Published in final edited form as:

Eur J Neurosci. 2010 November ; 32(9): 1528–1536.

Knockdown of Orexin Type I Receptor in Rat Locus Coeruleus increases REM Sleep during the Dark Period

Lichao Chen¹, James T. McKenna¹, Yunren Bolortuya¹, Stuart Winston¹, Mahesh M. Thakkar², Radhika Basheer¹, Ritchie E. Brown¹, and Robert W. McCarley¹

¹ Research Service, VA Boston Healthcare System, Brockton, MA 02301, USA Department of Psychiatry, Harvard Medical School, Brockton, MA 02301, USA

² Harry S. Truman Memorial Veterans Hospital, Dept of Neurology, University of Missouri, Columbia 65210, USA

Abstract

The locus coeruleus (LC) regulates sleep/wakefulness and is densely innervated by orexinergic neurons in the lateral hypothalamus. Here we used small interfering RNAs (siRNAs) to test the role of LC orexin type 1 receptor (OxR1) in sleep-wake control. In sleep studies, bilateral Ox1R siRNA injections led to an increase of time spent in REM sleep which was selective for the dark (active) period, peaked at approximately 30% of control during the second dark period after injection and then disappeared after 4 days. Cataplexy-like episodes were not observed. The percentage time spent in wakefulness and NREM sleep and the power spectral profile of NREM and REM sleep were unaffected. Control animals, injected with scrambled siRNA, had no sleep changes post-injection. Quantification of the knockdown revealed that unilateral microinjection of siRNAs targeting OxR1 into the rat LC on 2 consecutive days induced a 45.5% reduction of OxR1 mRNA in the LC two days following the injections when compared to the contralateral side receiving injections of control (scrambled) siRNAs. This reduction disappeared 4 days after injection. Similarly, unilateral injection of OxR1-siRNA into the LC revealed a marked (33.5 %) reduction of Ox1R receptor staining 2 days following injections. In contrast, both the mRNA level and immunohistochemical staining for tyrosine hydroxylase were unaffected. Our results indicate a modest knockdown of OxR1 is sufficient to induce observable sleep changes. Moreover, orexin neurons, by acting on OxR1 in the LC, play a role in the diurnal gating of REM sleep.

Keywords

Hypocretin; narcolepsy; RNA interference; Diurnal; siRNA

Introduction

The locus coeruleus (LC) promotes arousal by means of widespread projections to the forebrain and neocortex (Berridge, 2008). Noradrenergic neurons in the LC are most active during wakefulness, reduce firing during NREM sleep, and cease activity during REM sleep (Aston-Jones and Bloom, 1981; Foote et al, 1980; Hobson et al, 1975; Rasmussen et al, 1986). Correspondingly, the release of noradrenaline (NE) declines progressively from waking to NREM sleep, and reaches its' lowest levels during REM sleep (Shouse et al, 2000). Pharmacological or electrical activation of the LC leads to wakefulness and an

inhibition of rapid eye movement (REM) sleep (Gottesmann, 2008; McCarley, 2004; Pal and Mallick, 2007). However, the endogenous mechanisms which regulate the activity of LC neurons and their control of sleep-wake states are still incompletely understood.

Orexins (hypocretins) are peptides that are produced in the perifornical region of the hypothalamus (PFH) (De Lecea et al, 1998; Sakurai et al, 1998). Abnormalities of orexinergic systems have been implicated in the sleep disorder narcolepsy (Mignot, 2001), which involves dysregulation in the timing of sleep-wake states and the abnormal appearance of REM sleep-like phenomena during waking. Two isoforms of orexins (orexin-A and -B, or hypocretin-1 and hypocretin-2) are derived from proteolytic cleavage of a precursor peptide (prepro-orexin, or prepro-hypocretin) and exert their actions through two types of G-protein-coupled receptors (OxR1 and OxR2, or Hcrt1 and Hcrt2) (De Lecea et al, 1998; Sakurai et al, 1998). Orexin neurons are most active during wakefulness and are relatively quiet during NREM and REM sleep (Lee et al, 2005; Mileykovskiy et al, 2005; Takahashi et al, 2008).

The LC receives the densest extrahypothalamic innervation of the orexinergic system (Date et al, 1999; Hagan et al, 1999; Horvath et al, 1999; Mintz et al, 2001; Novak and Albers, 2002; Peyron et al, 1998) and LC neurons exclusively express OxR1 (Bourgin et al, 2000; Hervieu et al, 2001; Marcus et al, 2001; Trivedi et al, 1998; Zhang et al, 2004). Orexins excite LC neurons via Ox1R (Hagan et al, 1999) and local or icv injection of orexin-A into the LC suppresses REM sleep (Bourgin et al, 2000; Piper et al, 2000). As expected, the selective OxR1 antagonist SB-334867-A reverses the suppression of REM sleep by orexin-A application into the LC (Smith et al, 2003).

RNA mediated interference (RNAi) is a mechanism for highly selective and reversible gene suppression (Fire et al, 1998). Application of double-stranded, short interfering RNAs (siRNA) consisting of 21–23 nucleotides with a symmetric overhang of 2–3 nucleotides promotes RNAi in mammals without inducing an immune response (Elbashir et al, 2001). Previously, we microinjected siRNAs targeting prepro-orexin mRNA into the rat perifornical hypothalamus to knock down (KD) this gene and found a persistent increase in REM sleep during the dark (active) period (Chen et al, 2006). Here, we applied the same technique to assess the role of OxR1 in LC on sleep and wakefulness.

Methods

Animals

Adult Sprague Dawley male rats (300–350g; Charles River Laboratories, Wilmington, MA) were housed under a 12:12 h light-dark cycle (lights on: 0700 hr; lights off: 1900 hr) at 22±1°C. Food and water were provided ad libitum. All animals were treated in accordance with the Association for Assessment of Laboratory Animal Care policy on care and use of laboratory animals and all efforts were made to minimize the number of animals used. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Research Committee of the Boston Veterans Administration Healthcare System.

siRNA

A pool of 3 siRNAs (equal concentration) was used (Table 1). All of them were designed individually to target OxR1 (OxR1-siRNA). A corresponding control pool of 3 siRNAs with no homology to known rat genes (Ctrl-siRNA) was used. All siRNAs were annealed and HPLC purified (Applied Biosystems, Foster City, CA).

Assessment of sleep-wake effects of siRNA injection into the LC—To determine the effects of the specific knockdown of OxR1 on sleep-wakefulness standard EEG/EMG recording and analysis techniques were used (Chen et al., 2006). *Bilateral* injections of OxR1-siRNA ($N=7$) or Ctrl-siRNA ($N=6$) were made into the LC (Paxinos G and Watson C, 2000) on two consecutive days and sleep-wakefulness monitored for 6 subsequent days.

Surgery for implantation of sleep recording electrodes and guide cannulae: Under sterile surgical conditions, each rat was implanted with standard EEG and EMG recording electrodes. One EEG screw was implanted 2.0 mm anterior to Bregma and 1.5 mm lateral to the central suture. The second screw was implanted 5.0 mm posterior to Bregma and 4 mm lateral to the central suture. Bilateral EMG electrodes were placed in the nuchal muscles. Bilateral guide cannulae (Plastics One, Roanoke, VA) were implanted 1 mm above LC for later microinjections since the injection cannula extended 1 mm beyond the tip of the guide cannula.

Microinjections and sleep recordings: Recording cables were connected one day post-surgery. After at least a week of recovery and habituation to the recording cage, a 24-hour baseline sleep recording beginning at dark onset was obtained. For the next two days, 4 hr before dark onset (15:00), the rats were removed from the recording cage and gently swaddled in a towel during bilateral injection (approximately 2 min) with either OxR1-siRNA or Ctrl-siRNA (0.03 nmol per side). Twenty-four hour sleep recordings lasted a minimum of 6 days afterwards (Icelus, Mark Opp, University of Michigan, Ann Arbor, MI, USA). In our preliminary study, animal behavior was video-recorded using an infrared camera for 8 h per dark period. However, since no cataplexy/sleep onset REM (SOREM)-like episodes were identified, we discontinued this practice.

Histology: For identification of injection sites, rats were deeply anaesthetized with sodium pentobarbital and transcardially perfused with saline followed by 4% formaldehyde. The brain was isolated and placed overnight in 4% formaldehyde, transferred to 20% sucrose for cryoprotection, and coronal sections were cut at 30 μ m on a freezing microtome. One series of sections, encompassing the rostro-caudal extent of LC, was mounted onto chrom-alum slides. Cresyl violet staining was performed following standard procedures (Paxinos G and Watson C, 2000). Briefly, slides were exposed to Fisherbrand CitriSolv, followed by alcohols of descending concentration. Slides were dipped in water, and then stained in 0.5% cresyl violet for 15 minutes. Slides were dehydrated in a series of ascending alcohols, then placed in CitriSolv, and coverslipped. Animals which did not have injections localized correctly to the LC were excluded from subsequent analysis.

Data Analysis: The sleep data were recorded in 12 hour blocks (light and dark periods) and were scored visually offline in 10 sec epochs of Wakefulness, NREM and REM using standard criteria (Chen et al, 2006). The amount of time spent in each state was calculated along with number of episodes, as well as average duration of each behavioral state for each 12 hour block. Additionally, we calculated average REM sleep latency (defined as time from onset of REM sleep to the preceding wakefulness) and average REM sleep to REM sleep cycle duration (R-R cycle, defined as time between the beginning of a REM sleep episode to the beginning of the next REM sleep episode

Assessment of OxR1-knockdown: real time PCR—The extent and time course of knockdown (KD) was assessed quantitatively by performing real time PCR to examine OxR1 and tyrosine hydroxylase (TyH) mRNA levels.

Surgery and siRNA injections: Under isoflurane inhalation anesthesia, a group of 9 rats were implanted with bilateral guide cannulae (Plastics One, Roanoke, VA, USA) with tips 1 mm above the LC for later microinjections. After a one week recovery period, OxR1-siRNAs were unilaterally injected (0.03 nmol in 0.3 μ l water) into one side of the LC using an injection cannula with tips extending 1 mm beyond the guide cannula. Ctrl-siRNA was injected on the contralateral side. A second injection was repeated 24 hr later. 48 hours after treatment the animals were sacrificed by decapitation using a guillotine. The brain area containing the LC was punched out with a gauge 11 (2.4 mm internal diameter) micropunch, placed on dry ice, and subsequently stored at -80°C until processed for RNA extraction and real-time PCR. A separate group of 8 rats received the same treatment except that the brain tissues were collected 4 days after the last siRNA injection.

RNA extraction: The LC tissue samples were first homogenized (Dounce homogenizer) in TRIzol® reagent (0.5 ml per sample) at 4°C and incubated for 5 min at room temperature (RT). Subsequently, 100 μ l of chloroform was added, the mixture was vortexed for 15 s and then left for 2 min at RT. Next, the samples were centrifuged at 12,000 g for 15 min at 4°C and the supernatant of each sample was transferred to a new tube containing 1 μ l of glycogen. Isopropyl alcohol (250 μ l) was added to precipitate the RNA. The samples were subsequently centrifuged at 12,000 g for 15 min at 4°C . The supernatant was discarded and the pellets were washed twice with 75 % ethanol, air-dried, resuspended in 50 μ l of RNase-free water, and stored at -80°C .

cDNA preparation: First strand cDNA preparation was carried out with Superscript II enzyme (Invitrogen, Carlsbad, CA, USA). RNA (2 μ l) was heated with 1 μ l random hexamers, 2 μ l of 10 mM dNTP Mix, and 6 μ l DEPC-treated water at 65°C for 5 min. This mixture was then put on ice. A mixture of 2 μ l 10 X reverse transcription buffer, 4 μ l 25 mM MgCl_2 , 2 μ l 0.1 M DTT, 1 μ l RNase OUT™ was subsequently added, and the samples were incubated at 25°C for 2 min. Next, 1 μ l (50 units) of Superscript II was added to each sample and incubated at 42°C for 50 min followed by 75°C for 15 min. 1 μ l of RNase H was added and each sample was incubated for 15 min at 37°C .

Real time PCR: Each sample was run in duplicate in the Applied Biosystems real time PCR machine (Model 7300, Applied Biosystems, Foster City, CA). The primer/probe sets from the TaqMan Gene Expression Assays with efficiency of 100% for rat OxR1, TyH and endogenous control 18S RNA were used (OxR1: Rn00565032_m1; TyH: Rn00562500_m1_m1 Eukaryotic 18S rRNA Endogenous Control: 4333760T). The PCR reaction mixture contained 2X TaqMan® Universal PCR Master Mix (25 μ l) 20X TaqMan® Gene Expression Assay (2.5 μ l), cDNA (4 or 8 μ l) and water with a total volume at 50 μ l. The amplification was run by 40 cycles of denaturation at 95°C followed by annealing/ extending at 60° . We employed the comparative Ct method ($\Delta\Delta\text{Ct}$ method) to calculate relative changes in mRNA levels (Livak and Schmittgen, 2001). The ΔCt values were determined by subtracting the reference 18S RNA values from the target gene Ct values. The fold-change between the mRNA expression levels of the OxR1-siRNA treated and the Ctrl-siRNA treated samples was calculated using the formula $2^{-(\Delta\Delta\text{Ct})}$, where $\Delta\Delta\text{Ct}$ equals the difference in ΔCt between the control and the experimental sample. The expression level of OxR1 or TyH in Ctrl-siRNA treated samples was normalized to 100% and their respective levels in OxR1 treated samples were expressed as percentage of control.

Assessment of OxR1-knockdown: immunohistochemical analysis—To verify the knockdown of OxR1 *protein* we performed immunohistochemical labeling of OxR1 and TyH following OxR1-siRNA injection into the LC on one side of the brainstem and Ctrl-

siRNA injection on the other side. The same surgical and injection protocol (i.e. 2 injections on consecutive days) was followed as used in the PCR and sleep studies (see above).

Immunofluorescence double-labeling of OxR1 and TyH: For immunohistochemistry, coronal rat brain sections were cut as described in the above histological study. Brain sections containing LC were incubated in 0.5% Triton X-100 and 5% normal donkey serum in PBS for 1 hour followed by incubation in a solution of rabbit anti-OxR1 primary antibody (1:100, AB3092, produced against a 16-amino acid peptide near the N-terminus of rat OxR1 and a 16-amino acid peptide near the C-terminus of human OxR1, Millipore, Billerica, MA, USA) in 0.5% Triton, 1% normal donkey serum, and PBS for 2 days. The sections were treated with Cy3-conjugated donkey anti-rabbit secondary antibody (red, 1:400, 711-165-152, Jackson ImmunoResearch, West Grove, PA, USA) for 4 hours. Sections were then incubated overnight with sheep anti-TyH primary antibody (1:300, AB1542, produced against rat pheochromocytoma TyH, Millipore, Billerica, MA, USA). Finally, sections were incubated in donkey anti-sheep FITC (green, 1:100, 713-095-147, Jackson ImmunoResearch, West Grove, PA, USA) for 3 hours. All incubations were done at 4°C. Sections were mounted, dried, and coverslipped with Vectashield (Vector Laboratories, Burlingame, CA, USA).

Both primary antibodies have been widely used and extensively published, eg. TyH antibody (Hamlin et al, 2008; Kaufling et al, 2009; Marchant et al, 2007; Ramer, 2008) and OxR1 antibody (Beiras-Fernandez et al, 2004; Bengtsson et al, 2007; Espana et al, 2003; Magga et al, 2006). The anti-TyH antibody detects a 62 kDa band on Western blot from PC12 cells stimulated with okadaic acid (manufacturer's data). The specificity of this antibody was supported by our observation that TyH staining was only seen in the LC, but not in surrounding areas. The anti-OxR1 antibody recognizes a 50 kDa band from rat brain lysate and the distribution of OX1R immunoreactivity closely resembles its mRNA expression in rat brain (Suzuki et al, 2002). Regions expressing OxR1 immunoreactivity were similar to that previously reported in the rat (Bourgin et al, 2000; Greco and Shiromani, 2001; Hervieu et al, 2001), including the brainstem motor nuclei, inferior colliculi, locus coeruleus, dorsal raphe nucleus, laterodorsal tegmental nucleus, dorsal tegmental nucleus, pontine nucleus caudalis, Barrington's nucleus, ventral cochlear area, and olivary nuclei. Non-specific staining was not evident when TyH and OxR1 primary antibodies were omitted.

Quantification of immunohistochemical staining: Following immunohistochemical stains, four representative sections encompassing the rostro-caudal extent of LC were evaluated per case. For quantification of OxR1 receptor knockdown, cases were evaluated if staining for both TyH and OxR1 were successful (signal could be distinguished from background staining for both stains on the side receiving scrambled siRNA). Following this guideline, staining from 5 animals was analyzed. 20X magnification images of the LC were captured using a Zeiss Axioplan 2 microscope and Slidebook 3 software (Intelligent Imaging Innovations, Denver, CO, USA). TyH (tyrosine hydroxylase)-positive cells were identified by the presence of green fluorescence in the cytoplasm (fluorescein, excitation:emission 488:509 nm). OxR1 receptor labeling exhibited punctuate red fluorescence (Cy3, excitation:emission wavelength = 590:617 nm). Photographs of the LC were taken, switching between the two fluorescent filter sets, providing individual photographs (TyH or OxR1 receptor) of the same fields. All photographs were obtained using an exposure time of 780 ms for anti-TyH labeling, and an exposure time of 3 s for anti-OxR1 receptor labeling.

Image analysis was performed using Adobe Photoshop, Version 6 (Adobe Systems, Mountain View, CA), and adapted from previously published techniques (Lehr et al, 1997; Lehr et al, 1999; Mofidi et al, 2003; Vilaplana and Lavialle, 1999). Immunostaining

intensity/luminance for TyH-positive neurons and OxR1 receptors was determined by subtracting background values from foreground TyH or OxR1 receptor values. Luminance values across the four slices per case were determined for each side (left, Ctrl-siRNA; right, OxR1-siRNA). Mean staining intensity values were represented in arbitrary units (AU) according to the histogram measure of Photoshop (see supplemental methods).

Statistics: SigmaStat version 1.0 (Jandel Cooperation, Sanfael, CA, USA) was used for statistical calculation. PCR and immunohistochemical staining data were analyzed by non-directional *t*-tests comparing TyH and OxR1 receptor values and differences between Ctrl-siRNA and OxR1-siRNA treated sides. Sleep data were analyzed by one-way repeated measures (RM) analysis of variance (ANOVA) followed by post-hoc Bonferroni-corrected *t*-tests. Values were considered significant if $p < 0.05$.

Results

Sleep changes after siRNA-Ox1R injection into the LC

In the days following the last injection, the OxR1-siRNA treated animals ($N=7$) showed a significant increase ($F_{3,18} = 6.1$, $P=0.004$; Figure 1; Table 2) in the amount of time spent in REM sleep exclusively during the dark (active) period for the first 2 post-injection nights (an increase of 27 and 35%, respectively) compared to the baseline (pre-injection night). Further analysis revealed that this increase was due primarily to an increase in episode number ($F_{6,12} = 9.2$, $P=0.004$, Table 3). Neither REM sleep duration nor REM sleep to REM sleep cycle duration (time between REM sleep episodes) were appreciably altered. There was also no significant change in latency to REM sleep (time between previous waking to REM sleep) (Table 3). Furthermore, we compared the EEG power spectrum for the baseline night and the second post-injection night (when the largest REM sleep change was observed) and found that the characteristics of the EEG power distribution was unaltered for both NREM and REM sleep in OxR1-siRNA treated rats (Figure 2). In our preliminary study, we conducted nightly video recordings after siRNA injections and found neither behavioral nor polysomnographic signs of cataplectic attacks. In contrast to OxR1-siRNA injected rats ($N=6$), the Ctrl-siRNA treated (bilateral injections) rats did not have significant sleep changes after injection (Table 2).

As in our previous study (Chen et al., 2006), sleep, especially REM sleep, was affected during the two injection nights (Table 2) in both OxR1-siRNA and Ctrl-siRNA treated rats. As this effect is not specific and likely due to handling and the immediate mechanical effects of the injection, these data were not included in the analysis.

Verification of selective KD of Ox1R mRNA

Following unilateral injection of OxR1-siRNA into the LC area on two consecutive days on one side and Ctrl-siRNA injected into the contralateral side, a significant reduction of OxR1 mRNA (45.5%) was detected 48 hrs later (Figure 3, top panel, $t_{16} = -2.83$, $P=0.01$), i.e. at the timepoint with maximal REM sleep changes. There was no reduction of OxR1 mRNA 96 hrs (4 days) after the last OxR1-siRNA injection (at the same timepoint when sleep changes had returned to baseline; Figure 3, bottom panel). In contrast, there was no significant change in the non-targeted TyH mRNA levels between the OxR1-siRNA treated group and the Ctrl-siRNA treated group.

Verification of selective KD at the protein level—To confirm that there is a loss of Ox1R protein at the time when sleep changes were observed, we performed immunohistochemistry for TyH and OxR1 in animals that received unilateral (left) side Ctrl-siRNA injections, and contralateral right side OxR1-siRNA injections over 2 days ($N=5$).

Immunofluorescence labeling (Figure 4) revealed a marked (33.5 %) reduction in OxR1 immunoreactivity on the OxR1-siRNA injected side compared to the side treated with Ctrl-siRNA (6.4 ± 0.6 AU Ctrl-siRNA side vs. 4.3 ± 0.5 AU OxR1-siRNA side; $t_8 = 2.740$, $P = 0.03$); in contrast, there was not a significant reduction of TyH immunoreactivity (56.6 ± 2.6 AU Ctrl-siRNA side vs. 53.9 ± 1.9 AU OxR1-siRNA side).

Discussion

Our results show that a modest knockdown of OxR1 in the LC (45.5 % reduction of mRNA, 33.5 % reduction of protein) was sufficient to induce increased REM sleep for 2 consecutive nights, whereas other behavioral stages were unaffected. The ultradian cycle of REM sleep was unchanged but the diurnal distribution of REM sleep was altered resulting in more REM sleep episodes during the dark (active) phase. The power spectral characteristics of NREM and REM sleep were unaffected by the siRNA injections.

Technical considerations

Injections of OxR1-siRNA on two consecutive days induced a KD of Ox1R mRNA of 45.5%, detected by real-time PCR, and protein KD of 33.5%, evaluated by means of immunohistochemistry. Our current finding in terms of mRNA reduction is comparable to previous reports. We and others have demonstrated that microinjection of a cocktail of siRNAs against prepro-orexin induced a specific decrease of prepro-orexin mRNA expression and related behavioral changes (Chen et al, 2006; Johnson et al, 2010). In our previous study (Chen et al, 2006), we observed a 59% reduction in prepro-orexin mRNA and a persistent REM sleep increase for 4 nights following a single injection of siRNA. A similar *in vivo* application of siRNA targeting tumor necrosis factor alpha induced a 37% KD (Taishi et al, 2007). In preliminary studies, we found that a single injection of OxR1-siRNA induced a 26% KD of Ox1R mRNA (data not shown). The larger effect produced by an additional injection is probably reflective of a slower turnover rate of receptor proteins when compared to the more substantial reduction produced by a single injection of siRNA targeting the pro-peptide for the neurotransmitter. A vector based RNAi could be even more effective; however, it has been reported that *in vivo* RNAi via viral vectors cause toxic effects in blood, liver, lung and brain (An et al, 2006; Chiosea et al, 2007; Grimm et al, 2006; McBride et al, 2008; Swoap et al, 2004). We also found a consistent reduction of OxR1 immunohistochemical staining in the LC following two OxR1-siRNA injections indicating that the knockdown of mRNA is translated into a comparable knockdown of the protein. Importantly, both mRNA and protein levels of TyH in the LC were not affected, demonstrated by our real-time PCR and immunohistochemistry results, validating the specificity of the knockdown. Furthermore, the reduction of OxR1 mRNA was reversed 4 days after OxR1-siRNA injection.

Effect of LC inactivation on REM sleep

A number of previous studies have investigated the effect of inactivation of the LC on REM sleep. Pharmacological manipulations of the LC provide generally supportive evidence of an inhibitory role in REM sleep regulation (McCarley, 2007). Cooling of the LC increased REM sleep (Cespuglio et al, 1982) whereas microinjection of the GABA_A receptor antagonist picrotoxin into the LC reduced REM sleep (Kaur et al, 1997). Similarly, microinjection or icv application of a somatostatin antagonist, which leads to disinhibition of LC neurons, decreases REM sleep in rats (Toppila et al, 2000).

In our study we chose to use siRNAs against the OxR1 to functionally inactivate the orexinergic input. Compared to traditional gene KO techniques, siRNA induced KD has the advantage of being reversible, with a duration usually starting one day after administration

and lasting for several days, providing a longer window to study the function of the targeted gene compared to pharmacological experiments (Dillon et al, 2005; Ryther et al, 2005). In addition, targeting specific siRNAs reduces the possibility of affecting neighboring cells, or other genes in the same cell (Hough et al, 2003; Miyagishi et al, 2003). Thus, siRNA induced KD provides a unique opportunity to specifically manipulate one segment of the sleep regulating circuits while minimizing the effects on other portions of the network. Our experiments are in line with the pharmacological experiments described above suggesting an inhibitory effect of LC neurons on REM sleep generation but our effect was more long-lasting, with the increase in REM sleep being present for 2 days following the last injection. This allowed us to determine that the suppressive action of LC is mainly if not exclusively during the active period and due to an increase in the number of REM episodes.

The Orexin system mediates circadian/diurnal control of LC activity and REM sleep

LC neurons are more active during the dark period than the light period in rats (Aston-Jones et al, 2001). The high level of LC firing during the active (dark) period, however, was attenuated by the OxR1 antagonist, SB-334867-A (Aston-Jones et al, 2001). Our finding with KD of OxR1 in the LC that the REM sleep increase only occurred during the dark period also suggests a circadian/diurnal role for the orexin system in the LC in REM sleep regulation. These findings are consistent with manipulations of the whole orexin system. Thus, increased REM sleep in the dark period was shown in studies of orexin or orexin receptor knockout mice (Chemelli et al, 1999; Mochizuki et al, 2004; Willie et al, 2003); rats and mice with ablation of orexin neurons through expression of a cytotoxic transgene, ataxin-3 (Beuckmann et al, 2004; Hara et al, 2001; Zhang et al, 2007); rats with chemical destruction of neurons containing orexin receptors in the hypothalamus (Gerashchenko et al, 2001; Gerashchenko et al, 2003); as well as rats with prepro-orexin expression knockdown (Chen et al, 2006). Our current finding suggests that this increase in REM sleep is, at least partially, mediated via the OxR1 in the LC.

What is the mechanism by which circadian/diurnal control of LC neurons is achieved? Orexin neurons receive both direct and indirect projections from the suprachiasmatic nucleus (SCN) (Abrahamson et al, 2001; Yoshida et al, 2006). There is anatomical evidence of an indirect SCN projection to the LC via a relay from the dorsal medial hypothalamus (DMH), which is enriched with orexin neurons (Aston-Jones et al, 2001; Deurveilher and Semba, 2005; Gompf and Aston-Jones, 2008). The increased firing of LC neurons in the active (dark) period was abolished with bilateral lesion of the DMH (Aston-Jones et al, 2001). Furthermore, LC neurons started to increase their firing right before dark onset and this increase was correlated with increased c-Fos expression in orexin neurons (Gompf and Aston-Jones, 2008). Finally, ablation of LC neurons by DSP-4 (Gonzalez and Aston-Jones, 2006), or by anti-dopamine- β -hydroxylase conjugated saporin, a ribosomal inactivating protein (Blanco-Centurion et al, 2004), blunted the circadian amplitude of the sleep wake cycle.

Cataplexy, a sudden loss of muscle tone resembling the muscle atonia seen during REM sleep, is a prominent feature in many cases of narcolepsy associated with loss of orexins. During canine cataplexy, it has been shown that neurons in the locus coeruleus cease activity (Wu et al, 1999). However, in our study cataplectic attacks were not observed, suggesting that reduced orexin 1 mediated excitation of LC neurons alone is not sufficient to trigger this symptom.

In conclusion, our experiments are consistent with the view that SCN signals, relayed via orexin neurons and acting on OxR1 in the LC, control the circadian distribution of REM sleep. Our data that the increased REM sleep occurrence during the dark period, especially during the second night, was due to more frequent occurrence of REM episodes, but not to

altered REM duration or REM-REM interval length further support the model that the LC gates the probability of the REM sleep oscillator being turned on without modifying the fundamental properties of the oscillator, as previously proposed (Massaquoi and McCarley, 1992).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by VA (Merit Award to RWM) and NIMH grants 062522, 39683, 01798, NS059831 (MMT). The authors would like to thank Mike Leonard and Courtney Earle for their technical assistance.

Abbreviations

LC	locus coeruleus
DBH	dopamine β -hydroxylase
DMH	dorsal medial hypothalamus
DSP-4	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
KD	knockdown
KO	knockout
NE	norepinephrine
PFH	perifornical region of the hypothalamus
OxR1	orexin type 1 receptor
REM	rapid eye movement
R-R cycle	REM sleep to REM sleep cycle duration
RNAi	RNA interference
SCN	suprachiasmatic nucleus
siRNA	small interfering RNAs
TyH	tyrosine hydroxylase

Reference List

- Abrahamson EE, Leak RK, Moore RY. The suprachiasmatic nucleus projects to posterior hypothalamic arousal systems. *Neuroreport*. 2001; 12:435–440. [PubMed: 11209963]
- An DS, Qin FX, Auyeung VC, Mao SH, Kung SK, Baltimore D, Chen IS. Optimization and functional effects of stable short hairpin RNA expression in primary human lymphocytes via lentiviral vectors. *Mol Ther*. 2006; 14:494–504. [PubMed: 16844419]
- Aston-Jones G, Bloom FE. Activity of norepinephrine-containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle. *J Neurosci*. 1981; 1:876–886. [PubMed: 7346592]
- Aston-Jones G, Chen S, Zhu Y, Oshinsky ML. A neural circuit for circadian regulation of arousal. *Nat Neurosci*. 2001; 4:732–738. [PubMed: 11426230]
- Beiras-Fernandez A, Gallego R, Blanco M, Garcia-Caballero T, Dieguez C, Beiras A. Merkel cells, a new localization of prepro-orexin and orexin receptors. *J Anat*. 2004; 204:117–122. [PubMed: 15032918]

- Bengtsson MW, Makela K, Sjoblom M, Uotila S, Akerman KE, Herzig KH, Flemstrom G. Food-induced expression of orexin receptors in rat duodenal mucosa regulates the bicarbonate secretory response to orexin-A. *Am J Physiol Gastrointest Liver Physiol*. 2007; 293:G501–G509. [PubMed: 17585016]
- Berridge CW. Noradrenergic modulation of arousal. *Brain Res Rev*. 2008; 58:1–17. [PubMed: 18199483]
- Beuckmann CT, Sinton CM, Williams SC, Richardson JA, Hammer RE, Sakurai T, Yanagisawa M. Expression of a poly-glutamine-ataxin-3 transgene in orexin neurons induces narcolepsy-cataplexy in the rat. *J Neurosci*. 2004; 24:4469–4477. [PubMed: 15128861]
- Blanco-Centurion C, Gerashchenko D, Salin-Pascual RJ, Shiromani PJ. Effects of hypocretin2-saporin and antidopamine-beta-hydroxylase-saporin neurotoxic lesions of the dorsolateral pons on sleep and muscle tone. *Eur J Neurosci*. 2004; 19:2741–2752. [PubMed: 15147308]
- Bourgin P, Huitron-Resendiz S, Spier AD, Fabre V, Morte B, Criado JR, Sutcliffe JG, Henriksen SJ, de LL. Hypocretin-1 modulates rapid eye movement sleep through activation of locus coeruleus neurons. *J Neurosci*. 2000; 20:7760–7765. [PubMed: 11027239]
- Cespuglio R, Gomez ME, Faradji H, Jouvet M. Alterations in the sleep-waking cycle induced by cooling of the locus coeruleus area. *Electroencephalogr Clin Neurophysiol*. 1982; 54:570–578. [PubMed: 6181980]
- Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, Richardson JA, Williams SC, Xiong Y, Kisanuki Y, Fitch TE, Nakazato M, Hammer RE, Saper CB, Yanagisawa M. Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell*. 1999; 98:437–451. [PubMed: 10481909]
- Chen L, Thakkar MM, Winston S, Bolortuya Y, Basheer R, McCarley RW. REM sleep changes in rats induced by siRNA-mediated orexin knockdown. *Eur J Neurosci*. 2006; 24:2039–2048. [PubMed: 17067300]
- Chiosea S, Jelezcova E, Chandran U, Luo J, Mantha G, Sobol RW, Dacic S. Overexpression of Dicer in precursor lesions of lung adenocarcinoma. *Cancer Res*. 2007; 67:2345–2350. [PubMed: 17332367]
- Date Y, Ueta Y, Yamashita H, Yamaguchi H, Matsukura S, Kangawa K, Sakurai T, Yanagisawa M, Nakazato M. Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine and neuroregulatory systems. *Proc Natl Acad Sci US A*. 1999; 96:748–753.
- De Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FS, Frankel WN, van den Pol AN, Bloom FE, Gautvik KM, Sutcliffe JG. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci USA*. 1998; 95:322–327. [PubMed: 9419374]
- Deurveilher S, Semba K. Indirect projections from the suprachiasmatic nucleus to major arousal-promoting cell groups in rat: implications for the circadian control of behavioural state. *Neuroscience*. 2005; 130:165–183. [PubMed: 15561433]
- Dillon CP, Sandy P, Nencioni A, Kissler S, Robinson DA, Van Parijs L. Rnai as an experimental and therapeutic tool to study and regulate physiological and disease processes. *Annu Rev Physiol*. 2005; 67:147–173. [PubMed: 15709955]
- Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells 609. *Nature*. 2001; 411:494–498. [PubMed: 11373684]
- Espana RA, Valentino RJ, Berridge CW. Fos immunoreactivity in hypocretin-synthesizing and hypocretin-1 receptor-expressing neurons: effects of diurnal and nocturnal spontaneous waking, stress and hypocretin-1 administration. *Neuroscience*. 2003; 121:201–217. [PubMed: 12946712]
- Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*. 1998; 391:806–811. [PubMed: 9486653]
- Footo SL, Aston-Jones G, Bloom FE. Impulse activity of locus coeruleus neurons in awake rats and monkeys is a function of sensory stimulation and arousal. *Proc Natl Acad Sci US A*. 1980; 77:3033–3037.

- Gerashchenko D, Blanco-Centurion C, Greco MA, Shiromani PJ. Effects of lateral hypothalamic lesion with the neurotoxin hypocretin-2-saporin on sleep in Long-Evans rats. *Neuroscience*. 2003; 116:223–235. [PubMed: 12535955]
- Gerashchenko D, Kohls MD, Greco M, Waleh NS, Salin-Pascual R, Kilduff TS, Lappi DA, Shiromani PJ. Hypocretin-2-saporin lesions of the lateral hypothalamus produce narcoleptic-like sleep behavior in the rat. *J Neurosci*. 2001; 21:7273–7283. [PubMed: 11549737]
- Gompf HS, Aston-Jones G. Role of orexin input in the diurnal rhythm of locus coeruleus impulse activity. *Brain Res*. 2008; 1224:43–52. [PubMed: 18614159]
- Gonzalez MM, Aston-Jones G. Circadian regulation of arousal: role of the noradrenergic locus coeruleus system and light exposure. *Sleep*. 2006; 29:1327–1336. [PubMed: 17068987]
- Gottesmann C. Noradrenaline involvement in basic and higher integrated REM sleep processes. *Prog Neurobiol*. 2008; 85:237–272. [PubMed: 18514380]
- Greco MA, Shiromani PJ. Hypocretin receptor protein and mRNA expression in the dorsolateral pons of rats. *Brain Res Mol Brain Res*. 2001; 88:176–182. [PubMed: 11295245]
- Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, Davis CR, Marion P, Salazar F, Kay MA. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature*. 2006; 441:537–541. [PubMed: 16724069]
- Hagan JJ, Leslie RA, Patel S, Evans ML, Wattam TA, Holmes S, Benham CD, Taylor SG, Routledge C, Hemmati P, Munton RP, Ashmeade TE, Shah AS, Hatcher JP, Hatcher PD, Jones DN, Smith MI, Piper DC, Hunter AJ, Porter RA, Upton N. Orexin A activates locus coeruleus cell firing and increases arousal in the rat. *Proc Natl Acad Sci US A*. 1999; 96:10911–10916.
- Hamlin AS, Clemens KJ, McNally GP. Renewal of extinguished cocaine-seeking. *Neuroscience*. 2008; 151:659–670. [PubMed: 18164822]
- Hara J, Beuckmann CT, Nambu T, Willie JT, Chemelli RM, Sinton CM, Sugiyama F, Yagami K, Goto K, Yanagisawa M, Sakurai T. Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. *Neuron*. 2001; 30:345–354. [PubMed: 11394998]
- Hervieu GJ, Cluderay JE, Harrison DC, Roberts JC, Leslie RA. Gene expression and protein distribution of the orexin-1 receptor in the rat brain and spinal cord. *Neuroscience*. 2001; 103:777–797. [PubMed: 11274794]
- Hobson JA, McCarley RW, Wyzinski PW. Sleep cycle oscillation: reciprocal discharge by two brainstem neuronal groups. *Science*. 1975; 189:55–58. [PubMed: 1094539]
- Horvath TL, Diano S, van den Pol AN. Synaptic interaction between hypocretin (orexin) and neuropeptide Y cells in the rodent and primate hypothalamus: a novel circuit implicated in metabolic and endocrine regulations. *J Neurosci*. 1999; 19:1072–1087. [PubMed: 9920670]
- Hough SR, Wiederholt KA, Burrier AC, Woolf TM, Taylor MF. Why RNAi makes sense. *Nat Biotechnol*. 2003; 21:731–732. [PubMed: 12833082]
- Johnson PL, Truitt W, Fitz SD, Minick PE, Dietrich A, Sanghani S, Traskman-Bendz L, Goddard AW, Brundin L, Shekhar A. A key role for orexin in panic anxiety. *Nat Med*. 2010; 16:111–115. [PubMed: 20037593]
- Kauffling J, Veinante P, Pawlowski SA, Freund-Mercier MJ, Barrot M. Afferents to the GABAergic tail of the ventral tegmental area in the rat. *J Comp Neurol*. 2009; 513:597–621. [PubMed: 19235223]
- Kaur S, Saxena RN, Mallick BN. GABA in locus coeruleus regulates spontaneous rapid eye movement sleep by acting on GABAA receptors in freely moving rats. *Neurosci Lett*. 1997; 223:105–108. [PubMed: 9089684]
- Lee MG, Hassani OK, Jones BE. Discharge of identified orexin/hypocretin neurons across the sleep-waking cycle. *J Neurosci*. 2005; 25:6716–6720. [PubMed: 16014733]
- Lehr HA, Mankoff DA, Corwin D, Santeusano G, Gown AM. Application of photoshop-based image analysis to quantification of hormone receptor expression in breast cancer. *J Histochem Cytochem*. 1997; 45:1559–1565. [PubMed: 9358857]
- Lehr HA, van der Loos CM, Teeling P, Gown AM. Complete chromogen separation and analysis in double immunohistochemical stains using Photoshop-based image analysis. *J Histochem Cytochem*. 1999; 47:119–126. [PubMed: 9857219]

- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
- Magga J, Bart G, Oker-Blom C, Kukkonen JP, Akerman KE, Nasman J. Agonist potency differentiates G protein activation and Ca²⁺ signalling by the orexin receptor type 1. *Biochem Pharmacol*. 2006; 71:827–836. [PubMed: 16430869]
- Marchant NJ, Densmore VS, Osborne PB. Coexpression of prodynorphin and corticotrophin-releasing hormone in the rat central amygdala: evidence of two distinct endogenous opioid systems in the lateral division. *J Comp Neurol*. 2007; 504:702–715. [PubMed: 17722034]
- Marcus JN, Aschkenasi CJ, Lee CE, Chemelli RM, Saper CB, Yanagisawa M, Elmquist JK. Differential expression of orexin receptors 1 and 2 in the rat brain. *J Comp Neurol*. 2001; 435:6–25. [PubMed: 11370008]
- Massaquoi SG, McCarley RW. Extension of the Limit Cycle Reciprocal Interaction Model of REM cycle control. An integrated sleep control model. *J Sleep Res*. 1992; 1:138–143. [PubMed: 10607042]
- McBride JL, Boudreau RL, Harper SQ, Staber PD, Monteys AM, Martins I, Gilmore BL, Burstein H, Peluso RW, Polisky B, Carter BJ, Davidson BL. Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: implications for the therapeutic development of RNAi. *Proc Natl Acad Sci US A*. 2008; 105:5868–5873.
- McCarley RW. Mechanisms and models of REM sleep control. *Arch Ital Biol*. 2004; 142:429–467. [PubMed: 15493547]
- McCarley RW. Neurobiology of REM and NREM sleep. *Sleep Med*. 2007; 8:302–330. [PubMed: 17468046]
- Mignot E. A commentary on the neurobiology of the hypocretin/orexin system. *Neuropsychopharmacology*. 2001; 25:S5–13. [PubMed: 11682267]
- Milevskiy BY, Kiyashchenko LI, Siegel JM. Behavioral correlates of activity in identified hypocretin/orexin neurons. *Neuron*. 2005; 46:787–798. [PubMed: 15924864]
- Mintz EM, van den Pol AN, Casano AA, Albers HE. Distribution of hypocretin-(orexin) immunoreactivity in the central nervous system of Syrian hamsters (*Mesocricetus auratus*). *J Chem Neuroanat*. 2001; 21:225–238. [PubMed: 11382534]
- Miyagishi M, Hayashi M, Taira K. Comparison of the suppressive effects of antisense oligonucleotides and siRNAs directed against the same targets in mammalian cells. *Antisense Nucleic Acid Drug Dev*. 2003; 13:1–7. [PubMed: 12691531]
- Mochizuki T, Crocker A, McCormack S, Yanagisawa M, Sakurai T, Scammell TE. Behavioral state instability in orexin knock-out mice. *J Neurosci*. 2004; 24:6291–6300. [PubMed: 15254084]
- Mofidi R, Walsh R, Ridgway PF, Crotty T, McDermott EW, Keaveny TV, Duffy MJ, Hill AD, O'Higgins N. Objective measurement of breast cancer oestrogen receptor status through digital image analysis. *Eur J Surg Oncol*. 2003; 29:20–24. [PubMed: 12559071]
- Novak CM, Albers HE. Localization of hypocretin-like immunoreactivity in the brain of the diurnal rodent, *Arvicanthis niloticus*. *J Chem Neuroanat*. 2002; 23:49–58. [PubMed: 11756009]
- Pal D, Mallick BN. Neural mechanism of rapid eye movement sleep generation with reference to REM-OFF neurons in locus coeruleus. *Indian J Med Res*. 2007; 125:721–739. [PubMed: 17704548]
- Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. Academic Press; New York: 2000.
- Peyron C, Tighe DK, van den Pol AN, de LL, Heller HC, Sutcliffe JG, Kilduff TS. Neurons containing hypocretin (orexin) project to multiple neuronal systems. *J Neurosci*. 1998; 18:9996–10015. [PubMed: 9822755]
- Piper DC, Upton N, Smith MI, Hunter AJ. The novel brain neuropeptide, orexin-A, modulates the sleep-wake cycle of rats. *Eur J Neurosci*. 2000; 12:726–730. [PubMed: 10712652]
- Ramer MS. Anatomical and functional characterization of neuropil in the gracile fasciculus. *J Comp Neurol*. 2008; 510:283–296. [PubMed: 18634004]
- Rasmussen K, Morilak DA, Jacobs BL. Single unit activity of locus coeruleus neurons in the freely moving cat. I. During naturalistic behaviors and in response to simple and complex stimuli. *Brain Res*. 1986; 371:324–334. [PubMed: 3697761]

- Ryther RC, Flynt AS, Phillips JA III, Patton JG. siRNA therapeutics: big potential from small RNAs. *Gene Ther.* 2005; 12:5–11. [PubMed: 15496962]
- Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell.* 1998; 92:573–585. [PubMed: 9491897]
- Shouse MN, Staba RJ, Saquib SF, Farber PR. Monoamines and sleep: microdialysis findings in pons and amygdala. *Brain Res.* 2000; 860:181–189. [PubMed: 10727641]
- Smith MI, Piper DC, Duxon MS, Upton N. Evidence implicating a role for orexin-1 receptor modulation of paradoxical sleep in the rat. *Neurosci Lett.* 2003; 341:256–258. [PubMed: 12697296]
- Suzuki R, Shimojima H, Funahashi H, Nakajo S, Yamada S, Guan JL, Tsurugano S, Uehara K, Takeyama Y, Kikuyama S, Shioda S. Orexin-1 receptor immunoreactivity in chemically identified target neurons in the rat hypothalamus. *Neurosci Lett.* 2002; 324:5–8. [PubMed: 11983281]
- Swoap SJ, Weinshenker D, Palmiter RD, Garber G. Dbh(–/–) mice are hypotensive, have altered circadian rhythms, and have abnormal responses to dieting and stress. *Am J Physiol Regul Integr Comp Physiol.* 2004; 286:R108–R113. [PubMed: 12969876]
- Taishi P, Churchill L, Wang M, Kay D, Davis CJ, Guan X, De A, Yasuda T, Liao F, Krueger JM. TNFalpha siRNA reduces brain TNF and EEG delta wave activity in rats. *Brain Res.* 2007; 1156:125–132. [PubMed: 17531209]
- Takahashi K, Lin JS, Sakai K. Neuronal activity of orexin and non-orexin waking-active neurons during wake-sleep states in the mouse. *Neuroscience.* 2008; 153:860–870. [PubMed: 18424001]
- Toppila J, Niittymäki P, Porkka-Heiskanen T, Stenberg D. Intracerebroventricular and locus coeruleus microinjections of somatostatin antagonist decrease REM sleep in rats. *Pharmacol Biochem Behav.* 2000; 66:721–727. [PubMed: 10973509]
- Trivedi P, Yu H, MacNeil DJ, Van der Ploeg LH, Guan XM. Distribution of orexin receptor mRNA in the rat brain. *FEBS Lett.* 1998; 438:71–75. [PubMed: 9821961]
- Vilaplana J, Lavialle M. A method to quantify glial fibrillary acidic protein immunoreactivity on the suprachiasmatic nucleus. *J Neurosci Methods.* 1999; 88:181–187. [PubMed: 10389664]
- Willie JT, Chemelli RM, Sinton CM, Tokita S, Williams SC, Kisanuki YY, Marcus JN, Lee C, Elmquist JK, Kohlmeier KA, Leonard CS, Richardson JA, Hammer RE, Yanagisawa M. Distinct narcolepsy syndromes in Orexin receptor-2 and Orexin null mice: molecular genetic dissection of Non-REM and REM sleep regulatory processes. *Neuron.* 2003; 38:715–730. [PubMed: 12797957]
- Wu MF, Gulyani SA, Yau E, Mignot E, Phan B, Siegel JM. Locus coeruleus neurons: cessation of activity during cataplexy. *Neuroscience.* 1999; 91:1389–1399. [PubMed: 10391445]
- Yoshida K, McCormack S, Espana RA, Crocker A, Scammell TE. Afferents to the orexin neurons of the rat brain. *J Comp Neurol.* 2006; 494:845–861. [PubMed: 16374809]
- Zhang JH, Sampogna S, Morales FR, Chase MH. Distribution of hypocretin (orexin) immunoreactivity in the feline pons and medulla. *Brain Res.* 2004; 995:205–217. [PubMed: 14672810]
- Zhang S, Lin L, Kaur S, Thankachan S, Blanco-Centurion C, Yanagisawa M, Mignot E, Shiromani PJ. The development of hypocretin (orexin) deficiency in hypocretin/ataxin-3 transgenic rats. *Neuroscience.* 2007; 148:34–43. [PubMed: 17618058]

Figure 1.

REM

NREM

Figure 2.

Figure 3.

Figure 4.

Table 1

Sequences for OxR1-siRNAs and ctrl-siRNAs

	OxR1-siRNA	control siRNA
Sense	5'- GCGAUUAUCUCUAUCCGAAtt - 3'	5'- UUCCGAACUGUGCAACCGAtt - 3'
Antisense	5'- UUCGGAUAGAGAUAAUCGCgc - 3'	5'- UCGGUUGCACAGUUCGGAAtg - 3'
Sense	5'- UCAAUGUCCUAAAAAGAGUtt - 3'	5'- CUCUAGUUGC UAAUCCGGCtt - 3'
Antisense	5'- ACUCUUUUAAGGACAUUGAgg - 3'	5'- GCCGGAUUAGCAACUAGAGtg - 3'
Sense	5'- GCAAAUUUCGGGAGCAGUtt - 3'	5'- CUCAUAUGACCUCCUUGUGtt - 3'
Antisense	5'- AACUGCUC CCGAAA UUUGCca - 3'	5'- CACAAGGAGGUCAUAUGAGtc - 3'

The overhangs at the 3' ends are in lower case.

Table 2

Sleep % in OxR1-siRNA and ctrl-siRNA treated rats

Period (12 hr)	OxR1-siRNA (N=7)				Ctrl-siRNA (N=6)			
	Mean (± SEM) % time spent in				Mean (± SEM) % time spent in			
	Wakefulness	NREMS	REMS		Wakefulness	NREMS	REMS	
Night 0	72.2±1.5	23.9±1.8	3.9±0.4		73.2±2.3	22.5±1.7	4.3±0.7	
Inj-Night 1	68.6±1.5	25.5±1.5	5.8±0.6		66.0±3.2	27.6±3.0	6.5±0.5	
Inj-Night 2	67.6±1.9	26.9±1.7	5.4±0.5		67.8±4.0	25.4±2.7	6.8±1.5	
Night 1	71.2±2.1	23.9±1.8	4.9±0.8*		69.3±0.6	26.6±0.7	4.1±0.4	
Night 2	68.9±2.2	25.9±2.0	5.2±0.6*		69.5±2.3	25.8±1.5	4.7±1.4	
Night 3	71.6±2.1	24.0±1.9	4.4±0.7					
Day 0	31.7±2.1	58.6±2.1	9.7±0.6		26.7±1.1	63.5±1.1	9.8±1.1	
Inj-Day 1	29.6±1.9	61.1±1.8	9.3±0.9		30.6±1.8	60.2±1.8	9.2±0.9	
Inj-Day 2	32.5±2.6	58.3±2.0	9.2±0.7		34.0±2.6	57.6±2.5	8.4±1.1	
Day 1	27.0±1.9	63.7±2.2	9.2±0.9		29.0±1.6	60.9±1.2	10.1±1.3	
Day 2	30.5±2.7	59.4±3.0	10.1±1.2		28.9±1.9	59.9±2.5	11.2±0.1	
Day 3	28.9±1.7	59.1±2.7	12.0±1.5					

Percentage of REM sleep increased on nights 1 and 2 in animals treated with OxR1 siRNA. Night= 7 PM to 7 AM; Day = 7 AM to 7 PM; Night 0 or Day 0 = Pre-injection day or night. Inj-Night 1-2 = Injection Nights; Inj-Day 1-2 = Injection Days. Nights 1-3 = Post-injection nights; Day 1-3 = post-injection days;

* $P < 0.05$ versus Night 0 (post hoc Bonferroni's test).

Table 3

REM sleep parameters during the night in OxR1-siRNA injected animals.

siRNA	Period	REM sleep			
		Number	Duration	Latency	R-R Cycle
OxR1	Night 0	18.9±2.0	1.4±0.08	5.0±0.7	38.8±6.8
	Night 1	21.0±2.9	1.7±0.15	4.3±0.2	31.6±4.7
	Night 2	25.1±2.8*	1.5±0.08	4.1±0.2	28.2±3.4

Compared to Night 0, the number of REM sleep episodes increased during Night 2. Night= 7 PM to 7 AM; Night 0 = Pre-injection night. Nights 1–2 = Post-injection nights; Duration and latency and REMS-REMS cycle duration (R-R cycle) are all expressed as minutes. REM sleep latency is defined as the time between the beginning of a REM sleep episode and the preceding wakefulness episode. R-R cycle is defined as from the beginning of a REM sleep episode to the beginning of the next REM sleep episode. All data are mean ± SEM.

* $P<0.05$ versus Night 0 (post hoc Bonferroni's test).