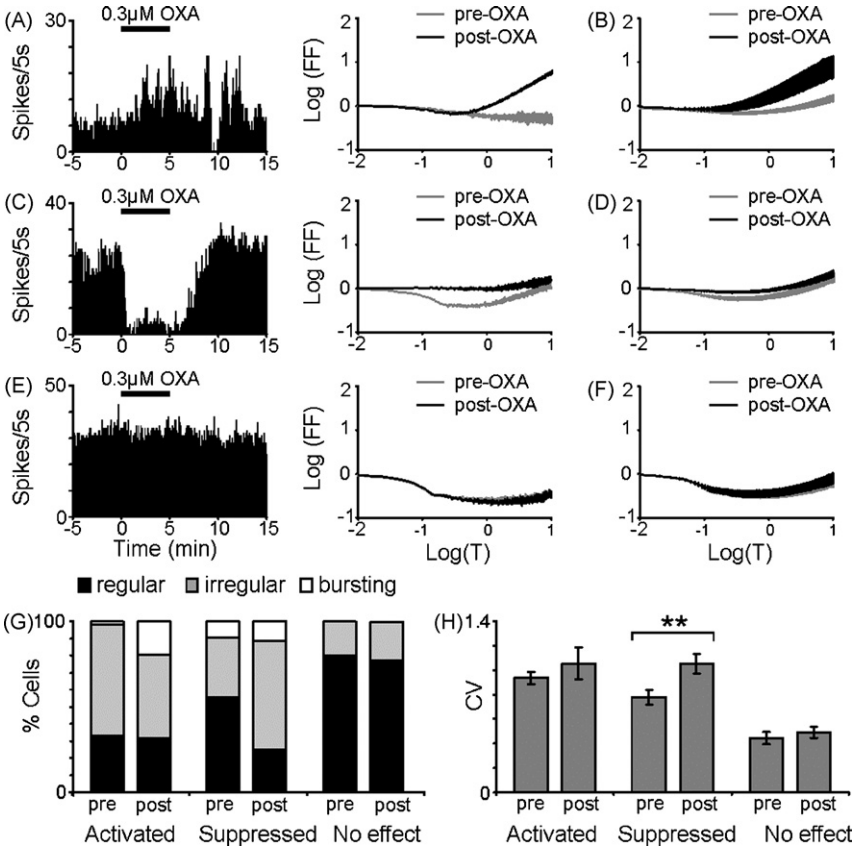

Fig. 3. OXA alters the firing pattern of SCN neurons. Panel (A, left) shows an integrated firing rate histogram for an OXA-activated cell and the FF profiles (right) before (grey line) and after (black line) OXA application. Note that initially the cell was classified as irregular firing, whereas during the OXA response the cell adopted a bursting pattern. Panel (B) shows the mean (±S.E.M.) FF profiles for all activated cells. Panel (C) shows an integrated firing rate histogram and the FF profiles for an OXA-suppressed cell. In this case the cell became less regular during the OXA-response as indicated by flattening of the FF profile. Panel (D) shows the mean (±S.E.M.) FF profiles of all suppressed cells. Cells that did not respond to OXA did not demonstrate changes in firing pattern (E and F). Histogram in (G) shows the percentage of cells classified as regular, irregular or bursting before and during OXA responses. Bar chart in (H) shows the mean (±S.E.M.) coefficient of variation (CV) of the interspike interval distributions before and after OXA treatment. $^{**}P < 0.01$, Paired t-test.

treatment (37/45, ~80%), a significantly higher proportion than during the projected day (45/140, 32%; χ^2-test $P < 0.05$), indicating that SCN neuronal firing patterns varied in a circadian manner. However, the relationships between OXA response and neuronal firing patterns were maintained when projected night cells were removed from the analysis (not shown).

As a positive control, we examined the effects of NMDA (20 μM) which evoked vigorous increases in firing rate in 47/61 cells (77%, activation: 475 ± 109% over baseline), including 14/15 cells that were unresponsive to OXA treatment. Thus, the lack of response to OXA was not attributable to impaired drug delivery to the SCN slice. A small number of cells (6/61) decreased their firing rate following NMDA treatment. Unlike OXA, NMDA-induced increases in firing rate did not promote power law-type spiking behavior (α pre: 0.22 ± 0.04, post: 0.30 ± 0.05; $P > 0.05$) but instead slightly increased the regularity of spike firing (CV pre: 1.04 ± 0.09, post: 0.87 ± 0.09; $P < 0.05$).

A different sample of 24 cells was tested for responses to OXB (0.1–1 μM). Twelve cells (50%) were suppressed and 8 cells (33%) were activated (suppressions: 69 ± 11%, activations: 130.0 ± 70% vs. baseline values). Four cells that did not respond to OXB were all activated by subsequent treatment with NMDA (20 μM). The average duration of the OXB-evoked responses was similar to those of OXA (~11 min), as were changes in firing patterns following drug treatment (not shown). Since OXB's neurorphysiological actions were indistinguishable from those of OXA, we did not pursue further studies with OXB.

Drug-induced changes in firing pattern can reflect direct effects on the recorded neuron or transynaptic effects mediated via other neurotransmitters [4]. To address this possibility, we used a cocktail of ionotropic glutamate and GABA receptor antagonists (50 μM D-AP5/2 μM NBQX/20 μM bicuculline). This cocktail significantly increased spontaneous activity in 17 of 24 cells for which we assessed the transition (Fig. 4A; 271 ± 78% baseline) and in another three cells firing was suppressed (70 ± 15%) indicating that most neurons were under the influence of GABAergic and/or glutamatergic tone. However, the firing patterns of these cells were not significantly altered by the antagonist cocktail (CV pre: 0.80 ± 0.02, post: 0.75 ± 0.02; α pre: 0.31 ± 0.01, post: 0.36 ± 0.01; $P > 0.05$).

Eighteen cells were tested with 0.3 μM OXA in the presence and absence (control condition) of the antagonist cocktail, 15 of which exhibited responses of similar magnitude under both conditions (Fig. 4; activation: 58 ± 16% vs. 54 ± 18%, $n = 6$; suppression: 54 ± 16% vs. 62 ± 12%, $n = 7$; two unresponsive under both conditions). Of the remaining cells, two failed to respond to OXA in the presence of the antagonists but did under control conditions (one supression/one activation) and one was activated in the presence of the antagonists but suppressed in their absence. A further 10 cells were tested with OXA in the presence of the antagonist cocktail only. We pooled data from all cells tested with 0.3 μM OXA ($n = 28$) to assess drug-induced changes in firing pattern. We found a similar relationship between the firing patterns of SCN neurons in the presence of the antagonist cocktail and subsequent response to OXA as under control conditions, with OXA-activated cells more likely to express irregular firing patterns and unresponsive cells more likely to exhibit regular firing patterns (Fig. 4E). As observed under control conditions, OXA-induced suppressions were associated with a decrease in the regularity of spike firing (Fig. 4C–F). In contrast, OXA-induced activations were no longer associated with altered firing patterns in the presence of the glutamate/GABA antagonists (Fig. 4A, B, E and F; α pre: 0.34 ± 0.14, post: 0.39 ± 0.13).

These data demonstrate that OXA and OXB regulate the firing rate of rat SCN neurons and that these effects occur largely independently of changes in ionotropic glutamate or GABA receptor

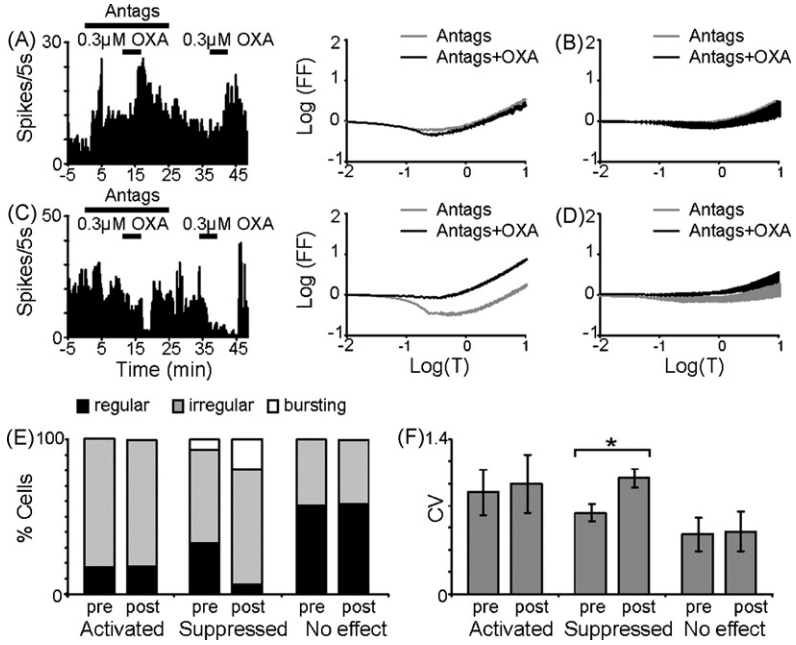

Fig. 4. OXA alters SCN neuronal firing rates in the presence of antagonists of ionotropic glutamate and GABA receptors. Panel (A, left) shows a firing rate histogram from an SCN neuron that was activated by OXA in the presence and absence of a cocktail of the ionotropic glutamate and GABA receptor antagonists (Antags: 50 μM D-AP5/2 μM NBQX/20 μM bicuculline). Despite the change in firing rate, the pattern of spikes from this neuron did not change during the response in the presence of the antagonists (A, right). Mean (±S.E.M.) FF profiles from all OXA-activated cells indicate that this treatment no longer alters firing pattern in the presence of the antagonists (B; compare with Fig. 3B). Panel (C, left) shows a firing rate histogram from an SCN neuron that was suppressed by OXA in the presence and absence of the antagonist cocktail. This cell exhibited a substantial change in firing pattern during the OXA-response (C, right). Mean (±S.E.M.) FF profiles from all OXA-suppressed cells demonstrate a similar effect of this peptide in the presence of the antagonists to that observed under control conditions (D; compare with Fig. 3D). Histogram in (E) shows the percentage of cells classified as regular, irregular or bursting before and during OXA responses in the presence of the antagonist cocktail. Bar chart in (F) shows the mean (±S.E.M.) coefficient of variation (CV) of the interspike interval distributions before and after OXA treatment in the presence of the antagonists. *$P < 0.05$, Paired t-test.

signaling. Detailed analysis of the firing patterns of SCN neurons indicate orexins also substantially alter the temporal structure of spiking in these cells and that some of these effects do involve changes in glutamatergic or GABAergic synaptic input. The predominant action of bath-applied OXA or OXB, noted in ∼50% of SCN cells tested, was suppression in firing rate. These findings are consistent with data from a preliminary study in which the most frequently observed response to OXA in the rat SCN was suppression of neuronal activity [12]. Collectively these data contrast with the mainly excitatory effects of orexin or hypocretin peptides reported previously in other parts of the rodent CNS [27] and may relate to the proposed antagonistic role between SCN neuronal activity and behavioral activity [5]. Indeed, sleep deprivation which is well-established to activate orexin neurons also suppresses SCN neuronal activity [11] and our results suggest a possible mechanism via which enhanced orexin release could suppress the SCN.

The receptor mechanisms mediating these actions of orexins in the rat SCN are unclear, however, neuroanatomical studies report extensive OX-R1 expression in the rat SCN region [14,19], which has been localized to both arginine-vasopressin-containing and vasoactive intestinal polypeptide-containing neurons, two of the major neurochemical cell types in the rat SCN [1]. These data are consistent with the high proportions of SCN neurons responding to orexin in our study. Taken together with our data indicating that blocking ionotropic GABA and glutamate receptors had very little effect on the ability of cells to respond to OXA, these anatomical studies suggest that orexins act directly on many SCN neurons to regulate their firing rate. However, we cannot exclude the possibility that some of the orexin-induced firing rate changes observed here were the result of trans-synaptic mechanisms that did not involve glutamate or GABA signaling.

In addition to examining the effects of OXA on firing rate, we classified SCN neuronal firing patterns using FF profiles. A previous study [17] used this approach to demonstrate that ∼90% of rat SCN neurons exhibit firing patterns that display 1/f-type power law behavior. We only observed this type of spiking behavior in regular and bursting cells (∼55% of cells sampled). This may reflect differences in experimental protocols as we recorded at 37 °C while Kim et al. [17] maintained their SCN slices at room temperature. The most common firing pattern we found, which we describe as irregular, did not exhibit power law behavior. Based on our observations that (1) during the projected night most SCN neurons fire irregular spike patterns and (2) orexins alter SCN neuronal firing patterns, we believe that individual SCN neurons can switch between these different types of spiking behavior. The functional significance of these different firing modes is unclear, although recent studies have demonstrated 1/f behavior in heart rate and locomotor activity [15,16] that is partially dependent on the SCN. In the case of heart rate, the scaling exponent decreases during the projected night, a phase where a reduced proportion of SCN cells displayed power law-type spiking behavior. Thus, SCN neuronal firing patterns may influence the temporal structure of various physiological and behavioral parameters, and by altering these patterns, orexins may modulate these aspects of physiology.

The relative contributions of cell intrinsic and network mechanisms in shaping SCN neuronal firing patterns are still not yet known. One study suggests that $GABA_A$-receptor mediated signaling is responsible for irregular firing of SCN neurons [18] and other studies indicate that regularity of firing is positively correlated with firing rate and that depolarization can increase the regularity of firing [28]. A parsimonious explanation is that cell intrinsic mechanisms that regulate cell membrane potential, and network effects involving GABA and other neurochemicals, interact to produce the observed firing patterns. We found that most cells that did not respond to OXA exhibited regular firing patterns, while most cells that were activated by OXA expressed irregular firing patterns. Thus, the mechanisms that give rise to these different types of spiking behavior may also influence the cells ability to respond to OXA. Further, we found that OXA-induced activations were associated with more 'bursty' firing patterns, while suppressions were associated with a decrease in the regularity of spike firing. In the case of suppressions, these effects were maintained during blockade of ionotropic glutamate/GABA receptors. In contrast, OXA-activated cells no longer displayed altered firing patterns in the presence of the antagonist cocktail, despite comparable changes in the magnitudes of mean firing rate to control conditions. Similarly, the antagonists alone evoked robust changes in mean firing rate without consistently altering firing pattern. We conclude that (1) changes in firing rate can occur independently of changes in firing pattern, (2) signaling via ionotropic glutamate/GABA receptors plays only minor roles in determining basal SCN neuronal firing patterns under our experimental conditions, but (3) that these systems may be recruited by OXA to modulate some of its effects within the SCN.

Existing data show that the SCN drive a daily rhythm in OXA levels [10], which may occur via an indirect output [9] from the SCN to the tuberal hypothalamus. As orexin neurons also innervate other brain areas in the thalamus and brainstem [20,21,26] that convey arousal-promoting information to the SCN [13,22], our neurophysiological data indicate an additional pathway through which arousal state may be communicated to the SCN clock. In summary, we demonstrate robust electrophysiological effects of orexins on the rat SCN consistent with the proposed roles of these neuropeptides in regulating behavioral state.

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References

[1] M. Bäckberg, G. Hervieu, S. Wilson, B. Meister, Orexin receptor1 (OX-R1) immunoreactivity in chemically defined neurons of the hypothalamus: focus on orexin targets involved in control of food and water intake, Eur. J. Neurosci. 15 (2002) 315–328.
[2] G.S. Bhumbra, A.N. Inyushkin, K. Saeb-Parsy, A. Hon, R.E.J. Dyball, Rhythmic changes in spike coding in the suprachiasmatic nucleus, J. Physiol. 563 (2005) 291–307.
[3] T.M. Brown, J.R. Banks, H.D. Piggins, A novel suction electrode recording technique for monitoring circadian rhythms in single and multiunit discharge from brain slices, J. Neurosci. Methods 156 (2006) 173–181.
[4] T.M. Brown, E. McLachlan, H.D. Piggins, Angiotensin II regulates the activity of mouse suprachiasmatic nuclei neurons, Neuroscience 154 (2008) 839–847.
[5] T.M. Brown, H.D. Piggins, Electrophysiology of the suprachiasmatic circadian clock, Prog. Neurobiol. 82 (2007) 229–255.
[6] A.N. Coogan, N. Rawlings, S.M. Luckman, H.D. Piggins, Effects of neurotensin on discharge rates of rat suprachiasmatic nucleus neurons *in vitro*, Neuroscience 103 (2001) 663–672.
[7] D.J. Cutler, R. Morris, V. Sheridhar, T.A.K. Wattam, S. Holmes, S. Patel, J.R.S. Arch, S. Wison, R.E. Buckingham, M.L. Evans, R.A. Leslie, G. Williams, Differential distribution of orexin-A and orexin-B immunoreactivity in the rat brain and spinal cord, Peptides 20 (1999) 1455–1470.
[8] Y. Date, Y. Ueta, H. Yamashita, H. Yamaguchi, S. Matsukura, K. Kangawa, T. Sakurai, M. Yanagisawa, M. Nkazato, Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine and neuroregulatory systems, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 748–753.
[9] S. Deurveilher, K. Semba, Indirect projections from the suprachiasmatic nucleus to major arousal-promoting cell groups in rat: implications for the circadian control of behavioural state, Neuroscience 130 (2005) 165–183.
[10] T. Deboer, S. Overeem, N.A. Visser, H. Duindam, M. Frölich, G.J. Lammers, J.H. Meijer, Convergence of circadian and sleep regulatory mechanisms on hypocretin-1, Neuroscience 129 (2004) 727–732.
[11] T. Deboer, L. Détári, J.H. Meijer, Long term effects of sleep deprivation on the mammalian circadian pacemaker, Sleep 30 (2007) 257–262.
[12] B. Farkas, I. Vilagi, L. Détári, Effect of orexin-A on discharge rate of rat suprachiasmatic nucleus neurones *in vitro*, Acta Biol. Hung. 53 (2001) 435–443.

[13] M.E. Harrington, The ventral lateral geniculate nucleus and the intergeniculate leaflet: interrelated structures in the visual and circadian systems, Neuosci. Biobehav. Rev. 21 (1999) 705–727.
[14] G.J. Hervieu, J.E. Cluderay, D.C. Harrison, J.C. Roberts, R.A. Leslie, Gene expression and protein distribution of the orexin-1 receptor in the rat brain and spinal cord, Neuroscience 103 (2001) 777–797.
[15] K. Hu, F.A.J.L. Scheer, P. Ivanov, R.M. Buijs, S.A. Shea, The suprachiasmatic nucleus functions beyond circadian rhythm generation, Neuroscience 149 (2007) 508–517.
[16] K. Hu, F.A.J.L. Scheer, R.M. Buijs, S.A. Shea, The endogenous circadian pacemaker imparts a scale-invariant pattern of heart rate fluctuations across time scales spanning minutes to 24 h, J. Biol. Rhythms 23 (2008) 265–273.
[17] S.I. Kim, J. Jeong, Y. Kwak, Y.I. Kim, S.H. Jung, K.J. Lee, Fractal stochastic modeling of spiking activity in suprachiasmatic nucleus neurons, J. Comput. Neurosci. 19 (2005) 39–51.
[18] N.I. Kononenko, F.E. Dudek, Mechanism of irregular firing of suprachiasmatic nucleus neurons in rat hypothalamic slices, J. Neurophysiol. 91 (2004) 267–273.
[19] J.N. Marcus, C.J. Aschkenasi, C.L. Lee, R.M. Chemelli, C.B. Saper, M. Yanagisawa, J.K. Elmquist, Differential expression of orexin receptors 1 and 2 in the rat brain, J. Comp. Neurol. 435 (2001) 6–25.
[20] P.A. McGranaghan, H.D. Piggins, Orexin A-like immunoreactivity in the hypothalamus and thalamus of the Syrian hamster (*Mesocricetus auratus*) and Siberian hamster (*Phodopus sungorus*), with special reference to circadian structures, Brain Res. 904 (2001) 234–244.
[21] E.M. Mintz, A.N. van den Pol, A.A. Casano, H.E. Albers, Distribution of hypocretin-(orexin) immunoreactivity in the central nervous system of Syrian hamsters (*Mesocricetus auratus*), J. Chem. Neuroanat. 21 (2001) 225–238.
[22] L.P. Morin, Serotonin and the regulation of mammalian circadian rhythmicity, Ann. Med. 31 (1999) 12–33.
[23] N. Mrosovsky, Locomotor activity and non-photic influences on circadian clocks, Biol. Rev. Camb. Philos. Soc. 71 (1996) 343–372.
[24] T. Nambu, T. Sakurai, K. Mizukami, Y. Hosoya, M. Yanagisawa, K. Goto, Distribution of orexin neurons in the adult rat brain, Brain Res. 827 (1999) 243–260.
[25] C.M.A. Pennartz, M.T.G. De Jeu, A.M.S. Guerstsen, A.A. Sluiter, M.L.H.J. Hermes, Electrophysiological and morphological heterogeneity of neurons in slices of rat suprachiasmatic nucleus, J. Physiol. 506 (1998) 775–793.
[26] C. Peyron, D.K. Tighe, A.N. van den Pol, L. de Lecea, H.C. Heller, J.G. Sutcliffe, T.S. Kilduff, Neurons containing hypocretin (orexin) project to multiple neuronal systems, J. Neurosci. 18 (1998) 9996–10015.
[27] T. Sakurai, The neural circuit of orexin (hypocretin): maintaining sleep and wakefulness, Nat. Rev. Neurosci. 8 (2007) 171–181.
[28] A.M. Thomson, D.C. West, Factors affecting slow regular firing in the suprachiasmatic nucleus in vitro, J. Biol. Rhythms 5 (1990) 59–75.