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Novel Analysis of Sleep Patterns in Rats Separates Periods of Vigilance Cycling from Long Duration Wake Events

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Abstract

Rats are polyphasic sleepers. However, a formal definition of when one sleep episode ends and another begins has not been put forth. In the present study we examine the distribution of wake episode durations and based on this distribution conclude there are multiple components of wake. If the wake episode exceeds 300 sec the wake episode is assigned to long duration wake (LDW), if the episode is less than 300 sec it is assigned to brief wake (BW). Further support for this separation was found in close analysis of the EEG power spectrum in BW versus LDW. We then used LDW episodes to separate one sleep episode from another. We term the sleep episodes vigilance cycling (VC) because the rat is cycling between the vigilance states of brief wake (BW), slow-wave sleep (SWS), and rapid-eye movement sleep (REMS). We find that the characteristics of VC are different in the light period versus the dark period. We further find that as VC episodes progress, SWS pressure lessens, but the amount of time spent in REMS increases. These findings suggest that VC episodes are regulated and meaningful to the sleep behavior of rats. The use of the concepts of LDW and VC provides additional insights into the description of sleep patterns in rats that may be important in the development of a complete description of sleep behavior in this animal.

Keywords

power-law distribution; exponential distribution; brief wake; slow-wave sleep; rapid-eye movement sleep; arousal

INTRODUCTION

A common animal model in sleep research is the rat. An aspect to the rat as a model for sleep is that they are polyphasic sleepers. This creates several issues when studying sleep in rats. For example, neurobiological models of the control of sleep must account for the kinetic patterning of vigilance states and how they change at different times of the day. In addition, when comparing the sleep pattern in rats to humans, typically a monophasic sleeper with one ~8 h sleep session per day, the factors that control the length of sleep episodes are likely to be similar but executed in a different manner. Further, in humans a common unit that is measured is a sleep cycle which involves the time from one rapid-eye movement sleep (REMS) episode to another (REMS-REMS cycle). However, in rats, vigilance states frequently cycle through slow-wave sleep (SWS) to wake without REMS episodes. This limits the usefulness of REMS-

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REMS cycle as a parameter for sleep in a rat. An important parameter to help fully describe the polyphasic pattern in a rat that has not been defined is when a sleep episode begins and ends.

When rats sleep they cycle through vigilance states of wake, SWS, and REMS. However, close examination of the wake episodes in these animals reveals that many of these wake episodes are relatively brief and interspersed among SWS and REMS episodes, and some of these wake episodes are of a long duration without disturbances of SWS or REMS. Although these long wake periods are few in number, they can account for significant amounts of time spent in wake, even in the light period. Like rats, other animals, including humans, have brief awakenings during their sleep periods. Lo et al. [15] found that the distribution of wake episode durations of brief duration followed a power law with similar coefficients for humans, cats, rats, and mice. This is in contrast to sleep times (REMS plus SWS) uninterrupted by wake, which follow an exponential distribution which differ among animals [15]. However, to our knowledge a definition of what separates brief wake (BW), that which occurs among SWS and REMS episodes, from long-duration wake (LDW), that would separate one sleep episode from another, has not been put forth. Defining this parameter would enable study of the factors that regulate this sleep period in rats.

In the present study we present an argument that durations of BW and LDW separate from each other at ~300 seconds. Using this definition we show that sleep patterns in rats can be separated into periods of LDW, and between LDW is a period we have termed vigilance cycling (VC) since the animal is rapidly cycling between SWS, REMS, and BW. We find that the pattern of VC is different in the light period and dark period, and further, that SWS pressure decreases, but REMS pressure increases, as the VC period continues.

METHODS

Subjects

To be able to have sufficient number of observations of any rare events (e.g., long wakes in the light period), we used recordings from a relatively large sample size (26 male Sprague-Dawley rats ~350 g at the start of the measurements). These recordings had been collected over a two years period of time as control values in various projects. The rats were housed individually at 22 ± 2 °C with a 12h:12h light:dark cycle. The treatment of the rats was done with the approval of the Institutional Animal Care and Use Committee of Washington State University.

Instrumentation for sleep recordings

At least one week prior to any sleep recordings the animals were instrumented by standard procedures as previously reported [10]. Briefly, under ketamine (87 mg/kg) and xylazine (13 mg/kg) anesthesia, 3 EEG electrodes (Plastics One, Roanoke, VA) were implanted, one in the left frontal (5 mm A and 2 mm L, from bregma), one over the right parietal (-5 mm A and 6 mm L, from bregma), and a ground electrode over the left occipital cortex (-11 and 4 mm L, from bregma). One EMG electrode was implanted in dorsal neck muscle.

Sleep recordings

During the period of data collection sleep recordings were made in enclosed environmental chambers (4 rats in individual cages within a chamber, each chamber ~6 ft × 2 ft × 6 ft; width × depth × height) with constant room air flow through the chamber. The interior of the chamber was illuminated by a 15 watt light (12h: 12h light:dark cycle) and maintained at 22 ± 2 °C. Rats were habituated to the chambers for at least 3 days before starting sleep recordings. Any animal care was performed during the last hour of the light period, and we routinely do not

include any analysis from this period of time because of the interruption of the records and disturbances associated with animal husbandry.

Collection and analysis of EEG and EMG signals

The EEG (electroencephalogram) and EMG (electromyogram) signals were collected through a wire harness tethered to an overhead multi-channel commutator (Plastics One, Roanoke, VA). The signals were amplified by polygraph amplifiers (Grass-Telefactor, Inc., West Warwick, RI) and digitized at 128 Hz through a computer software program (SleepSign for Animals, Kissei America Inc.). The EEG/EMG recordings were analyzed with either 4-, 8-, or 12-sec epochs and each epoch was assigned to a particular vigilance state: wake, SWS, or REMS by standard criteria embedded in the software program. All scoring was visually inspected to ensure correct assignment of vigilance states and to eliminate any epochs with artifact noise. In addition to assigning vigilance state, the software program enabled use to analyze the EEG waveforms by fast Fourier Transform (FFT) within each epoch so that we could examine the power of the component frequencies within the EEG.

Curve fitting

Histograms of the probability density and cumulative probability density of wake episode durations for the light period and dark period were generated from the pooled results from all 26 animals. In the main analysis (8-sec epochs used for scoring sleep records) binning for the histogram was in 8 sec increments. When 4 or 12 sec epochs were used for scoring, histogram binning was in 4 or 12 sec increments, respectively. Probability density distributions were then determined by dividing the number of observed events in anyone bin by the total number of observed events. The cumulative probability density was determined by totaling all events equal to or greater than the bin duration, and then dividing that number by the total number of observed events (thus in the cumulative probability density the first bin in the distribution had a value of 1.0).

Long wake events in the cumulative probability density (cumPD) plots were fit to an exponential function:

$$\text{cumPD} = \text{amplitude} * \exp(-X/\tau)$$

where X is the event durations, amplitude is the amplitude of the cumPD at the y-axis, and τ is the time constant of the fit. Both the amplitude term and τ were allowed to vary during the fits. For brief wake episode durations a plot of the probability density (PD) of the wake episodes were fit to a power function:

$$\text{PD} = \text{amplitude} * X^{\alpha}$$

where X is the event durations, amplitude is the amplitude of the PD at the y-axis, and α is the power coefficient. The amplitude term and the power coefficient α were allowed to vary in the fits. Fits were evaluated by TableCurve 2D software (SYSTAT Software, Richmond, CA) which used a non-linear least squares procedure (Levenburg-Marquardt algorithm). Fits were evaluated by visual inspection, and the statistical parameters returned by the fitting program (correlation coefficient, standard error, and F statistic).

Statistical Analysis

Paired t-tests were used to compare light period derived values to dark period derived values. However, because of the one hour period reserved for animal care, and because we express time series data in 2-h blocks, there was only 10 hours (five 2-h blocks) of light period recording vs 12 hours (six 2-h blocks) of dark period recording. Thus for some comparisons, normalized values (either expressed as a percent or the light period value normalized to 12 hours) were used. Paired t-tests were also used in the analysis of first quarter parameters versus fourth

quarter parameters within vigilance cycling periods. To compare the distribution of vigilance states when different epoch durations were used in analysis of the sleep records, we used a one-way analysis of variance with repeated measures at each time point. To compare the power of frequency components in the EEG we used a two-way repeated measures analysis of variance (factors of frequency and period of data collection). All analysis of variance (ANOVA) were performed with SigmaStat version 3.5 software (SYSTAT Software, Richmond, CA), and t-tests were performed with Microsoft Office Excel 2003.

RESULTS

Sleep patterns in rats

Hypnograms from two representative sleep recordings are shown in Fig. 1. In the dark period the rats have many brief periods of sleep activity (SWS with some REMS) separated by long periods of wake. Within the sleep periods are many brief awakenings. In contrast, in the light period, the number of periods in which the rat remains awake for extended periods of time is greatly reduced. In the periods between these few long wake periods the rats cycle relatively rapidly through SWS, REMS, and brief periods of wake. In subsequent discussion of the results, the long periods of wake between periods of sleep activity will be referred to as long-duration wake (LDW), the brief periods of wake within sleep activity will be referred to as brief wake (BW), and the periods of sleep activity separated by LDW episodes will be referred to as vigilance cycling (VC).

Separation of BW from LDW

If one wants to analyze the characteristics of VC in the records, the critical issue is to determine a length of time when a wake episode shifts from BW to LDW. To accomplish this we examined the distribution of wake episodes when results were combined from all 26 rats (Fig. 2). (See supplementary Fig. S1 for a summary of vigilance state distributions with time of day for this group of rats.) We worked with a combined data set because some events (e.g., LDW in the light period) were relatively rare in any one rat (2–4 times during a light period) to make conclusions drawn from such few observations relatively unstable (e.g., curve fitting equations to two points). When the distribution of wake episode durations were expressed as a cumulative probability density, and the results plotted on a semi-logarithmic scale, the distribution clearly shows a biphasic nature (Fig. 2A). This was true in both the dark period and the light period. In order to separate the two distributions of wake durations, we fit the long events to an exponential starting at events 500 sec in duration or longer, thus concentrating on the distribution of long duration events at a time that the distribution of the brief events would be complete. The time constants (τ) of the fits to the distribution of long wake events were similar in both the dark period and light period (~1000 sec). The fit to the distribution of the light period was not as good as that to the dark period. The deviation from the fit in the light period occurs mainly at extremely long periods of time where events are very rare. In addition, the logarithmic plot tends to exaggerate the magnitude of the deviation of the fit when the absolute y-value is very small.

To fit the distribution of the brief events we expressed the results as a probability density and plotted the results on a log-log scale (Fig. 2B). We did this in order to be able to directly compare our results with those of Lo et al. [15] who found a power function best fit the distribution of the probability density of brief wake episode durations in mice, rats, cats, and humans. We also restricted our fits to the data from 24 sec to 360 sec. The reasons for this restriction are that this is the range that Lo et al. fit their results, and the 24 sec time point is the first common time point in the analysis when we used different epoch durations for analysis of the sleep records (see below). As is seen (Fig. 2B) the distribution of brief wake episodes are linear on the log-log plot over this range and the coefficients of the fits (-2.29 in the dark period and

−2.38 in the light period) are in excellent agreement with each other and with the results of Lo et al. [15] (their coefficients ranged from −2.0 to −2.3). Also, as in the results of Lo et al. [15], the episode durations less than 24 sec fall below the line of the fit, and the durations greater than 360 sec lie mostly above the line of the fit.

We used these results to select a time point that would serve to separate BW from LDW. An expansion of the fit to the cumulative probability density (Fig. 2C) reveals that the data begin to deviate from the fitted line starting around 300 sec, so in the subsequent analyses we selected 300 sec as the time point that separates BW from LDW. Using this separation time we have divided the state of the rat into periods of LDW and periods of VC (the cycling that occurs between LDW episodes). The resulting analysis is shown in Fig. 1 as the lines drawn above the hypnograms for the individual rats (lines indicate periods of VC). In some instances, two periods of LDW would be separated by a single SWS episode (e.g., Fig. 1, top hypnogram, period of time between 4 and 5 hours). We analyzed the results either by allowing such brief sleep episodes to separate LDW or not separate LDW, and the results of the fits to the wake episode durations were barely affected (the distribution of the cumulative probability density was still biphasic on the semi-logarithmic plot with time constants of fits to the longer wake events shifted by 50–100 sec, with virtually no change in the power coefficients in the fits to the probability density; data not shown). Thus we decided to ignore these brief episodes of SWS with the rule that if no cycling occurred (only a single SWS event), then the LDW episode was considered as a single episode. The reason this rule had minimal effect is that on average, this rule caused us to eliminate only one event per rat in the dark period, and only one event in all 26 rats in the light period. However, eliminating these microsleep events provided more consistency to the average characteristics of VC episodes because the brief sleep episode was not included in the final averaged values for the characteristics of VC (i.e., these few brief episodes were outliers; data not shown).

Characteristics of LDW and VC

Summarized in Table 1 are the characteristics of LDW and VC when 300 sec is used as the cut-off between BW and LDW. In the light period there are fewer LDW episodes with a much smaller percent of time spent in LDW. Interestingly, the average duration of a LDW was independent of light or dark period. In contrast to the similar length of LDW episodes in the light and dark periods, VC episodes in the light period are extremely long compared to the dark period. There are also fewer number of VC episodes in the light period. In spite of the drastically reduced number of VC episodes in the light period, the extremely long nature of the VC episodes in the light period caused the percent time spent in VC to double in the light period compared to the dark period.

The characteristics of the vigilance states within periods of VC can also be analyzed (Table 1). In the light period we found that the average episode duration for both SWS and REMS within VC significantly increased relative to the dark period. Remarkably, the average duration of BW episodes was independent of the light versus dark period. On the other hand, the percentage of time that the animal spent in BW during VC was significantly greater in the dark period. Also, the percentage of time spent in REMS was greater in the light period, but the percentage of time in SWS was only slightly different between the light and dark periods. Thus the characteristic of VC in the light period (the major rest time for rats) are that the VC episodes are relatively long, the average duration of individual SWS and REMS episodes are also relatively long, the percentage of BW is less, and the percentage of REMS is greater. These observations are all consistent with the VC events in the light period having a more intense nature of sleep than those that occur in the dark period.

We have also explored the data set using separation times briefer or longer than 300 sec. When a time less than 300 sec was used (100 sec) the periods of VC became highly fragmented (data

not shown). The reason for this is that in any individual rat the number of wake episodes that occur that are less than 300 sec begins to increase at rapid rate as the time is shortened, thus producing frequent breaks in the VC periods even in the light period (data not shown). Thus we conclude that 300 sec is a minimal time for the separation. On the other hand, we have explored the results with a separation time as long as 500 sec and on the whole we obtain results very similar to those found with the 300 sec separation time (data not shown). The stability of the result when using the larger separation time results from the relatively few number of events that are actually observed between 300 and 500 sec in the record of any one rat, and thus the longer separation time removes at most two to four events from the record (e.g., in the upper hypnogram of Fig. 1 the relative short LDW events at 3–4 h period, around 6 h, and between 15–16 h would be eliminated and in the lower hypnogram the LDW events at around 4 h, 8 h, 10 h, and 20 h would be eliminated). Elimination of these few events has only minor effects on the average characteristics of the VC (a slight reduction in number but a slight lengthening of duration; data not shown).

Alternative primary analysis parameters

An issue that arises from this analysis is whether the results we observe are dependent on the specific criteria by which we analyzed the raw sleep records. One such issue is the duration of the epoch used in the analysis of the raw sleep records. To investigate this issue we reanalyzed the records of the animals in the dark period with 4 and 12 sec epochs (see supplementary Fig. S2 for the effect of changing epoch duration on the distribution of vigilance states with time), and then subjected the resulting distribution of wake episodes to the same curve fitting routine as for the results with the 8 sec epoch (supplementary Fig. S3). We found in each case that the distribution still had a biphasic nature in the semi-logarithmic plot and that the time constant of the long duration events were very similar (supplementary Fig. S3A; brief single SWS events surrounded by LDW ignored as described above). The fit to the power function of the probability density was also similar (supplementary Fig. S3B). Finally, as with the results with the 8 sec epoch, the cumulative probability density began to deviate from the fit to the exponential equation at ~300 sec, thus reinforcing the conclusion that a 300 sec cut-off was a good value to separate BW events from LDW (supplementary Fig. S3C).

Another factor we examined was manipulation of short events that fell off the line of the power fit (Fig. 2B). To eliminate these short events from the record we invoked a three-epoch rule in which to register as a state transition, the new state had to be stable for at least 3 consecutive epochs (or 24 sec in the 8 sec epoch data set). If a new state did not last at least three epochs, the epoch(s) in question was assigned the state of the preceding epoch. As with the re-analysis with different epoch durations, the inclusion of the 3-epoch rule did not significantly alter the results of the analysis (supplementary Fig. S4). The cumulative probability density still was biphasic with a time constant for the LDW events of ~1000 sec (supplementary Fig. S4A); the BW events were still fit by a power distribution with a power coefficient of approximately -2 (supplementary Fig. S4B); and the brief events began to deviate from the exponential fit at around 300 sec (supplementary Fig. S4C). Further, we analyzed the resulting pattern of VC and LDW using the records scored with the 3-epoch rule (see Supplementary Table S1) and the results of the analysis are virtually indistinguishable from the analysis of the data when a single 8 sec epoch was used except that the average durations of BW, SWS, and REMS episodes are longer, as would be expected if the numerous 8 and 16 sec events are ignored (37%, 43%, and 77% of all wake events were <24 sec for the 8-sec, 12-sec, and 4-sec epoch scoring, respectively). Thus the distribution of wake events into BW and LDW, with a break point of 300 sec (or 500 sec) is independent of a number of arbitrary parameters used to analyze the raw sleep records. This demonstrates that the division of BW and LDW is a biological phenomenon and not an artifact of the arbitrary selection of parameters used in the analysis.

EEG of wake events versus REMS

We next examine the EEG behavior of BW and LDW. We first wanted to establish that the EEG power spectrum of either BW or LDW was not similar to REMS. We compared the distribution of the power in the frequency bands 1–25 Hz in episodes assigned to BW and LDW compared to those from episodes assigned to REMS (Fig. 3A and 3B). As can be seen, epochs assigned to REMS had a relatively high power in the theta band (5–8 Hz) and relatively low power in the delta band (1–4 Hz) compared to either of the two components of wake. Thus both components of wake were clearly different from REMS.

EEG of BW versus LDW

Up to this point we have separated BW from LDW only based on the kinetic distribution of wake episode duration. While such a distinction suggests that the neural networks that underlie the stability of BW and LDW are different, it is also possible that BW and LDW differ in other ways, such as the characteristics of the EEG. However, comparison of the EEG in between BW and LDW is complicated by the unknown relationship between BW and LDW. For example, it is not known if an animal always awakens into BW and then transitions into LDW, or whether the animal can enter directly into LDW from SWS or REMS. To address these issues we first compared the average EEG of all BW epochs to LDW epochs when LDW epochs less than 300 sec were excluded from the analysis. If BW precedes LDW, this would eliminate any contamination of the average EEG from LDW epochs by BW epochs that occur early in the LDW episode. By this approach we found that the EEG of BW had significantly greater power in the frequency bands from 1–16 Hz (Fig. 3A–D).

Since this analysis demonstrated subtle differences between the EEG of BW and the EEG of LDW, we could then address the issue as to whether a BW episode precedes a LDW episode by examining the EEG power in epochs at the beginning of LDW episodes. We collected the power of the frequency bands from each progressive epoch starting with the first epoch of a BW or LDW episode, and then combined the values into groups that included epochs 1–3, 4–6, 7–12, 13–18, 19–24, 25–30, and 31–36, thus covering the entire 300 sec period of BW episodes and the portion of the LDW episodes excluded in the prior analysis (results were also segregated into light period and dark period). To determine if the power spectrum of the EEG throughout the 300 sec period were similar within BW and LDW, we compared the power spectrum of the 1–3 epoch group to all successive groups within BW or LDW. For BW in both the light and dark period, we found that in epochs 1–3 the EEG power spectrum was elevated compared to subsequent groups primarily in the 1–13 Hz range (supplementary Fig. S5). For LDW we found that epochs 1–3 had higher power primarily in the 4 to 12 Hz bands (supplementary Fig. S6), but only in the dark period. For LDW in the light period the 1–3 epoch group was not different other groups, but because there are so few LDW episodes in the light period, the data are very noisy, and thus any conclusion from this observation must be interpreted with caution. These findings demonstrate that the EEG power spectrum in the 1–3 epoch group (the first 24 sec of the episodes) is different from all subsequent times within BW or LDW episodes.

We next compared epochs 4–6 with all subsequent groups within BW and LDW. In the dark period we found, with the exceptions of few frequencies here and there, that there were no differences between the power spectrums from the 4–6 epoch group and subsequent epoch groups (supplementary Figs. S7 and S8). In the light period the 4–6 epoch group did show some minor differences compared to subsequent groups in both LDW and BW (supplementary Figs. S7 and S8), but when the 7–12 epoch group was used as the bases for comparison, the differences in BW were no longer significant, and the differences in LDW were not different with the exception of a few individual frequencies in some groups (data not shown). These results demonstrate that once the 24 sec period at the beginning of an episode has been passed

(perhaps a little longer than 24 sec in the light period), the EEG power spectrum was stable and constant within a BW or LDW episode.

We next compared the EEG power spectrum of all groups between BW and LDW (supplementary Fig. S9). We found that the BW groups had higher power components for every comparison similar to that found when we compared all BW episodes to all LDW episodes when LDW episodes that occurred less than 300 sec from the beginning were excluded (Fig. 3A–3D). These findings demonstrate that the animals did not pass through a BW episode at the beginning of a LDW episode, but rather entered directly into a LDW episode from the prior sleep period.

An additional parameter we examined was whether there are any differences between BW and LDW in bands >25 Hz. Up to 25 Hz the power was greater in BW compared to LDW, although this was not statistically significant above ~16 Hz (Fig. 3A–3D). This pattern (BW>LDW) continued into high frequency bands up to ~30 Hz where the power in LDW began to systematically exceed that found in BW, although the differences were not statistically significant (supplementary Fig. S10).

A final parameter we examined was whether the power spectrum of the EEG was different in BW between light and dark periods. We found that there were differences at a few frequencies, but that these did not appear systematic but rather seemed more likely to be due to random fluctuations (BW in light had more power at 1 and 6 Hz but BW in the dark had more power at 8 and 9 Hz; supplementary Fig. S11). This finding, along with the prior finding that BW episode durations are similar in the dark and light period (Table 1), suggests that BW is similar regardless of its occurrence in the dark period or light period.

Sleep pressure during VC episodes

Finally, we hypothesized that if VC events are a type of sleep unit for the rat, there is likely to be changes in the characteristics of the SWS or REMS events over the course of an individual VC event (similar to the decreased depth of SWS and increase REMS that occurs in humans as the sleep period progresses during the night). To examine this issue we analyzed the average duration of BW, SWS, and REMS events, and the percent time spent in each vigilance state, as well as the power in the EEG spectrum during SWS episodes over the course of individual VC events. For BW, SWS, and REMS event durations we restricted the analysis to the first quarter and fourth quarter of VC events. Further, we restricted our analysis to VC events of at least 2000 sec in duration for two reasons. First, for us to be able to evaluate individual episodes durations, we wanted each quarter we analyzed to contain at least 3 episodes for the averaged value, and with the 2000 sec event duration this held true for SWS and BW, but REMS episode numbers frequently were less than 3 (this also forced us to not analyze REMS-REMS cycle). Second, we reasoned that for there to be a significant shift in these characteristics enough time had to elapse so that sleep intensity could lessen over the VC, and this was unlikely to occur in brief VC events. The 2000 sec event duration limitation forced us to ignore almost all events in the dark period, so we only show analysis from events in the light period. Further, the 2000 sec event limitation forced us to drop, on average, about 1–2 events from the light period record of each rat, but these shorter events only represented $1.8 \pm 0.5\%$ of the total time spent in VC.

We found that the average duration of SWS episodes and the percent of time spent in SWS decreased in the fourth quarter of the VC events compared to the first quarter of the VC events (Fig. 4, top set of panels, black bars). We also found that the percent of time spent in REMS increased in the fourth quarter versus the first quarter of VC episodes (Fig. 4, middle set of panels, black bars). We also found that the duration of BW episodes decreased in the fourth quarter versus the first quarter, but that the percent of time spent in BW did not change (Fig. 4, bottom set of panels, black bars). Finally, we found that the power of the slow wave

components during SWS episodes were smaller in the fourth quarter versus the first quarter, whereas the high frequency components were not different (Fig. 5). We extended the analysis of the power spectrum to include the second and third quarter periods and found that there was a smooth progression towards lower power in the 1–7 Hz bands as time in VC progressed (supplemental Fig. S12). These findings; shorter SWS episodes, less percent of time spent in SWS, and less power in the slow wave components of the EEG spectra during SWS, all support the idea that sleep pressure is lessened at the end of a VC compared to the beginning of a VC. Further, just as in human sleep at the end of the night, the amount of REMS increased at the end of the VC compared to the start of the VC. A noticeable trend in all vigilance states was the tendency for episode durations to decrease as the VC continued. Thus at the end of the VC the cycling between states becomes progressively faster.

A final issue with regard to this analysis is that it is well known that in rats sleep pressure is greatest at the beginning of the light period. Thus it is possible that the trends we observed in our analysis of first quarter versus fourth quarter behavior within VC events is mostly due to the high sleep pressure that exists at the beginning of the light period (first VC event of the light period), and is not a change observed over individual VC events that occur later in the light period. To examine this issue we reanalyzed the results, only we dropped the first VC event of the light period from the analysis (Fig. 4, gray bars in all panels; Fig. 5, filled triangles in the lower panel). In all cases the trends we observed when all VC events were included remained significant when the first VC event of the light period was dropped from the analysis. In one case, REMS episode duration, the trend toward decreased durations became significant once the first VC event was dropped (Fig. 5, middle left panel). These findings provide further support to our contention that regardless of the time during the light period that a VC event occurs, as the VC event progresses SWS pressure is reduced, REMS becomes more prominent, and the rapidity of cycling increases.

DISCUSSION

Our findings indicate that BW and LDW are different components of wake and a time between 300 and 500 sec can be used to separate them. The distinction between these two components is based upon both the stability of the component in time and the slight differences in the EEG power expressed within each component. Use of this break point enabled us to describe a sleep unit for rats, VC, that produces an additional descriptor for the polyphasic sleep patterns found in this animal. We also found that the characteristics of VC were different in the light period and dark period, and within individual VC episodes, especially long VC episodes in the light period, we found that SWS pressure waned but the percent of time in REMS increased.

Multiple components of wake

Our results also indicate that wake events occur in more modes than the two we have described above. In our analysis events less than 24 sec fell off the fitted power distribution, and these events account for a significant number of the total wake events. In addition, we also found that these brief periods had slight but significantly more power in their EEG compared to other components of wake, especially in the frequency bands between 5 and 15 Hz. Previous investigators have observed ultra-short awakenings (cut-off times from 2 to 16 sec) and they are known to occur in both humans [1,11] and rats [6,14]. Whereas some interpret these ultra-short events to be a sign of fragmentation of sleep [1], Halasz et al. [11] interpret these ultra-short events to be preparatory for future vigilance states. Halasz et al. [11] base their conclusion on the observation that synchronized EEG wake events occur in humans descending into deep slow-wave sleep, whereas desynchronized EEG wake events occur when ascending out of deep slow-wave sleep. However, the synchronized/desynchronized EEG patterns in these brief wake events observed in humans are more complex than what we have observed in rats.

Multiple components of wake create a potential for confusion when discussing these components. For example, Lo et al. [15] refer to events that align exactly with our BW events as arousal, but Franken et al. [6] refer to ultra-short wake events (8–16 sec in duration) as brief wakes, with Halasz et al. [11] and Lena et al. [14] referring to the brief wake events they investigate as arousal or micro-arousal, respectively. We selected to call the wake events that fall on the power distribution as brief wake events to avoid confusion with the arousal terminology of investigators that study shorter events; events that are likely to serve a different function than BW [11].

Additional findings that support the separation of arousal from other components of wake are the results of Horner et al. [12] who showed in rat immediately after wakening (3–10 sec) the animal has less pre-pulse inhibition to an audio signal compared to the animal in established wake (>30 sec). These authors concluded that this finding indicates the animal has less filtering of sensory inputs and increased responsiveness to external cues immediately after wakening, and further supports that the state immediately after wakening is neurophysiologically distinct from that which occurs in established wake.

It is of interest to note that when we eliminated these arousal events from the sleep record by use of the 3-epoch rule, there was little effect on the characteristics we determined for BW, LDW, or VC. This finding supports the conclusion that BW and LDW are the critical wake components in the determination of VC events.

Power-law distribution of BW

Our finding that brief wakes are well fit to a power-law in the dark period as well as the light period extends the analysis by Lo et al. [15]. Lo et al. concluded that finding a scale-invariant power-law behavior suggested that sleep-wake transitions involves fractal-like phenomena observed in systems undergoing phase transitions or self-organized criticality. However, the power-law distribution arises in rats and mice by P21 [3,4]. These latter investigators concluded that the basis for the power-law distribution must require additional neuronal circuits that develop as the rat matures and is not present in all circumstances of sleep-wake transitions. Our finding of the power-law distribution in the circadian phase outside the normal rest period indicates that the power-law behavior is generalized to both light and dark periods in adult animals.

BW versus LDW

Just as with arousal versus other components of wake, the stability and EEG power for BW and LDW are different from each other. Because the EEG and EMG patterns exhibited in both BW and LDW are very similar, it seems logical that the same basic neural activity supports both components of wake. However, the increased stability observed in LDW implies that a separate neural component must be involved in stabilization of these wake periods. In support of this idea Mochizuki et al. [17] found in orexin knockout mice that long wake events were selectively reduced whereas shorter wake events became more plentiful, indicating that orexinergic neurons are part of the network that supports LDW. On the other hand, Blanco-Centurion et al. [2], using various saporin constructions to lesion cholinergic, histaminergic, and noradrenergic neurons, found brief events (<1 min) were greatly reduced but that long duration wake events were not altered. Furthermore, different lines of inbred mice have significant differences in the distribution of wake episode durations [7]. These studies support the idea that there are different underlying neuronal circuits that regulate wake event durations of different lengths.

As previously mentioned, another distinction we observed between BW and LDW are the subtle differences in EEG power. While the differences in the EEG are not distinct enough to

assign epochs to different components of wake, they are distinct enough for us to determine that the animals do not need to transit through a BW episode to enter into a LDW event. We found that there was higher power in low to mid-frequency bands in the EEG of BW episodes. It is possible that the higher power in low frequency bands in BW implies that if the animal enters into wake and SWS pressure has not been completely dissipated, the animal soon falls back into SWS. However, if SWS pressure has dissipated in the prior sleep period, the animal wakes and enters into a LDW period. This suggestion does not preclude the possibility that an animal could enter into a LDW episode from a BW episode if, during the BW episode, the animal becomes aware of external cues necessitating attention of a long duration.

Role of LDW and BW in sleep regulation models

The flip-flop model put forward by Saper and colleagues [20] has been presented as an explanation for the control of sleep-wake transitions. In this model a positive feedback due to mutual inhibition between sleep promoting GABAergic neurons in the ventrolateral preoptic nucleus (VLPO) and several arousal systems produces a bistable situation in which only the states of wake and sleep are stable. However, the study of animals lesioned with saporin constructs to destroy the forebrain cholinergic neurons, midbrain histaminergic neurons, and brainstem noradrenergic neurons has been interpreted as evidence against the flip-flop hypothesis since no enduring changes in the percentage of wake was observed [2]. Our results suggest an alternative explanation for these findings. While these lesions did not produce much loss of overall wakefulness, they did produce a dramatic loss of brief wake events. As alluded to earlier, perhaps the wake components supported by the lesioned areas are either arousal or BW events rather than all wake events. Thus these short wake events were severely altered by loss of these activational systems, but since LDW may involve other neurocircuitry, as long as the animal can activate these additional circuits, LDW episodes will occur. Although LDW are few in number, they account for the majority of time spent in wake in the dark period and a significant portion in the light period. Thus something that eliminates the frequency of BW but not LDW might not be expected to dramatically reduce overall wakefulness in an animal.

Saper and colleagues [5,21] have extended their original flip-flop model to also include a circuit that involves the dorsal medial nucleus of the hypothalamus (DMH). The DMH receives a variety of inputs such as circadian signals from the suprachiasmatic nucleus and feeding cues from the arcuate nucleus, and it projects to the lateral hypothalamus where orexinergic neurons are found [21]. They suggest that the circuitry involved in the DMH enables the animal to coordinate wakefulness with circadian and environmental demands be they visceral sensory, cognitive, or emotional in nature. This would suggest that what we have labeled LDW is stabilized by the output of the DMH.

In support of this interpretation are the results of lesions of the orexin system. Whereas lesions of multiple activational systems resulted in loss of the number of BW episodes with long wake periods relatively intact [2], loss of orexinergic neurons or action results in fragmentation of long wake episodes [8,17,22]. Furthermore, orexin knockout mice continue to exhibit power-law behavior, supporting the idea that BW events are not influenced by orexinergic neurons [3]. However, there must be additional circuits beyond orexin that help maintain wakefulness during LDW because even with loss of orexin, animals still have periods of LDW which enable them to interact with their environment. The nature of these additional wake stabilizing mechanisms remain to be identified.

Purpose of BW and LDW

While it is clear that LDW episodes are needed to enable the animal to interact with its environment, the need for BW episodes is more opaque. It should be noted that although BW episodes can be as long as 300 sec, the vast majority of these events are less than 100 sec. Lo

et al. [15] found that whereas the behavior of BW was invariant from different size mammals, the time constant of the exponential fits to sleep (SWS plus REMS) increased with size of the animal. They suggest that the greater frequency of awakenings observed in small animals enable these animals to more frequently monitor their environment, which may provide survival advantage in a small prey species. Perhaps it is the arousal period that allows the animal to monitor its environment, consistent with heightened sensory awareness as previously described by Horner et al. [12]. Then depending on the mix of internal (e.g., SWS pressure) and external (e.g., danger) cues, the animal can fall directly back to SWS (wake events <24 sec), or enter into a BW episode in which undissipated SWS pressure eventually causes the animal to fall back into SWS, or, if cues are appropriate (no SWS pressure, hunger, demanding external events, etc.) the animal enters into a LDW episode.

Vigilance cycling

The separation of BW from LDW results in the identification of a period between LDW episodes we termed VC, which we suggest is a sleep unit in rat. Support of this distinction was found in the differences between behavior within VC in light and dark periods, and that sleep pressure during the VC episode waned. However, the analysis of sleep pressure within VC episodes was not performed on VC episodes in the dark period and in the light period brief VC episodes were ignored. While these decisions were made for practical analytic reasons, some additional issues are worth considering. The amount of total VC in the light period that occurred within these brief episodes was miniscule (<2%), and these episodes were only ignored in the analysis of sleep pressure within VC, but were included in the calculation of average values for VC in this period. These brief episodes typically occur near the beginning or end of other VC episodes and become separate because of a relatively short LDW event that separates them from the prolonged VC episode that follows or has just ended. Thus they may arise out of an occasional event that has been assigned to LDW but really is a BW event (note that the cut-off is not an absolute, and because of this there is undoubtedly some events that are misclassified).

In regards to VC in the dark period, whether sleep pressure dissipates during each event remains difficult to assess. However, this does not dismiss the idea that these events are without purpose to the animal. Humans show napping behavior in the light period and these sleep episodes, even if very brief, can be refreshing even though it might be difficult to document that sleep pressure waned over the episode [13].

Conclusions

The kinetic analysis of vigilance states presented in this manuscript separates behavioral wake in at least three components; arousal, BW, and LDW, and defines a sleep unit in rats labeled VC. We contend that this unit of sleep is akin to the 8-hr sleep period in humans, and provides another descriptor of sleep patterns in this animal. It should be noted that like rats, humans also have brief wake episodes during their sleep period [15]. Current neurobiological models of sleep and wake are very good at defining the neurocircuits that control sleep-wake transitions; however they have not yet provided details that have the power to explain the kinetic control of these transitions. It is likely that sleep patterns are not generated by mechanisms within the sleep-wake [20] and REMS flip-flop switches [16] but by the actions of influences that impinge on the flip-flop switches [21]. For example, the circadian variation in SWS episode duration is likely due to the balance between sleep homeostatic drive, perhaps mediated by the accumulation of adenosine in the forebrain [19] or cytokines [9], with circadian and environmental cues generated by the hypothalamic integrator in the DMH [21], both of which impinge on the VLPO, which in turn, controls the sleep-wake flip-flop switch [18]. Our analysis provides a step in defining the kinetic behavior that ultimately will need to be accounted for in these models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Representative hypnograms from two animals (B3 and G3). The shaded regions in this figure and subsequent figures indicates the dark period. W, S, and R indicate times in wake, slow-wave sleep and rapid-eye movement sleep, respectively. Bars over the state hypnogram at the level indicated by VC are periods that were classified as vigilance cycling (see text for further explanation).

Figure 2.

Cumulative probability density and probability density plots of the distribution of wake event durations. Circles illustrate results from the dark period (5,070 total events) and downward triangles indicate events from the light period (4,871 total events). In all plots the light period data is offset (one log unit in **A** and **B**, half log-unit in **C**) on the vertical axis for clarity. In all graphs the filled symbols indicate the data used to fit equations. In **A** and **C** an exponential equation was used to fit events >500 sec (the small vertical lines in graph **A** indicate 500 sec mark), and the solid line through the data points illustrates the fit of the equation to the data. The time constants (τ) from the fits (in sec) are indicated on the graphs. In **B** a power function was used to fit events from 24–360 sec and the line through the data points indicates the fit of

the equation to the data. The coefficients of the power term (α) are indicated on the graphs. In **C** (an expansion of the plot in **A**) the vertical line that intersects the two fits indicates the 300 sec time point.

Figure 3.

Spectral power of the component frequencies in the EEG for wake (open symbols) and REMS (filled symbols). In **A** and **B** upward triangles are from wake epochs in LDW after 300 sec from the start of the episode, and open circles are from wake epochs in BW. In **C** and **D** the power is expressed as a ratio of power in BW over power in LDW (normalized for each individual animal). The dashed line in **C** and **D** is for reference indicating equal power. Data are expressed as average $\pm$ S.E.; $N = 26$. The lines and numbers over the plotted data points in **C** and **D** indicate the frequencies that are significantly different ($p < 0.05$; pairwise multiple comparisons by Holm-Sidak method).

Figure 4.

Comparisons of episode durations for the indicated states (left hand panels) and percent time in the indicated state (right hand panels) for the first quarter of VCs and fourth quarter of VCs. Only results from VC >2000 sec were used. Results are from the light period only. Solid bars include results from all VCs, whereas the gray bars indicates the results after the first VC of the light period was dropped from the analysis (see text for explanation). Results were calculated for each individual rat and then expressed as the average $\pm$ S.E.; N = 26. Asterisks indicate significant differences (* < 0.05; ** < 0.005; paired t-tests).

Figure 5.

Top panel: Spectral power of the component frequencies of the EEG from SWS epochs in the first quarter of VCs (filled circles) and last quarter of VCs (open circles). VCs were selected as described in Fig. 4. Asterisks indicate significant differences between filled and open symbols ($p < 0.05$; pairwise multiple comparisons by Holm-Sidak method). Bottom panel: Normalization of the spectral power of the component frequencies of the EEG from SWS epochs (power of the first quarter expressed relative to the fourth quarter). Filled circles include results from all VCs, whereas filled upward triangles are results after the first VC of the light period was removed from the analysis. The dashed line is for reference indicating equal power. Asterisks indicate significant differences between first and fourth quarter values for filled

circles and # indicates significant differences between first and fourth quarter values for filled triangles ($p < 0.05$; pairwise multiple comparisons by Holm-Sidak method). Values were calculated for each rat and the values expressed as averages $\pm$ S.E.; $N = 26$.

Table 1
Summary of the characteristics of VC and LDW.

parameter	dark period	light period
<u>Long Duration Wake</u>		
# of LDW episodes:	18.0 ± 1.0	5.3 ± 0.3 **
Duration of LDW:	1420 ± 90	1300 ± 80
% time in LDW:	55.0 ± 1.6	15.2 ± 0.9 **
LDW (% of total wake):	76.8 ± 1.8	44.1 ± 2.2 **
<u>Vigilance Cycling</u>		
# of VC episodes:	18.3 ± 1.0	5.1 ± 0.3 **
Duration of VC:	1130 ± 80	7600 ± 380 **
% time in VC:	44.5 ± 1.6	84.8 ± 0.9 **
<u>Brief Wake</u>		
BW episode duration:	39.8 ± 1.4	38.9 ± 1.3
BW (% of VC):	37.5 ± 1.9	22.7 ± 0.8 **
<u>Slow-Wave Sleep</u>		
SWS episode duration:	60.7 ± 2.8	103.6 ± 4.0 **
SWS (% of VC):	55.7 ± 1.5	60.4 ± 0.8 *
<u>REMS</u>		
REMS episode duration:	60.8 ± 2.9	88.2 ± 3.1 **
REMS (% of VC):	6.8 ± 0.6	16.9 ± 0.4 **

Durations are reported in sec. # of episodes (LDW and VC) in the light period have been normalized to 12 h. Percent time in LDW or VC is with respect to total time. LDW (% of total wake) is percent of total wake (LDW + BW) that is LDW. Episode durations and percent of VC for BW, SWS, and REMS are with respect to periods of VC only. Values are averages ± SE; N = 26. Asterisks: significantly different from dark period (

*
p<0.05,

**
p<0.005, paired t-test).