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Expression of a Poly-Glutamine-Ataxin-3 Transgene in Orexin Neurons Induces Narcolepsy–Cataplexy in the Rat

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The sleep disorder narcolepsy has been linked to loss of hypothalamic neurons producing the orexin (hypocretin) neuropeptides. Here, we report the generation of transgenic rats expressing a human ataxin-3 fragment with an elongated polyglutamyl stretch under control of the human *prepro-orexin* promoter (*orexin/ataxin-3* rats). At 17 weeks of age, the transgenic rats exhibited postnatal loss of orexin-positive neurons in the lateral hypothalamus, and orexin-containing projections were essentially undetectable. The loss of orexin production resulted in the expression of a phenotype with fragmented vigilance states, a decreased latency to rapid eye movement (REM) sleep and increased REM sleep time during the dark active phase. Wakefulness time was also reduced during the dark phase, and this effect was concentrated at the photoperiod boundaries. Direct transitions from wakefulness to REM sleep, a defining characteristic of narcolepsy, occurred frequently. Brief episodes of muscle atonia and postural collapse resembling cataplexy were also noted while rats maintained the electroencephalographic characteristics of wakefulness. These findings indicate that the *orexin/ataxin-3* transgenic rat could provide a useful model of human narcolepsy.

Key words: **cataplexy; electroencephalography; electromyography; sleep; REM; lateral hypothalamus**

Introduction

Narcolepsy is a debilitating disorder, affecting 20–60 per 100,000 adults (Mignot, 1998; Overeem et al., 2001; Beuckmann and Yanagisawa, 2002). A cardinal symptom is excessive daytime sleepiness, manifested particularly as attacks of somnolence at inappropriate times. The latency for rapid eye movement (REM) sleep is also notably reduced, and the presence of pathologically short transitions from wakefulness to REM sleep is a diagnostic criterion for the disorder. Other symptoms of narcolepsy include hypnogogic and hypnopompic hallucinations and cataplexy. Cataplexy is characterized by sudden attacks of muscle weakness, frequently triggered by strong emotions (Bassetti and Aldrich, 1996) with patients remaining conscious during an attack (Scrima, 1981; Billiard, 1985; Gerhardstein et al., 1999; Taheri et al., 2002). The symptoms of narcolepsy have been considered an intrusion of REM sleep-related phenomena into wakefulness, indicating that the disorder may be one of vigilance state boundary control (Saper et al., 2001).

The concurrent discoveries that the autosomal recessive form of canine narcolepsy is caused by a mutation in the orexin receptor-2 gene (Lin et al., 1999; Hungs et al., 2001) and that *orexin*$^{-/-}$ mice exhibit a phenotype similar to human narcolepsy (Chemelli et al., 1999) provided insight into the pathophysiology of narcolepsy. In narcolepsy patients, a dysfunction or loss of orexin-containing neurons in the hypothalamus was subsequently confirmed (Peyron et al., 2000; Thannickal et al., 2000). Orexin-expressing neurons constitute a highly delimited population in the perifornical region of the lateral hypothalamus (LH) (de Lecea et al., 1998; Peyron et al., 1998; Sakurai et al., 1998; Nambu et al., 1999; Chou et al., 2001). Two neuropeptides, orexin-A and -B (hypocretin-1 and -2), are derived from the *prepro-orexin* gene and act at two G-protein-coupled receptors, orexin receptor-1 and -2 (de Lecea et al., 1998; Sakurai et al., 1998). In the human, mutations of the *prepro-orexin* or *orexin receptor* genes appear to be extremely rare (Peyron et al., 2000; Gencik et al., 2001). However, undetectable to very low levels of orexin-A neuropeptide in the CSF have been described in most patients with narcolepsy–cataplexy, whereas orexin-A levels of patients presenting with other disorders were comparable with those of healthy controls (Nishino et al., 2000, 2001; Ripley et al., 2001; Mignot et al., 2002). The number of orexin neurons is

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therefore likely to be diminished in narcoleptic patients (Peyron et al., 2000; Thannickal et al., 2000), although the cause for this apparent neuronal degeneration remains undetermined (Lin et al., 2001; Taheri et al., 2002).

In an attempt to mimic the loss of orexin neurons, we recently created transgenic mice in which orexin neurons expressed a cytotoxic gene product (a truncated form of human ataxin-3) under control of the human *prepro-orexin* promoter (Hara et al., 2001). This resulted in degeneration of orexin-containing neurons and a narcoleptic phenotype. We have now expressed the *orexin/ataxin-3* transgene in the rat, a species more widely used for physiological and pharmacological studies, and we describe here the initial histological and phenotypic characterization of the transgene expression in this species.

Materials and Methods

Generation of transgenic rat lines. The *orexin/ataxin-3* transgene expresses an N-terminally truncated human ataxin-3 protein containing a Q_{77} polyglutamine stretch under control of the human *prepro-orexin* promoter (Hara et al., 2001). The C terminus of the transgene contains a Myc oncogene epitope for histological examination. The transgene was injected into pronuclei of fertilized Wistar rat eggs to generate founder animals, which were bred to produce ten *orexin/ataxin-3* transgenic lines. Two lines were chosen for additional evaluation. Although one line is described here in detail, both lines displayed an essentially comparable phenotype. Lines were maintained in the hemizygous state for these studies. The transgene was propagated in a Mendelian manner, indicating no embryonic lethality. Throughout the study, animals were housed under a constant 12 hr light/dark cycle with free access to food and water. All experimental procedures were approved by the Institutional Animal Care and Research Advisory Committee of the University of Texas Southwestern Medical Center and were strictly in accordance with National Institutes of Health guidelines.

Genotyping. PCR was performed on genomic DNA from tail biopsies. Primers used were 5′-GCA GCG GCC ATT CCT TGG-3′ and 5′-CAG CGT AAT CTG GAA CAT CGT ATG GG-3′ against the *orexin/ataxin-3* transgene and 5′-GCA CCG AAG ATA CCA TCT CTC CGG ATT GC-3′ and 5′-GAC TCT GGA TCC GCC CCG GGG CGC TAA AGC-3′ against the endogenous rat *prepro-orexin* gene as an internal control.

Immunohistochemistry. All histological examinations were performed on male hemizygous transgenic rats (N3 back-cross generations into Sprague Dawley) and their male wild-type littermates. Male rats (wild-type and hemizygous transgenic littermates 4, 7, 10, 13, and 17 weeks of age) were deeply anesthetized with chloral hydrate (3.5 gm/kg, i.p.) and transcardially perfused with 150 ml of ice-cold PBS (11 U/ml heparin) and 150 ml of 4% (w/v) paraformaldehyde in PBS, pH 7.4. Brains were rapidly dissected and postfixed for 12 hr in 4% (w/v) paraformaldehyde in PBS, pH 7.4, at 4°C before being cryoprotected by equilibrating them in 30% sucrose in PBS. After cyroprotection, samples were frozen and sectioned (30 μm) with an SM2000R sliding microtome (Leica, Nussloch, Germany). Sections were then stored at 4°C in 0.004% sodium azide in PBS before histochemical staining.

For orexin/Myc double-immunohistochemistry, free-floating sections were rinsed with PBS three times for 5 min, followed by 0.3% hydrogen peroxide for 30 min to quench endogenous peroxidases. Sections were then rinsed again with PBS three times for 5 min. Nonspecific binding sites were blocked with 3% (v/v) normal horse serum (Vector Laboratories, Burlingame, CA) with 0.25% Triton X-100 in PBS for 2 hr at room temperature. After blocking, sections were incubated with Myc monoclonal antibody (Ab) (9E10; 1:400 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) against the transgene product for 12 hr at 4°C. As a secondary Ab, biotinylated horse anti-mouse Ab (Vector Laboratories) at a 1:600 dilution in 0.25% Triton X-100/PBS was applied for 2 hr. Subsequently, sections were incubated with horseradish peroxidase streptavidin at a 1:500 dilution in 0.5% Triton X-100/PBS for 1 hr, followed by application of $NiSO_4$-enhanced DAB solution (60 μl of 10% $NiSO_4$ per 2 ml of DAB; Dako, High Wycombe, UK) for 7–10 min at room temperature to create a dark brown-to-black precipitation. Orexin immunohistochemistry (light brown DAB staining without nickel enhancement) was performed as described previously (Chemelli et al., 1999) after Myc staining was completed. Sections were then mounted onto coated slides, dehydrated through an ascending ethanol/xylene series, and embedded using Permount (Fisher Scientific, Houston, TX).

Vigilance state determination. For behavioral evaluation, the control group consisted of male wild-type littermates of transgenic rats (N3 back-cross generations into Sprague Dawley) as well as of age- and weight-matched nonrelated male wild-type Sprague Dawley rats (Charles River Laboratories, Wilmington, MA). At 14 weeks of age, rats (8 hemizygous *orexin/ataxin-3* transgenic rats, 20 wild-type controls) were anesthetized (40 mg/kg ketamine, 4 mg/kg xylazine, 1 mg/kg acepromazine, i.p.) and surgically implanted with recording electrodes under sterile conditions. Miniaturized electrodes were affixed to the skull using a glass ionomer dental cement (Ketac Cem; ESPE, Norristown, PA), such that the electrodes just penetrated the skull and touched the dura. EEG signals were recorded unilaterally from fronto-occipital electrode pairs, positioned 1.5 mm rostral and 1.45 mm lateral from bregma, and 3.1 mm caudal and 1.45 mm lateral from bregma. EMG signals were concurrently recorded from two flexible wires, insulated except at the tips, and implanted bilaterally by blunt dissection into the nuchal musculature. The rats were tethered to a counterbalanced arm (Instech Laboratories, Plymouth Meeting, PA) that allowed virtually unrestricted mobility of the animals and exerted minimal weight. All rats recovered from surgery and habituated to the recording conditions for 3 weeks before recording commenced.

EEG–EMG signals were recorded under controlled conditions (12 hr light/dark cycle; 24 ± 1°C) for three consecutive days as described previously (Chemelli et al., 1999). Signals were digitized at 250 Hz, recorded to hard disk, and subsequently archived to optical media for off-line analysis. Concurrently with EEG–EMG recording, the behavior of the rats was videotaped for the first 4 hr of each dark phase using an infrared light source. For vigilance state analysis, EEG–EMG data were classified visually into 20 sec epochs by two independent observers blinded as to genotype. Standard criteria for rodent vigilance state measurements were used (Radulovacki et al., 1984), and results were summarized in terms of non-REM (NREM) sleep, REM sleep, and wakefulness. Epochs of wakefulness were not discriminated for active and quiet wakefulness. Summary vigilance state data for each rat were averaged over 3 d, before the results were grouped according to genotype, and analyzed by alternate Welch *t* test. The criterion for rejection of the null hypothesis was $p < 0.05$.

The following vigilance state parameters for the 12 hr dark and light periods were derived from the summary data for each rat before averaging by genotype. Total times spent in wakefulness, NREM, and REM sleep were derived by summing the total number of 20 sec epochs in each state. The number of episodes of wakefulness, NREM, and REM sleep was counts of the number of episodes of that state. Because we adopted a 20 sec epoch duration for vigilance state analysis, the minimum duration for an episode to be included in this analysis was 10 sec. Mean episode durations for wakefulness, NREM, and REM sleep were determined by dividing the total time spent in each state by the number of episodes of that state. Mean inter-REM interval was derived by determining the interval from the start of one REM sleep episode to the start of the next REM sleep episode and calculating the average of these intervals. A minimum of 10 sec of REM sleep was required before an episode was counted.

Mean REM sleep latency was determined by averaging the time elapsed from the beginning of a continuous NREM sleep episode to the beginning of the subsequent REM sleep episode. This calculation was also based on the 20 sec episode duration. Thus, if >10 sec of wakefulness occurred during an NREM sleep episode, the calculation was restarted and the interval was derived from the beginning of the NREM sleep episode that commenced after the intrusion of wakefulness. REM sleep latency as calculated here is thus sensitive to vigilance state fragmentation, because a brief arousal that lasted >10 sec was sufficient to reset the

calculation. Similarly, >10 sec of NREM sleep before the REM sleep episode was required for the latency to be included in the average. Thus, the minimum latency in this calculation was 20 sec, and episodes of direct transition to REM sleep were not counted.

Episodes of abnormal transition from wakefulness to REM sleep are considered a defining characteristic of narcolepsy. For this initial study in the rat, we defined an abnormal REM sleep transition as an REM sleep episode that followed a minimum of 60 sec of wakefulness with <20 sec of intervening NREM sleep. The former criterion ensured that brief arousals just before an REM sleep episode were not counted as abnormal transitions. The total count of such events, defined as the number of episodes of abnormal transition to REM sleep, was determined.

The EEG frequency distribution was analyzed by power spectral analysis [i.e., fast Fourier transform (FFT)] from 1 to 32 Hz using SleepSign 2.0 (Kissei Comtech, Tokyo, Japan). For derivation of power spectra for each vigilance state, FFT data for 150–200 representative artifact-free 4 sec epochs per rat (50 epochs for cataplexy per rat) from the 72 hr continuous EEG recordings were averaged for the transgenic rats and their wild-type littermates. Data were then normalized to a spectral density function by dividing each bin by the total average power of all epochs for that rat over the recording period to allow comparison between individual animals. Statistical analysis was by Student's *t* test, and the null hypothesis was rejected at $p < 0.05$.

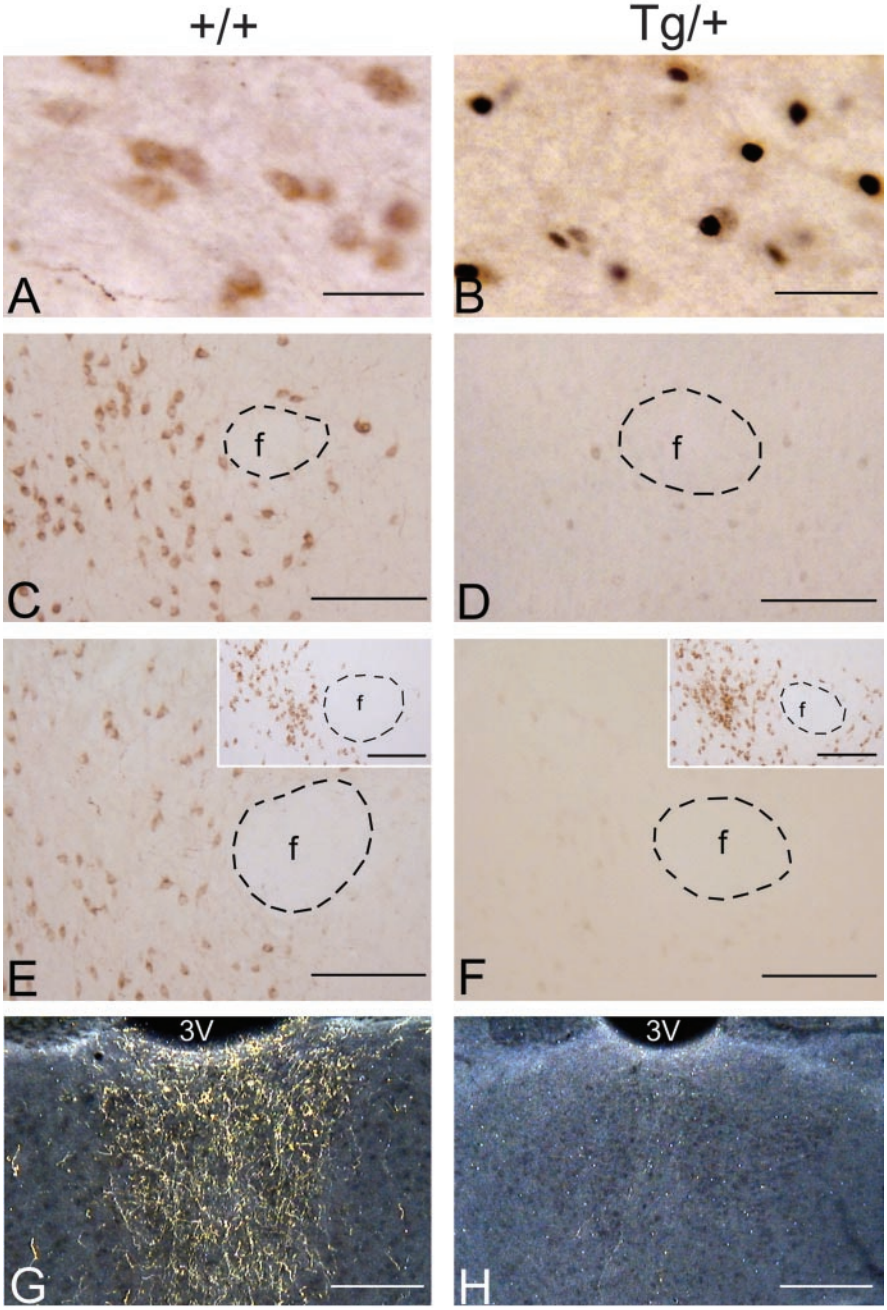

Figure 1. *A–H*, Colocalization of orexin neuropeptide and the *orexin/ataxin-3* transgene product (*A*, *B*) and disappearance of orexin-positive neurons (*C–F*) and projections (*G*,*H*) in *orexin/ataxin-3* hemizygous transgenic rats. The LH region of wild-type rats (*A*) and *orexin/ataxin-3* hemizygous transgenic littermates (*B*) was stained using antibodies against orexin-A (brown) and against the Myc tag of the transgene (black). All transgenic animals showed nuclear staining of the transgene product only in orexin neurons. Wild-type animals showed a normal distribution of orexin neurons in the LH at 4 weeks (*C*) and 17 weeks (*E*) of age. In contrast, *orexin/ataxin-3* transgenic littermates showed a notable reduction of orexin-immunoreactivity by 4 weeks of age (*D*) and a virtually complete loss of immunoreactivity at 17 weeks of age (*F*). No difference in MCH neuronal population in the perifornical area could be found at 17 weeks between wild-type rats (*E*, inset) and their *orexin/ataxin-3* hemizygous transgenic littermates (*F*, inset). Dense orexin-containing projections in the thalamic paraventricular nucleus of wild-type animals (*G*) were undetectable in transgenic animals at 17 weeks of age (*H*). f, Fornix; 3V, third ventricle. Scale bars: *A*, *B*, 40 μm; *C–H*, insets, 200 μm. *A–F*, Bright-field microscopy; *G*, *H*, dark-field microscopy.

Results

Generation of *orexin/ataxin-3* transgenic rats

Expression of the *orexin/ataxin-3* transgene was examined by immunohistochemical staining against the Myc tag at 4 weeks of age. We observed ectopic expression of the transgene outside the LH in 4 of 10 lines; we discarded these lines. In the line described here, the transgene product was colocalized exclusively in orexin-expressing neurons of the LH as verified by double immunostaining against Myc and orexin-A (Fig. 1*A*,*B*). In this line, ectopic expression outside the LH was absent throughout the brain (data not shown). By 4 weeks of age, the number of orexin-expressing cells was notably diminished in the perifornical region of the LH compared with wild-type littermate controls (Fig. 1*C*,*D*). The number of orexin-positive cells continued to decline at 7, 10, and 13 weeks of age (data not shown). At 17 weeks, the age of vigilance state recording, essentially no orexin-positive neurons could be found in the LH (Fig. 1*E*,*F*). However, at this age, immunostaining against Myc revealed picnotic remnants of cell nuclei that were positive for the *orexin/ataxin-3* transgene product in the LH, despite the complete lack of orexin-like immunoreactivity (data not shown).

Orexin-containing neuronal projections were also examined. The thalamic paraventricular nucleus is an area that, *inter alia*, receives a dense orexinergic innervation (Fig. 1*G*). In transgenic rats, no orexin-positive projections could be found at 17 weeks of age in this thalamic nucleus (Fig. 1*H*). Other brain regions, including the tuberomam-

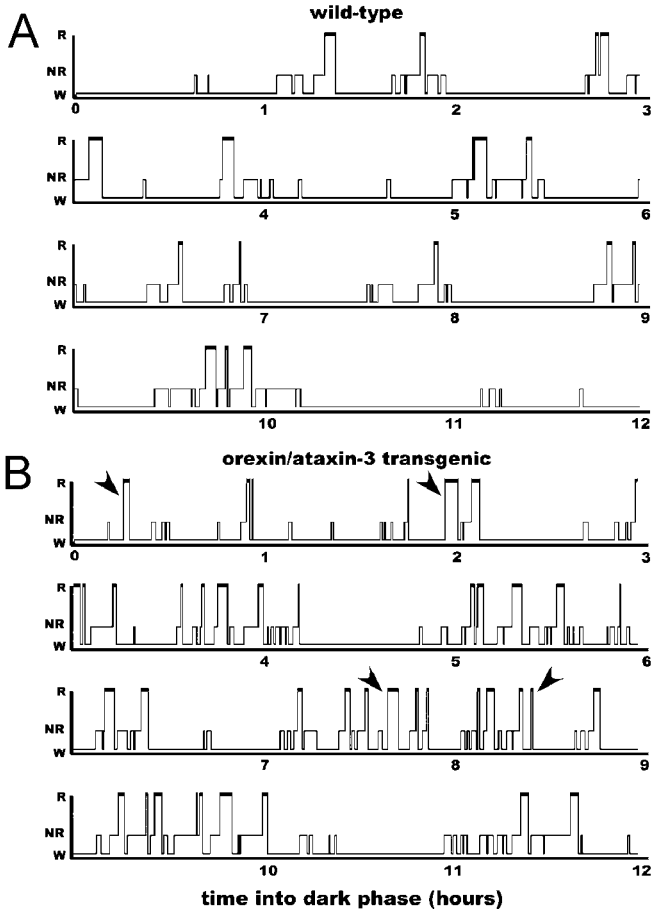

Figure 2. *A*, *B*, Representative dark-phase hypnograms of a wild-type rat (*A*) and an *orexin/ataxin-3* hemizygous transgenic littermate (*B*). The hypnogram of the transgenic animal shows more rapid cycling between vigilance states. It also exhibits direct transitions from wakefulness to REM sleep (indicated by arrowheads). W, Wakefulness; NR, NREM sleep; R, REM sleep.

millary nucleus, central gray, perifornical nucleus, arcuate nucleus, laterodorsal and pedunculopontine tegmental nuclei, locus coeruleus, and raphe nuclei, were also examined and similarly found to be lacking orexin projections.

Neurons expressing melanin-concentrating hormone (MCH) are intermingled with orexin neurons in the LH but form a distinct neuronal population (Elias et al., 1998). No difference in MCH expression was observed between *orexin/ataxin-3* transgenic animals (Fig. 1*F*, inset) and their wild-type littermates at 17 weeks of age (Fig. 1*E*, inset). Thus, the disappearance of orexin expression in the LH was not the result of a generalized lesion or widespread cell degeneration in that area. In summary, the immunohistochemical evaluation of *orexin/ataxin-3* hemizygous transgenic rats indicated a selective disappearance of orexin-containing neurons in the LH, combined with a concomitant loss of orexinergic projections throughout the brain.

Vigilance state characteristics of *orexin/ataxin-3* rats

REM sleep dysregulation

A hallmark characteristic of narcolepsy is dysregulation of REM sleep, and *orexin/ataxin-3* transgenic rats showed marked deviations from the wild-type phenotype in this sleep stage. A representative hypnogram during the dark, active phase in wild-type rats is displayed in Figure 2*A*, and the corresponding hypnogram for a transgenic rat is displayed in Figure 2*B*. Bouts of REM sleep in the wild-type rats were always preceded by an NREM sleep episode that usually lasted for several minutes: the mean REM sleep latency was longer during the dark phase in these rats (2.9 ± 0.2 min vs 3.8 ± 0.2 min for transgenic and wild-type rats, respectively; $p = 0.002$) (Table 1). In contrast, episodes of abnormal transition to REM sleep could be observed in all *orexin/ataxin-3* transgenic rats, primarily during the dark phase, whereas this was found only once in one wild-type control rat during a total of ~1500 hr of recording time. *Orexin/ataxin-3* transgenic rats had 3.8 ± 0.8 episodes showing abnormal transition to REM sleep per 12 hr dark phase and 0.3 ± 0.1 of these episodes per 12 hr light phase. Episodes of abnormal transition were therefore less frequent than normal REM sleep events (42.9 ± 2.8 and 27.9 ± 2.6 REM sleep events during dark and light phase, respectively) (Table 1). The mean duration of these episodes of REM sleep after an abnormal transition was 119 ± 11 and 68 ± 15 sec for the dark and light phases, respectively. Therefore, these durations were comparable with normal REM sleep episode durations (90.5 ± 2.2 and 67.1 ± 4.8 sec during the dark and light phase, respectively) (cf. Table 1).

Figure 3 shows representative traces of EEG–EMG recording from an *orexin/ataxin-3* transgenic rat. Normal transitions from wakefulness to NREM sleep (Fig. 3*A*) were indistinguishable from those in wild-type rats. However, as noted above, abnormal transitions to REM sleep (in this example, a direct transition without intervening NREM sleep) could be observed frequently in transgenic animals (Fig. 3*B*). Power spectral analysis of the EEG did not reveal any differences in the frequency spectra of the vigilance states between *orexin/ataxin-3* transgenic and wild-type rats (Fig. 4*A*, *B*). Furthermore, in *orexin/ataxin-3* rats, the average power spectrum of REM sleep episodes after an abnormal transition was indistinguishable from the corresponding spectrum for those REM sleep episodes that were preceded by NREM sleep (Fig. 4*C*). Thus, an REM sleep episode after an abnormal transition was essentially identical to a normal REM sleep episode with respect to both the EEG frequency distribution and episode duration.

The time spent in REM sleep during the dark phase in *orexin/ataxin-3* transgenic animals was approximately twice that recorded in the wild-type controls (64.2 ± 3.0 vs 32.9 ± 2.6 min for transgenic and wild-type rats, respectively; $p < 0.0001$) (Table 1). This resulted from the increase in the number of REM sleep episodes (42.9 ± 2.8 vs 23.4 ± 1.9 for transgenic and wild-type rats, respectively; $p < 0.0001$). In the light phase, however, overall REM sleep time was significantly less in the transgenic rats than in the wild-type controls (30.6 ± 2.6 vs 50.4 ± 2.6 min for transgenic and wild-type rats, respectively; $p < 0.0001$), resulting from a decrease in the number of REM sleep episodes (27.9 ± 2.6 vs 41.8 ± 2.4 for transgenic and wild-type rats, respectively; $p = 0.001$). In contrast to these differences in REM sleep episode number, the mean duration of REM sleep episodes was not significantly different between the transgenic and wild-type rats in either the dark or light phases (Table 1). The hourly distribution of REM sleep time across the 24 hr also was strikingly different between the genotypes (Fig. 5*A*). In fact, during only 6 hr, primarily clustered around the photoperiod boundaries were the hourly REM sleep times not significantly different between the wild-type and transgenic rats ($p > 0.05$). Therefore, the genotypes not only demonstrated the opposite circadian pattern in the incidence of REM sleep, as also evidenced by the total time spent in this stage during the light and dark 12 hr periods (Table 1), but the transgenic rats also did not show the gradual increase in REM

Table 1. Vigilance state parameters recorded from *orexin/ataxin-3* hemizygous (Tg/+) and wild-type control (+/+) rats

	REM sleep		NREM sleep		Awake	
	+/+	Tg/+	+/+	Tg/+	+/+	Tg/+
24 hr						
Total time[a] (min)	83.3 ± 2.9	94.8 ± 3.6*[b]	663 ± 13	691 ± 15	691 ± 14	651 ± 18
Episode duration (sec)	78.2 ± 2.6	80.9 ± 2.7	231 ± 5	223 ± 9	291 ± 18	238 ± 24
Number of episodes	65.5 ± 3.4	71.0 ± 4.0	174 ± 5	189 ± 11	148 ± 5	172 ± 13
REM latency (min)	5.8 ± 0.3	4.5 ± 0.2**				
Inter-REM interval (min)	22.5 ± 1.2	20.0 ± 1.3				
Light phase						
Total time (min)	50.4 ± 2.6	30.6 ± 2.6**	493 ± 6	497 ± 7	175 ± 6	191 ± 9
Episode duration (sec)	73.9 ± 2.8	67.1 ± 4.8	305 ± 8	330 ± 17	135 ± 7	147 ± 13
Number of episodes	41.8 ± 2.4	27.9 ± 2.6**	98.8 ± 2.9	92.2 ± 4.7	78.0 ± 2.4	80.9 ± 5.9
REM latency (min)	7.4 ± 0.3	7.2 ± 0.5				
Inter-REM interval (min)	15.8 ± 0.9	24.5 ± 1.7**				
Dark phase						
Total time (min)	32.9 ± 2.6	64.2 ± 3.0**	170 ± 9	194 ± 17	516 ± 10	460 ± 19*
Episode duration (sec)	86.4 ± 4.8	90.5 ± 2.2	137 ± 5	121 ± 7	490 ± 52	321 ± 37*
Number of episodes	23.4 ± 1.9	42.9 ± 2.8**	75.5 ± 3.9	96.7 ± 6.8*	70.0 ± 3.8	91.5 ± 7.4*
REM latency (min)	3.8 ± 0.2	2.9 ± 0.2**				
Inter-REM interval (min)	42.7 ± 8.5	17.8 ± 1.3**				

[a]Total time spent in each state (minutes), episode duration (seconds), number of episodes, REM latency, and interval between successive REM sleep episodes (in minutes) over 24 hr is itemized separately for the light and dark phases.
[b]Significant differences between (+/+) ($n = 20$) and (Tg/+) ($n = 8$) rats are indicated with two asterisks (**$p \leq 0.01$) or one asterisk (*$0.01 < p < 0.05$). Data are expressed as mean ± SEM.

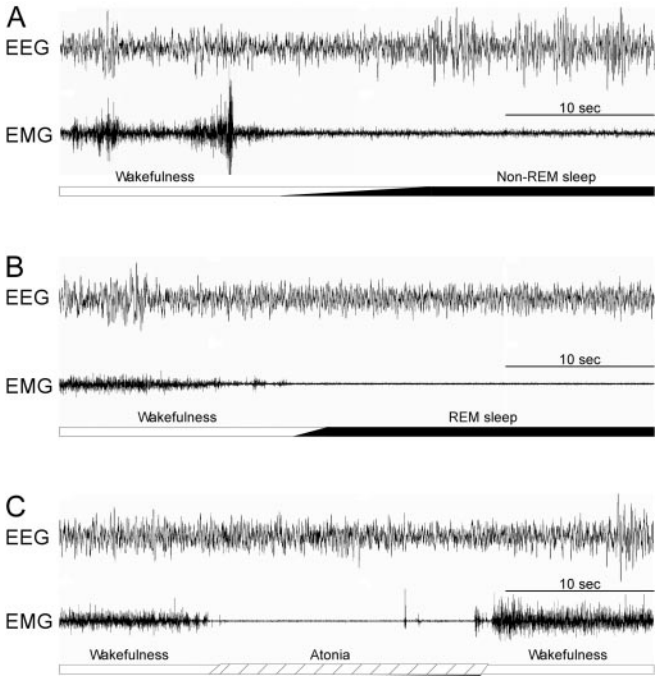

Figure 3. Representative EEG–EMG recordings from an *orexin/ataxin-3* hemizygous transgenic rat. *A*, Normal transition from wakefulness to NREM sleep. The EEG signal increases in amplitude and slows in frequency, whereas neck muscle tone diminishes in the EMG signal. *B*, Abnormal direct transition from wakefulness to REM sleep. The EEG signal starts with the typical mixed-frequency, low-amplitude, wakefulness pattern, which directly gives way to the regular low-amplitude pattern of REM sleep, dominated by θ activity in the 6–10 Hz range, with concomitant complete neck muscle atonia. *C*, Cataplexy-like event. Although the EEG characteristic of wakefulness remains unchanged, a sudden and transient complete neck muscle atonia occurs, after which normal muscle activity suddenly resumes. Note that θ activity in the EEG signal is less than that recorded during REM sleep, and that no visual indications of NREM sleep are apparent during this interval.

sleep time during the 12 hr light period that was a hallmark of REM sleep in the wild-type rats.

Fragmentation of wakefulness and NREM sleep

The hypnogram of a wild-type rat (Fig. 2*A*) showed prolonged periods of wakefulness with occasional sleep episodes. In contrast, *orexin/ataxin-3* transgenic rats showed marked fragmentation of vigilance states during the normally active dark phase, characterized by more rapid cycling between wakefulness and sleep (Fig. 2*B*). This fragmentation during the dark phase is reflected in an increased number of wakefulness episodes (91.5 ± 7.4 vs 70.0 ± 3.8 for transgenic and wild-type rats, respectively; mean ± SEM; $p = 0.03$), NREM sleep episodes (96.7 ± 6.8 vs 75.5 ± 3.9; $p = 0.02$), and REM sleep episodes (42.9 ± 2.8 vs 23.4 ± 1.9; $p < 0.0001$) (Table 1). However, although the transgenic rats had a greater number of wakefulness episodes, the reduced mean episode duration (321 ± 37 vs 490 ± 52 sec for transgenic and wild-type rats, respectively; $p = 0.014$) resulted in less time spent awake (460 ± 19 vs 516 ± 10 min for transgenic and wild-type rats, respectively; $p = 0.03$). We also noted a corresponding tendency toward increased NREM sleep time in the transgenic rats (194 ± 17 vs 170 ± 9 min for transgenic and wild-type rats, respectively; $p = 0.23$) (Table 1). A plot of the hourly distribution of wakefulness time demonstrated that the difference between the genotypes in time spent awake was concentrated primarily at the photoperiod boundaries (Fig. 5*B*). Figure 5*B* also shows that, even in terms of the hourly distribution, wakefulness time during the light period was very similar in both genotypes.

Behavioral analysis

Behavior was monitored for 4 hr by infrared video recording, starting at the onset of the dark phase, while EEG–EMG signals were recorded simultaneously. Subsequent analysis of the video recordings revealed that episodes of abnormal transition to REM sleep occasionally occurred during motivated behavior, such as ambulation or drinking (cf. supplemental video 1, available at www.jneurosci.org). Each abnormal transition to REM sleep was associated with a sudden loss of muscle tone and concomitant loss of posture. Rocking movements along the body axis, as described in *orexin*$^{-/-}$ mice (Chemelli et al., 1999) and in saporin-lesioned rats (Gerashchenko et al., 2001), were not observed. Behavioral arrests associated with these abnormal REM sleep episodes in the transgenic rats were always abruptly terminated with the resumption of full mobility and purposeful behavior. As noted above, the EEG power spectrum for these episodes of REM

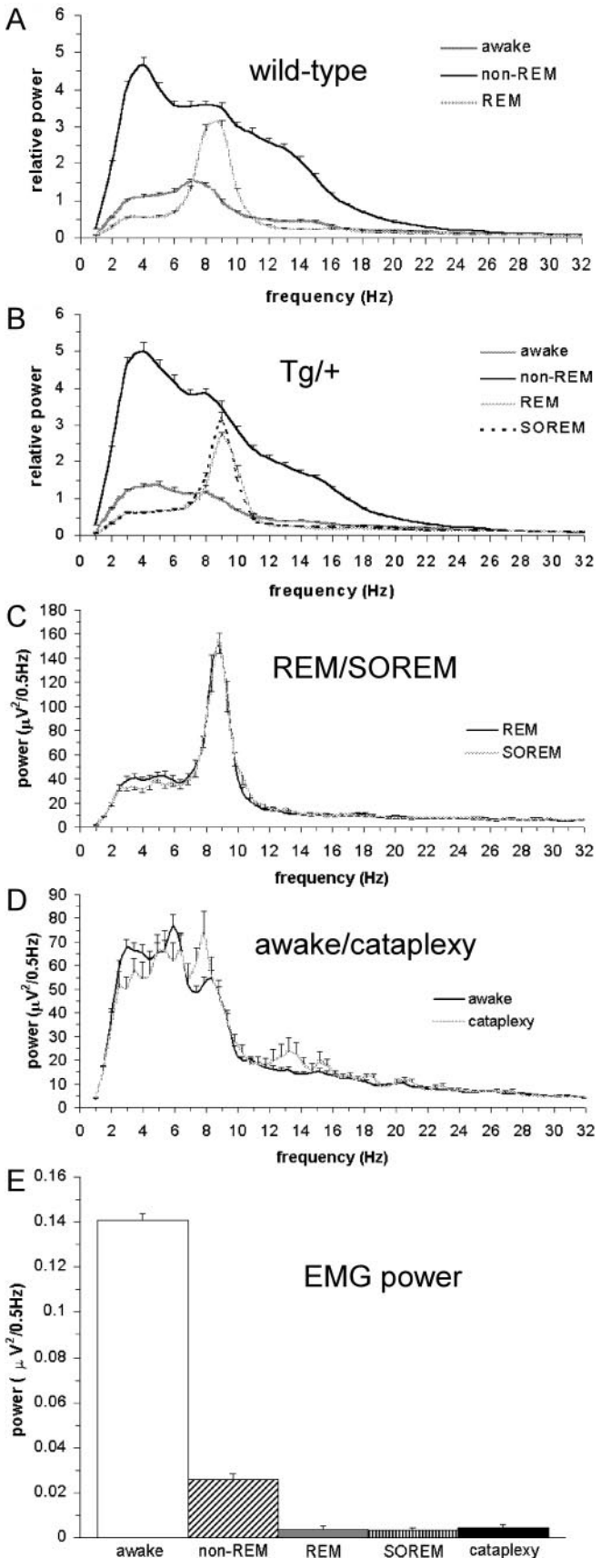

Figure 4. Power spectra of EEG–EMG recordings from wild-type rats and their *orexin/ataxin-3* hemizygous transgenic littermates. *A*, Representative EEG power spectra of wakefulness, NREM sleep, and REM sleep in a wild-type rat. *B*, Representative EEG power spectra of an *orexin/ataxin-3* transgenic littermate. SOREM (i.e., sleep-onset REM) episodes designate those

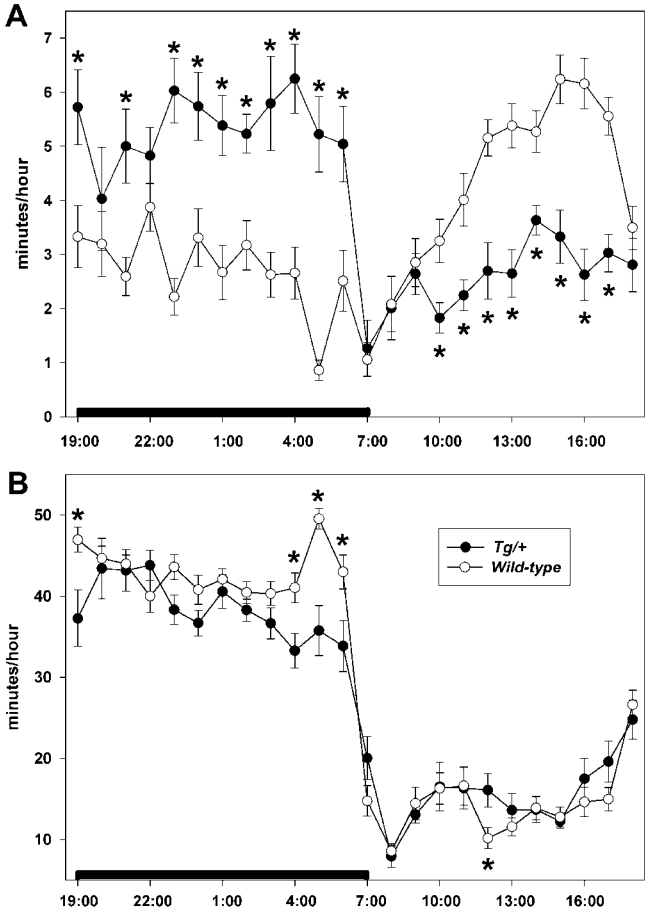

Figure 5. *A*, *B*, Time spent each hour (in minutes; mean ± SEM) in REM sleep (*A*) and wakefulness (*B*) for wild-type rats and their *orexin/ataxin-3* hemizygous transgenic littermates. Significant differences between the genotypes (*t* test; $p < 0.05$) are marked by asterisks. The dark period is denoted by the horizontal bar.

sleep after an abnormal transition was not different from the spectrum recorded during normally occurring REM sleep (Fig. 4*C*).

In addition to typical behavioral arrests associated with abnormal transitions to REM sleep, another type of postural collapse was observed (cf. supplemental video 2, available at www.jneurosci.org). These latter episodes could not be behaviorally differentiated from an episode of abnormal transition by video photography alone. As in abnormal transitions to REM sleep, the rat showed a sudden and complete loss of muscle tone, and purposeful behavior ceased (Fig. 3*C*). However, power spectral analysis of the corresponding EEG and EMG signals revealed that *orexin/ataxin-3* transgenic rats had this loss of muscle tone at a time when the EEG maintained a pattern very similar to that recorded during normal wakefulness (Fig. 4*D*). These arrests occurred both during the dark (4.7 times per 12 hr) and light (2.8

←

REM sleep episodes that follow an abnormal transition from wakefulness. Note that the mean EEG frequency distribution of SOREM episodes is essentially identical to that recorded during normally occurring REM sleep in the same animal. The signal amplitudes in *A* and *B* have been normalized in each animal to allow comparison across animals. *C*, *D*, Representative absolute EEG power spectra of REM sleep and SOREM episodes (*C*) and of wakefulness and cataplexy-like events in an *orexin/ataxin-3* hemizygous transgenic rat (*D*). *E*, Average EMG signal power recorded during the designated vigilance states from a representative *orexin/ataxin-3* hemizygous transgenic rat. Data are expressed as mean ± SEM.

times per 12 hr) phases and were generally short in duration (dark phase, 12 ± 1 sec; light phase, 20 ± 4 sec). As displayed in Figure 4*E*, muscle tone, when compared with either normal wakefulness or NREM sleep, was markedly reduced and became essentially absent during REM sleep, REM sleep after an abnormal transition, and these episodes of behavioral collapse with wakefulness-like EEG. In contrast to human and canine cataplectic events, however, which are often triggered by emotional stimuli (Aldrich, 1992; Riehl et al., 1998), we could not reliably elicit behavioral arrests in the *orexin/ataxin-3* transgenic rats using external emotive stimuli.

Discussion

Although canine and murine models of narcolepsy have been established previously (Mitler et al., 1976; Chemelli et al., 1999, 2000; Hara et al., 2001; Kisanuki et al., 2000, 2001; Willie et al., 2003), human narcolepsy is likely to result from a gradual degradation of orexin neurons and consequent loss of orexin expression. Here, we adapted to the rat the technique for producing *orexin/ataxin-3* transgenic mice, in which orexin neurons were genetically ablated by selective expression of a cytotoxic poly-Q-ataxin-3 protein (Hara et al., 2001). An alternative approach was used by Gerashchenko et al. (2001), who infused orexin-B conjugated to the ribosome-inhibiting cytotoxic protein saporin into the LH of wild-type rats. Orexin-expressing neurons presumably possess orexin autoreceptors, and the infusion of saporin thus resulted in a loss of these neurons and a narcoleptic-like phenotype. However, other LH cell populations that express orexin receptors, including MCH and adenosine-deaminase-containing neurons, were also lesioned by this technique (Gerashchenko et al., 2001). Moreover, a recent electrophysiological study showed that orexin activates orexin neurons via glutamatergic interneurons (Li et al., 2002). Because orexin B-saporin might also ablate these interneurons, this approach cannot be considered a precise model of human narcolepsy, in which orexin neurons appear to be affected in a highly specific way.

The rat model described here thus combines the selective targeting of orexin-expressing neurons and a gradual loss of these cells during ontogenesis, with the advantages of a species that is widely adopted for physiological and pharmacological studies. The most important characteristics of this model are: decreased wakefulness during the normally active dark phase; fragmentation of wakefulness and NREM sleep patterns; shortened REM sleep latency; episodes of abnormal transition to REM sleep; differences in total REM sleep time; and cataplexy-like behavioral arrests, during which the animals show muscle atonia comparable with REM sleep but remain awake as judged by spectral analysis of the EEG. Additionally, vigilance state fragmentation during the dark phase indicates that transgenic rats are unable to maintain prolonged periods of wakefulness. Ongoing activity was more frequently interrupted by bouts of NREM sleep than in the wild-type controls. However, despite these marked differences in sleep during the dark phase, the time spent in wakefulness and NREM sleep over a 24 hr period remained unchanged in the transgenic rat. Overall, these results are directly comparable with vigilance state observations in narcoleptic humans, and we conclude that the phenotype of the *orexin/ataxin-3* transgenic rat closely resembles narcolepsy.

Cataplexy, in particular, is difficult to provoke in a clinical setting in narcoleptic patients, and in most cases, the diagnosis of cataplexy is based on self-evaluation by the patient. The mechanisms of cataplexy have been intensely studied to date in narcoleptic dogs (Riehl et al., 1998; Fujiki et al., 2002). Elicited by emotional stimuli or food presentation, cataplectic attacks in these dogs result in complete muscle atonia, frequently affecting the whole body. Concurrently, the animals are awake and remain aware of their surroundings (Nishino and Mignot, 1997). Analysis of *orexin*$^{-/-}$ mice also demonstrated the existence of episodes of postural collapse, during which the mice remained conscious as judged by behavioral observation (Willie et al., 2003). Detailed characterization of the abnormal state transitions in the *orexin/ataxin-3* transgenic rat are now required, but we noted similarities between these existing descriptions of cataplexy and a particular type of behavioral arrest in these rats.

The striking difference between the wild-type and *orexin/ataxin-3* transgenic rats in the hourly distribution of REM sleep time throughout the 24 hr period contrasts with the similarity of the corresponding wakefulness times, especially during the light phase. The wakefulness deficit in the transgenic rats during the dark, or active, period is essentially concentrated at the photoperiod boundaries and in particular at the end of this period. This time corresponds closely to the timing, in humans, of maximal circadian alertness, which consolidates wakefulness at the end of the active part of the daily cycle when the homeostatic drive for sleepiness is highest (Dijk and Czeisler, 1994). With a polyphasic sleep pattern and no single consolidated wakefulness bout, a similar alertness signal has not been investigated in the rat. However, our data in the wild-type animals demonstrate, just before the beginning of the sleep period, a significant increase in wakefulness, which is absent after the loss of orexin in the transgenic rats. Importantly, the circadian variation in the orexin signal in rat brain peaks at the same time as this increase in wakefulness, late in the active portion of the daily cycle (Taheri et al., 2000; Fujiki et al., 2001; Yoshida et al., 2001). Our data thus support the proposal that orexin contributes to the daily variation in wakefulness at the end of the active period (Mignot, 2001). Previous corroboration for this hypothesis came from Dantz et al. (1994), who used a forced desynchrony protocol to show that narcoleptic patients have a deficit in circadian wakefulness, even though their circadian pacemaker per se and homeostatic sleep drive are normal. Also, a recent study in the squirrel monkey has demonstrated that the maximal orexin signal in this species corresponds to the timing of maximum circadian alertness at the end of the active period (Zeitzer et al., 2003).

Comparison of the hourly distribution of REM sleep time between the genotypes indicates an apparently continuous effect of the loss of orexin on the expression of REM sleep. Thus, the absence of orexin in the transgenic rats results in increased REM sleep throughout the normally active phase and, consequentially, a reduced homeostatic drive for REM sleep during the light phase. In the wild-type rat, the homeostatic drive for REM sleep during the normal sleep phase is expressed as a gradual increase in the hourly times spent in REM sleep. Significantly, this distribution of REM sleep in the wild-type rats is inversely correlated with the diurnal variation of the orexin signal, which remains high throughout the dark period and reaches a minimum toward the end of the light phase in this species (Taheri et al., 2000; Fujiki et al., 2001; Yoshida et al., 2001). Together, these data suggest that the release of orexin has an inhibitory influence on the appearance of REM sleep.

Importantly, the difference in REM sleep time between the genotypes was attributable to a change in the number of episodes of REM sleep, whereas the REM sleep bout length remained unchanged. This indicates that orexin normally inhibits the onset of an episode of REM sleep but, once initiated, it does not affect the characteristics of that episode. Kiyashchenko et al. (2002) re-

ported that orexin release is highest during active wakefulness and REM sleep. This result left open the possibility that orexin also might be involved in the generation of the REM sleep state. The current data now preclude this possibility and suggest that the link to orexin is through ongoing motor patterns, whether they are expressed as in active wakefulness or inhibited as in REM sleep. We therefore speculate that an important function of orexin is to inhibit the appearance of REM sleep and particularly when the brain state, including cortical activation, is most similar during active wakefulness. For this reason, when orexin is absent, the REM sleep switch appears biased (Saper et al., 2001; Sutcliffe and de Lecea, 2002), and direct transitions from wakefulness to REM sleep can occur.

In summary, our data show that the presence of orexin impacts vigilance state control in two ways. First, it may act as a circadian arousal signal to enhance alertness at the end of the normal wake period when the homeostatic drive for sleepiness is reaching its maximum. Second, it appears to inhibit the onset of REM sleep episodes. Orexin is excitatory to the brainstem monoaminergic cells, which are quiescent during REM sleep (i.e., the REM-off cells) (Hagan et al., 1999; Horvath et al., 1999; Brown et al., 2001), providing a mechanism by which the neuropeptide could influence REM sleep initiation. The diurnal variation in orexin levels, closely coupled to ongoing motor activity (Kiyashchenko et al., 2002), thus provides a basis for the variation in the expression of REM sleep throughout the nychthemeron. In this regard, it is interesting to note that McCarley and Massaquoi (1992), during development of a model of the REM sleep oscillator, postulated the existence of a circadian control factor that excited monoaminergic REM-off cells to prevent the occurrence of REM sleep and so influence the diurnal distribution of the state. Our data indicate that orexin could be a factor that plays such a role in REM sleep expression.

We conclude that this model of narcolepsy in the rat is likely to prove useful for research into the pathophysiology of the disorder and for the discovery and validation of pharmacological treatments based on orexin neurotransmission. With its ease of breeding propagation, in which one allele of the *orexin/ataxin-3* transgene is sufficient to yield the narcoleptic phenotype combined with extensive knowledge of neurophysiology and neuropharmacology in the rat, this model will be valuable in continuing investigations into narcolepsy.

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