 Increasing length of wakefulness and modulation of hypocretin-1 in the wake-consolidated squirrel monkey

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Zeitzer JM, Buckmaster CL, Lyons DM, Mignot E. Increasing length of wakefulness and modulation of hypocretin-1 in the wake-consolidated squirrel monkey. Am J Physiol Regul Integr Comp Physiol 293: R1736–R1742, 2007. First published August 8, 2007; doi:10.1152/ajpregu.00460.2007.—The neuropeptides hypocretins (orexins), the loss of which results in the sleep disorder narcolepsy, are hypothesized to be involved in the consolidation of wakefulness and have been proposed to be part of the circadian-driven alertness signal. To elucidate the role of hypocretins in the consolidation of human wakefulness we examined the effect of wake extension on hypocretin-1 in squirrel monkeys, primates that consolidate wakefulness during the daytime as do humans. Wake was extended up to 7 h with hypocretin-1, cortisol, ghrelin, leptin, locomotion, and feeding, all being assayed. Hypocretin-1 (P < 0.01), cortisol (P < 0.001), and locomotion (P < 0.005) all increased with sleep deprivation, while ghrelin (P = 0.79) and leptin (P = 1.00) did not change with sleep deprivation. Using cross-correlation and multivariate modeling of these potential covariates along with homeostatic pressure (a measure of time awake/asleep), we found that time of day and homeostatic pressure together explained 44% of the variance in the hypocretin-1 data (P < 0.001), while cortisol did not significantly contribute to the overall hypocretin-1 variance. Locomotion during the daytime, but not during the nighttime, helped explain < 5% of the hypocretin-1 variance (P < 0.05). These data are consistent with earlier evidence indicating that in the squirrel monkey hypocretin-1 is mainly regulated by circadian inputs and homeostatic sleep pressure. Concomitants of wakefulness that affect hypocretin-1 in polyphasic species, such as locomotion, food intake, and food deprivation, likely have a more minor role in monophasic species, such as humans.

orexin; ghrelin; leptin; homeostasis

HYPOCREtin (orexin)-expressing neurons, located in the lateral hypothalamus, are thought to be primarily involved in the maintenance of wakefulness (28). Loss of these neurons in humans leads to the sleep disorder narcolepsy (15, 21), which is primarily characterized by excessive daytime sleepiness. Other narcolepsy symptoms include cataplexy (rapid eye movement(REM) sleep-like muscle atonia during wakefulness typically triggered by strong emotions), rapid sleep onset into REM sleep, sleep paralysis(REM sleep-like atonia at the onset or offset of sleep), hypnagogic hallucinations (dream-like hallucinations at sleep onset), and disturbed nocturnal sleep. Based on the above and the observation that hypocretin antagonists increase sleep and REM sleep in humans (5), the primary downstream effect of hypocretin in humans may be the promotion of wakefulness, the consolidation of sleep and wake states, and the inhibition of REM sleep. In dogs and mice with hypocretin cell loss or hypocretin receptor-2 abnormalities, a phenotype similar to human narcolepsy is observed, suggesting a major sleep regulatory effect in these species as well. Data also suggest an important role for hypocretin in food anticipatory activity, stress, and modulation of the reward system, but studies suggesting such additional functions have been conducted in rodents.

A more detailed examination of the physiologic role of hypocretins in the control of human wakefulness, however, has been hampered as hypocretins are only found in significant concentrations in brain tissue and cerebrospinal fluid (CSF). Furthermore, if hypocretins are involved in the consolidation of wake into a single, daily episode in humans, use of mice and rats, which are polyphasic (i.e., have multiple, distributed wake bouts during both light and dark, although more occurring during darkness), may be inappropriate as models of human hypocretin physiology. We have, therefore, developed a primate model in which to examine the regulation of hypocretin release. Squirrel monkeys (Saimiri sciureus) are small, New World primates that, like humans and very few other species of mammals, consolidate wake into a single, daily episode (2, 10, 16). CSF is easily obtainable from the cerebellomedullary cistern (cisterna magna), which obviates most issues of temporal delay between hypocretin release and collection (11, 25), such as occurs when CSF is sampled from the lumbar sac (9).

Using this model, we have demonstrated previously that CSF hypocretin-1 concentrations are maximal at the end of the waking period of these primates (27), corresponding to the time of peak circadian drive for wake (10). A similar profile has been found in rats using in vivo dialysis or cisternal CSF measurements (11, 25). It has been hypothesized that individuals who lack significant production of hypocretins (i.e., narcoleptics) have a deficit in this circadian drive for wake (7).

Our data, however, also indicated that the circadian system is not solely responsible for control of hypocretin-1 release as a brief extension of wakefulness increases CSF hypocretin-1 concentrations (27). A similar effect of sleep deprivation was also found in canines (23) and rats (8, 14), indicating that the activation of hypocretin tone by sleep deprivation is found across all species examined to date. It is also in line with human clinical data indicating that a major abnormality in hypocretin-deficient narcoleptic patients is an inability to withstand small amounts of sleep deprivation. Indeed, human narcoleptic patients typically feel refreshed in the morning or after a nap (i.e., in the absence of a sleep debt) but rapidly become sleepy a few hours thereafter, perhaps when the

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hypocretin system should be activated to provide a much-needed compensation for the mounting sleep debt. Whether further activation at the end of the day, as reflected by higher hypocretin release, is solely driven by homeostatic pressure (an appetitive process that reflects greater sleep need with greater time awake) or driven by both circadian and homeostatic influences is still unknown.

Other behaviors have been found to modulate hypocretin tone, most notably changes in metabolic cues (i.e., food deprivation), emotional stimulation, and increased locomotion. Unfortunately, however, all of these studies have been done in polyphasic animals. Increased cisternal CSF hypocretin-1 after exercise, for example, has been found in rats (13) and canines (13, 23). Similarly, a 24-h food deprivation was shown to increase CSF hypocretin-1 levels in rats (11), a finding of notable importance as locomotor activation induced by food deprivation is abolished in hypocretin knockout mice (3). Furthermore, in mice, hypocretin neurons have been shown to sense glucose at physiological levels as well as respond to the feeding hormones leptin and ghrelin (24). In rodents, it is likely that food deprivation induces metabolic changes that lead to hypocretin neuron activation and subsequent wake promotion for the search of food. In contrast with these findings, however, 24-h food deprivation in canines (23) and 12-h food deprivation in squirrel monkeys (28) did not change CSF hypocretin-1 levels, suggesting interspecies differences.

A difficulty in interpreting rodent and canine data lies in the polyphasic nature of sleep regulation in these animals. Indeed, movement, feeding, and stress are concomitants of wakefulness in these species such that it is difficult to modulate these behaviors for long periods of times without simultaneously changing the amount of wakefulness. An illustration of this complexity can be found in a recent study (29) where we found that increased food intake and decreased locomotion in hypocretin cell-deficient mice could be explained by reduced wake bout length. Similarly, in wake-consolidated monkeys, we were able to reduce or increase locomotion without affecting wakefulness and found only a very small modulation of hypocretin levels by activity (26). These results suggest that locomotion has a minor role in the activation of hypocretin tone in species with wake-consolidation and a looser association between locomotion and wakefulness.

To extend our previous findings and to gain a better understanding of the physiology underlying the sleep and circadian control of hypocretin release, we examined the effects of increasing lengths of sleep deprivation at night on CSF hypocretin-1 in squirrel monkeys. A subjective rating of sleepiness as the result of sleep deprivation was also estimated and correlated with CSF hypocretin-1 changes. Given the proposed role of hypocretins in the regulation of feeding and metabolism (17), we also examined the influence of two feeding-related hormones, leptin and ghrelin, and the potential influences of these on hypocretin-1 in this primate model.

METHODS

Six adult female squirrel monkeys (Saimiri sciureus sciureus) housed as a single group at the Stanford University Research Animal Facility were used in this study. The group was placed in a 12:12-h light-dark schedule (lights on at 07:00) with constant temperature and humidity, and food and water available ad libitum. Throughout the protocol during the dark period, a dim (25 W) yellow light was constantly illuminated to enable visual contact between the researchers and the monkeys. Preliminary data indicated that this stimulus did not affect the pattern of circadian entrainment.

The monkeys typically awoke at 07:00 and assumed a sleeping position at 20:00. Sleep/wake patterns and entrainment to the light-dark schedule were confirmed through analysis of activity data, obtained from actigraphs attached to each monkey’s collar (Activwatch; Minimitter, Bend, OR), which were collected throughout the protocol. Baseline blood and CSF samples were obtained at 19:00, 22:00, 01:00, and 03:00; monkeys were able to sleep in their home cages until data were collected at each of the time points. Blood and CSF samples were also obtained at 22:00, 01:00, and 03:00 after 2, 5, and 7 h of wake extension (sleep deprivation), respectively. Monkeys were kept awake during these periods through investigator interaction. When a monkey would enter into a sleeping position (typically crouching on an upper perch), the investigator would rattle the exterior cage or cage lock, which would cause the monkey to move and remain awake. Each such interaction with a monkey was recorded. The total number of interactions during each hour was summed for all events, and the percentage of those times that a given monkey needed to be stimulated was calculated. From this percentage, the monkeys could be ranked hourly as to the most difficult to keep awake (greatest percentage of stimuli directed at this monkey) to the easiest to keep awake. The average rank across each sleep deprivation was also calculated. At the end of each 30-min period of observation, investigators also ranked how difficult they felt each monkey was to keep awake. Correlations between sleepiness and change in hypocretin-1 during sleep deprivation were made with Spearman rank correlation coefficient (available at: http://www.fon.hum.uva.nl/Service/Statistics/RankCorrelation_coefficient.html). Instances of feeding and drinking during the wake extension were also recorded, although the rarity of these events during the sleep deprivation precluded meaningful statistical analyses.

At each sample collection, monkeys were transferred from their home cage in which the wake extensions took place to a transport box and then moved to an adjacent procedure room. The transport box was covered in opaque vinyl to reduce light stimulation to the monkeys. Monkeys were individually removed from the transport box and given a nuxivone of 5.0 mg/kg ketamine. Following induction of the anesthetic state, CSF (200 μl) was obtained from the cisternal CSF samples were assayed for cortisol (Diagnostic Products, Los Angeles, CA) and hypocretin-1 (custom primary antibody and kit; Phoenix Pharmaceuticals, Belmont, CA) (see Ref. 27 for assay details). CSF hypocretin-2 has not yet been detected in squirrel monkeys (27) and was not assayed. Blood samples assayed for leptin and ghrelin. Except for a single published abstract (6), we could find no instance in the literature of ghrelin or leptin being assayed in a squirrel monkey. To determine leptin and ghrelin concentrations, we adapted existing, commercially available radioimmunoassays that were intended to assay human blood (human leptin RIA kit; ALPCO Diagnostics, Windham, NH, and human ghrelin RIA kit; Phoenix Pharma-
cteicals, Belmont CA). In preliminary tests, we found that undiluted concentrations of squirrel monkey plasma contained very low concentrations of leptin and very high concentrations of ghrelin. Preliminary testing indicated that squirrel monkey plasma samples needed to be diluted at a ratio of 6.25:1 (84 μl buffer + 16 μl sample) to obtain values that were on the ghrelin standard curve, but no dilution was necessary (100 μl sample) when assaying leptin concentrations. Preliminary testing also indicated that the high sensitivity protocol needed to be used when assaying leptin concentrations and that a new standard curve (ranging from 0.02 to 0.2 ng/ml) needed to be established. Results were reproducible, showed linearity with serial dilution, had low intrasample variability, and showed expected relationships with weight and feeding. Nonspecific binding was low (<5%) for both assays, and percentage-bound binding was always > 20%.

To determine the interrelatedness of hypocretin-1 and other aspects of sleep/wake physiology, in addition to the current data, we included previously published hypocretin-1, cortisol, and locomotion data (26, 27). Locomotion was calculated as the integral of the actigraph-recorded activity (in arbitrary units) occurring from 4:55–7:37 min before the CSF was collected, which is the time of peak correlation between CSF hypocretin-1 concentrations and locomotor activity (26). The homeostatic pressure was calculated for each monkey at each time point. Homeostatic pressure is a mathematical abstraction of prior sleep/wake history and has been calculated by a variety of methods. We use one of the iterative methods (1). According to this model, during wake, homeostatic pressure (S) accumulates as a function: 

\[ S_t = S_{t-1} + r \times (S_t - S_{t-1}) \]

where \( r \) is a rise factor equal to \( e^{-\Delta t} \times \tau_w \) and \( \tau_w \) is the rise time constant (18.2 h). During sleep, S dissipates as a function: 

\[ S_t = S_{t-1} - d \times (S_t - S_{t-1}) \]

where \( d \) is a decay factor equal to \( e^{-\Delta t} \times \tau_d \) and \( \tau_d \) is the decay time constant (4.2 h). Thus, homeostatic pressure accumulates and dissipates in a logarithmic fashion. Outliers from a normal distribution were removed from both the cortisol and locomotion data sets (see Extreme Studentized Deviate, available at: http://graphpad.com/quickcalcs/Grubbs1.cfm) in the modeling procedures, although all data were used in correlation analyses and in the comparison of the model to the raw data (see below). Based on the results of fitting individual variables to hypocretin-1 data, both sequential and simultaneous fitting were performed using the Levenberg-Marquardt method.

Data were analyzed using Microsoft Excel (version 10), Microcal Origin (version 6.1), and web-based java scripts (see text for specific links). Data are presented as means ± SE unless otherwise noted.

RESULTS

With increasing duration of sleep deprivation, monkeys were increasingly difficult to keep awake, yet all monkeys were kept awake throughout each of the sleep deprivation periods. Without intervention (i.e., in the presence of normal sleep/wake habits), CSF hypocretin-1 concentrations at 19:00 were 1,344 ± 125.8 pg/ml and further increased to a peak of 1,705 ± 130.1 pg/ml at 22:00. Concentrations then progressively fell to 1,251 ± 115.4 pg/ml at 01:00 and 975.4 ± 71.79 pg/ml at 03:00. During each of the sleep deprivations, hypocretin-1 concentrations were maintained at peak concentrations: 1,689 ± 199.6 pg/ml at 22:00 (2-h sleep deprivation), 1,621 ± 160.8 pg/ml at 01:00 (5-h sleep deprivation), and 1,510 ± 106.2 pg/ml at 03:00 (7-h sleep deprivation) (Fig. 1). This represented a change of \(-0.2326 ± 10.01\%\), 31.32 ± 11.91%, and 62.98 ± 22.76% after 2, 5, and 7 h of sleep deprivation, respectively, compared with time-matched baseline. Sleep deprivation samples were significantly different from baseline samples (\( F = 10.3, P < 0.01 \); ANOVA for correlated samples, see http://faculty.vassar.edu/lowry/anovalu.html). Post hoc testing indicates that the change observed after 2 h of sleep deprivation was not significant (\( P = 0.93\), Student’s paired t-test), but those observed after 5 or 7 h of sleep deprivation were significant (\( P < 0.05\), paired Student’s paired t-test). Hypocretin-1 levels during sleep deprivation were similar between 22:00 and 01:00 (\( P = 0.54\), Student’s paired t-test), but did decline at 03:00, after 7 h of sleep deprivation, compared with the 22:00 samples (\( P < 0.05\), Student’s paired t-test).

CSF cortisol concentrations were also elevated during sleep deprivation conditions compared with time-matched baseline. Cortisol concentrations at 19:00 were 9,802 ± 1,713 μg/dl, declined to 6,419 ± 1,773 μg/dl at 22:00 and to 5,639 ± 1,271 μg/dl at 01:00, and then began a surge upward at 03:00 to 10.44 ± 1,900 μg/dl. During the sleep deprivation, cortisol concentrations were elevated to 14.55 ± 3.904 μg/dl at 22:00 (2-h sleep deprivation), 26.77 ± 3.869 μg/dl at 01:00 (5-h sleep deprivation), and 22.38 ± 3.285 μg/dl at 03:00 (7-h sleep deprivation) (Fig. 2). This represented a change vs. the time-matched baseline of 196 ± 91.3%, 750 ± 391%, and 135 ± 34.3% after 2, 5, and 7 h of sleep deprivation, respectively. Sleep deprivation samples were significantly different from baseline samples (\( F = 40.0, P < 0.001\); ANOVA for correlated samples). Post hoc testing indicates that the change observed after 2 h of sleep deprivation was not significant (\( P = 0.055\), Student’s paired t-test), but changes observed after 5 or 7 h of sleep deprivation were significant (\( P < 0.005\), Student’s paired t-test).

Plasma ghrelin concentrations in the squirrel monkey were 1,660 ± 171 pg/ml at 19:00 baseline. Levels trended upward with time and were 1,830 ± 240 pg/ml, 1,940 ± 479 pg/ml, and 2,240 ± 220 pg/ml at 22:00, 01:00, and 03:00, respectively; these values, however, were not significantly different from one another (\( F = 1.35, P = 0.30\), ANOVA for correlated samples). Ghrelin levels were 2,090 ± 252 pg/ml,
Plasma leptin concentrations in the squirrel monkey were not correlated with body mass index (BMI; kg/m²) at 19:00 baseline. Levels trended down-earlier shown, leptin was correlated with ghrelin. Cortisol and leptin also both exhibited time-of-day dependence.

We had two measures of sleepiness, defined as the number of times each monkey needed to be disturbed to maintain wake and the investigator’s overall impression of sleepiness. We ascertained whether monkey sleepiness during sleep deprivation could be explained by relative change in hypocretin-1 by comparing the rank order of monkey alertness to the rank order of percentage change in hypocretin (i.e., the difference between sleep-deprivation concentrations of hypocretin-1 and time-matched baseline). Sleepiness data obtained by both methods of calculation (percentage of prompts and investigator impression) were well correlated to each other at each of the three sleep deprivation time points (r > 0.89, P < 0.05, Spearman rank correlation coefficient). However, only after the 5-h sleep deprivation did we observe a good correlation between a measure of sleepiness (prompts) and change in hypocretin-1 (r = 0.9429, P < 0.05, Spearman rank correlation coefficient). At this time point, correlation between sleepiness by investigator impression and change in hypocretin-1 was also nearly significant (r = 0.83, P = 0.058, Spearman rank correlation coefficient). After both 2 and 7 h of sleep deprivation, neither measure of sleepiness was well correlated with change in hypocretin-1 (−0.03 < r < 0.37, P > 0.49, Spearman rank correlation coefficients). Changes in sleepiness also failed to correlate with changes in cortisol (−0.77 < r < 0.14, P > 0.10, Spearman rank correlation coefficients).

In previously published experimental protocols, we systematically examined the effects of covarying locomotion and stress on hypocretin-1 concentrations and found a small effect of locomotion and no effect of cortisol on hypocretin-1 concentrations. We included these data sets (published in Refs. 26 and 27) with our current data set in an attempt to best measure correlations between hypocretin-1 and factors that may be upstream of its production. From the various studies, we had 240 hypocretin-1 concentrations and the associated time of collection and homeostatic pressure for each sample, which were taken from 51 different monkeys. We also had concentrations of, from contemporaneous samples, 239 CSF cortisol samples, 42 plasma leptin samples, and 42 plasma ghrelin samples. In addition, we had locomotor data associated with 125 of these samples (n = 18 monkeys).

In an attempt to examine the interrelationship between hypocretin and variables that have been purported to be important in the regulation of hypocretin physiology, the first step in our analysis was to do a simple cross-correlation with these data. We attempted to get the best and physiologically most appropriate relationship possible and tested linear, logarithmic, sinusoidal, Gaussian, and exponential relationships between variables. As can be seen in Table 1, many of these variables were significantly interrelated. Hypocretin-1 was correlated with time of day, homeostatic pressure, locomotion, and serum leptin levels. Homeostatic pressure was correlated with time of day and locomotion, cortisol was correlated with leptin, and as earlier shown, leptin was correlated with ghrelin. Cortisol and leptin also both exhibited time-of-day dependence.

![Fig. 2. CSF cortisol concentrations during baseline and sleep deprivation. CSF samples were obtained after baseline (white bars) or sleep deprivation (black bars) conditions. No significant change was observed at 22:00 after 2 h of sleep deprivation, but there was a significant difference (F) between time-matched baseline and sleep deprivation values at 01:00 (5-h sleep deprivation) and 03:00 (7-h sleep deprivation).](http://ajpregu.physiology.org/)

2,180 ± 430 pg/ml, and 1,920 ± 282 pg/ml after 2 h (22:00), 5 h (01:00), or 7 h (03:00) of sleep deprivation, respectively; these values were also not significantly different from one another (F = 0.240, P = 0.79, ANOVA for correlated samples). There was no significant difference at any of the time points between baseline and sleep deprivation (P > 0.05, Student’s paired t-tests). There were no significant correlations between either the absolute or percentage change in ghrelin and the absolute or percentage change in hypocretin-1 at any of the sleep deprivation time points (r < 0.23, P > 0.38, Pearson correlations). Plasma ghrelin concentrations at the 19:00 baseline were not correlated with body mass index (BMI; kg/m², with length being the crown-rump distance) (r = −0.40, P = 0.43).

Plasma leptin concentrations in the squirrel monkey were 0.361 ± 0.0695 ng/ml at 19:00 baseline. Levels trended downward with time and were 0.256 ± 0.0551 ng/ml, 0.219 ± 0.0451 ng/ml, and 0.271 ± 0.0655 ng/ml at 22:00, 01:00, and 03:00 baselines, respectively; these values, however, were not significantly different from one another (F = 0, P = 1, ANOVA for correlated samples). Leptin levels were 0.273 ± 0.0690 ng/ml, 0.283 ± 0.0817 ng/ml, and 0.154 ± 0.0550 ng/ml after 2 h (22:00), 5 h (01:00), or 7 h (03:00) of sleep deprivation, respectively; these values were also not significantly different from one another (F = 0, P = 1, ANOVA for correlated samples). There was no significant difference between baseline and sleep deprivation after 2 (P = 0.62, Student’s paired t-test) or 5 (P = 0.38, Student’s paired t-test) h of sleep deprivation. Leptin concentrations after 7 h of sleep deprivation, however, were 51.4 ± 11.6% lower than the time-matched baseline (P < 0.001, Student’s paired t-test). Leptin values at 19:00 were significantly, positively correlated with monkey BMI (r = 0.857, P < 0.05), such that monkeys with greater BMI had higher concentrations of plasma leptin. There were no significant correlations between either the absolute or percentage change in leptin and the absolute or percentage change in hypocretin-1 at any of the sleep deprivation time points (r < 0.22, P > 0.39, Pearson correlation).
Using the results of the cross-correlation, we ranked the predictors of CSF hypocretin-1 concentrations as time > homeostatic pressure > locomotion > ghrelin > cortisol. There were, however, insufficient leptin and ghrelin data to include in further modeling. We therefore, first fit the hypocretin data using a time-of-day sinusoidal model: hypocretin-1 = \{A \times \sin(\pi \times (\text{time} - x_c) \times 2)\} + r, where A is amplitude of wave, x_c is mesor crossing, and r is mesor. The residuals from this (R1) were then fit to homeostatic pressure data (R1 = m_H \times \text{homeostatic pressure} + b_H, where m_H is slope and b_H is the R1 value of the x-axis crossing). Linear and logarithmic fits gave statistically indistinguishable fits, but since a linear fit uses two variables and our logarithmic fit uses four, we opted to present the linear fit as it is more parsimonious. Residuals from this fit (R2) were then fit to locomotor data (R2 = m_L \times \text{locomotion} + b_L, where m_L is slope and b_L is the R2 value of the x-axis crossing) and the cortisol data (R2 = m_C \times \text{locomotion} + b_C, where m_C is slope and b_C is the R2 value of the x-axis crossing), but no significant fits were achieved. Combining the constants, we achieved a final equation of: hypocretin-1 = \{A \times \sin(\pi \times (\text{time} - x_c) \times 0.0833)\} + (m_H \times \text{homeostatic pressure}) + c. Variables were estimated (±SD) as: A = 297 ± 24.7; x_c = 14.8 ± 0.309; m_H = 359 ± 89.2; c = 1,065 (R^2 = 0.44, P < 0.001). Using the same equation, we allowed the software to simultaneously fit the data without constraints and similar estimates (±SD) were derived: A = 223 ± 26.4; x_c = 15.5 ± 0.451; m_H = 531 ± 107; c = 1,012 ± 39.3 (R^2 = 0.45, P < 0.001).

While both the sequential and simultaneous equation fits well-predicted CSF hypocretin-1 concentrations, we further parsed the data into that collected during the normal waking (07:00–20:00) and sleeping (20:00–07:00) times. For data collected during the daytime, there was a good correlation between time of collection and hypocretin-1 (r = 0.73, P < 0.0001, sinusoidal fit), as well as between homeostatic pressure and hypocretin-1 (r = 0.72, P < 0.0001, Pearson correlation), but a poor correlation with locomotion (r = 0.06, P = 0.57, Pearson correlation) and cortisol (r = -0.09, P = 0.24, Pearson correlation). Using a sequential fit as described above, we found that either the linear fit to homeostatic pressure (R^2 = 0.51, P > 0.001) or the sinusoidal fit to time of day (R^2 = 0.53, P < 0.001) could fit the hypocretin-1 data, but not both (note, as defined, homeostatic pressure only has a meaningful distinction from time of day if sleep and wake are modulated, and they are not in our current daytime samples). Although there was no correlation between raw hypocretin-1 data and locomotion data, locomotion data were significantly able to explain some of the remaining variance after fitting the data with time of day (R^2 = 0.045, P < 0.05, linear fit). Cortisol data were not able to significantly explain the residuals from the time of day fit or the residuals remaining after the fit to the locomotion data (P > 0.65, linear fits).

For data collected during the nighttime, there was a good correlation between time of collection and hypocretin-1 (r = 0.40, P < 0.001, sinusoidal fit), as well as between homeostatic pressure and hypocretin (r = 0.49, P < 0.0001, Pearson correlation). As with the daytime data, there was no correlation between hypocretin-1 and cortisol (r = 0.06, P = 0.58), but unlike the daytime fit, there was a strong correlation with locomotion (r = 0.53, P < 0.01, Pearson correlation). Using a sequential fit as described above, we found that time of day significantly fit the hypocretin-1 data (R^2 = 0.16 P < 0.001, sinusoidal regression) and that homeostatic pressure significantly fit the residuals from the time of day fit (R^2 = 0.16, P < 0.001, linear regression). Despite the correlation between locomotion and hypocretin-1, once time of day and homeostatic pressure were taken into account, locomotion no longer significantly explained any of the variance in the hypocretin-1 data (P = 0.34, linear regression). Cortisol too (P = 0.45, linear regression) was unable to fit significantly the residuals from either the fit to the homeostatic or time-of-day data.

**DISCUSSION**

We have shown that CSF concentrations of hypocretin-1 increase in response to prolonged sleep deprivation, such that the elevated levels that are present at the end of the wake time are maintained for at least 5 h. After 7 h, hypocretin-1 levels are slightly lower than their peak, but remain significantly higher than time-matched baseline. Secondarily, we also observed that these changes in hypocretin-1 during sleep deprivation did not consistently correlate with subjective or objective measures of sleepiness or effort to go to sleep, nor did they correlate with change in the appetite hormones leptin and ghrelin. Furthermore, by modeling our expanded data set, we confirmed that in the wake-consolidated squirrel monkey, cortisol and locomotion have little, if any, impact on CSF hypocretin-1 concentrations.

In most mammalian species, wakefulness is not consolidated into a single, daily bout under standard laboratory conditions (polyphasic wake). Under certain circumstances, however, wakefulness in these species can be greatly consolidated. For example, the presence of a running wheel increases the consolidation of wakefulness in mice (22). Thus, if hypocretin neuron activity is involved in the wake consolidation process, it is not surprising to find that hypocretin-1 concentrations can be affected by wake-promoting activities, such as motivated locomotion in polyphasic species (13, 23). Squirrel monkeys,
as is the case with humans, have a single, daily episode of wakefulness that occurs whether externally stimulated or not. Thus, our finding of little to no correlation between locomotor activity levels or cortisol concentrations and hypocretin-1 concentrations is not surprising. It may be the case that hypocretin-1 concentrations increase throughout the daytime in monophasic species, such as the squirrel monkey and human, relatively independent from the concomitant activities of wakefulness. In polyphasic species, it may be that there are circadian and homeostatic drives onto hypocretin that are more strongly modulated by the activities of wakefulness and, in such a manner, can modify the wake state in a feed-forward mechanism.

In rats, circadian cyclicity of hypocretin-1 release is dependent on the presence of an intact suprachiasmatic nucleus (8, 30). Given that an extension of wakefulness can modify hypocretin-1 concentrations, homeostatic processes dependent on sleep and wake history are also influences on hypocretin activity. At 22:00, 2 h after normal sleep onset, hypocretin-1 concentrations were elevated in the monkeys. This elevation during sleep may be due to a temporal delay between the release of hypocretin-1 and its equilibration in the cisternal CSF. An even greater delay would explain the midsleep peak of hypocretin-1 concentrations found in human lumbar CSF (12, 18). We did not observe a significant difference in hypocretin-1 concentrations after 2 h of sleep deprivation (i.e., at 22:00), which may be due to an inability to drive hypocretin-1 concentration higher than the normally observed peak. We are not aware of any published report in which hypocretin-1 concentrations could be raised above the normal, diurnal peak levels. Alternatively, it may be that 2 h of sleep deprivation is an insufficient stimulus to cause a significant change in hypocretin-1 concentrations or that there is too great a temporal delay for us to observe these changes. One of the future questions that needs to be answered is the relative contribution of homeostatic and circadian mechanisms on hypocretin-1 concentrations, as well as the process by which hypocretin declines during sleep (i.e., is it the lack of wake or the presence of sleep or a particular aspect of sleep physiology that causes a decline in hypocretin-1 concentrations). The interaction of homeostatic pressure and the circadian clock also must be examined during the daytime to fully understand this question. We also examined the role of appetite hormones leptin and ghrelin in our primate model. As expected, there was a good correlation between leptin and BMI, and between leptin and ghrelin. Given that in humans, sleep restriction causes an increase in ghrelin and decrease in leptin concentrations (19), we had expected ghrelin to be elevated during the sleep deprivation and leptin to decrease during the sleep deprivation. We did not, however, see such a pattern, except that the greatest amount of sleep deprivation (7 h) did result in a significant decrease in plasma leptin. This may be due to our use of a single sleep deprivation period, as opposed to several days of modifying sleep behavior or to a lack of statistical power in our data set in which the effects on these hormones was a secondary hypothesis. In our monkeys, the concentrations of leptin were very low and the concentrations of ghrelin were very high, compared with humans. The low leptin concentrations are likely due to the low body fat content of the female squirrel monkey (4), while the elevated ghrelin concentrations are consistent with our observation that the squirrel monkeys spend much of their day feeding, which is in line with field studies of foraging in this species (20). It may be due to their thin coat of hair and low body fat content that the squirrel monkey needs a large caloric intake to maintain homeothermy.

In summary, our data show that hypocretin-1 concentrations are responsive to extended changes in sleep and wake through an interaction of homeostatic and circadian mechanisms and that concomitants of wakefulness, such as cortisol and locomotion, contribute little if any influence on hypocretin-1.

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