Adrenergic Signaling Plays a Critical Role in the Maintenance of Waking and in the Regulation of REM Sleep

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Adrenergic signaling plays a critical role in the maintenance of waking and in the regulation of REM sleep. J Neurophysiol 92: 2071–2082. 2004. First published June 9, 2004; 10.1152/jn.00226.2004. Many experiments have suggested that the adrenergic system is important for arousal and the regulation of sleep/wake states. Electrophysiological studies have found strong correlations between the firing of adrenergic neurons and arousal state. Lesions of adrenergic neurons have been reported to cause changes in sleep/wake regulation, although findings have been variable and sometimes transient. To more specifically address the role of adrenergic signaling in sleep/wake regulation, we performed electroencephalographic and electromyographic recordings in mice with a targeted disruption of the gene for dopamine β-hydroxylase, the enzyme that converts dopamine to noradrenaline. These mice are unable to synthesize the endogenous adrenergic ligands norepinephrine and epinephrine. The mutant mice sleep ∼2 h more each day. The decrease in waking is due to a considerable decrease in the duration of waking bouts in spite of an increase in the number of waking bouts and transitions from sleep to waking. In contrast, the amount of rapid-eye-movement (REM) sleep is only half that in control mice due to a decrease in the number and duration of REM sleep bouts. Delta power is selectively increased in the mutant mice, and there is much less variation in non-REM sleep delta power over 24 h. After 6 h of total sleep deprivation during the first half of the light period, there is no rebound recovery of sleep time (e.g., delta power over 24 h). After 6 h of total sleep deprivation during the first half of the light period, there is no rebound recovery of sleep time (e.g., delta power over 24 h).

Introduction

Many studies have suggested that the broadly projecting adrenergic system of the CNS may play an important role in sleep/wake regulation (Berridge and Waterhouse 2003; Jouvet 1969; Pace-Schott and Hobson 2002). Neurons of the locus coeruleus (LC), the largest adrenergic nucleus, fire at their highest rates during active waking, less during quiet waking, even less during non-rapid-eye-movement (NREM) sleep, and cease firing altogether during REM sleep (Aston-Jones and Bloom 1981; Foote et al. 1980; Hobson et al. 1975). Microdialysis studies examining extracellular norepinephrine (NE) levels support these results (Park 2002; Shouse et al. 2000). Intracerebroventricular injections of NE or epinephrine (E) can elicit prolonged wakefulness (Cordeau et al. 1971; Matsuda 1968) but also can cause marked behavioral depression (Feldberg and Sherwood 1954; Haley and McCormick 1957). Importantly, pharmacologically decreasing LC activity decreases arousal as measured by electroencephalographic (EEG) recordings in anesthetized rats, whereas increasing LC activity increases the level of EEG arousal (Berridge and Foote 1991; Berridge et al. 1993). In addition, NE has pronounced effects on hypothalamic (Gallopin et al. 2000), thalamic (McCormick et al. 1991), and basal forebrain regions (Berridge and Foote 1996; Berridge and O’Neill 2001; Mallick and Alam 1992) thought to be critical for the regulation of cortical arousal. Finally, activation of a number of transcription factors and immediate early genes associated with waking are thought to be positively regulated by the adrenergic system (Cirelli and Tononi 2000; Cirelli et al. 1996).

The preceding studies are suggestive but do not address the actual roles of endogenous adrenergic signaling in spontaneous sleep/wake regulation, however. To address this, various approaches have been used to interfere with adrenergic signaling in unanesthetized animals. Results from these studies have been contradictory (Table 1). Potential caveats of these approaches are the specificity and efficacy of the treatment used to implicate NE/E. NE synthesis inhibitors either reduce dopamine simultaneously (e.g., a-methyl-p-tyrosine, a tyrosine hydroxylase inhibitor), or interfere with other copper-dependent enzymes in addition to dopamine β-hydroxylase (DBH) (Allain and Kri 1991; Delmaestro and Trombetta 1995; Grassi Zucconi et al. 2002). Electrolytic and neurotoxic lesions of brain stem adrenergic neurons or their projections can be incomplete and nonspecific and often are transient because of recovery mechanisms (Abercrombie and Zigmond 1989; Acheson et al. 1980; Chiodo et al. 1983; Gage et al. 1983; Hughes and Stanford 1998). The use of selective adrenergic receptor antagonists can be instructive, but they must be applied at the correct location, dose, and time and be for the appropriate receptor subtype(s) of which there are at least nine (Hoffman and Lefkowitz 1996). For these reasons, we created a model with which to investigate the roles of adrenergic signaling that avoids many of these issues by generating mice with a targeted disruption of the dopamine β-hydroxylase gene (Dbh). Genetic ablation of this enzyme results in the complete absence of NE/E in the mutant (Dbh−/−) mice (Thomas et al. 1995, 1998) without disturbing the innervation pattern of the adrenergic neurons themselves (Jin et al., 2004). Here we investigate the regulation of sleep/wake state and sleep homeostasis in these mice.

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Animals

The mice were hybrids of C57BL/6J and 129/SvCPJ. Dbh<sup>+/−</sup> males were mated with Dbh<sup>+/−</sup> males and treated with 100 µg/ml each of phenylephrine and isoproterenol (Sigma, St. Louis, MO) from embryonic day (E)9.5 to E16.5 and 2 mg/ml of L-threo-3,4-dihydroxyphenylethyl-2-bromobenzylamine; IP, intraperitoneal; Synch, EEG synchronization was observed at times. —, no change; ↑, increase with treatment; ↓, decrease with treatment; *, changes were transient; †, examined behavioral state over only 90 min.

**Methods**

**Spontaneous locomotor activity**

Each mouse (total of 16 for each genotype) was placed in an opaque Plexiglas box (38 × 38 × 38 cm) at the beginning of the light period. The activity of four mice in adjacent boxes was simultaneously recorded using the initial-reference-frame tracking mode of the SMART image analysis system (San Diego Instruments, San Diego, CA), in conjunction with a Sony Camcorder (CCD-TRV57) in infrared recording mode. Continuous infrared illumination was provided by two infrared bulbs (75 W, Coralife reptile nightlight) ~1 m above the boxes. Food and water were provided ad libitum in one corner of the box. Ground cloth was provided in one well of a 12-well tissue culture dish taped to the floor (whole food pellets could not be used because their dislocation would disturb tracking of the mice). Cotton was provided as bedding material. The boxes were cleaned at visible-light-hours (12 h cycle) between days while the mice were housed in a holding cage. Activity reaches baseline within 4 h of the mice being placed in the boxes the first day.

**Surgery**

Mice separate from those used for activity monitoring were implanted with four EEG electrodes and two electromyographic (EMG) electrodes under anesthesia (~70 mg/kg pentobarbital sodium ip). Four holes were made in the skull for EEG electrodes with a hand-held 1-mm drill bit (2 mm bilateral to midline and 1.5 mm posterior to bregma; 3 mm bilateral to midline and 2 mm anterior to lambda). Ball-type EEG electrodes were inserted into the holes over the surface of the neocortex, and EMG electrodes were placed under the nuchal musculature. Electrodes were anchored to the skull with dental ceramic (P-10, 3M, Minneapolis, MN), and the recording leads were counter-weighted. EEG and EMG electrodes were fashioned as described (Veasey et al. 2000). Briefly, electrodes were formed from Teflon-coated silver wire (Φ = 0.33 mm), soldered to gold socket contacts (Plastics One, Roanoke, VA) and pushed into a 6-pin plug (363 plug, Plastics One), which was then attached to a fully rotating commutator (SLC6, Plastics One). Mice were injected immediately postsurgery with 5 µg/kg gentamycin.

**EEG/EMG recordings**

Mice were allowed 10–14 days to recover from surgery and were habituated to the recording apparatus (standard Plexiglas mouse cages, 17 × 28 cm) for 2–3 days before experimentation. Recordings of EEG and EMG signals were obtained under a 12-h light/dark cycle (lights on from 8 AM to 8 PM; soft white light, 60 W, 840 lm, ~1 m above cages). EEG signals were filtered at 0.3 and 30 Hz (1/2 amplitude, 6 dB/octave) and EMG signals were filtered at 1 and 100 Hz (12 A5 amplifier, Astro-Med, West Warwick, RI), and both of these signals were amplified and sampled at 100 Hz. Combinations of four EEG electrodes were viewed polygraphically (Grass 12-32-35S, Neurodata Acquisition, Astro-Med) using an electrode selector board (12 PB 36 Electrode Selector, Astro-Med) to determine the optimal recording sites for best identifying waking, NREM and REM sleep. The electrode derivations were randomly distributed within and between genotypes. Signals were processed with an A/D board (Converter 4801A, ADAC) and acquired with a PC using ACQ 3.4 software (Benington et al. 1994; Veasey et al. 2000). EEG and EMG signals were recorded simultaneously and stored on optical disks. Before the beginning of the recordings, a calibration signal (50 µV) was recorded on all of the EEG and EMG channels.

**Sleep latency**

The latency to quiet wakefulness and NREM sleep were measured. Five hours after lights-on, mice were subjected to 20 min of sleep deprivation by gentle handling. After this initial deprivation, mice were left undisturbed for 10 min. The times for a mouse to enter a 10-s

### TABLE 1. Summary of effects of adrenergic lesions on behavioral state

<table>
<thead>
<tr>
<th>Reference</th>
<th>Lesion</th>
<th>Species</th>
<th>W</th>
<th>N</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jouvet (1969)</td>
<td>LC, Surgical</td>
<td>Cat</td>
<td></td>
<td></td>
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<tr>
<td>Jones et al. (1973)</td>
<td>LC, Electolytic</td>
<td>Cat</td>
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<tr>
<td>Jones et al. (1977)</td>
<td>LC, Electolytic</td>
<td>Cat</td>
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<tr>
<td>Hartmann et al. (1971)</td>
<td>6-OHDA, ICV</td>
<td>Rat</td>
<td></td>
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<tr>
<td>Laguzzi et al. (1972)</td>
<td>6-OHDA, ICV</td>
<td>Cat</td>
<td></td>
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<tr>
<td>Matsuyama et al. (1973)</td>
<td>6-OHDA, ICV</td>
<td>Rat</td>
<td></td>
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<tr>
<td>Hansen and Whishaw (1973)</td>
<td>6-OHDA, ICV</td>
<td>Rat</td>
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<tr>
<td>Howard and Breese (1974)</td>
<td>6-OHDA, ICV</td>
<td>Cat</td>
<td>↑</td>
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<tr>
<td>Panksepp et al. (1973)</td>
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<td>Cat</td>
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<tr>
<td>Lidbrink (1974)</td>
<td>6-OHDA, D/VAB</td>
<td>Rat</td>
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<tr>
<td>Leconte and Hennevin (1981)</td>
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<td>Rat</td>
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<td>Gonzalez et al. (1998)</td>
<td>DSP-4, IP</td>
<td>Rat</td>
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</table>

Not all studies examined effects over time, so some changes may have been transient but are not indicated as such. LC, locus coeruleus; 6-OHDA, 6-hydroxydopamine; ICV, intracerebroventricular; VAB, ventral adrenergic bundle; D/VAB, dorsal and ventral adrenergic bundles; DSP-4, N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine; IP, intraperitoneal; Synch, EEG synchronization was observed at times. —, no change; ↑, increase with treatment; ↓, decrease with treatment; *, changes were transient; †, examined behavioral state over only 90 min.
bout of inactivity and a 10-s bout of NREM sleep were determined. Mice that did not enter quiet waking or fall asleep during this period were assigned quiet waking or sleep latencies of 10 min, respectively. The mice were then subjected to 10 min of additional sleep deprivation, and latencies were again determined. This was repeated for a total of five measurements from each mouse. The mean of these measurements was used as the latency for that individual when determining group statistics.

**Sleep deprivation**

Three consecutive 24-h recordings of EEG and EMG signals were obtained. The first 2 days of recordings served as baseline, and on day 3, mice were subjected to 6 h total sleep deprivation beginning at lights-on, when they normally initiate their most intensive sleep period (Franken et al. 1999). EEG and EMG signals were recorded for the remaining 18 h. Deprivation was attained by making noise, introducing objects into the cage, and gently blowing air whenever the animals looked drowsy, attempted to engage in a sleeping posture, or when the EEG showed signs of low-frequency signals. Sleep/wake states were scored for the next 12 h after deprivation, and these data were compared with the baseline recordings from the same mice.

**Data analysis**

Behavioral states were classified off-line as waking or NREM or REM sleep on the basis of the EEG and EMG signals. Twenty-three mice from each genotype had recordings deemed sufficiently good to permit characterization. States were determined for consecutive 10-s epochs by the visual inspection of the EEG and EMG signals, which were displayed on a PC screen. Scoring was aided by the display of the fast-Fourier transform of the EEG and the components of the EEG above and below 4 Hz for each epoch. Wake was characterized by low-amplitude, high-frequency EEG with a high EMG signal. NREM sleep was characterized by high-amplitude, low-frequency EEG with a low EMG signal. REM sleep was characterized by low-amplitude, high-frequency EEG with a high EMG signal. Arousal states were expressed as a percentage of total recording time. Baseline values are the average of the first 2 days of recording.

The distribution of bout duration in the arousal state was analyzed according to published criteria (Franken et al. 1999). Bouts were allotted to 1 of 10 bins of exponentially increasing size according to the published criteria (Franken et al. 1999). Bouts were then subjected to 10 min of additional sleep deprivation, and latencies were again determined. This was repeated for a total of five measurements from each mouse. The mean of these measurements was used as the latency for that individual when determining group statistics.

**Arousal states**

Arousal states were scored for the next 12 h after deprivation, and these data were compared with the baseline recordings from the same mice.

**Statistical analysis**

A mixed-model ANOVA was used to simultaneously model the between-subjects factor of genotype and the within-subjects factors, including time period, type of bout (for transitions among sleep/wake states), power frequency (from EEG power density transformations), and sleep deprivation. The within-subjects factors were modeled as nominal random effects to perform analyses without making specific assumptions about the trends over time, bout size, or frequency (Diggle et al. 2002; Singer 1998). Thus the mixed-effects model was estimated using restricted maximum likelihood (Diggle et al. 2002) as implemented in the SAS procedure Proc.Mixed (SAS 1999) to account for correlations among replications. To investigate the effect of sleep deprivation, underlying experimentally induced changes over time were assessed by comparison with a control group without deprivation. Thus the primary hypotheses involved genotype by sleep-deprivation interactions for each of the sleep/wake states.

A separate ANOVA was performed for each of the sleep/wake variables under consideration. The ANOVA determined whether there was a significant main effect for each factor in the model as well as any higher-order interactions. If no higher-order interactions were significant, a reduced model was retained. If there was a significant interaction involving genotype, separate analyses were performed for each genotype. Bonferroni-adjusted post hoc contrasts were computed to maintain the α level over the entire set of comparisons (Morrison 1983). For all graphs and tables, * is \( P < 0.05 \); ** is \( P < 0.01 \); *** is \( P < 0.001 \) for between genotype comparisons within the same condition or between sleep deprivation and baseline. All values are means ± SE.

**RESULTS**

**Spontaneous locomotor activity**

Because adrenergic signaling has been implicated in regulating arousal, we first examined spontaneous activity in unoperated mutant and control mice. To assess total activity levels and the diurnal variation of activity, spontaneous locomotor activity was recorded over a 2-day period after introduction into the recording apparatus. There were no differences between the genotypes in either overall activity or circadian regulation of activity (Fig. 1). Similar results were obtained when voluntary wheel-running was monitored in activity cages (not shown).

**Sleep/wake states**

Representative recordings for each state are shown for the two genotypes in Fig. 2. During 24 h, Dbh\(^{+/-}\) mice were...
awake for \( \sim 2 \) h more than \( Dbh^{-/-} \) mice (13.0 \( \pm \) 0.34 and 10.8 \( \pm \) 0.47 h, respectively, \( P < 0.001 \)). The decrease in waking in the mutants corresponded with a 3-h increase in NREM sleep \((Dbh^{-/-}, 9.4 \pm 0.27 \) h and \( Dbh^{-/-}, 12.4 \pm 0.45 \) h, \( P < 0.001 \)) and a nearly 1-h decrease in REM sleep \((Dbh^{-/-}, 1.57 \pm 0.12 \) and \( Dbh^{-/-}, 0.68 \pm 0.08 \) h, \( P < 0.001 \)). When expressed as a percentage of total sleep time, REM sleep accounted for 14.3\% in controls and only 5.2\% in the mutants.

Analysis of hour by hour data indicated that there were significant interactions between genotype and time of day for NREM sleep and REM sleep (Fig. 3). Because the last 6 h of the dark period appeared to be less affected by the absence of NE/E for waking and NREM sleep, the percent time in each behavioral state was analyzed in 6-h blocks. In this case, the interaction between genotype and time of day was significant for each behavioral state. Post hoc analysis indicated that the decrease in waking and the increase in NREM sleep were present at all times except the last 6 h of the dark (Fig. 3). While the decrease in REM sleep was significant at all time periods, there were significant changes in the magnitude of this difference with time of day.

To better understand the mechanism by which adrenergic signaling influences time in each behavioral state, the distribution of bouts for each state was analyzed according to duration and number. Over 24 h, the mean durations of waking bouts and REM sleep bouts were significantly decreased in the \( Dbh^{-/-} \) mice (Fig. 4A). Interestingly, the number of waking bouts and NREM sleep bouts were both increased in the \( Dbh^{-/-} \) mice, whereas there was a nonsignificant \( (P = 0.1) \) decrease in the number of REM sleep bouts (Fig. 4B). The decrease in waking bout duration combined with an increase in the number of waking bouts indicated that the \( Dbh^{-/-} \) mice entered waking more often but were not able to maintain waking as readily as control mice. To examine this more directly, the number of transitions to and from each state was also analyzed. The greatest number of transitions occurred between the two most abundant states, waking and NREM sleep. Both of these transitions (waking to NREM sleep and REM sleep)
NREM sleep to waking) were significantly increased in the Dbh−/− mice (Fig. 4C).

The preceding data indicated that the primary cause for decreased waking in the mutant mice was a decrease in the mean duration of waking bouts, which outweighed the increase in the number of bouts. To examine this more closely, the relative distribution of bout lengths was plotted for each state. Because significant differences in total state times by genotype varied with time of day (Fig. 3), this was analyzed in 6-h blocks. As a percentage, more time was spent in shorter duration bouts for NREM sleep as indicated by the mutants spent a significant difference in total state times by genotype (Fig. 4). For waking, the decrease in the time spent in long-duration bouts in the mutants was offset by the increase in the time spent in short-duration bouts only during the last 6 h of the dark period, in keeping with the observation that total waking during this period was not significantly different between genotypes (Fig. 3).

Sleep latency

Based on the findings of decreased waking and shorter bouts of waking in the mutant mice, we predicted that the latency to NREM sleep after a period of imposed waking would be significantly decreased in these mice. This was examined by waking the mice in the middle of the light phase and then keeping them awake with gentle handling for a short period (10–20 min) before allowing them to fall asleep again. The measurement was repeated several times for each mouse. There was no significant difference in the latency of the mutant mice to become inactive compared with the controls (Dbh+/−: 2.6 ± 1.0 min; Dbh−/−: 1.4 ± 0.2 min; n = 7 each, P > 0.2). Importantly, there was a significant decrease in the latency to NREM sleep in the mutant mice that was less than half that for controls (Dbh+/−: 6.7 ± 0.8 min; Dbh−/−: 3.1 ± 0.5 min; n = 7 each, P < 0.01).

Spectral analysis of EEG

To examine whether adrenergic signaling regulates global CNS electrical activity, we performed spectral analysis of the EEG data. Because the power spectrum differs between sleep/wake states and the amounts of these states differ between genotypes, we performed spectral analysis of the EEG data within each state. Analyses indicated that for each state there was a highly significant interaction between genotype and frequency (P < 0.0001, Fig. 6). When the power spectrum was grouped into delta, theta and sigma frequency ranges, there was a nonsignificant increase in absolute delta power during waking (P = 0.08) and a significant increase in absolute delta power during NREM sleep (P < 0.05) and in relative delta power during REM sleep (P < 0.01) in the Dbh−/− mice (Fig. 6). In addition, there were significant decreases in relative theta and sigma powers during REM sleep (P < 0.05). Differences between genotypes were also evident when inspecting individual EEG records, particularly for REM sleep (Fig. 2). A shift in the peak frequency of theta to lower frequencies was present in the Dbh−/− mice (Fig. 6). This was most apparent for REM sleep, where the peak for the Dbh−/− mice was 7.2 ± 0.14 Hz and the peak for the Dbh+/− mice was 6.1 ± 0.19 Hz (P < 0.001, n = 10 each). We also analyzed power spectra in 6-h blocks to examine whether there were time-of-day-dependent genotype differences as were observed for total time in each state.
behavioral state. Similar data were present in all four 6-h blocks for each behavioral state, including the last 6 h of the dark (not shown).

A potential concern arising from the preceding results is whether epochs of waking were incorrectly scored as NREM sleep in the mutant mice, leading to artifactually elevated levels of NREM sleep. For example, the increase in delta power going from waking to NREM sleep was 32% in controls but only 20% in mutants (Fig. 6). However, behavioral state scoring relies on the EMG as well as the EEG, and there was a significant difference in the mean integrated EMG between waking and NREM sleep in the mutant mice, the latter being only 30% of the former. To explore this further, state transitions during sleep latency analysis in which activity of the mouse and electrophysiological data were simultaneously recorded. State transitions between waking and NREM sleep were readily apparent in the Dbh−/− mice (Fig. 7). Transitions from waking to NREM sleep included short epochs of inactive waking, indicating that inactive waking could be distinguished from NREM sleep in the mutant mice. Together these observations suggest that behavioral state scoring of the Dbh−/− mice was reliable.

As an indicator of the regulation of basal sleep homeostasis, NREM sleep delta power was analyzed over 24 h. Control mice exhibited a decrease in NREM sleep delta power toward the end of the light period relative to the beginning (Fig. 8). They also exhibited an increase in NREM sleep delta power during the first half of the dark period. In contrast, Dbh−/− mice exhibited much less variation in NREM sleep delta power across 24 h with some increase occurring only toward the end of the dark period.

**Sleep deprivation**

To examine whether adrenergic signaling contributes to sleep homeostasis, mice underwent total sleep deprivation for 6 h and behavioral states were quantified during the subsequent 12 h. The interaction of genotype and sleep deprivation was statistically significant for all three behavioral states. In the control mice, the effect of sleep deprivation was manifest in several ways. For the first 3 h after sleep deprivation (still in the light period), there was a decrease in REM sleep relative to baseline (Fig. 9). For the first 3 h of the dark period, there was an increase in both NREM and REM sleep and a decrease in waking relative to baseline. Sleep returned to normal levels during the next 3 h of the dark period. In contrast, the mutant mice failed to exhibit significant changes in the amount of sleep after deprivation. NREM-sleep delta power has been shown to be elevated for 2–3 h immediately after sleep deprivation in mice (Franken et al. 1999), and this is thought to

**FIG. 5.** Distribution of bout durations. The data were analyzed in 6-h blocks due to the variation in phenotype according to time of day (Fig. 4). Left: examples of detailed bout duration distributions for the first 6-h light period. For each arousal state, the relative time at various bout lengths is shown. x-axis numbers are the lower limits for the number of epochs for bout durations in that bin; bout durations range from that number up to but not including the lower limit for the next bin. Bout durations were grouped into short (S) and long (L) for subsequent analysis of all 4 time blocks (right). For each genotype, percent time within each state totals 100%. For all states, the main effect of bout duration was P < 0.0001. For waking and REM sleep, P < 0.0001 for the interaction of genotype and bout duration, whereas for NREM sleep, P < 0.05 for the interaction (n = 23 for each genotype). Right: total time spent in S and L duration bouts in 6-h intervals as indicated above the bars. All main effects and interactions were P < 0.0001 except the interaction between genotype and time period (P < 0.01) and the main effect of short vs. long bout duration (not significant; n = 23 for each genotype).
reflect a change in the intensity of sleep during recovery from deprivation (Borbely 1982). Therefore NREM-sleep delta power was examined over the 3-h period immediately after sleep deprivation as another indicator for processes related to sleep homeostasis. The increase in NREM-sleep delta power after deprivation was significant in control (22.0 ± 6.0%, n = 6, P < 0.05) and mutant (27.2 ± 11.2%, n = 6, P < 0.05) mice but not between genotypes (P = 0.7).

DISCUSSION

This study determines the overall roles for adrenergic signaling in sleep/wake regulation by examining mice in which NE/E are absent due to targeted disruption of Dbh. Results from these mice provide strong support for the hypothesis that adrenergic signaling maintains arousal, specifically by extending the duration of periods of wakefulness in mice. Our results support a model whereby a primary effect of adrenergic signaling is to decrease the probability of making the transition from waking to NREM sleep. This is consistent with our observation of an increase in the number of transitions from waking to NREM sleep and a reduction in the duration of waking bouts in the Dbh−/− mice. It appears that adrenergic signaling is not required for the initiation of arousal because the number of transitions from NREM sleep to waking is significantly increased in the Dbh−/− mice. This is interesting because there is a short burst of activity in LC neurons just

FIG. 6. EEG power spectra over 24 h. Shown are the absolute spectra for each behavioral state and the relative spectra for REM sleep. The latter is shown because of the large variation in absolute power between mice for REM sleep. The main effect of frequency and the interaction of frequency and genotype were significant for each plot (P < 0.0001, n = 10 each), whereas the main effect of genotype was not. When power was analyzed by frequency range (delta: 1.0–4.1 Hz; theta: 5.1–9.0 Hz; sigma: 10.2–14.1 Hz), the interaction of frequency and genotype was significant for NREM sleep (P < 0.01) and REM sleep (P < 0.02) but not waking (P = 0.12).

FIG. 7. Representative state transitions in a Dbh−/− mouse. The mouse first became inactive at ~8 min and then entered NREM sleep 30 s thereafter as defined by a simultaneous rise in delta power and fall in the integrated EMG. About 1 min later the mouse woke up and briefly became active, simultaneous with a reversal of the EEG/EMG changes. The mouse then entered inactive waking again for 30 s, followed by NREM sleep for the remainder. Changes in delta power and integrated EMG clearly distinguish NREM sleep from waking.

FIG. 8. Variations in NREM sleep delta power. Absolute delta power during NREM sleep is shown across 24 h, relative to the 1st 3 h of the light period. The main effect of time of day and the interaction of time of day and genotype were significant (P < 0.002, n = 10 each), whereas the main effect of genotype was not.
before the transition from NREM sleep to waking, which suggested that LC neurons might have been partly responsible for the transition (Aston-Jones and Bloom 1981).

Variations in the presence and magnitude of the behavioral state phenotypes according to the time of day may indicate that adrenergic signaling plays a role in circadian output (Aston-Jones et al. 2001; Gonzalez et al. 1998). However, the major diurnal variation in activity and sleep/wake states was apparent for the Dbh+/− and Dbh−/− mice. It is also interesting to note that even though the Dbh−/− mice slept 2 h more per day, this had no impact on their locomotor activity, suggesting that regulation of sleep and activity could be dissociated. Because different mice and environments were used for the two measurements, we cannot rule out the possibility that this dissociation did not occur, however. Based on our results, we hypothesize that NE/E is most critical for maintaining arousal during quiet (inactive) waking and that in the absence of NE/E, a selective reduction in quiet waking reduces waking bout durations but does not alter overall activity.

Our results concerning sleep/wake regulation are not entirely consistent with any adrenergic lesion study (Table 1). The differences between these studies and ours are most likely related to differences in the methods of NE/E depletion with the lack of effects in some studies being due to insufficient or locally restricted depletion and the transient effects in other studies being due to the recovery of adrenergic function over time (Abercrombie and Zigmond 1989; Acheson et al. 1980; Chiodo et al. 1983; Gage et al. 1983; Hughes and Stanford 1998). In about one-third of the studies, EEG synchronization during waking was reported. The increase in delta power in the Dbh−/− mice is consistent with this. Overall, our data indicate that the adrenergic system regulates global CNS electrical activity, independent of sleep/wake state, and support pharmacologic studies suggesting a role for NE/E in regulating such activity (Cape and Jones 1998; Delagrange et al. 1989; Itil and Heller 1995; Berridge and Espana 2000; Hilakivi and Leppavuori 1984; Kleinlogel 1989; Makela and Hilakivi 1986; McCormick et al. 1991; Roubicek 1976; Torbati 1986).

Considering which adrenergic receptor(s) may be influencing waking and NREM sleep, regulation seems likely to be mediated at least in part by α1-adrenergic receptors. The α1-agonist methoxamine has been shown to increase waking (Hilakivi and Leppavuori 1984; Monti et al. 1988; Pellejero et al. 1984). However, the α1-selective antagonist prazosin has often been reported to have no effect on waking (Benington and Heller 1995; Berridge and Espana 2000; Hilakivi and Leppavuori 1984; Kleinlogel 1989; Makela and Hilakivi 1986; Pellejero et al. 1984). β-adrenergic receptors seem less likely to be involved because the β-selective agonist clenbuterol typically decreases waking and increases NREM sleep (Monti et al. 1988), whereas the β-selective antagonist propranolol either increases waking or has no effect (Betts and Alford 1985; Hilakivi 1983; Kostis et al. 1990; Lanfumey et al. 1985; Zamboni et al. 1990). More consistent wake-promoting effects have been observed with adrenergic agonists (α1 and/or β) applied locally to the medial preoptic area of the hypothalamus or the medial septum (Berridge and Foote 1996; Berridge and O’Neill 2001; Mallick and Alam 1992). However, locally applied antagonists do not support a role for tonic adrenergic
signaling in the medial preoptic area in maintaining arousal (Mallick and Alam 1992). The medial septum has not been studied in this manner.

An important region in sleep/wake regulation is the ventrolateral preoptic area (VLPO). This region is unusual in that it is active during sleep (Sherin et al. 1996). The VLPO is primarily composed of inhibitory neurons containing GABA and galanin that have robust projections to wake-active nuclei containing acetylcholine, histamine, serotonin, and NE. A bistable model of reciprocal inhibition has been proposed between the VLPO and several of the wake-active neurotransmitters, including NE (Gallop et al. 2000; Saper et al. 2001). Selective lesions of the VLPO cause chronic insomnia and result in shorter but more frequent bouts of NREM sleep (Lu et al. 2000). The Dbh−/− mice have an “opposite” phenotype of chronic somnolence and shorter but more frequent bouts of waking. This is consistent with the adrenergic system opposing the actions of the VLPO in a bistable reciprocal inhibition model (Saper et al. 2001). Adrenergic innervation to the hypothalamus, including the VLPO, is derived primarily from the A1 and A2 brain stem adrenergic nuclei (Palkovits et al. 1980).

Strengths of the Dbh−/− model include its specificity and completeness for the loss of NE/E. A potentially important drawback is the chronic nature of the model, which may lead to compensation or dysregulation of downstream systems. It would be interesting to compare results derived from the mutant mice to those from acute pharmacologic manipulations. In one study, a combination of α1- and β-adrenergic receptor antagonists was administered to investigate their effects on behavioral states following restoration of NE in the mutant mice (Hunsley and Palmiter 2003). No differences in total wake time were observed between vehicle and drug treatments, although EEG changes were apparent. It is difficult to make a direct comparison because our study measured spontaneous changes in behavioral state over a 2-day period in a familiar environment, whereas the pharmacologic study examined effects over a 30-min period during habituation to a novel environment.

It is also of interest to compare our results to a recently published analysis of sleep/wake regulation in Dbh−/− mice (Hunsley and Palmiter 2003). In that study, no differences in behavioral state times were observed. The basis for the differences in results from the two studies is not clear. They analyzed behavioral state in six pairs of mice during the first 6 h of the light and dark periods, 24 h after placing the mice in the recording apparatus. We analyzed 23 pairs of mice over a 48-h period 48–72 h after placing the mice in the recording apparatus. It is also possible that differences in the composition of the genetic background might contribute to the dissimilar results. While both colonies are a mix of C57BL/6J and 129/SvCPJ inbred strains, the proportion of each may have diverged, especially after rederivation of our colony after relocation. However, a follow-up study comparing nine pairs of Dbh+/+ and Dbh−/− mice over a 24-h period found significant decreases in waking and increases in NREM sleep that were similar in magnitude to our results (M. S. Hunsley and R. D. Palmiter, personal communication). Thus it seems likely that technical and possibly genetic factors may be relevant.

The decrease in REM sleep in the Dbh−/− mice is striking and, perhaps, surprising. That LC neurons essentially stop firing during REM sleep led to a hypothesis that the lack of adrenergic signaling plays a permissive role in the activation of REM sleep. If this hypothesis was correct, then one would expect that the Dbh−/− mice would have increased (or unaltered) REM sleep, which they do not. Therefore we believe that the regulation of REM sleep by adrenergic signaling is more complex. One hypothesis is that as NE/E levels fall during NREM sleep, the decrease in adrenergic activity would promote the initiation of REM sleep. A second possibility is that for REM sleep to occur, extracellular NE/E levels must be within a critical low (but nonzero) window and that levels above or below this window inhibit REM sleep. Microdialysis studies support this possibility because extracellular NE levels are at their lowest during REM sleep but are detectable (Park 2002; Shouse et al. 2000).

Our results indicating that some adrenergic signaling is necessary to facilitate REM sleep are supported by results from human studies. A small number of patients with sympathetic autonomic failure have congenital DBH deficiency, and EEG sleep studies have been performed on two of these patients. Their REM sleep time was lower than normal due to a decrease in the length of REM sleep bouts (Tulen et al. 1990). When these two patients were treated with L-DOPS to restore NE, REM sleep increased due to an increase in the duration of REM sleep bouts (Tulen et al. 1991). These data indicate that the changes in REM sleep were due to a physiological requirement for NE/E rather than sequelae arising from chronic adrenergic deficiency during development. Future studies examining behavioral states following restoration of NE in the Dbh−/− mice would further test this idea.

Our results indicating a role for adrenergic signaling in the regulation of REM sleep are also supported by some studies using adrenergic receptor antagonists. Specifically, the β antagonist propranolol has been shown to decrease REM sleep in rats, cats, and humans (Betts and Alford 1985; Hilakivi 1983; Lanfumey et al. 1985). Results with the α1 antagonist prazosin in rats have been inconsistent (Benington and Heller 1995; Kleinlogel 1989; Makela and Hilakivi 1986; Pellejero et al. 1984).

The greatly reduced variation in NREM-sleep delta power during baseline recordings suggested that sleep homeostasis might be altered in the Dbh−/− mice. To explore this further, we examined sleep homeostasis in the Dbh−/− mice after inducing sleep deprivation using gentle handling. Surprisingly, the increase in NREM sleep delta power during the recovery period immediately after deprivation was present in both genotypes. In contrast, the increase in NREM sleep and the alterations in REM sleep observed in control mice after deprivation were absent in the Dbh−/− mice. It is unlikely that changes in behavioral state (relative to baseline) after sleep deprivation in the mutants were masked by ceiling/floor effects. The mutant mice failed to increase NREM and REM sleep at 13–15 h, yet at 7–9 and 10–12 h, these mice exhibited considerably more NREM sleep than at 13–15 h, suggesting that the mice could have increased their NREM sleep at 13–15 h but did not. Likewise, the mutant mice failed to decrease REM sleep at 7–9 h. Yet at 13–15 h, these mice exhibited considerably less REM sleep than at 7–9 h, suggesting that the mice could have decreased their REM sleep at 7–9 h but did not.

Our findings with sleep deprivation are consistent with a study using rats in which CNS NE was reduced by the adrenergic neurotoxin N-(2-chloroethyl)-N-ethyl-2-bromoben-
zylamine (DSP-4). The normal increases in NREM and REM sleep after instrumental sleep deprivation were blunted in the DSP-4-treated rats (Gonzalez et al. 1996). Interestingly, the method of sleep deprivation was important because the recovery from methamphetamine-induced sleep deprivation was not altered by DSP-4. Thus adrenergic signaling may have its greatest impact on poststress sleep regulation rather than on sleep homeostasis per se.

It is interesting to compare the effects of NE/E deficiency on sleep/wake regulation to the effects of other neurotransmitter deficiencies. An important discovery in sleep/wake regulation was that of the orexin/hypocretin (Orx/Hcrt) neuropeptide system, the cell bodies of which are located in the posterior lateral hypothalamus (de Lecea et al. 1998; Sakurai et al. 1998). Although these neurons innervate many regions of the CNS, one of their most dense projections is to the LC, the adrenergic neurons of which are excited by Orx/Hcrt (Hagan et al. 1999). Importantly, deficiencies in the Orx/Hcrt system result in a narcoleptic phenotype where spontaneous, rapid transitions from waking to REM sleep become common and debilitating (Chemelli et al. 1999; Lin et al. 1999). Our results suggest that decreased adrenergic signaling could contribute to this phenotype because NE/E are important for inhibiting transitions from waking. However, our results also suggest that Orx/Hcrt projections to nonadrenergic neurons may be important for determining whether the transition from waking is made to NREM or REM sleep because of the absence of narcolepsy in the Dbh−/− mice (i.e., no increase in transitions from waking to REM sleep). This is consistent with recent findings indicating that mice lacking orexin receptor-2 exhibit only some of the phenotypes observed in mice lacking the Orx/Hcrt peptides. Specifically, both types of mutant mice have reductions in the duration of waking and NREM sleep bouts during the dark, but only the orexin-deficient mice exhibit increased transitions from waking to REM sleep and an increase in total REM sleep time in the dark (Willie et al. 2003). It seems likely then that the adrenergic system is one of several important downstream targets involved in the actions of Orx/Hcrt signaling to maintain arousal.

Another neurotransmitter system thought to be important for maintaining arousal is the histaminergic neurons of the hypothalamic tuberomammillary nuclei. Recently the gene for histidine decarboxylase, which is responsible for synthesizing histamine, was disrupted in mice. Surprisingly, this mutation had no effect on total waking times with the major effects being an increase in REM sleep during the day and a reduction in waking selectively during the light-dark transition period (Parmentier et al. 2002). In comparison, the changes in waking seen in the Dbh−/− mice are dramatic and highlight a critical and nonredundant role for the adrenergic system in sleep/wake regulation.

Finally, it is interesting to note that there is a similarity between the sleep/wake regulation phenotypes of the Dbh−/− mice and mice lacking the major isofoms of the transcription factor cAMP response element-binding protein (CREB). CREB hypomorphs exhibit a decrease in waking and an increase in NREM sleep that are primarily due to shorter bouts of waking even though there is an increase in the number of waking bouts (Graves et al. 2003). Others have shown that phosphorylation of CREB in the neocortex may depend on intact adrenergic signaling (Cirelli et al. 1996). Thus it is possible that at least part of the effects of adrenergic signaling on sleep/wake regulation could occur through transcriptional regulation. It would be interesting to examine CREB phosphorylation and CRE-mediated gene expression in the Dbh−/− mice during the day, at night, and after sleep deprivation in future studies.

ACKNOWLEDGMENTS

We thank A. Pack, S. Veasey, and G. Aston-Jones for discussions, L. Graves for technical advice, J. Cater for assistance with statistical analyses, D. C. Harris for technical assistance, and Sumitomo Pharmaceuticals for the generous gift of L-DOPS.

GRANTS

This work was supported by National Institutes of Health (NIH) Grants and the National Alliance for Research on Schizophrenia and Depression to S. A. Thomas; by NIH, the American Cancer Society, the John Merck Foundation, and the David and Lucile Packard Foundation to T. Abel; and by the National Institute of Mental Health Grant to K. Hellman.

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J Neurophysiol • VOL 92 • OCTOBER 2004 • www.jn.org


