Behavioral State Instability in Orexin Knock-Out Mice

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Narcolepsy is caused by a lack of orexin (hypocretin), but the physiologic process that underlies the sleepiness of narcolepsy is unknown. Using orexin knock-out (KO) mice as a model of narcolepsy, we critically tested the three leading hypotheses: poor circadian control of sleep and wakefulness, inadequate activation of arousal regions, or abnormal sleep homeostasis. Compared with wild-type (WT) littermates, orexin KO mice had essentially normal amounts of sleep and wake, but wake and non-rapid eye movement (NREM) bouts were very brief, with many more transitions between all behavioral states. In constant darkness, orexin KO mice had normal amplitude and timing of sleep–wake rhythms, providing no evidence for disordered circadian control. When placed in a new, clean cage, both groups of mice remained awake for ~45 min, demonstrating that, even in the absence of orexin, fundamental arousal regions can be engaged to produce sustained wakefulness. After depriving mice of sleep for 2–8 hr, orexin KO mice recovered their NREM and rapid eye movement sleep deficits at comparable rates and to the same extent as WT mice, with similar increases in EEG delta power, suggesting that their homeostatic control of sleep is normal. These experiments demonstrate that the fragmented wakefulness of orexin deficiency is not a consequence of abnormal sleep homeostasis, poor circadian control, or defective fundamental arousal systems. Instead, the fragmented behavior of orexin KO mice may be best described as behavioral state instability, with apparently low thresholds to transition between states.

Key words: orexin; hypocretin; narcolepsy; sleepiness; transitions; sleep

Introduction

Narcolepsy is characterized by chronic sleepiness in association with brief episodes of muscle weakness, known as cataplexy (for review, see Scammell, 2003). Approximately 90% of people with this common sleep disorder lack the hypothalamic neuropeptide orexin (hypocretin) (Mignot et al., 2002), and orexin knock-out (KO) mice have a phenotype very similar to that of the human disorder (Chemelli et al., 1999; Willie et al., 2003). These findings show that a loss of orexin is sufficient to produce narcolepsy, but the physiologic process that underlies the sleepiness of narcolepsy is unknown.

Three neurobiologic models have been hypothesized to account for this sleepiness. The orexin neurons heavily innervate and excite wake-promoting brain regions, such as the aminergic and cholinergic neurons of the brainstem, hypothalamus, and basal forebrain (Peyron et al., 1998; Hagan et al., 1999; Eggermann et al., 2001; Eriksson et al., 2001). In addition, orexin promotes wakefulness when injected near the locus ceruleus (LC) (Bourgin et al., 2000), and histaminergic signaling appears necessary for the wake-promoting actions of orexin (Huang et al.,

2001; Yamanaka et al., 2002). Thus, the sleepiness of orexin deficiency may simply be a consequence of inadequate activation of these fundamental arousal regions.

Abnormal sleep homeostasis is also hypothesized to cause the excessive sleepiness of narcolepsy (Tafti et al., 1992b; Besset et al., 1994). Narcoleptics often feel sleepy after just a few hours of wakefulness, and, after 24 hr of sleep deprivation, people with narcolepsy have shorter sleep latencies and more deep non-rapid eye movement (NREM) sleep than controls (Tafti et al., 1992a). Considered together, these observations suggest a heightened sensitivity to or a more rapid accumulation of sleep pressure in narcolepsy.

Other researchers have hypothesized that the sleepiness of narcolepsy is caused by impaired circadian control of sleep and wake (Kripke, 1976; Dantz et al., 1994; Broughton et al., 1998). Circadian rhythms are governed by the suprachiasmatic nucleus (SCN) and play an essential role in the timing of sleep and wakefulness (Klein et al., 1991; Dijk and Czeisler, 1995). The SCN may also help consolidate wakefulness because monkeys with lesions of the SCN have very short bouts of wake (Edgar et al., 1993), although this phenomenon is less apparent in SCN-lesioned rodents (Mistlberger et al., 1983). The orexin neurons are anatomically well positioned to time sleep–wake behavior because they receive direct and indirect projections from the SCN (Abrahamson et al., 2001; Lu et al., 2001; Chou et al., 2003), and they may relay this circadian information to arousal regions. People with narcolepsy have normal amounts of sleep over 24 hr (Nobili et al.,

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1995), but the broad distribution of sleep and the occurrence of rapid eye movement (REM) sleep at all times of day led to the idea that their sleepiness might be caused by a decrease in the amplitude of the circadian signals that time sleep and wakefulness (Broughton et al., 1998).

We critically tested these hypotheses by examining the sleep–wake behavior of orexin KO mice under conditions that assess endogenous circadian rhythms, the integrity of fundamental arousal systems, and the homeostatic control of sleep.

Materials and Methods

Animals

Founder orexin KO mice were on a C57BL/6J–129/SvEV background, and their offspring were backcrossed with C57BL/6J mice for six to eight generations. These experiments used seven male KO mice and eight wild-type (WT) male littermates, all 11 weeks old and weighing 26–28 gm. Mice were genotyped using PCR with a neo primer, 5′-CCGCTATCAGGACATAGCGTTGGC, or a genomic primer, 5′-GACGACGGCCTCAGACTTCTTGGG, and a genomic primer, 3′-TCACCCCTTGGGATAGCCCTTCC, common to KO and WT mice. All experiments were approved by the Institutional Animal Care and Use Committees of Beth Israel Deaconess Medical Center and Harvard Medical School.

Surgery and electroencephalogram–electromyogram recordings

Mice were anesthetized with ketamine–xylazine (100 and 10 mg/kg, i.p.) and implanted with electroencephalogram (EEG) and electromyogram (EMG) electrodes for polysomnogram recording. EEG signals were recorded using two ipsilateral stainless-steel screws (1.5 mm to the right of the sagittal suture, 1 mm anterior to bregma, and 1 mm anterior to lambda). EMG signals were acquired by a pair of multistranded stainless-steel wires inserted into the neck extensor muscles. These leads were attached to a 2 × 2 pin header that was secured to the skull using dental acrylic. A telemetric temperature transmitter (TA-F20; Data Sciences International, St. Paul, MN) was placed in the peritoneal cavity of all mice. Mice were housed individually for 9 d after surgery. They were then transferred to recording cages in a sound-attenuated chamber with a 12 hr light/dark (LD) cycle (30 lux; lights on at 7:00 A.M. and off at 7:00 P.M.) and a constant temperature (22–24°C). They had *ad libitum* access to food and water and acclimated to the recording cables for another 5 d.

The EEG–EMG signals were acquired using Grass Instruments (West Warwick, RI) model 12 amplifiers and digitized at 128 Hz using a sleep scoring system (Sleep Sign; Kissei Comtec, Matsumoto, Japan). The signals were digitally filtered (EEG, 0.3–30 Hz; EMG, 2–100 Hz) and semi-automatically scored in 10 sec epochs as wake, NREM, or REM sleep. This preliminary scoring was visually inspected and corrected when appropriate.

People with narcolepsy have cataplexy, defined as emotionally triggered episodes of muscle weakness with preserved consciousness, and orexin KO mice have similar events during active wake (previously referred to as “abrupt behavioral arrests”) (Chemelli et al., 1999; Willie et al., 2003). Although EEG theta activity and atonia occur in both cataplexy and REM sleep (Guilleminault et al., 1974; Mitler and Dement, 1977; Dyken et al., 1994; Willie et al., 2003), cataplexy is a distinct state, based on behavioral and physiologic criteria. In narcoleptic dogs, neurons of the LC, dorsal raphe, and tuberomammillary nucleus (TMN) fall silent during REM sleep, but, during cataplexy, the raphe and the TMN fire at moderate and high rates, respectively (John et al., 2004; Wu et al., 2004). Cataplexy in orexin KO mice is immediately preceded by active wake with grooming, climbing, and ambulation and is immediately followed by an abrupt resumption of activity (Chemelli et al., 1999; Willie et al., 2003).

Using infrared video recordings of five uninstrumented KO mice, housed together in a regular cage, we observed 5.7 episodes of cataplexy per hour, lasting an average of 52 ± 14 sec, during the first 2 hr of the dark period (a time when cataplexy is common). Because Willie and colleagues have shown that 80% of cataplexy is accompanied by EEG theta (4–9 Hz) activity (Willie et al., 2003), we counted cataplexy episodes

using EEG and EMG recordings alone, provided that the event (1) begins with an abrupt transition from active wake to atonia with EEG theta activity and (2) is terminated by an abrupt return from atonia and theta to active wake (supplemental Fig. 1, available at www.jneurosci.org/cgi/content/full/24/28/6291/DC1). Using this operational definition with EEG–EMG recordings, orexin KO mice had 5.2 occurrences of cataplexy, lasting an average of 67 ± 6 sec, during the first 2 hr of the dark period. On rare occasions, orexin KO mice had brief episodes of atonia during sustained wake that lacked clear theta or delta (0.5–4 Hz) activity, and these indeterminate epochs were scored as a fifth state. These atypical episodes accounted for only 0.2% of the total recording time and were omitted from additional analysis. WT mice never had cataplexy or this fifth state.

Analysis of sleep–wake bouts

To examine the ability of mice to maintain behavioral states, we analyzed the distribution of behavioral states as a function of bout length (Trachsel et al., 1991; Franken et al., 1999). No minimal criteria were used, and a bout could be as short as one epoch. All bouts of each state were separated into eight bins of increasing duration (< 30, 40–70, 80–150, 160–310, 320–630, 640–1270, 1280–2550, and >2560 sec). The amount of state in each bin was time weighted and normalized by the total amount of the state in each animal during the observation period and then averaged in groups to produce a time-weighted frequency histogram.

NREM and REM sleep latencies were calculated as the time from an intervention (cage change or the end of sleep deprivation) to the first epoch of NREM or REM sleep. To measure the duration of sleep cycles, we used an approach similar to that used previously in rats (Trachsel et al., 1991). We defined the sleep cycle as beginning at the onset of NREM sleep and as ending at the offset of REM sleep, allowing for brief awakenings that were no longer than 20 sec. Cycles were considered incomplete and were excluded from analysis if they lacked REM sleep or at least 30 sec of NREM before REM sleep. In both groups, the discarded NREM sleep amounted to 30–40% of total NREM sleep in the 12 hr light period, but only 10% of REM sleep was excluded.

EEG spectral analysis

EEG spectral power was calculated in 0.25 Hz bins using fast Fourier transformation of each 10 sec epoch. Power in the 0.5–25 Hz range of artifact-free epochs was averaged in groups across each behavioral state in the dark and light periods, and the mean values were plotted in 0.25 Hz bins. To measure the effects of sleep deprivation on EEG power, the total power in the delta range during NREM sleep or in the theta range during REM sleep was compared between the first 4 hr after sleep deprivation ended and the corresponding 4 hr interval on the baseline day. This interval was chosen to ensure adequate amounts of NREM and REM sleep for analysis.

Experimental design

To examine the ability of mice to maintain wakefulness, they were tested across a variety of conditions. Baseline recordings were on day 1, cage-change task on day 3, and sleep deprivation on days 6 (2 hr), 9 (4 hr), and 12 (8 hr). On day 15, we turned off the lights in the recording chambers and recorded free-running behavior on day 20. Sleep–wake behavior was analyzed in 10 sec epochs, and body temperature and locomotor activity were recorded every 5 min.

Sleep–wake behavior under a 12 hr LD cycle. To establish the baseline behavior of mice, EEG–EMG recordings were analyzed over 24 hr, beginning at the onset of the dark period (7:00 P.M.).

Behavior in constant darkness. Some researchers have hypothesized that the circadian promotion of wakefulness is impaired in narcolepsy (Kripke, 1976; Broughton and Mullington, 1994; Dantz et al., 1994; Broughton et al., 1998), so we housed mice in constant darkness (DD) for 5 d and then recorded behavior for 48 hr, beginning at 1:00 P.M. the next day. The free-running periods of individual animals were estimated using the body temperature rhythm during the 7 d of DD (Circadia; Behavioral Cybernetics, Cambridge, MA), and all data were aligned according to the circadian time of the animals. The amplitudes of sleep and wake rhythms were calculated using the circadian index ($\text{mean}_{\text{subjective night}} - \text{mean}_{\text{subjective day}} / \text{mean}_{24 \text{ hr}}$) (Lu et al., 2001; Chou et al., 2003).

Sleep–wake behavior with behavioral challenge. We used a cage-change task to examine the ability of mice to maintain wakefulness in a novel environment (Parmentier et al., 2002; Hunsley and Palmiter, 2003). On the regular LD cycle, mice were transferred to identical cages with clean bedding at 1:00 P.M., and behavior was assessed over the next 4 hr.

Total sleep deprivation. To examine the integrity of sleep homeostasis, all mice were sleep deprived by gentle handling for 2, 4, or 8 hr (Franken et al., 1999). The deprivation began at lights on, and behavior was recorded for the next 24 hr. To examine the kinetics of recovery sleep, we calculated the cumulative change in the amounts of NREM and REM sleep. Hourly amounts of NREM and REM sleep on the baseline day were subtracted from those on the sleep deprivation day, and the cumulative deficit was tallied over the recovery period. The NREM and REM sleep recovery rates were calculated for each animal using linear regression across the recovery period, and the rates were then averaged within groups.

Statistical analysis

All results are expressed as means $\pm$ SEM. Hourly changes in behavior, EEG power spectra, and time-weighted bout distributions were compared between WT and KO mice using two-way, repeated measures ANOVA with a *post hoc*, two-tailed Student's *t* test. Pairwise comparisons between WT and KO mice (e.g., sleep–wake amounts, numbers and duration of bouts, number and proportions of state transitions, sleep latencies, and rate and extent of recovery from sleep deprivation) were determined using unpaired, two-tailed Student's *t* tests. Within-group responses to sleep deprivation (dose-dependent effects on the amount and latency of recovery sleep, as well as EEG delta and theta power during sleep) were analyzed using paired, two-tailed Student's *t* tests.

Results

Baseline sleep–wake behavior on an LD cycle

The amounts of wake and sleep in orexin knock-out mice were nearly normal. Compared with WT littermates, orexin KO mice had the same hourly amounts of wake and NREM sleep, using a two-way ANOVA (Fig. 1A). Across time, orexin KO mice had slightly more REM sleep than WT mice (genotype $\times$ time, $F = 1.72$; $p = 0.02$), but this increase was much smaller than reported previously (Chemelli et al., 1999; Willie et al., 2003), most likely because we scored cataplexy as a separate state. When viewed as a percentage of total sleep time, orexin KO mice had slightly more REM sleep during the dark period (12.5 vs 10.3% in WT mice; $p = 0.04$). Nearly all episodes of cataplexy occurred during the dark period, particularly when the amount of REM sleep was $<4\%$ /hr. During the 12 hr dark and light periods, the mean amounts of sleep and wake were almost the same in WT and KO mice, except that, during the dark period, KO mice spent 3.8% of their time in cataplexy and had slightly less wake ($p < 0.05$) (Fig. 1B).

Although the amounts of sleep and wake were almost normal in orexin KO mice, the durations of these behaviors were very short (Fig. 2A). During the dark period, wake bouts lasted an average of only 3.4 min in orexin KO mice compared with 8.2 min in WT mice. Wake bouts were also shorter during the light period, although the difference was less striking. The short wake bouts of orexin KO mice were not merely a result of disruption of wakefulness by cataplexy because transitions into cataplexy accounted for only 15% of all transitions out of wakefulness, during the dark period. Orexin KO mice also failed to produce long bouts of NREM sleep at all times, although REM bouts were only significantly shorter during the light period. The mean duration of cataplexy did not change over time. As expected, episodes of wake and sleep occurred much more frequently, with approximately twice as many wake and NREM bouts during the dark period and smaller increases during the light period (Fig. 2B).

This inability to maintain long bouts of wake may be one of

Figure 1. Orexin KO mice have nearly normal amounts of wake and sleep on a 12 hr LD cycle. *A*, Orexin KO mice have normal hourly amounts of wake and NREM sleep and slightly more REM sleep. Cataplexy occurs almost exclusively during the dark period, especially during times of relatively little REM sleep. *B*, Across the 12 hr dark and light periods, WT and orexin KO mice have similar amounts of NREM and REM sleep. Orexin KO mice have slightly less wake during the dark period, perhaps because of cataplexy during this period. This and all other experiments used seven male orexin KO mice and eight WT male littermates. * $p < 0.05$; ** $p < 0.01$ compared with WT mice.

the most important elements of the narcolepsy phenotype because it could easily account for the daytime sleepiness of people with narcolepsy. To examine the maintenance of wakefulness in greater detail, we analyzed wake bouts during the dark period as a function of bout length (Fig. 2C). WT mice often had wake bouts lasting >2560 sec (42.7 min), particularly during the first few hours of the dark period. Orexin KO mice rarely produced any of these very long bouts but instead had many short- to mid-length bouts. When viewed as a percentage of the total amount of wake during the dark period, 82% of wake occurred in bouts lasting over 1280 sec (21.2 min) in WT mice, but this accounted for only 13% of wake in orexin KO mice. Instead, 80% of their wake occurred in bouts lasting 80–1270 sec (1.3–21.2 min).

The short sleep bouts of orexin KO mice were accompanied by short sleep cycles during the light period. A sleep cycle was defined as the time from the beginning of a NREM sleep episode to the end of the immediately subsequent REM sleep. The average sleep cycle in WT mice lasted 6.4 ± 0.1 min, with some cycles lasting up to 20 min (Fig. 2D). Orexin KO mice had 34% more sleep cycles during the 12 hr light period, and an average cycle lasted 4.0 ± 0.2 min ($p < 0.01$). When viewed as a percentage of all sleep cycles, orexin KO mice had many more cycles lasting < 3 min and fewer cycles lasting > 9 min (genotype $\times$ duration, $F = 12.89$; $p < 0.01$).

These short bouts of sleep and wake in orexin KO mice were accompanied by many more transitions between all states (Fig. 3). However, the relative distribution of transitions between wake, NREM, and REM sleep was normal in orexin KO mice, demonstrating that, with the exception of cataplexy, these mice do not have a bias for REM sleep or any other state.

Although orexin KO mice had fragmented NREM sleep, they had the same distribution of EEG power during NREM sleep, with most energy concentrated in the delta range (Fig. 4). Orexin KO mice also had a normal distribution of EEG power during REM sleep, with normal energy in the theta range. As reported previously (Willie et al., 2003), the spectral distribution of EEG power during cataplexy did not differ from that of REM sleep.

Sleep–wake behavior in constant darkness

Circadian rhythms generated by the SCN control the timing of wake and REM sleep, perhaps partly via projections to the orexin neurons that then relay this information to sleep-regulatory and wake-regulatory regions (Abrahamson et al., 2001; Aston-Jones et al., 2001; Lu et al., 2001; Chou et al., 2003). To determine whether the abnormal sleep–wake behavior of orexin KO mice is a consequence of poor circadian control, we studied mice that were free running in constant darkness.

The mean period of the free-running body temperature rhythm was 24.0 ± 0.1 hr in WT mice and 23.9 ± 0.1 hr in orexin KO mice. When analyzed as a function of circadian time, both groups showed similar free-running sleep–wake behavior, with no statistical difference in the hourly amounts of wake, NREM, or REM sleep (Fig. 5A). During the 12 hr subjective dark and light periods, the mean amounts of wake, NREM, and REM sleep were the same in WT and orexin KO mice (Fig. 5B), except that orexin KO mice had slightly less wake during the subjective light period. The amplitudes of sleep and wake rhythms were the same in both groups (no significant differences in the circadian indices of wake, NREM, and REM sleep). Compared with the LD condition (Fig. 1), both groups had less wakefulness and more NREM sleep at the beginning of the subjective dark period, thus slightly flattening their rhythms of sleep–wake behavior, as has been reported previously (Trachsel et al., 1986). In contrast to the LD condition, orexin KO mice had a small amount of cataplexy (20% of the daily total) during the subjective light period, but the total amount of cataplexy over 24 hr was unchanged.

Figure 2. Orexin KO mice have fragmented wakefulness and sleep on a 12 hr LD cycle. *A*, Orexin KO mice have very short bouts of wake and NREM sleep. REM bouts are slightly shorter only during the light period, and episodes of cataplexy last approximately as long as REM sleep bouts. *B*, Orexin KO mice have a greater number of wake and sleep bouts during the light and dark periods. *C*, During the dark period, most wakefulness in WT mice occurs in bouts longer than 1280 sec (21.3 min), but most wakefulness in orexin KO mice occurs in bouts lasting only 80–1270 sec (1.3–21.2 min). In this time-weighted frequency histogram, the number of bouts of wakefulness has been normalized by the amount of wakefulness occurring over this interval. *D*, After normalizing for the total number of cycles, orexin KO mice have more short sleep cycles and fewer long cycles during the light period. * $p < 0.05$; ** $p < 0.01$.

Figure 3. Orexin KO mice have more transitions between all behavioral states. The mean number of transitions between states is indicated along the arrows between states and by the thickness of the arrows. All transitions are significantly increased in orexin KO mice, and transitions into cataplexy account for only a small number of the transitions out of wakefulness. * $p < 0.05$; ** $p < 0.01$.

The mean duration of wake and NREM sleep bouts was much shorter at all times in the orexin KO mice, and REM bouts were shorter during the subjective light period (Fig. 6A). The number of sleep and wake bouts was substantially increased at all times (Fig. 6B). Analysis of wake bout lengths during the subjective dark showed a pattern very similar to that seen in LD (Fig. 6C): 66% of wake in WT mice occurred in bouts longer than 1280 sec, but 86% of wake in orexin KO mice lasted only 80–1270 sec, with no bouts longer than 2560 sec. This preservation of the rhythms

Figure 4. The distribution of EEG power during NREM and REM sleep is similar in WT and orexin KO mice. During cataplexy, the EEG spectra of orexin KO mice is very similar to that seen in REM sleep. The data include all artifact-free sleep and cataplexy epochs during the 12 hr dark period.

Figure 5. In constant darkness, orexin KO mice have nearly normal amounts of wakefulness and sleep. *A*, Orexin KO and WT mice have similar hourly amounts of wake, NREM, and REM sleep. As in the LD condition, most cataplexy occurs during the subjective dark period. *B*, Across the subjective dark and light periods, orexin KO mice have nearly normal amounts of wake, NREM, and REM sleep, except for slightly less wake during the subjective light period. * $p < 0.05$.

of sleep and wakefulness in constant darkness suggests that impaired circadian timing is not the cause of the brief bouts of wakefulness in orexin KO mice.

Effects of cage change

The propensity of orexin KO mice to have very short bouts of wakefulness could result from a defect in fundamental arousal mechanisms. For example, mice lacking histamine or norepinephrine cannot remain awake as long as WT littermates when placed in a new environment (Parmentier et al., 2002; Hunsley and Palmiter, 2003). To test the integrity of these funda-

mental arousal regions, we placed mice in a new, clean cage at 1:00 P.M. Both WT and orexin KO mice had sudden increases in wakefulness, accompanied by substantial increases in locomotor activity and body temperature (Fig. 7A). The mean latency to the onset of NREM sleep was 48.4 min in the orexin KO mice and 47.0 min in the WT mice (Fig. 7B). These values are in the same range as those observed in WT littermates of histamine- or norepinephrine-deficient mice. Wake bouts of this duration hardly ever occurred in undisturbed KO mice in the light period and were very rare even during the dark period (Fig. 2C). This ability of orexin KO mice to remain awake in a new environment contrasts sharply with the short sleep latencies seen in mice lacking histamine or norepinephrine (Parmentier et al., 2002; Hunsley and Palmiter, 2003).

Orexin KO mice did have some small differences in their response to cage change. First, the latency to the onset of REM sleep was shorter in orexin KO mice (52.2 min after cage change vs 68.9 min in WT mice; $p < 0.01$) (Fig. 7B). Second, WT mice were generally asleep during the 60–80 min period after the cage change, but orexin KO mice were primarily awake during this time, although their behavior was variable (genotype $\times$ time for the 120 min after cage change, $F = 2.78$; $p < 0.01$). Slightly higher levels of locomotor activity (genotype $\times$ time, $F = 3.07$; $p < 0.01$) and body temperature (genotype $\times$ time, $F = 3.60$; $p < 0.01$) accompanied this waking in KO mice. Thus, although orexin KO mice fall asleep as rapidly as WT mice, they wake soon after, perhaps reflecting their poor maintenance of sleep.

Sleep deprivation and recovery sleep

Some researchers have hypothesized that short sleep latencies in narcolepsy result from abnormal sleep homeostasis, with an inappropriately rapid accumulation or intense expression of sleep pressure (Tafti et al., 1992a,b; Besset et al., 1994). To examine sleep homeostasis, we gently handled mice to deprive them of sleep for 2, 4, or 8 hr, beginning at lights on. Gentle handling prevented the mice from sleeping almost completely for 2 hr, but, during the longer sleep deprivations, brief episodes of NREM sleep occurred in both groups. Specifically, during the 8 hr of sleep deprivation, WT and orexin KO mice spent a total of 13 and 15 min in NREM sleep, respectively ($p = \text{NS}$). More stimuli were required to maintain wake in the orexin KO mice because they had an average of 45 transitions into NREM sleep, whereas WT mice entered NREM sleep an average of 28 times ($p < 0.05$). However, the time course of these attempts to sleep was similar in the two groups, with most wake to NREM sleep transitions occurring after 6 hr of sleep deprivation.

In the hours after the sleep deprivation, all animals had more NREM sleep than on their baseline day. For example, after 8 hr of

Figure 6. Orexin KO mice have fragmented sleep and wake in constant darkness. *A, B*, Orexin KO mice have shorter and more frequent bouts of wake and NREM sleep, but these abnormalities are apparent in REM sleep only during the subjective light period. A few episodes of cataplexy occur in the subjective light period. *C*, During the subjective dark period, most wakefulness in orexin KO mice occurs in short- to mid-length bouts. ** $p < 0.01$.

Figure 7. Placing WT and orexin KO in new, clean cages at 1:00 P.M. increases wakefulness, body temperature, and locomotor activity. *A*, After an initial period of wakefulness, both groups return to sleep after 45 min, but, 60–90 min after cage change, orexin KO mice have more wakefulness and a small rise in body temperature. Orexin KO mice also have a smaller initial increase in locomotor activity. *B*, After cage change, orexin KO mice remain awake for over 45 min just as seen in WT mice, but they enter REM sleep more rapidly. * $p < 0.05$; ** $p < 0.01$.

sleep deprivation, both WT and orexin KO mice had 25% more NREM sleep in the subsequent 4 hr, and this rebound sleep persisted into the dark period (Fig. 8). The time course of recovery NREM sleep did not differ between WT and KO mice (genotype $\times$ time, $F = 1.53$; $p = 0.10$). REM sleep was recovered more slowly, perhaps because of the initial preponderance of NREM sleep, and orexin KO mice had slightly more REM sleep than WT mice did during a few hours of the dark period (genotype $\times$ time, $F = 2.77$; $p < 0.001$).

To examine the kinetics of recovery sleep, we calculated the cumulative change in the amounts of NREM and REM sleep compared with the baseline day. With 8 hr of sleep deprivation, both groups of mice accumulated NREM and REM sleep deficits of ~ 240 and 40 min, respectively (Fig. 9A). During the subsequent 16 hr recovery period, these deficits were partially recovered because the animals had higher than normal amounts of sleep. Both groups recovered NREM sleep at the same rate (WT, 6.9; KO, 8.5 min/hr recovery NREM sleep; $p = \text{NS}$) and to the same extent (deficits at the end of the recordings, -126.5 and -128.7 min in WT and KO mice; $p = \text{NS}$). WT and orexin KO mice also recovered their REM sleep deficits at the same rate (WT, 1.7; KO, 1.5 min/hr recovery REM sleep; $p = \text{NS}$) and to the same extent (-15.1 and -19.5 min in WT and KO mice; $p = \text{NS}$).

The duration of NREM sleep bouts often increases after sleep deprivation, and so we determined the length of NREM sleep bouts during the first 4 hr of recovery (Fig. 9B). After 8 hr of sleep deprivation, both groups had NREM sleep bouts that were $\sim 60\%$ longer than during the corresponding period on the baseline day (3:00–7:00 P.M.; WT means: 166 ± 7 and 288 ± 20 sec on the baseline and recovery days, condition $\times$ bouts, $F = 14.48$, $p < 0.001$; KO means: 121 ± 5 and 194 ± 21 sec, $F = 10.65$, $p < 0.001$). Although NREM sleep was more consolidated during recovery, KO mice still had fragmented NREM sleep (genotype $\times$ bouts, $F = 3.90$; $p < 0.001$), with more short bouts of NREM sleep (40–150 sec; $p < 0.01$) and fewer long bouts of NREM sleep (320–630 sec; $p < 0.01$) than WT mice. Thus, even after a period of prolonged wakefulness, orexin KO mice continued to have NREM sleep fragmentation.

Orexin KO mice had normal responses to sleep deprivation using several other measures of sleep homeostasis. With 2, 4, or 8 hr of sleep deprivation, WT and orexin KO mice produced similar amounts of rebound NREM and REM sleep in a dose-dependent manner (Fig. 10A). Sleep deprivation also shortened the latencies to enter NREM or REM sleep in both groups, with shorter latencies in the more sleep-deprived animals (Fig. 10B). Although KO mice tended to fall asleep sooner than WT mice after the sleep deprivation, this effect was statistically significant only in the very short latency to enter REM sleep after 8 hr of sleep deprivation. EEG delta power during NREM sleep may reflect homeostatic sleep need (Borbély and Tobler, 1996), and sleep deprivation dose dependently increased NREM sleep delta power during the first 4 hr of recovery, with no significant difference between WT and orexin KO mice (Fig. 10C). In both groups of mice, this increased delta power declined gradually over the first several hours of the recovery period (supplemental Fig. 2,

Figure 8. Orexin KO mice have nearly normal responses to 8 hr of sleep deprivation. *A*, Compared with the baseline day (open circles), WT mice have a marked increase in NREM sleep after 8 hr of sleep deprivation (filled circles). WT mice also have rebound REM sleep, primarily during the dark period. *B*, Orexin knock-out mice have normal rebound of NREM sleep but relatively more rebound REM sleep during the dark period. Although not statistically significant, cataplexy appears to be less frequent after sleep deprivation. Horizontal brackets mark the sleep deprivation (SD) period. * $p < 0.05$; ** $p < 0.01$ compared with the baseline day. † $p < 0.05$; †† $p < 0.01$ compared with WT mice on the sleep deprivation day.

Figure 9. After sleep deprivation, orexin KO mice recover NREM and REM sleep normally, but NREM sleep is still fragmented. *A*, When compared with the baseline day, both WT and orexin KO mice accumulate a NREM sleep deficit of ~240 min over the 8 hr of sleep deprivation. Both groups then recover from this deficit at the same rate and to the same extent. *B*, At baseline, orexin KO mice have shorter bouts of NREM sleep than WT mice. After 8 hr of sleep deprivation, both groups produce longer bouts of NREM sleep, but orexin KO mice still have shorter than normal NREM bouts. * $p < 0.05$; ** $p < 0.01$.

available at www.jneurosci.org/cgi/content/full/24/28/6291/DC1). As a control, EEG theta power in REM sleep was also measured and showed no changes. Thus, across a variety of measures, orexin KO mice appear to have normal homeostatic responses to sleep deprivation.

Discussion

Excessive daytime sleepiness or an inability to sustain wakefulness is the most disabling symptom for patients with narcolepsy,

and orexin KO mice have very short bouts of wakefulness. These experiments demonstrate that this inability to maintain wakefulness is probably not caused by poor circadian promotion of wakefulness, defective fundamental arousal systems, or abnormal homeostatic regulation of sleep. Because orexin-deficient mice have more transitions between all states, their phenotype may best be described as behavioral state instability, with apparently low thresholds to transition between all behavioral states.

Technical considerations

Across all conditions, we found that orexin KO mice have essentially normal amounts of wake, NREM, and REM sleep, just as seen in people and dogs with narcolepsy (Dantz et al., 1994; Broughton et al., 1998; Nishino et al., 2000). Previous studies of orexin KO mice reported increased REM sleep during the dark period (Chemelli et al., 1999; Willie et al., 2003), but REM sleep may have simply appeared increased because these studies did not separate cataplexy from REM sleep. We operationally defined cataplexy as atonia with EEG theta activity that is immediately preceded and followed by active wake. Without video recordings in all experiments, we may have overlooked some cataplexy, but 80% of all cataplexy episodes can be detected using these EEG and EMG criteria (Willie et al., 2003).

Circadian control of sleep–wake behavior is normal in orexin KO mice

The daytime sleepiness and fragmented sleep of narcolepsy could be caused by impaired circadian control (Kripke, 1976; Dantz et al., 1994; Broughton et al., 1998). Circadian signals help time and consolidate sleep–wake behavior, and the orexin neurons are well positioned to relay circadian information to state-regulatory regions (Abrahamson et al., 2001; Aston-Jones et al., 2001; Lu et al., 2001; Chou et al., 2003). In the absence of orexin, the amplitude of the circadian signals that time sleep–wake behavior might be reduced, resulting in brief episodes of wake, fragmented sleep, and inappropriately timed REM sleep.

Willie and colleagues showed that orexin KO mice have relatively normal timing of sleep and wake when housed in an LD cycle (Willie et al., 2003), but the light cycle might mask any underlying circadian defects. We studied orexin KO mice in constant darkness and found that their body temperature rhythm has a normal free-running period, indicating normal timing in the SCN. More importantly, the amplitude and timing of their sleep–wake rhythms was normal, demonstrating that orexin is unnecessary for the timing of sleep and

Figure 10. Orexin KO mice have normal, dose-dependent responses to sleep deprivation. *A*, The amount of rebound NREM and REM sleep in the 4 hr immediately after sleep deprivation increases in proportion to the duration of sleep deprivation. *B*, In both groups, the latency to enter NREM and REM sleep becomes shorter with increasing durations of sleep deprivation. Latencies are similar between groups, except that orexin KO mice enter REM sleep more rapidly after 8 hr of sleep deprivation. *C*, EEG delta power (0.5–4 Hz) during NREM sleep in the 4 hr after sleep deprivation increases with increasing duration of sleep deprivation in both groups. Theta power (4–9 Hz) during REM sleep does not change with sleep deprivation. * $p < 0.05$; ** $p < 0.01$ compared with other durations of sleep deprivation. †† $p < 0.01$ compared with WT mice.

wake. These results suggest that the fragmented sleep–wake behavior of orexin KO mice is not a consequence of poor circadian control.

Fundamental arousal mechanisms are intact in orexin KO mice

Wakefulness is promoted by ascending projections from aminergic and cholinergic nuclei, including noradrenergic neurons of the LC and histaminergic neurons of the TMN. Mice lacking norepinephrine or histamine fall asleep rapidly after a mild stress, such as transfer to a new cage (Parmentier et al., 2002; Hunsley and Palmiter, 2003), demonstrating that these arousal regions are necessary for responding to challenges. Because the orexin neurons send heavy, excitatory projections to the LC, TMN, and many other arousal regions (Peyron et al., 1998), the short bouts of wake in orexin KO mice might be a consequence of inadequate activation of these fundamental arousal regions.

To test this hypothesis, we transferred mice to a new cage in the middle of their sleep period. We found that orexin KO mice remained awake just as long as WT mice, and their sleep latency was quite similar to WT mice in other studies (Parmentier et al., 2002; Hunsley and Palmiter, 2003). Therefore, even in the absence of orexin, fundamental arousal regions, including the LC and TMN, can be sufficiently engaged by a mild stress for the sustained production of wakefulness. Nevertheless, we cannot conclude that aminergic activity is entirely normal in orexin KO mice under baseline, unstimulated conditions. Extracellular recordings from freely moving orexin KO mice are needed to determine whether the level and pattern of activity in aminergic neurons is normal.

NREM sleep homeostasis is normal in orexin KO mice

Increased sleep pressure from abnormal sleep homeostasis is also hypothesized to cause the excessive daytime sleepiness of narcolepsy. For example, the greatest propensity for napping occurs 2.5 hr earlier in narcoleptics than in control individuals who habitually take a nap (Broughton et al., 1998). In addition, after 24 hr of sleep deprivation, people with narcolepsy have shorter sleep latencies and more stage 3 and 4 NREM sleep compared with controls, suggesting a more rapid accumulation of or heightened sensitivity to sleep pressure in narcolepsy (Tafti et al., 1992b).

To examine this sleep homeostasis hypothesis, we deprived orexin KO mice of sleep and then examined their subsequent recovery. As expected, sleep deprivation dose dependently shortened the latencies to enter NREM or REM sleep, increased the

amounts of NREM and REM sleep, and increased NREM sleep EEG delta power in both WT and KO mice. Across these experiments, the responses of orexin KO mice were entirely normal, except they rapidly entered and had slightly more REM sleep than WT mice after 8 hr of sleep deprivation. In addition, orexin KO mice recovered their NREM and REM sleep deficits at the same rate and to the same extent as WT mice. Thus, consistent with some previous clinical studies (Volk et al., 1990; Dantz et al., 1994), the accumulation and expression of homeostatic “sleep drive” is normal in orexin KO mice.

Behavioral state instability

Our studies primarily focused on the poor maintenance of wakefulness in orexin KO mice, but these mice also have fragmented NREM sleep, even after 8 hr of sleep deprivation. In fact, orexin KO mice have considerably more transitions between all states, as has been noted in people and dogs with narcolepsy (de Barros-Ferreira and Lairy, 1976; Kaitin et al., 1986; Nishino et al., 2000; Mukai et al., 2003). Sleep cycles in orexin KO mice still generally progress from wake to NREM and then to REM sleep, and, with the exception of cataplexy, there is no bias toward any particular state. Thus, the increased number of transitions is not a consequence of pressure for NREM or REM sleep but may represent a process of behavioral state instability in which a breakdown of neural control processes reduces the thresholds to transition between all states.

Reduced activity in arousal regions could explain the frequent transitions from wake into NREM sleep. The orexin neurons may be persistently active during wakefulness (Alam et al., 2002; Li et al., 2002; Eggermann et al., 2003), helping to sustain activity in aminergic and cholinergic arousal regions. In the absence of orexin, these arousal regions may have reduced or vacillating activity, resulting in inappropriately low thresholds to transition into NREM sleep. For example, during the 8 hr of sleep deprivation, orexin KO mice had 60% more transitions into NREM sleep than WT mice, although their sleep homeostasis appears normal.

The frequent awakenings from NREM sleep in orexin KO mice are probably not caused by low sleep drive, although they typically enter sleep after short periods of wake. EEG delta power during NREM sleep rises in relation to sleep propensity (Borbély and Tobler, 1996), and orexin KO mice have normal NREM delta power. In addition, after accumulating substantial sleep pressure with 8 hr of sleep deprivation, their sleep was still fragmented, just as seen in people with narcolepsy (Tafti et al., 1992a,b). Awakenings from NREM sleep also might occur if orexin KO mice rapidly

dissipate sleep pressure, but this appears unlikely because they recovered their NREM deficit at a normal rate after 8 hr of sleep deprivation.

More likely, NREM sleep is disrupted by uncoordinated activity in sleep–wake systems. The orexin neurons are probably inactive during NREM sleep (Estabrooke et al., 2001; Alam et al., 2002), but excitation of arousal regions by orexin during wake might induce lasting changes in neuronal activity that help persistently silence these regions during sleep. Lack of orexin signaling might cause noisy or disorganized activity in downstream aminergic nuclei, which would sporadically inhibit the sleep-producing neurons of the ventrolateral preoptic area (Sherin et al., 1996; Gallopin et al., 2000; Saper et al., 2001). This noise could result in a faster sleep cycle or simply more random state changes. Perhaps, such disorganized activity underlies the ambiguous or “intermediate” sleep of narcolepsy in which atonia or saccadic eye movements occur during NREM sleep and bursts of muscle activity occur during REM sleep (de Barros-Ferreira and Lairy, 1976; Schenck and Mahowald, 1992). Electrophysiologic studies of state control neurons in narcoleptic animals will be needed to determine whether the activity of sleep–wake regulatory regions is truly disorganized.

Long before the discovery of orexin, Broughton hypothesized that the fundamental problem in narcolepsy was a loss of the “neurochemical glues” that help integrate neuronal activity to produce stable sleep–wake behavior (Broughton et al., 1986). Most likely, he was correct because a loss of orexin appears to lower the thresholds to transition between behavioral states, producing the fragmented wakefulness and sleep of narcolepsy.

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