On-line detection of sleep-wake states and application to produce intermittent hypoxia only in sleep in rats

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Disorders of sleep and breathing constitute a major public health problem (32, 36). Obstructive sleep apnea, for example, affects ~4% of adults (47) and causes recurrent asphyxia and arousals from sleep. Sleep apnea syndromes are associated with important clinical consequences such as excessive daytime sleepiness; impaired driving and work performance (15, 19); hypertension (10); increased risk for stroke, angina, and myocardial infarction (42); and suppressed ventilatory and arousal responses to altered blood gases and airway occlusions (9, 24).

There is currently major interest in determining the potential role of stimuli involved with sleep-disordered breathing (e.g., intermittent hypoxia) and the mechanisms underlying the adverse physiological consequences (33). Rats have been the most used animal model for such studies because of their particular suitability for chronic instrumentation and experiments in sleep. For example, the effects of repetitive intermittent or chronic hypoxia applied for several hours during the day on sleep-wake states (28, 34, 40), blood pressure and cardiovascular responses (3, 16, 17, 20, 26), and ventilation have been studied (30, 34). The effects of repetitive arousal stimuli on cardiovascular responses have also been reported in rats (4).

The applicability of such studies, however, to the understanding of the adverse physiological consequences of sleep-related breathing disorders is limited because the intermittent stimuli in all of those studies were applied without reference to sleep-wake states and only for several hours during the day (3, 16, 17, 20, 26, 28, 34, 40). Such protocols are typically employed because nocturnal rodents like rats tend to consolidate their sleep into the light phase, thus increasing the likelihood that stimuli would be applied in sleep. However, the robust ultradian rhythm of the sleep-wake cycle in rats (e.g., Ref. 43 and see RESULTS) ensures that there are prolonged periods of wakefulness even during the light phase, thereby complicating the assumption that the intermittent stimuli are likely to be applied in sleep. Moreover, even if the intermittent hypoxic episodes happened to be applied coincidentally with sleep episodes, it would also be highly unlikely that the offset of hypoxia would also be coincident with arousal as occurs in clinical disorders. Limiting stimuli only to the light phase also restricts the number of stimuli that can potentially be applied in sleep because the same ultradian rhythm of sleep-wake patterns also ensures that there are prolonged periods of sleep even in the dark phase in rats (e.g., Ref. 43 and see RESULTS). Moreover, because hypoxic stimuli also promote wakefulness (28, 34, 40) and can phase shift or depress circadian rhythms (23, 31), it is also conceivable that repetitive hypoxia in the light phase (3, 16, 17, 20, 26) may result in rats simply shifting their sleep into the
dark phase when the stimuli are not applied. These caveats, therefore, confound the interpretation that the intermittent hypoxic stimuli used in previous studies were applied in sleep in a fashion that mimics the clinical situation of sleep-disordered breathing. Rather, these observations suggest that criteria to ensure application of stimuli during sleep and removal of stimuli at arousal are a prerequisite to studying the effects of specific respiratory-related stimuli on sleep mechanisms and other physiological processes in a way that most effectively models the clinical sleep-related breathing problems.

Continuous visual scoring of sleep-wake states from polygraphic records and manual application of stimuli in sleep, however, is obviously not practical for chronic animal experiments that can last many hours or days. For these reasons, Bergmann et al. (6, 7) originally pioneered a computer algorithm for on-line detection of sleep-wake states in behaving rats from chronic electroencephalogram (EEG) and neck electromyogram (EMG) recordings. This algorithm has been used extensively in rats to apply stimuli exclusively in sleep in studies of full or partial sleep deprivation (6, 7). However, original validation of this algorithm was restricted to the light phase of the light-dark cycle and was performed in few rats (7). Validation data were presented only in the light phase in that study, most likely because constant illumination is routinely used in sleep deprivation experiments to dampen or eliminate circadian rhythms (6, 41). However, such criteria for detection of sleep-wake states in constant illumination may not be readily applicable to studies using the normal light-dark cycle. In addition, restricting analysis of EEG frequencies to <15 Hz precludes analysis of a significant portion of EEG frequencies that change across sleep-wake states in rats.

Given these caveats, the aims of the present study were to develop and validate a simple computer algorithm to accurately detect sleep-wake states on-line in freely behaving rats using an algorithm that would be applicable in both the light and dark cycles. Development and validation of this algorithm would also include measures of EEG frequencies across an appropriate range to include those expected to be significantly modulated by sleep-wake states. Triggering of voltage outputs on sleep detection would also fulfill the requirement of a system capable of applying respiratory-related stimuli (e.g., hypoxia) exclusively in sleep and removing those stimuli at arousal. The hypothesis is that parameters detected by frequency and amplitude analysis of the EEG and neck EMG can distinguish sleep-wake states on-line, irrespective of light-dark cycle. Because such analyses are now readily performed using a variety of commercial systems, such a simple sleep-detection algorithm would facilitate studies investigating the impact of respirato-

METHODS

Animals and Surgical Preparation

Studies were performed on 12 adult male Sprague-Dawley rats (mean body wt: 315 g; range: 290–420 g; Charles River Laboratories). Each rat was housed individually, maintained on a 12:12-h light-dark cycle, and had access to food and water ad libitum. Six animals were studied on an 1100–2300 light and 2300–1100 dark cycle, whereas the other six were studied on the reverse schedule. The rats on the 1100–2300 light cycle had been acclimatized to this regimen for an average of 10.0 days (range, 6–14 days), and the rats on the reverse schedule had been acclimatized for an average of 13.5 days (range, 12–21 days).

Sterile surgery was performed under general anesthesia induced by intraperitoneal ketamine (85 mg/kg) and xylazine (15 mg/kg). Before surgery, the rats were also given buprenorphine (0.03 mg/kg), atropine (1 mg/kg), and sterile saline (3 ml, 0.9%). To fix body position, the rats were placed in a stereotypic apparatus (Kopf model 962) with blunt ear bars. An anesthesia mask was placed over the snout, and halothane in air was administered as necessary for the remainder of the surgery. Body temperature was measured with a rectal probe and maintained between 36 and 38°C with a heating pad (BAS, West Lafayette, IN). All surgical procedures were approved by the Animal Care Committee of the University of Toronto.

Two stainless steel screws attached to insulated wire were implanted in the skull over the frontal-parietal cortex to record the EEG. One of these electrodes was placed ~2 mm anterior and 2 mm to the right of bregma, and the other ~3 mm posterior and 2 mm to the left of bregma. Two insulated, multistranded stainless steel wires bared at the tips were sutured onto the dorsal cervical neck muscles to record the EMG. Electrode leads were connected to amphenol pins inserted into a miniature plug (STC-89PI-220ABS, Carleton University, Ottawa, Ontario) affixed to the skull with dental acrylic and anchor screws. The rats recovered for an average of 14.1 ± 3.7 days (range, 7–20 days) before the onset of experiments.

Recording Procedures

For electrophysiological recording, a lightweight shielded cable was connected to the plug on the rat's head that was attached to a counterbalanced swivel that permitted free movement. All rats were studied in their home cages in a noise-attenuated environment, free from interruption. For purposes of habituation, they were connected to the recording apparatus at least 1 day before the experiments. The signals were routed to a Grass model 79D polygraph with 7P511 amplifiers. The EEG and neck EMG were filtered between 1 and 100 and between 10 and 1,000 Hz, respectively, and were recorded on chart paper at 5 mm/s.

Computerized Analysis of EEG and EMG Signals

A personal computer (IBM-compatible 386, 16 MHz) received the EEG and EMG signals after analog-to-digital conversion at a sampling rate of 300 Hz (Lab Master DMA, Arrow Electronics, Techmar, OH). In 6-s epochs, the computer then analyzed the periods in the EEG signal, then derived the corresponding frequencies, and calculated the percent signal in each of six bandwidths: δ2 (0.5–2 Hz), δ1
The EEG signal for a given input was a coefficient of variation (i.e., 100/0.0001, was also linearly related to the input voltage (99.3), although it was modified slightly in this study for delivery of stimuli (see below). The accuracy for detecting frequencies in the EEG was examined using signals from a function generator (Wavetek, San Diego, CA). Sine waves equivalent to inputs of 10–500 μV and frequencies of 1–25 Hz were used in accordance with the amplitudes present in the rat EEG (see RESULTS), and the range of frequency bands was analyzed (see above). For a 100-μV peak-to-peak sine wave, the average signal detection accuracy for all frequency bands (δ through to 7.5–13.5 Hz), α (13.5–20 Hz), and β1 (20–30 Hz), The peak-to-peak EEG amplitude and the moving average of the EMG signal were also determined. EEG analysis was performed using a modification of the interval histogram method (27). In this analysis, the amplitude of the EEG signal was divided into 32 equally spaced horizontal slice lines. A period was measured as the time interval between two points at which the same slice line crossed consecutive positive-going slopes of the EEG signal (27). A histogram was then constructed for these intervals, and from this histogram the percent distribution of frequencies was calculated. The analysis software used in the present study is similar to that previously used in dogs (21, 25), although it was modified slightly in this study for delivery of stimuli (see below).

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### Protocol

The rats were connected to the cable and swivel apparatus at least 1 day before the experiments. On the day of the experiment, the EEG and EMG signals were calibrated on chart and computer, and these signals were then monitored continuously. Except for brief periods to download and backup the data, computerized sampling was continuous for the remainder of the experiment.

### Analysis

Wakefulness and non-rapid eye movement (non-REM) and rapid eye movement (REM) sleep were determined visually in 10-s epochs from the chart record using standard EEG and EMG criteria (22). Periodic observations of the animal were also performed as an aid to determination of sleep-wake states, and appropriate notations were made on the chart. In summary, in wakefulness, the rats lay quietly, and the EEG displayed low-voltage, high-frequency activity. During non-REM sleep, the EEG was dominated by high-voltage, low-frequency activity. During REM sleep, the EEG was dominated by low-voltage, high-frequency activity, and neck muscle EMG was silent except for occasional muscle twitches. Brief arousals from sleep lasting from 3 to 10 s were also identified from the EEG and EMG signals (1). From the chart records, sleep efficiency (sleep time/recording time), the percentage of wakefulness, non-REM and REM sleep, and the number of arousals per hour were calculated. These values were also calculated from the computerized records, and, in addition, EEG frequencies and EEG and EMG amplitudes were determined from the computer. When the results of the computerized analysis were compared with visual scoring, both records were time aligned, and direct comparisons of the judgments of wakefulness and non-REM and REM sleep were performed. Also, in six rats chosen randomly (3 studied in the dark phase and 3 studied in the light phase), the percentages of wakefulness and non-REM and REM sleep were also determined by a second independent human scorer without reference to the results of the first scorer. For these rats, comparisons between scorers were performed for the data collected over the whole of the 6-h experimental period in each rat.

### Statistical Analysis

The analyses performed for each statistical test are included in the text where appropriate. For all comparisons, differences were considered significant if the null hypothesis was rejected at a level of $P < 0.05$ using a two-tailed test. Where post hoc comparisons were performed after ANOVA with repeated measures, the Bonferroni corrected $P$ value was used to infer statistical significance. For two-way ANOVA, the two factors were light-dark cycle and sleep-wake states (i.e., wakefulness and non-REM and REM sleep). Analyses were performed using SigmaStat (Jandel Scientific, San Rafael, CA). Data are presented as means ± SE, unless otherwise indicated.

### RESULTS

The overall sleep architecture was determined from visual analyses of the chart records to facilitate later comparisons with the computerized records. The chart recorder was run continuously for an average of 5 h 48 min ± 13 min for the rats studied in both the light and dark phases. As such, validation data were recorded for ~6 h per rat per day per lighting condition. For the rats studied in the light, the percentage of the recording time spent in wakefulness and non-REM and REM sleep was 26.9 ± 2.7, 66.3 ± 2.5, and 6.7 ± 1.3%, compared with 69.7 ± 2.3, 23.5 ± 1.8, and 6.7 ± 0.7% for the dark, respectively. There was a significant effect of light-dark cycle on sleep-wake states ($F(1, 10) = 7.68$, $P = 0.020$) with the differences being significant for wakefulness and non-REM sleep ($t(10) = 14.84$ and 14.04, respectively, both $P < 0.001$) but not for REM sleep ($t(10) = 0.01$, $P = 0.989$).
**Determination of Criteria for On-line Sleep Detection**

**EEG frequencies across sleep-wake states.** Figure 1 shows an example of the raw EEG and EMG signals across sleep-wake states and the distribution of EEG frequencies for the group of 12 rats. Note the typical shift to slower EEG frequencies in non-REM sleep compared with waking and REM. The frequency bands that showed the most obvious changes were the $\delta_1$ (2–4 Hz) and $\theta$ (4–7.5 Hz) bands, which showed clear increases in non-REM sleep compared with waking and REM, whereas the $\beta_2$ (20–30 Hz) band showed clear decreases in non-REM.

For the group, $\delta_1$ activity was consistently different across sleep-wake states [$F(2,20) = 137.6, P < 0.0001$], being significantly increased in non-REM sleep compared with wakefulness and REM [$t(11) = 11.7$ and $16.0$, respectively, both $P < 0.001$]. Similarly, $\theta$ activity was consistently affected by sleep-wake states [$F(2,20) = 90.2, P < 0.0001$], being significantly increased in non-REM sleep compared with waking and REM [$t(11) = 11.4$ and $11.9$, respectively, $P < 0.001$]. The relative increase in $\theta$ activity in non-REM sleep, however, was much less than the increase in $\delta_1$ activity, which nearly doubled in non-REM sleep compared with wakefulness and REM (Fig. 1). A decrease in fast EEG frequencies in non-REM sleep was also reflected by major changes in $\beta_2$ activity [$F(2,20) = 263.5, P < 0.0001$], with significant decreases in non-REM sleep compared with wakefulness and REM [$t(11) = 20.76$ and $18.87$, respectively, both $P < 0.001$; see Fig. 1].

Most importantly, these effects of sleep-wake states on the distribution of EEG frequencies were not dependent on whether the rats were studied in the light or dark phases ($P > 0.309$); i.e., the changes in $\delta_1$, $\theta$, and $\beta_2$ activities in non-REM sleep were consistent across the light-dark cycle.

These data showed that there were consistent and highly significant changes in the mean values of $\delta_1$, $\theta$, and $\beta_2$ frequencies across sleep-wake states. Figure 2 shows, however, that there was some overlap between populations of individual EEG frequencies within a rat that somewhat blurred this apparent discrete separation of sleep-wake states based on average frequency values alone. However, this overlap between populations of EEG frequencies was reduced by calculating the ratio of $\beta_2$ to $\delta_1$ activity ($\beta_2/\delta_1$), which better reflected the overall shift to increased slower frequencies and decreased faster frequencies in non-REM sleep compared with when the individual frequency bands were considered alone (Fig. 2).

Figure 3 shows the group mean data from 12 rats for the changes in $\beta_2/\delta_1$ activity, EEG amplitude, and neck EMG amplitude across sleep-wake states. There were consistent and highly significant differences in $\beta_2/\delta_1$ activity between states [$F(2,20) = 75.66, P < 0.0001$], and importantly this effect was independent of whether the rats were studied in the light or dark phases [$F(1,10) = 1.18, P = 0.302$]. The $\beta_2/\delta_1$ activity was significantly decreased in non-REM sleep compared with waking and REM [$t(11) = 9.5$ and 11.5, respectively]

**Figure 1.** Top: example of the raw electroencephalogram (EEG) and neck electromyogram (EMG) activities recorded in wakefulness and non-rapid eye movement (non-REM) and rapid eye movement (REM) sleep in these chronically instrumented rats. Bottom: group mean ± SE data from 12 rats for the changes in EEG frequencies in wakefulness (solid bars), non-REM sleep (open bars), and REM sleep (shaded bars). Note the major increases in slow EEG frequencies in non-REM sleep compared with wakefulness and REM (increased $\delta_1$ and $\theta$ activity) and decreased faster frequencies (decreased $\beta_2$ activity). See text for further details.
respectively, both \( P < 0.001 \); Fig. 3A], whereas values were not statistically different between REM sleep and wakefulness \( t(11) = 2.0, P > 0.05 \); Fig. 3A).

**EEG amplitudes across sleep-wake states.** There was an effect of sleep-wake states on EEG amplitudes \( F(2,20) = 123.0, P < 0.0001 \), with EEG amplitude being significantly increased in non-REM sleep compared with wakefulness and REM \( 311.2 \pm 22.3 \) vs. \( 169.7 \pm 11.8 \) and \( 183.0 \pm 12.4 \) \( \mu V \), respectively; \( t(11) = 14.2 \) and 12.9, respectively, both \( P < 0.001 \); Fig. 3B]. However, EEG amplitudes were not statistically different between REM sleep and waking \( t(11) = 1.3, P > 0.10 \); Fig. 3B]. Importantly, there was also an overall effect of light-dark cycle on EEG amplitude \( F(1,10) = 6.78, P = 0.026 \), with overall amplitudes being greater in the dark phase \( 189.5 \pm 17.3 \) vs. \( 253.1 \pm 17.3 \) \( \mu V \); \( P < 0.05 \). Nevertheless, there was no significant interaction between sleep-wake state and light-dark cycle on EEG amplitude \( F(2,20) = 2.29, P = 0.128 \), showing that the direction of change in EEG amplitude across sleep-wake states was consistent across light-dark cycle, although the absolute EEG amplitudes differed between lighting conditions.

**Neck EMG across sleep-wake states.** Neck EMG varied significantly across sleep-wake states \( F(2,20) = 153.3, P < 0.0001 \) and decreased from wakefulness to non-REM sleep [mean decrease = 62.6%; \( t(11) = 25.8, P < 0.001 \); Fig. 3C] and was further decreased in REM [mean decrease = 83.7%; \( t(11) = 34.46, P < 0.001 \); Fig. 3C]. Importantly, for neck EMG there was no statistically significant interaction between sleep-wake state and light-dark cycle \( F(1,10) = 3.44, P = 0.09 \).

**Choice of algorithm and threshold criteria to separate sleep-wake states.** The above results showed that, irrespective of the light-dark cycle, the \( \beta_2/\delta_1 \) activity distinguished non-REM sleep from both wakefulness and REM, and neck EMG effectively distinguished REM sleep from wakefulness. Based on these results, \( \beta_2/\delta_1 \) and neck EMG were then incorporated into a simple two-step algorithm to distinguish among these three sleep-wake states. This algorithm is shown in Fig. 4.

**Validation of Criteria for On-line Sleep Detection**

Each rat was studied on 2 separate days to determine the accuracy of the algorithm in detecting sleep-wake states. Day 1 was used to select appropriate thresholds for \( \beta_2/\delta_1 \) and neck EMG to distinguish sleep-wake states according to the algorithm in Fig. 4. These thresholds were chosen after continuous monitoring of sleep-wake states on chart and the corresponding computer-generated values of \( \beta_2/\delta_1 \) and neck EMG.

The \( \beta_2/\delta_1 \) thresholds used to distinguish non-REM sleep from wakefulness and REM were not statistically different for the rats studied in the dark and light phases \( 2.32 \pm 0.45 \) (SD) vs. \( 1.97 \pm 0.29; t(10) = 1.62, P = 0.136, \) unpaired \( t \)-test] as were the EMG thresholds \( t(10) = 0.27 \) and 1.62; \( P = 0.80 \) and 0.40, unpaired \( t \)-tests]. After choosing the appropriate thresholds on
algorithm in Fig. 4 was 94.5 ± 1.0% for wakefulness, 96.2 ± 0.8% for non-REM sleep, and 92.3 ± 1.6% for REM sleep (Table 1). Because these figures refer to unequivocally identified sleep-wake periods, computer detection accuracies were also calculated for periods more difficult to identify definitively by visual inspection, e.g., periods of drowsiness, transitions to REM sleep, and brief arousals from sleep. Incorporating these additional, less definitive periods at the transitions between states, also identified blindly without reference to the computer record, reduced the detection accuracy of the computer algorithm to 88.0 ± 2.4% for wakefulness, 86.3 ± 2.1% for non-REM sleep, and 89.6 ± 1.5% for REM sleep. Figure 5 shows that these additional errors in computerized detection were clustered at values of β2/β1 close to the threshold used to separate the sleep-wake states, i.e., periods of transitional EEG activity. Scoring accuracies for periods of wakefulness and non-REM and REM sleep by computer were the same in the light and dark phases (mean difference = 0.8 ± 1.7%).

Table 1 shows the accuracy of computerized detection of sleep-wake states and the associated errors for all visually scored epochs. The errors in the detection of wakefulness typically occurred during drowsy periods between wakefulness and sleep. The errors in detection of non-REM sleep tended to occur at transitions between non-REM and REM sleep as the EEG frequency became more mixed before the onset of muscle atonia. The errors in REM sleep detection by computer were in the interpretation of some REM body twitches as wakefulness. In addition, some REM sleep β2/β1 values were occasionally in the range of non-REM sleep (see Fig. 2).

Overall sleep architecture was also calculated from the computerized judgments of sleep-wake states and compared with the same periods matched from the chart record scored by blinded visual inspection. The percentages of wakefulness determined from the computer and chart were not statistically different [mean difference = 0.58 ± 4.42 (SD) %; t(11) = 0.457, P = 0.657, paired t-test]. The percentages of non-REM sleep determined from the computer algorithm and chart records were also not statistically different [mean difference = 0.58 ± 4.88 (SD) %; t(11) = 0.414, P = 0.687]. In contrast, there was a small overdetection of REM sleep by the computer algorithm [mean difference = 2.81 ± 3.20 (SD) %; t(11) = 3.038, P = 0.01]. Sleep efficiencies determined by the computerized and chart records were not statistically different [mean difference = 0.58 ± 4.42 (SD) %; t(11) = 0.457, P = 0.657, paired t-test]. Unlike sleep architecture, however, the numbers of arousals determined by computerized detection were different from the number determined by the standard visual criteria. Over the recording period, an average of 113.7 ± 13.6 brief arousals per hour (i.e., those lasting one computer epoch of 6 s) were detected by the computer, whereas only 34.3 ± 8.0 were detected visually [t(11) = 10.654, P < 0.001, paired t-test]. For arousals lasting two computer epochs, an average of 45.3 ± 10.5 arousals per hour were detected by computer, whereas 20.5 ±
3.0 were detected visually \[ t(11) = 2.806, P = 0.017, \text{paired } t\text{-test}. \]

For overall judgments of sleep-wake states, the agreement between the two independent human scorers was 96.2 ± 1.7% for wakefulness, 93.2 ± 1.9% for non-REM sleep, and 98.3 ± 1.6% for REM sleep. The discrepancies between scorers were due to determination of transitions from quiet wakefulness to non-REM sleep and determination of brief arousals from sleep.

Table 1. Accuracy of computerized sleep detection compared with visual scoring in 12 rats

<table>
<thead>
<tr>
<th>Human Judgment</th>
<th>Computer Judgment</th>
</tr>
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<tbody>
<tr>
<td>Awake</td>
<td>94.5 ± 1.0</td>
</tr>
<tr>
<td>Non-REM</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>REM</td>
<td>2.2 ± 0.6</td>
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</tbody>
</table>

\begin{align*}
\text{Awake:} & \quad 94.5 \pm 1.0 \\
\text{Non-REM:} & \quad 2.3 \pm 0.6 \\
\text{REM:} & \quad 2.2 \pm 0.6
\end{align*}

Values are means ± SE. REM, rapid eye movement; non-REM, non-rapid eye movement.

Application to apply stimuli exclusively in sleep. Figure 6 shows an example of the hypoxic stimuli applied in sleep and removal of hypoxia at arousal. A total of 270 hypoxic stimuli were applied in the three rats over

Fig. 4. Flow chart to show the series of decisions based on previously determined threshold criteria for the judgment of sleep-wake states. If the EEG signal shows predominantly slow frequencies relative to high frequencies (i.e., low \( \beta_2/\alpha_1 \)) and the neck EMG is below a high-amplitude threshold, then the rat is in non-REM sleep. If the EEG is dominated by slow frequencies, but the EMG is above the high-amplitude threshold, then the rat is in active wakefulness (i.e., EEG movement artifact associated with grooming). In contrast, if the EEG shows predominantly high frequencies relative to low frequencies (i.e., high \( \beta_2/\alpha_1 \)) and the neck EMG is below a low-amplitude threshold, then the rat is in REM sleep. If the EEG is dominated by fast frequencies, but the EMG is above the low-amplitude threshold, then the rat is awake.

Values of EEG and EMG amplitudes and of EEG frequencies in each bandwidth [i.e., \( \delta_2 (0.5–2 \text{ Hz}) \), \( \delta_1 (2–4 \text{ Hz}) \), \( \theta (4–7.5 \text{ Hz}) \), \( \alpha (7.5–13.5 \text{ Hz}) \), \( \beta_1 (13.5–20 \text{ Hz}) \), and \( \beta_2 (20–30 \text{ Hz}) \)] are written to disk along with a time stamp and sleep-wake judgment for each epoch for subsequent analysis.

Fig. 5. Errors in computerized detection as a function of \( \beta_2/\alpha_1 \) EEG frequencies around the threshold level used to distinguish between wakefulness and non-REM sleep. Group mean ± SE data from 12 rats are shown. Note that the errors in computerized detection are clustered at values of \( \beta_2/\alpha_1 \) frequencies close to threshold, i.e., periods of transitional EEG activity.
a 4-h period in the light phase (1030–1430). Of these stimuli, 93.7% were correctly applied in sleep (80.4% in non-REM sleep and 13.3% in REM), 2.2% were incorrectly applied in wakefulness, and 4.1% were applied in drowsiness as judged by an EEG pattern intermediate between light sleep and quiet wakefulness. Furthermore, 94.4% of these hypoxic stimuli were correctly removed at arousal from sleep, whereas 4.4% were incorrectly removed in sleep (3.7% in non-REM sleep and 0.7% in REM), and 0.7% were removed in drowsiness. The time spent in 10% O2 after the triggering of stimuli averaged 45.6 ± 3.37 s for non-REM sleep and 72.2 ± 8.7 s for REM sleep.

DISCUSSION

This study describes an algorithm for on-line detection of sleep-wake states in rats and its use in applying hypoxic stimuli exclusively in sleep. In previous studies in rats, application of episodic hypoxia has typically been performed for several hours during the day without reference to sleep-wake states (e.g., Refs. 3, 16, 17, 20, 26). For the reasons outlined in the Introduction, applying stimuli without reference to sleep has major drawbacks if such studies are designed to mimic episodic hypoxia associated with sleep-disordered breathing and determine the mechanisms underlying the adverse physiological consequences.

**EEG and EMG Criteria to Recognize Sleep-Wake States On-line in Rats**

EEG frequencies in the δ1 (2–4 Hz) and β2 (20–30 Hz) bands showed consistent changes in non-REM sleep compared with wakefulness and REM sleep. This increase in slow EEG frequencies in non-REM sleep, and decrease in fast frequencies, was better reflected in the β2/δ1, which showed a larger percent change in non-REM sleep compared with when the other frequency bands were considered alone (Figs. 1 and 3). The β2/δ1 also varied consistently with sleep-wake states, but not light-dark cycle, making it a robust parameter to distinguish non-REM sleep. The experimentally derived threshold values of β2/δ1 used in the present sleep-detection algorithm were not statistically different between the light and dark phases. These results highlight two important factors that distinguish this algorithm from that described by Bergmann et al. (6, 7). In those previous studies, the EEG was analyzed 15 Hz, such that significant changes in β2 activity could not be observed, and validation data were only presented in the light phase (6, 7).

Like δ activity, θ also increased in non-REM sleep compared with REM (Fig. 1). This unexpected result may have been because of the frequency range used to distinguish θ activity. The range of 4–7.5 Hz was based on the range of Kuwahara et al. (27), whose method we also followed for our EEG analysis. However, unlike for the δ band, there appears to be no uniform frequency range used to define θ, e.g., 5–10, 5–15, 6–9, 5.5–8, and 6.25–9 Hz have been used (6, 7, 39, 43). In our case, we did not see an increase in θ activity in REM sleep compared with non-REM in the 4- to 7.5-Hz range, but an increase in EEG frequencies in REM was observed in the 7.5- to 13.5-Hz band (Fig. 1), which partially overlaps the θ range used by some investigators (6, 7, 39, 43). A second set of EEG electrodes placed more medially may have improved our detection of θ.
rhythm (6, 7). Nevertheless, with our electrode placements, the EEG signals in REM sleep could be clearly distinguished from wakefulness (Fig. 1) and had the same visual appearance as in other studies. Our choice of one set of EEG electrodes was simply for practical reasons to reduce the number of electrodes during surgery. However, despite the use of only one set of EEG electrodes, the algorithm was accurate in detecting sleep-wake states and applying hypoxic stimuli in sleep. A second set of electrodes for long-term studies, however, has the advantage of redundancy in case one pair becomes dislodged or malfunctions.

EEG amplitude also showed characteristic and highly significant changes in non-REM sleep (Fig. 3). However, EEG amplitudes in non-REM sleep were affected by light-dark cycle. This observation fits with the demonstrated time-of-day dependence of homeostatic sleep regulation that produces high-intensity, non-REM sleep at the beginning of the sleep phase after prolonged wakefulness, which then diminishes over time (8, 43). Nevertheless, this observation also shows that EEG amplitude would not be a reliable criterion to distinguish sleep-wake states in rats in experiments that cover several hours, because the ability to detect a difference between non-REM sleep and the other states would vary with time of day and the light-dark cycle. This result highlights another important factor that distinguishes the present algorithm from that described by Bergmann et al. (6, 7), where integrated EEG amplitude is a primary criterion to separate sleep-wake states. The choice of EEG amplitude in those previous studies resulted from the original validations being performed in the light phase (7), because constant illumination is routinely used in sleep deprivation experiments to dampen circadian rhythms (6, 41). Accordingly, the accuracy and/or general applicability of EEG amplitude as a robust criterion was not apparent in those studies, as validation data were not presented for the dark phase (7). Based on our analysis in the light and dark phases, EEG amplitude was not chosen as a criterion in the decision tree of our algorithm (Fig. 4). Nevertheless, EEG amplitude does feature in one part of the EEG analysis because it allows the calculation of EEG frequencies from the distribution of periods (see METHODS).

Therefore, our EEG data showed that the $\beta_2/\delta_1$ EEG activity was a robust criterion to distinguish non-REM sleep from wakefulness or REM in both the light and dark phases. However, no other EEG parameter effectively distinguished between the sleep-wake states across light-dark cycle, especially wakefulness from REM sleep, which shared similar overall EEG characteristics (Figs. 1–3). In contrast, neck EMG showed reliable and highly consistent decreases in activity from wakefulness to REM sleep (Fig. 3) that were independent of the light-dark cycle. As such, the $\beta_2/\delta_1$ EEG activities and the neck EMG were used in combination in a simple two-step algorithm to separate sleep-wake states on-line after appropriate thresholds were chosen.

### On-line Detection of Sleep-Wake States

There are a variety of techniques to analyze EEG and EMG signals, such as spectral and interval histogram frequency analyses, autoregressive modeling, fuzzy logic, neural network approaches, and detection of EEG and EMG amplitudes after integration (e.g., see Refs. 6, 11, 35, 38, 39). Review of the major differences and assumptions among techniques is outside the scope of the present discussion. However, it is worth mentioning that the interval histogram method was chosen for EEG analysis in this study because it does not assume that the EEG signal is stationary, unlike another commonly used technique such as fast-Fourier transform. However, this distinction may not be overly significant for practical purposes in the range of frequencies that are important in this algorithm. For example, although differences among techniques exist, robust changes in $\delta$ and $\beta$ frequencies have been recorded across the night with both period amplitude analysis and fast-Fourier transform (44). Because one of the main aims of this paper was to identify EEG frequency characteristics that can be used to distinguish sleep-wake states on-line, for the purposes of applying stimuli exclusively in sleep, our use of these frequency data led to the present algorithm that fulfilled these criteria. It remains to be determined whether this same algorithm can be used with other frequency-detection software using different techniques.

Using the algorithm shown in Fig. 4, the accuracy of detecting unequivocally identified sleep-wake states was 94.5% for wakefulness, 96.2% for non-REM sleep, and 92.3% for REM sleep. This degree of accuracy, especially for REM sleep, is similar or improved compared with other computerized systems typically employed in rodents (e.g., Refs. 5, 7, 13, 39, 45, 46) and also compared with an earlier, similar algorithm used in dogs (21, 25).

In the present study, the accuracies of computerized detection were also calculated for periods more difficult to identify definitively by visual criteria, such as drowsiness, transitions to REM sleep, and brief arousals. Incorporating these additional less definitive periods reduced the detection accuracy of the computer algorithm to 88.0% for wakefulness, 86.3% for non-REM sleep, and 89.6% for REM sleep. Although including all available data reduced computerized detection accuracies compared with visual scoring, this was a necessary and required comparison because such continuous data would occur during an on-line experiment. Nevertheless, this level of computer accuracy is still improved or is at least comparable to other systems that typically did not include such transitional states in the analyses and validation (39). Indeed, a significant strength of this study is that the parameters used in the algorithm to separate sleep-wake states were derived from extensive analysis of EEG and EMG signals in a relatively large number of rats studied in both the light and dark phases. This degree of validation is more stringent than that described in most previous studies (7, 39), and its importance is...
highlighted by the observation that the analysis revealed parameters that were, and were not, robust enough to distinguish sleep-wake states in long-term experiments covering both the light and dark cycles. As such, it was not sufficient that a parameter such as EEG amplitude showed consistent and highly significant changes across sleep-wake states; these changes had to persist across states and be independent of the light-dark cycle to be identified and used as robust criteria for sleep detection.

Although validation in the present study was more systematic than in most previous experiments, validation in the light and dark phases for each rat was for only 6 h/day. However, we do not think that this 6-h continuous validation (i.e., rather than 24 h) significantly detracts from the main results. First, the major outcome of the study was that the threshold criteria used to separate sleep-wake states using the computer algorithm were sufficiently accurate to apply hypoxic stimuli exclusively during sleep. Because these threshold criteria also did not change significantly between the light and dark phases, the ability of the system to detect sleep and deliver stimuli only in sleep would not be expected to change appreciably over time to significantly affect most experimental protocols. Moreover, the 6-h validation was long enough for large changes in δ activity to occur over the sleep phase (8, 43), and yet the threshold criteria for sleep-detection were still accurate and similar to the accuracies at other times of the light-dark cycle compared with visual scoring. As such, although non-REM sleep may have higher δ activity at the onset of the light phase, the threshold criteria used in the computer algorithm to actually distinguish non-REM sleep from the other states remained sufficiently stable. By analogy, although deep non-REM sleep is dominant in the early phase of sleep in humans, the visual criteria used to actually determine sleep onset and distinguish it from wakefulness does not vary as the same visual criteria are used to separate sleep-wake states across the night.

The percentages of wakefulness and non-REM sleep scored by the computer and the blinded human scorer were indistinguishable, although the percentage of REM sleep was overdetected by the computer by an average of 2.8%. In contrast, arousals from sleep were detected significantly more often than with visual scoring. Such overdetection of arousals has also been observed in other automated systems (e.g., Ref. 12). It remains to be determined whether this difference in arousal detection is due to conventional visual scoring methods (e.g., Ref. 1) being insensitive to the subtle EEG changes that may indicate a true transient waking EEG, or whether computerized detection methods are oversensitive to short-term EEG fluctuations.

Errors in computerized detection tended to be clustered at the threshold values used to separate sleep-wake states (Fig. 5), i.e., periods of transitional activity. Errors also tended to occur at times when EEG activity became temporally dissociated from neck EMG. It is normal, however, for EEG and EMG signals to exhibit somewhat different time courses before achieving the typical patterns stereotypical of wakefulness and non-REM and REM sleep (2, 29). In the present study, such normal variations in neck EMG activity were observed, with some low-EMG activity in wakefulness being in the range more typical of sleep, and conversely with some high-EMG activity in sleep being in the range more typical of waking (Fig. 2F). Sudden changes in body position, especially with arousal, were also associated with bursts of neck EMG activity that decayed slowly compared with the development of sleep, as judged by EEG criteria. These observations highlight the difficulty in constraining the dynamic changes exhibited by EEG and EMG signals across the full spectrum of behavioral states to one of only three classes of sleep-wake states and is a problem encountered in all computerized sleep-detection systems (see Ref. 35 for discussion). Nevertheless, computerized sleep detection with the algorithm shown in Fig. 4 was sufficiently high to comply with the primary aim of this study, i.e., to develop a system capable of applying hypoxic stimuli in periods of sleep and removing those stimuli at arousal (Fig. 6).

Summary

The results of the present study show that EEG frequencies in the high (20–30 Hz)- and low (2–4 Hz)-frequency bands effectively distinguished non-REM sleep from wakefulness and REM in freely behaving chronically instrumented rats. In addition, neck EMG effectively distinguished REM sleep from wakefulness. Importantly, these criteria were sufficiently robust to be applicable in both the light and dark cycles. Incorporation of these parameters into a simple two-step algorithm then showed that sleep-wake states could be accurately detected on-line. The algorithm was also coupled to a system for triggering hypoxic stimuli exclusively in sleep. Because the ability to perform frequency and amplitude analysis of the EEG and neck EMG signals is readily performed using a variety of commercial systems, incorporation of these parameters into such an algorithm will facilitate studies investigating the consequences of respiratory stimuli applied exclusively in sleep in such a way that it more effectively mimics sleep-related breathing disorders.

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