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REM sleep changes in rats induced by siRNA-mediated orexin knockdown

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Abstract

Short interfering RNAs (siRNA) targeting prepro-orexin mRNA were microinjected into the rat perifornical hypothalamus. Prepro-orexin siRNA-treated rats had a significant (59%) reduction in prepro-orexin mRNA compared to scrambled siRNA-treated rats 2 days postinjection, whereas prodynorphin mRNA was unaffected. The number of orexin-A-positive neurons on the siRNA-treated side decreased significantly (23%) as compared to the contralateral control (scrambled siRNA-treated) side. Neither the colocalized dynorphin nor the neighbouring melanin-concentrating hormone neurons were affected. The number of orexin-A-positive neurons on the siRNA-treated side did not differ from the number on the control side 4 or 6 days postinjection. Behaviourally, there was a persistent (~ 60%) increase in the amount of time spent in rapid eye movement (REM) sleep during the dark (active) period for 4 nights postinjection, in rats treated with prepro-orexin siRNA bilaterally. This increase occurred mainly because of an increased number of REM episodes and decrease in REM-to-REM interval. Cataplexy-like episodes were also observed in some of these animals. Wakefulness and NREM sleep were unaffected. The siRNA-induced increase in REM sleep during the dark cycle reverted to control values on the 5th day postinjection. In contrast, the scrambled siRNA-treated animals only had a transient increase in REM sleep for the first postinjection night. Our results indicate that siRNA can be usefully employed in behavioural studies to complement other loss-of-function approaches. Moreover, these data suggest that the orexin system plays a role in the diurnal gating of REM sleep.

Keywords

diurnal consolidation; hypocretin; perifornical hypothalamus; RNA interference; sleep and wakefulness

Introduction

Orexins (also known as hypocretins; de Lecea *et al.*, 1998; Sakurai *et al.*, 1998) are peptides that are produced mainly in the perifornical region of the hypothalamus (PFH). Two isoforms of orexins (orexin-A and -B) are derived from proteolytic cleavage of a precursor peptide (prepro-orexin) and they act on two G-protein-coupled receptors.

Although orexins are involved in multiple functions, the most studied one is their role in the regulation of sleep–wakefulness and in the aetiology of sleep-related disorders, especially narcolepsy (Taheri *et al.*, 2002). Increased rapid eye movement (REM) sleep in the dark period and cataplexy is generally observed in orexin- or orexin receptor-knockout mice, as well as in mice or rats in which a truncated Machado–Joseph disease gene product, ataxin-3, has been used to destroy the orexin neurons (Chemelli *et al.*, 1999; Hara *et al.*, 2001; Willie *et al.*, 2003; Beuckmann *et al.*, 2004). Chemical destruction of neurons containing orexin receptors in the PFH had similar effects (Gerashchenko *et al.*, 2001). Although these experiments provide much insight into the roles of orexins in sleep, they also have potential confounding factors. Developmental compensation is always a concern in constitutive gene-knockout studies. Chemical lesions destroy other cells, including melanin-concentrating hormone (MCH) neurons, which also participate in REM sleep regulation (Verret *et al.*, 2003). While ataxin-3-induced cell death is highly specific, it destroys other neuromodulators present in orexinergic neurons such as dynorphin (Chou *et al.*, 2001), which works with orexins to indirectly excite the histaminergic system (Eriksson *et al.*, 2004).

RNA-mediated interference is a sequence-specific gene suppression induced by double-stranded RNA (Fire *et al.*, 1998). Small double-stranded RNAs having 21–23 nucleotides with a symmetric overhang of two or three nucleotides are capable of inducing RNA interference. These double-stranded RNAs are often termed short interfering RNAs (siRNAs; Elbashir *et al.*, 2001).

The finding that siRNA mediates RNA interference forms the basis of a powerful new technique to complement the traditional arsenal of loss-of-function techniques. siRNA avoids the laborious creation and maintenance of genetically modified animals and the potential pitfall of developmental compensation in constitutive knockout animals. Unlike inducible knockouts, siRNA effects are reversible, with a duration usually starting 1 day after administration and lasting for several days, providing a good window for studying the function of the targeted gene (Dillon *et al.*, 2004; Ryther *et al.*, 2005). Unlike traditional lesion techniques, siRNA does not affect neighbouring cells and, unlike techniques causing cellular degeneration, such as ataxin-3, siRNA is more specific, not affecting all genes. Finally, compared with antisense, a technology we have previously used, siRNA appears to be more effective and specific and does not require potentially toxic chemical modifications, and its effects last longer (Hough *et al.*, 2003; Miyagishi *et al.*, 2003).

Although widely used *in vitro*, siRNA applications in *in vivo* mammalian brain are relatively rare and, to our knowledge, the use of siRNA is novel in sleep research and in studies of the orexin system. In this study, we examined the effect of siRNA targeting the prepro-orexin gene on orexin-A immunoreactivity and sleep–wakefulness in rats.

Methods

Experimental design

Our first two sets of experiments were designed to verify the specificity of prepro-orexin siRNA treatment and to examine the degree of orexin peptide and mRNA knockdown, respectively, following prepro-orexin siRNA administration. Our third experiment was designed to evaluate the effect of prepro-orexin siRNA treatment on the behavioural states of the animals.

Animals

Adult Sprague–Dawley male rats (300–350 g; Charles River Laboratories, Wilmington, MA, USA) were housed under 12 : 12 h light : dark cycle (lights on at 07.00, off at 19.00 h) 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 minimise 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.

For experiments 1 and 3, rats were anesthetized with an i.p. cocktail of ketamine (90 mg/kg) and xylazine (10 mg/kg). They were then killed with an overdose of pentobarbital (100mg/kg) followed by transcardiac perfusion. For experiment 2, rats were anesthetized with isoflurane and decapitated for tissue collection.

siRNA

A pool of three siRNAs (equal concentration) was used (Table 1). All siRNAs were designed individually to target prepro-orexin (prepro-orexin siRNA). A corresponding control pool of three siRNAs with scrambled sequences and no homology to known rat genes (scrambled siRNA) was used. All siRNAs were annealed and HPLC-purified (Ambion, Austin, TX, USA). A pool of siRNAs was used because it largely circumvents the task of testing each siRNA individually, reduces possible nonspecific effects (Dharmacon Co., private communication) and may increase target knockdown (Akaneya *et al.*, 2005).

Experiment 1

We first verified the knockdown of prepro-orexin peptide by performing orexin-A immunohistochemistry. Next, we verified the specificity of prepro-orexin siRNA effect by double-labelling of orexin-A and MCH (localized to the same region, but not colocalized) and orexin-A and dynorphin B (colocalized in orexin neurons). Finally, we quantified the degree of orexin peptide knockdown by counting orexin-A-positive neurons.

Methodological considerations—One of the major limitations of using immunohistochemical techniques to count the number of orexin-positive neurons from sampled sections is the potential variability due to differences in the site of injection, differences in the number of orexin-positive neurons in different sections and differences in the immunohistochemical staining. Therefore, for immunohistochemistry, we performed both the siRNA and the scrambled treatment in the same animal on homotopic contralateral PFH sites. In each animal, we counted orexin neurons in two sections both from the siRNA-treated ipsilateral side and the scrambled siRNA-treated contralateral side and compared them with each other. We believe that this experimental design controls for any variability in the number of orexin neurons between animals in sampled sections.

Surgery and siRNA injections—Under general anaesthesia, prepro-orexin siRNAs were unilaterally injected (0.03 nmol in 0.3 μ L water) into one side of the orexinergic PFH using a Hamilton syringe. Scrambled siRNA was injected on the contralateral side. Coordinates (Paxinos & Watson, 1998) for the PFH target site were: AP, -3.3; ML, 1.5; and DV, 8.3 mm. Based on our initial, preliminary dose-finding immunohistochemistry and behavioural state data, the maximal prepro-orexin siRNA effect was found to be on day 2 postinjection but returned to normal values on day 5 postinjection. Therefore, we killed the animals at three different time points, namely days 2, 4 and 6, and verified the effect and specificity of prepro-orexin siRNA. For histology, 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 then transferred to 20% sucrose for cryoprotection, and coronal sections (30 or 40 μ m) were cut on a freezing microtome.

Orexin-A immunohistochemistry—Orexin-A immunohistochemistry was performed in four animals, killed 2 days postinjection. One-third of the sections were used for orexin immunohistochemistry and another one-third for orexin-A and MCH double labelling. Free-floating sections were successively incubated in 0.3% Triton X-100 (2 h), 3% normal donkey serum in PBS (1 h), and overnight at 4 °C with rabbit antiorexin-A primary IgG antibody (1 : 2000; Peninsula Laboratories, San Carlos, CA, USA) and 0.3% Triton X-100. After incubation in secondary donkey antirabbit IgG antibody (1 : 400; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h, sections were stained using the Vectastain ABC kit and the DAB substrate kit (Vector Laboratories, Burlingame, CA, USA) following the manufacturer's instructions.

Immunofluorescence double-labelling of orexin-A and MCH—Sections were incubated in 0.25% Triton X-100 and 5% normal donkey serum in PBS for 1 h followed by streptavidin/biotin blocking 15 min each (Streptavidin/Biotin Blocking Kit (Vector)). A mixture of rabbit antiorexin-A IgG antibody (1 : 300, Peninsula Laboratories, San Carlos, CA, USA) and chicken anti-MCH IgY antibody (1 : 300, Chemicon International, Temecula, CA, USA) were used to incubate sections overnight. The sections were then incubated with biotinylated donkey antirabbit IgG antibody (1 : 300, Jackson ImmunoResearch) for 1 h, Cy3 conjugated streptavidin (1 : 3000, Jackson ImmunoResearch) for 30 min and FITC conjugated goat antichickens IgY (1 : 300, Jackson ImmunoResearch) antibody for 1 h. Sections were mounted, dried, and coverslipped with Vectorshield (Vector). Orexinergic neurons were identified with Cy3 (red fluorescence) labelling whereas MCH neurons were identified with FITC (green fluorescence) labelling.

Immunofluorescence double-labelling of orexin-A and dynorphin B—In a separate group of animals ($n = 9$), we performed orexin-A and dynorphin B double labelling on day 2, day 4 and day 6 postinjection. The protocol for double labelling of orexin-A and dynorphin B was similar to the protocol described above except that monoclonal mouse antiorexin-A IgG antibody (1 : 300; R & D systems, Minneapolis, MN, USA) and rabbit antidynorphin B IgG antibody (1 : 2400; EMD Biosciences, San Diego, CA, USA) were used as primary antibodies, and biotinylated donkey antirabbit IgG antibody (1 : 300; Jackson ImmunoResearch) and donkey antimouse IgG antibody conjugated to FITC (1 : 300; Jackson ImmunoResearch) were used as secondary antibodies. The specificity of the rabbit antidynorphin B IgG antibody had been verified by preabsorption assay (EMD Biosciences, personal communication) and has been used previously (Shirayama *et al.*, 2004). Dynorphin B-immunoreactive neurons were identified with Cy3 (red fluorescence) labelling whereas orexin-A-containing neurons were identified with FITC (green fluorescence) labelling.

Microscopy and quantification of orexin-A and dynorphin B—Fluorescence microscopy was carried out with an Axioplan 2 microscope and Slidebook software (Slidebook 4.1; Intelligent Imaging Innovations, Denver, CO, USA). In each animal, every third section was double-labelled for orexin-A and dynorphin B; of these, the two sections with the highest quantity of orexin-A immunoreactivity were selected for bilateral counting. We used the profile of the neuronal cell body and the presence of the fluorescence in the cytoplasm to count neurons and counted all the orexin-A- and dynorphin B-positive neurons in the selected sections (Thakkar *et al.*, 2002).

Experiment 2

We next verified the effectiveness and selectivity of siRNA-induced knockdown of the prepro-orexin message by performing real-time PCR for prepro-orexin and dynorphin.

Methodological considerations—Although immunohistochemical detection of remaining protein expression is currently widely used as an effective assay in gene-knockdown experiments (Turchi & Sarter, 2001; Thakkar *et al.*, 2003; Bhargava *et al.*, 2004) as well as in ataxin-3-induced degeneration (Beuckmann *et al.*, 2004), the immunological technique does not allow the quantification of the remaining protein expression even with the use of stereology. For example, a neuron with partial reduction in prepro-orexin peptide would still be detected and counted as an orexin-A-immunoreactive neuron although the intensity might vary. To overcome these limitations, we used real-time PCR to confirm and quantify the effectiveness of siRNA-induced down-regulation of the prepro-orexin message.

Surgery and siRNA injections—Under general anaesthesia, in a group of nine rats prepro-orexin siRNAs were bilaterally injected (0.03 nmol in 0.3 μ L water) into the orexinergic PFH. A separate group of nine rats were bilaterally injected with scrambled siRNA into the PFH. Forty-eight hours after treatment the animals were killed by decapitation using a guillotine. The hypothalamus was quickly dissected out and placed on dry ice and subsequently stored at -80°C until processed for RNA extraction and real-time PCR.

RNA extraction and real-time PCR—The hypothalamic 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 and 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 and resuspended in 50 μ L RNase-free water and stored at -80°C until further analysis.

Reverse transcription for cDNA preparation was carried out with Superscript II (Invitrogen) enzyme. RNA (2 μ L) was heated with 1 μ L random hexamers, 2 μ L 10 mM dNTP mix and 6 μ L DEPC-treated water at 65°C for 5 min and then put on ice. A mixture of 2 μ L 10 $\times$ reverse transcription buffer, 4 μ L 25 mM MgCl_2 , 2 μ L 0.1 M DTT and 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. Next, 1 μ L of ribonuclease H was added and each sample was incubated for 15 min at 37°C .

Each sample was run in duplicate in the Applied Biosystems real-time PCR machine (Model 7700; Applied Biosystems, Foster City, CA, USA). The primer and probe sets from the TaqMan Gene Expression Assays, with efficiencies of 100% for rat prepro-orexin, dynorphin and endogenous control 18S RNA, were used (prepro-orexin: Rn00565995_m1; prodynorphin: Rn00571351_m1; Eukaryotic 18S rRNA Endogenous Control: 4333760T). The PCR reaction mixture contained 2 $\times$ TaqMan® Universal PCR Master Mix (25 μ L), 20 $\times$ 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 and extending at 60°C . The relative quantification was performed using the comparative threshold cycle method ($\Delta\Delta\text{Ct}$ method; Livak & Schmittgen, 2001). The ΔCt values were determined by subtracting the reference 18S RNA values from the target gene Ct values. The change between the mRNA expression levels of the experimental (prepro-orexin siRNA-treated) and the control (scrambled siRNA-treated) samples was expressed as $2^{-(\Delta\Delta\text{Ct})}$, where $\Delta\Delta\text{Ct}$ equals the difference in ΔCt between the control and the experimental sample.

Experiment 3

To determine the effects of prepro-orexin siRNA on behavioural states of the animals, we performed bilateral injections of prepro-orexin siRNA into the PFH and monitored sleep and wakefulness. In a separate group, bilateral injections of scrambled siRNA (control) was performed and sleep–wakefulness was monitored.

Surgery and implantation of sleep-recording electrodes and guide cannulae—

Under sterile surgical conditions, each rat was implanted with standard EEG and EMG recording electrodes. Bilateral guide cannulae (Plastics One, Roanoke, VA, USA) were implanted 1 mm above the target sites for later microinjections.

Microinjections and sleep recordings—Recording cables were connected 1 day postsurgery. After at least a week of recovery and habituation to the recording cage, a 24-h baseline sleep recording beginning at dark onset was obtained. The next day, 4 h before dark onset (15.00 h), the rats were removed from the recording cage and gently swaddled in a towel during bilateral injection (~ 2 min) with either prepro-orexin siRNA or scrambled siRNA (0.03 nmol per side). Twenty-four-hour sleep recordings lasted a minimum of 6 days (Icelus Software, Mark Opp, University of Michigan, Ann Arbor, MI, USA; and Gamma Software, Astromed-Grass, Warwick, RI, USA); animal behaviour was video-recorded using an infrared camera for 8 h per dark period.

Histology—At the end of experiments, animals were killed and their orexinergic zones were identified by orexin-A immunohistochemistry (described above). Animals with injection sites > 1 mm AP and/or DV outside the zone of orexin-A-immunoreactive neurons were considered ‘missed hits’. Data were obtained from 12 rats; six were bilaterally treated with prepro-orexin siRNA and six with bilateral scrambled siRNA. Rats ($n = 2$) with injections outside the target zone were analysed separately.

Data analysis—The sleep data were recorded in 12-h blocks (light and dark periods) and were scored visually off-line in 10-s epochs of Wakefulness, non-REM (NREM) and REM using standard criteria (Thakkar *et al.*, 2003). The amount of time spent in each state was calculated along with number of episodes and average duration of each behavioural state for each 12-h block. Additionally, we calculated average REM sleep latency (defined as time from onset of REM sleep to the preceding wakefulness) and average REM-to-REM interval (defined as time between two REM episodes). Cataplexy or sleep onset REM (SOREM)-like episodes were identified by the concomitant presence of desynchronized, low-amplitude EEG with theta activity and a sudden reduction in muscle tone that was preceded by > 30 s of wakefulness. The average duration and average number of cataplexy or SOREM-like episodes was also calculated.

Statistics—While sleep data were analysed by one-way repeated-measures (RM) ^{ANOVA} followed by *post hoc* Bonferroni-corrected *t*-tests, neuronal counts were analysed using paired *t*-test and PCR data were analysed with Student's *t*-test.

Results

Experiment 1

The prepro-orexin siRNA-injected (unilateral) side demonstrated a marked reduction in orexin-A immunoreactivity (Fig. 1; $n = 4$) on day 2 as compared to the side that received scrambled siRNA (control). Double immunofluorescence labelling of MCH and orexin-A revealed that there was a marked reduction in orexin-A immunoreactivity on the prepro-orexin siRNA-injected side (Fig. 2) as compared with the side treated with scrambled siRNA; in contrast,

there was no reduction in MCH immunoreactivity on the prepro-orexin siRNA-injected side as compared with scrambled siRNA side.

Double immunofluorescence labelling of dynorphin B and orexin-A revealed that all orexin-A-immunoreactive neurons were double-labelled and showed dynorphin B immunoreactivity. Dynorphin B immunoreactivity was also found in many other nonorexin-positive neurons (Fig. 3). Similar findings were also reported by two previous studies (Bayer *et al.*, 2002 and Harthoorn *et al.*, 2005). On the prepro-orexin siRNA-injected (unilateral) side, the number of orexin-A-labelled neurons was significantly reduced 2 days after receiving the prepro-orexin siRNA treatment, as compared with the scrambled siRNA side (23% reduction; $n = 5$, mean $\pm$ SEM 219 ± 29 vs. 285 ± 21 ; $P < 0.05$, $t = 3.3$, paired t -test; Fig. 3, Table 2). In contrast, there was no reduction in dynorphin B neurons on the prepro-orexin siRNA side as compared with the scrambled siRNA side ($n = 3$, mean $\pm$ SEM 1283 ± 143 vs. 1331 ± 153 ; Table 3). The prepro-orexin siRNA-treated side did not show any significant change in orexin-A-immunoreactive neurons on days 4 ($n = 3$, mean $\pm$ SEM 145 ± 21 vs. 144 ± 41) and 6 ($n = 3$, mean $\pm$ SEM 213 ± 47 vs. 175 ± 42) postinjection as compared with scrambled siRNA (control) side.

Experiment 2

Real-time PCR data showed that there was a 58.5% down-regulation of prepro-orexin mRNA (Fig. 4; $n = 9$, $P < 0.05$, Student's t -test) in the siRNA-treated (bilateral injections) group: mean, 41.5% of control; range, 29.8–57.9%; controls being the scrambled siRNA-treated (bilateral injections) group (mean, 100%; range, 81.4–122.8%). Although there was still a slight decrease (16.7%) in prodynorphin in the siRNA-treated group, the change was not statistically significant and its range of variation overlapped with that of the control group.

Experiment 3

The prepro-orexin siRNA-treated animals (bilateral injections) showed a significant increase ($n = 6$, $F_{6,30} = 6.1$, $P < 0.01$, one-way RM ANOVA; see Fig. 5, Table 4) in the amount of time spent in REM sleep exclusively during the dark (active) period after prepro-orexin siRNA administration (bilateral) as compared to the baseline (preinjection night). The increase persisted over the first four postinjection nights (all $P < 0.05$, *post hoc* test). In contrast, the scrambled siRNA-treated (bilateral injections) rats (controls) had only a transient increase in REM sleep during the first postinjection night ($n = 6$, $F_{3,15} = 6.9$, $P < 0.01$, one-way RM ANOVA; Fig. 5 for the first night postinjection ($P < 0.05$, *post hoc* test) as compared to the baseline (preinjection night), perhaps due to cellular trauma from the microinjection and a consequent decrease in orexinergic neurotransmission.

Further analysis of REM sleep characteristics revealed that the increase in the amount of time spent in REM sleep after prepro-orexin siRNA injection was mainly due to an increase in the number of REM sleep episodes ($n = 6$, $F_{6,30} = 3.5$, $P < 0.05$, one-way RM ANOVA; Table 5) and rapid REM-to-REM sleep cycling ($F_{6,30} = 4.0$, $P < 0.01$, one-way RM ANOVA; Table 5). There was no significant change in average REM duration or latency to REM sleep.

In scrambled siRNA-treated animals, the transient REM sleep increase was also due to an increased number of episodes and a shortened REM sleep interval ($P < 0.05$ for the first night postinjection; Table 5). The transient increase in REM sleep was only observed with injections in the PFH; there were no REM sleep changes in rats ($n = 2$) where the injections were in nonorexinergic zone (see Fig. 6).

In contrast to the REM sleep enhancement during the dark period, during the light (inactive) period neither the prepro-orexin siRNA- or scrambled siRNA-treated rats showed any change

in REM sleep compared to the baseline during the first three postinjection days (Fig. 7). In addition, neither group of animals showed any statistically significant changes in wakefulness or NREM sleep during the dark or the light period (Table 4).

Cataplexy or SOREM-like events were observed in three of the six prepro-orexin siRNA-treated rats and occurred almost exclusively during the dark period (only one cataplexy or SOREM-like episode was observed during the light period in one rat on postinjection day 2). The cataplexy or SOREM behaviour was always followed by wakefulness. Cataplexy or SOREM-like episodes were not present in: (i) rats before prepro-orexin siRNA injection; (ii) scrambled siRNA-treated animals, and (iii) in animals with prepro-orexin siRNA injections in nonorexinergic zones. The average percentage of time spent in cataplexy or SOREM-like episodes in the prepro-orexin siRNA-treated rats in the dark period was very short: baseline, 0%; night 1, 0.07%; night 2, 0.34%; night 3, 0.03%; night 4, 0.01%; night 5, 0.06%; and night 6, 0%.

Discussion

The results of our study demonstrate the following. (i) PFH administration of prepro-orexin siRNA against the prepro-orexin gene caused a persistent and significant increase in REM sleep exclusively during the dark period without significantly affecting any other behavioural state. The effect on REM sleep was mainly due to a significant increase in the number of REM sleep episodes and a reduction in the REM sleep to REM sleep interval. There was no significant change in average REM duration or latency to REM sleep. Furthermore, there was no change in any of the behavioural states during the light period. (ii) In contrast, in scrambled siRNA-treated rats, except for a transient increase in REM sleep during first postinjection night, there was no change in any behavioural state during either the light or the dark period.

The effectiveness and the specificity of prepro-orexin siRNA treatment on the targeted protein and message was verified by the following. (i) Orexin-A immunohistochemistry showed a significant loss of orexin-A immunoreactivity on day 2 postinjection (when the behavioural effects of prepro-orexin siRNA were maximal) in and around the site of prepro-orexin siRNA injection, as compared with that of scrambled siRNA injection (Fig. 1); MCH and dynorphin B immunoreactivity was unaffected (Figs 2 and 3), suggesting that there was no global cell loss or protein synthesis down-regulation after siRNA administration. (ii) Real-time PCR showed a marked reduction in the prepro-orexin message in the prepro-orexin siRNA-treated site as compared with that of the scrambled siRNA-treated side (Fig. 4). Furthermore, the expression of prodynorphin was unaffected. (iii) The effect was site-specific and injections outside the orexinergic PFH did not have any effect on REM sleep. (iv) The behavioural effects of bilateral siRNA injections observed on day 4 postinjection were probably due to reduced orexins at the terminals, which may take time to normalise. (v) Our sleep and video recording data are also highly congruent with orexin down-regulation with the time course shown by the immunohistochemical changes, and the recovery of prepro-orexin immunoreactivity after 4–6 days after injection supports the absence of a generalised toxic effect on PFH orexinergic neurons.

Use of siRNA to suppress prepro-orexin expression

Our study is, to our knowledge, the first to employ siRNA to elucidate the role of orexins in the regulation of behavioural state. Accompanied by appropriate controls, siRNA technology appears to have advantages over other techniques as it provides a highly specific and reversible inhibition of expression of a particular gene.

Numerous studies, including ours, indicate that naked siRNA are taken up by neurons *in vivo* without transfection reagents or viral vectors, and produce target gene knockdown

(Makimura *et al.*, 2002; Shishkina *et al.*, 2004; Thakker *et al.*, 2004; Thakker *et al.*, 2005). Furthermore, naked siRNA have the advantage of not eliciting interferon and other nonspecific responses (Heidel *et al.*, 2004; Fedorov *et al.*, 2005).

Both the timing of effects and the dose of siRNA used are consistent with the notion that siRNA can achieve the same gene-suppressive effects in 2–4 days with a dose $\geq$ 100-fold lower than that required for inhibition by antisense oligonucleotides (Hough *et al.*, 2003; Miyagishi *et al.*, 2003). We observed the effects of siRNA after 1 day, even though the dose (0.03 nmol) in our experiment was $>$ 100-fold lower than the dose used in previous siRNA infusion and antisense studies (Dorn *et al.*, 2003; Thakker *et al.*, 2004, 2005).

Finally, we note that a microinjection approach may further reduce the chance of nonspecific effects by limiting the spread of siRNA to other brain regions. We found a single treatment (bilateral injection) of prepro-orexin siRNA produced a significant knockdown ($\sim$ 59%) of the prepro-orexin message; this was similar to other continuous siRNA infusion studies where the knockdown ranged from 30 to 50% (Dorn *et al.*, 2003; Thakker *et al.*, 2004, 2005). Moreover, this down-regulation of orexin expression, in turn, induced 4 days of behavioural state alteration, with a maximal effect at 2 days postinjection. This appears to be in accord with data from most RNA interference studies and a recent extensive literature review, which noted that siRNA effects on mRNA peaked at 36–48 h and started to disappear at $\sim$ 96 h (Ryther *et al.*, 2005).

Our study and others have found that a single microinjection of siRNA into the brain worked well (Makimura *et al.*, 2002; Bhargava *et al.*, 2004). In contrast, chronic infusion either did not work (Isacson *et al.*, 2003) or needed several days and a very high dose to work (Dorn *et al.*, 2003; Thakker *et al.*, 2004, 2005). Local infusion of siRNA may decrease the required dose for effectiveness (present study) while, in the cited infusion studies, intracerebroventricular or intrathecal application of siRNA may have decreased the effectiveness. Additionally, those previous infusion studies have targeted receptors, ion channels or transporters whose turnover rate is probably lower than that of orexin neurotransmitter peptides.

Orexin system and the diurnal regulation of REM sleep

Our major finding is that REM sleep increases after prepro-orexin siRNA administration occurred exclusively during the dark period. This strongly suggests a diurnal regulatory role of orexin. There is anatomical evidence of a suprachiasmatic nucleus-to-PFH projection (Deurveilher & Semba, 2005). In the rat, orexin release is higher during the dark (active) phase than the light (inactive) phase and this consolidation is disrupted by lesions of the circadian pacemaker in the suprachiasmatic nucleus (Deboer *et al.*, 2004). In the squirrel monkey, which has consolidated periods of sleep and waking similar to humans, orexin release is higher during the latter third of the day (active period), indicating that in primates the orexin system may act to oppose accumulating sleep drive during the day (Zeitzer *et al.*, 2003). Furthermore, orexin neurons are most active during wakefulness and are relatively quiet during NREM and REM sleep (Alam *et al.*, 2002; Lee *et al.*, 2005; Mileykovskiy *et al.*, 2005).

Our behavioural results of altered diurnal distribution of REM-like events are strikingly similar to data obtained from rats in which orexin-containing neurons are destroyed postnatally by progressive degeneration (Beuckmann *et al.*, 2004). In this study, the most dramatic change in REM-like events was the difference in diurnal distribution; REM-like events (including REM sleep and cataplexy or SOREM) were increased approximately two-fold over the wild type in the normally REM-poor dark period. However, there are two important interpretative differences between prepro-orexin siRNA-induced orexin knockdown and ataxin-3-induced cell death. First, although postnatal neurodegeneration avoids the problem of developmental compensation the degenerative process can take several weeks, which may give other sleep

regulatory systems enough time to compensate. Second, orexin neurons contain other neuromodulators, such as dynorphin, which are involved in sleep regulation (Eriksson *et al.*, 2004) and whose effects cannot be disambiguated from those of orexin in the degeneration methodology.

Although Mochizuki *et al.* (2004) found only a slight increase in REM percentage in the dark period, cataplexy-like events occurred frequently during the dark period in their constitutive orexin-knockout mice. This study raised an important theoretical consideration (Mochizuki *et al.*, 2004). Do increased REM-like events (cataplexy) caused by the reduction or absence of orexins during the dark cycle represent an alteration in the diurnal distribution of REM components or, alternatively, an abnormal dark period expression of cataplexy, without affecting the diurnal distribution of REM sleep? Based on their findings, Mochizuki *et al.* (2004) favour the latter interpretation, but their data do not rule out an alteration in the diurnal distribution of all REM components (including cataplexy) in orexin-knockout animals. Additionally, their use of constitutive orexin-knockout animals had the potential confound of developmental compensation. In contrast, in the present study a selective knockdown of orexin neurotransmission showed that the diurnal distribution of full-criteria REM sleep, as well as REM components (cataplexy or SOREM) was altered. It is as if the removal of orexin (in prepro-orexin siRNA-treated animals) greatly increases the probability of the REM oscillator being turned on, allowing it to run during its normal suppression during the active period (see review of REM oscillator mathematical modelling by Massaquoi, McCarley and colleagues in Steriade & McCarley, 2005). Our finding that the increase in the amount of REM sleep was due to the increase in REM sleep bouts and decrease in REM-to-REM sleep interval further support this conjecture.

Thus, taken together, previous and current data support the hypothesis that the diurnal control of the distribution of REM sleep and REM-component phenomena and their consolidation into the light (inactive) phase is under orexinergic control.

Indeed, it has been shown that a failure to consolidate REM sleep into the sleep (inactive) circadian phase is highly characteristic of human narcolepsy (Passouant *et al.*, 1969). These data also argue against the hypothesis that orexin acts as a necessary factor controlling the intracycle events of the wake–NREM–REM sleep cycle, as this cycle occurs in orexin-deficient animals and humans. Rather, it seems that orexin is best viewed as a modulatory, consolidating factor shaping the diurnal (circadian) phase of occurrence of REM sleep.

Summary

A relatively low dose of prepro-orexin siRNA induced a visible knockdown of orexin peptide immunoreactivity and robust REM sleep changes during the inactive period, suggesting that orexins may play a role in diurnal gating of REM sleep. Our approach indicates that simple and straightforward siRNA administration can be used as a convenient yet powerful tool with broad behavioural study applications.

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Abbreviations

Ct
threshold cycle

MCH

	melanin-concentrating hormone
NREM	non-REM
PFH	perifornical region of the hypothalamus
REM	rapid eye movement
RM	repeated-measures
siRNA	short interfering RNA
SOREM	sleep-onset REM

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Fig. 1. A representative photomicrograph shows that the side (left) with unilateral injection of siRNA against prepro-orexin mRNA evinces reduced orexin-A immunoreactivity in the perifornical region of the hypothalamus as compared to the side (right) injected with scrambled siRNA. Similar results were observed in another three animals. F, fornix; MT, mammillothalamic tract.

Fig. 2.

Double immunofluorescence labelling of MCH (FITC, green) and orexin-A (Cy3, red) neurons in a representative photomicrograph revealed that the prepro-orexin siRNA-injected (unilateral) side (left) had fewer orexin-A-positive neurons than the scrambled siRNA (control)-injected side (right) on day 2 postinjection. In contrast, the siRNA-injected side (left panel) and the scrambled siRNA-injected side (right panel) had a similar number of MCH neurons. F, fornix.

Fig. 3.

Double immunofluorescence labelling of orexin-A (FITC-labelled, green) and dynorphin B (Cy3-labelled, red) in a representative photomicrograph reveals that all orexin-A-immunoreactive cells show dynorphin B immunoreactivity but not vice versa. The prepro-orexin siRNA-injected side had fewer orexin-A-positive neurons (left side) than the scrambled siRNA (control)-injected side (right side) on day 2 postinjection. In contrast, the prepro-siRNA-injected side (left) and the scrambled siRNA-injected side (right) had similar levels of dynorphin B staining.

Fig. 4.

Bilateral injection of siRNA against prepro-orexin into the PFH induced a significant decrease in prepro-orexin mRNA but not prodynorphin mRNA ($n = 9$) when compared to scrambled siRNA-treated (bilateral) rats ($n = 9$). Prepro-orexin and prodynorphin mRNA levels in scrambled siRNA-injected rats were normalized to 100%. Their respective levels in prepro-siRNA-injected rats were expressed as percentages of the control. Ranges of gene expression were calculated using $2^{(-\Delta\Delta Ct)} \pm \text{SEM}$. * $P < 0.05$.

Fig. 5. REM sleep percentages (filled bars) during the dark period after prepro-orexin siRNA injection (bilateral, $n = 6$, A) or scrambled siRNA injection ($n = 6$, B). There was a significant increase in full-criteria REM sleep in prepro-orexin siRNA-treated animals over the first four nights following injection while scrambled siRNA-treated rats only had a transient change in REM sleep during the first postinjection night. * $P < 0.05$ for REM sleep values vs. baseline.

Fig. 6.

Percentage of REM sleep after prepro-siRNA injection (bilateral) into sites outside the orexinergic zone (mean of two rats). Note the percentage of REM on the two postinjection nights is about the same as that on the baseline night. The insert shows the microinjection site (arrow, guide cannula lesion) from one rat; the site is dorsal and posterior to the orexinergic zone of the PFH. Although the section was processed for orexin-A immunohistochemistry, no orexin cells are present. F, fornix; mt, mammillothalamic tract.

Fig. 7. REM sleep percentage during the light period in animals treated with siRNA injection (bilateral, $n = 6$, A) or scrambled siRNA injection (bilateral, $n = 6$, B) showed no significant differences from baseline. Data are expressed as mean $\pm$ SEM.

Table 1

Sequences for siRNAs and scrambled siRNAs

	Prepro-siRNA	Scrambled siRNA
Sense	5'-GAGCUAGAGCCAUAUCC TT -3'	5'-UUCCGAACUGUGCAACCG ATT -3'
Antisense	5'-AGGGAUAUGGCUCUAGC U TG-3'	5'-UCGGUUGCACAGUUCGGA AT G-3'
Sense	5'-GAGUCUGAACCCGUCUUC TT -3'	5'-CUCUAGUUGC ¹ AAUCCGG CT T-3'
Antisense	5'-AGAAGACGGGUUCAGAC U TG-3'	5'-GCCGGAUUAGCAACUAGAG GT G-3'
Sense	5'-GUCUGAACCCGUCUUC U TA TT -3'	5'-CUCAUAUGACCUCCUUGUG TT -3'
Antisense	5'-AUAGAAGACGGGUUCAG ACT C-3'	5'-CACAAGGAGGUCAUAUGAG TC -3'

The overhangs at -3' ends are in bold.

Table 2

Number of orexin-A-positive neurons in the prepro-siRNA- and scrambled siRNA-treated sides on day 2

Animal	Orexin-positive neurons	
	siRNA	Scrambled
201	266	321
202	272	335
203	155	215
204	140	277
205	262	276
Mean $\pm$ SEM	219 $\pm$ 29 *	284.8 $\pm$ 21

Data are expressed as the number of orexin-positive neurons in individual animals on the siRNA-treated side.

* $P < 0.05$ vs. the scrambled siRNA-treated side.

Table 3

Number of dynorphin B-positive neurons in the siRNA- and scrambled siRNA-treated sides on day 2

Animal	Dynorphin B-positive neurons	
	siRNA	Scrambled
201	1567	1633
202	1115	1141
203	1167	1220

Data are expressed as the number of dynorphin B-positive neurons in individual animals.

Table 4

Sleep percentages in prepro-siRNA- and scrambled siRNA-treated rats

Period (12-h)	Time (%)			
	siRNA (n = 6)		Scrambled (n = 6)	
	W	NREM	REM	W
Night 0	75.9 ± 3.2	20.7 ± 2.9	3.4 ± 0.5	73.8 ± 1.6
Night 1	69.9 ± 2.1	24.4 ± 1.8	5.6 ± 0.4 *	69.1 ± 2.7
Night 2	68.1 ± 3.4	26.0 ± 3.2	5.6 ± 0.5 *	70.7 ± 1.4
Night 3	70.6 ± 3.1	24.2 ± 2.6	5.2 ± 0.6 *	73.4 ± 2.7
Night 4	67.7 ± 2.7	26.4 ± 2.6	5.9 ± 0.6 *	
Night 5	70.6 ± 1.8	24.8 ± 0.8	4.6 ± 0.2	
Night 6	72.6 ± 2.6	22.8 ± 2.3	4.5 ± 0.5	
Day 0	32.6 ± 1.6	59.2 ± 1.5	8.3 ± 1.0	32.0 ± 2.6
Day 1	32.9 ± 1.9	58.6 ± 2.2	7.9 ± 0.8	33.3 ± 3.2
Day 2	32.2 ± 2.8	59.8 ± 2.5	8.6 ± 1.3	31.6 ± 3.5
Day 3	30.5 ± 2.3	61.2 ± 2.2	8.7 ± 0.9	31.8 ± 2.6

Night, 19.00–07.00 h; day, 07.00–19.00 h; Night 0 or Day 0, preinjection day or night; nights 1–6, postinjection nights; days 1–3, postinjection days. W, wakefulness.

* P < 0.05 vs. Night 0.

Table 5

REM sleep and cataplexy or SOREM during the night

siRNA	REM sleep			Cataplexy or SOREM		
	Period	Number	Duration	Latency	R-R cycle	Duration
Prepro	Night 0	17.7 ± 2.8	1.4 ± 0.04	4.9 ± 0.6	39.8 ± 5.6	0.0 ± 0.0
	Night 1	24.8 ± 2.3	1.6 ± 0.07*	5.2 ± 0.7	25.9 ± 1.4*	0.5 ± 0.3
	Night 2	26.2 ± 3.8	1.6 ± 0.20*	5.7 ± 0.6	26.3 ± 4.7*	2.2 ± 0.2
	Night 3	23.5 ± 3.3	1.6 ± 0.04*	6.1 ± 0.9	29.5 ± 3.7	0.3 ± 0.3
	Night 4	28.2 ± 3.4	1.5 ± 0.07*	6.4 ± 0.7	24.2 ± 3.0*	0.2 ± 0.2
	Night 5	24.0 ± 1.5	1.4 ± 0.07	5.3 ± 0.3	27.2 ± 1.5*	0.7 ± 0.4
Control	Night 6	22.0 ± 3.2	1.5 ± 0.09	5.8 ± 0.8	32.0 ± 3.7	0.0 ± 0.0
	Night 0	16.2 ± 2.3	1.6 ± 0.09	5.3 ± 1.0	42.0 ± 7.1*	0.0 ± 0.0
	Night 1	25.7 ± 3.1*	1.5 ± 0.05	5.2 ± 0.8	25.1 ± 2.4*	0.0 ± 0.0
	Night 2	22.2 ± 1.7	1.2 ± 0.03	5.3 ± 0.8	30.2 ± 3.0	0.0 ± 0.0
	Night 3	18.2 ± 1.3	1.3 ± 0.04	6.6 ± 1.7	35.6 ± 2.5	0.0 ± 0.0

Night, 19:00–07:00 h; Night 0, preinjection night; nights 1–6, postinjection nights; Duration, Latency and R-R cycle values are all expressed in min. All data are mean ± SEM. 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 the average interval between two REM sleep episodes; $n = 6$ for each group.

* $P < 0.05$ vs. Night 0.