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CONVERGENCE OF CIRCADIAN AND SLEEP REGULATORY MECHANISMS ON HYPOCRETIN-1

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Abstract—Hypocretin is a potential regulator of sleep and wakefulness and its levels fluctuate with the day–night cycle with high levels during the animal's activity period. Whether the daily fluctuations are driven endogenously or by external light cycles is unknown. We investigated the circadian and homeostatic regulation of hypocretin in the absence of environmental light cycles. To this purpose we performed repetitive samplings of cerebrospinal fluid in rats through implanted microcannulas in the cisterna magna and determined hypocretin-1 levels by radioimmunoassay. These experiments were also performed in rats that received a lesion of the suprachiasmatic nucleus (SCN), a major pacemaker for circadian rhythms in mammals. The results showed sustained rhythmicity of hypocretin in constant dim red light in control animals. SCN-lesioned animals showed no circadian rhythms in hypocretin and mean hypocretin levels were remarkably low. The results indicate that the SCN is indispensable for rhythmicity in hypocretin and induces a daily increase in hypocretin levels during the animal's active phase. Additional sleep deprivation experiments were carried out to investigate homeostatic regulation of hypocretin. Hypocretin levels increased in response to sleep deprivation in both control and SCN-lesioned animals, demonstrating that sleep homeostatic control of hypocretin occurs independently from the SCN. Our data indicate that the circadian pacemaker of the SCN and sleep homeostatic mechanisms converge on one single sleep regulatory substance.

Key words: narcolepsy, orexin, suprachiasmatic nucleus.

The hypothalamus has long been implicated in the regulation of food intake, sleep, body weight and energy balance (Taheri et al., 2002). Hypocretin-1 and 2 (also known as orexin A and B) are neuropeptides derived from the same precursor and their expression is restricted to a few thousand neurons of the lateral hypothalamus (Peyron et al., 1998; Willie et al., 2001). Initial interest in these neuropeptides concentrated on their role in food intake. Since then an increasing number of functions have become associated with hypocretin signaling, such as the cardiovascular system, metabolic rate, locomotor activity, body temperature and a wide variety of hormones (for review see Willie et al., 2001; Taheri et al., 2002). Experiments in animals and humans have demonstrated that hypocretin deficiency causes narcolepsy (Chemelli et al., 1999; Lin et al., 1999; Nishino et al., 2000; Peyron et al., 2000; Thannickal et al., 2000; Hara et al., 2001; Ripley et al., 2001). The primary symptoms of narcolepsy are excessive daytime sleepiness, difficulty to maintain wakefulness as well as sleep, and cataplexy, a sudden onset of muscle atonia elicited by emotional excitation (Overeem et al., 2001; Scammell, 2003). The identification of hypocretin deficiency as the primary cause of narcolepsy has stimulated research exploring the role of the hypocretin system in sleep regulation in general.

Sleep and waking alternate in cycles of 24 hours that result from interactions between the circadian timing system and homeostatic sleep regulatory mechanisms (Borbély and Achermann, 2000). An important circadian pacemaker resides in the suprachiasmatic nucleus (SCN) of the hypothalamus (Takahashi et al., 2001). Experiments performed under light-dark conditions have shown that hypocretin-1 levels in rat cerebrospinal fluid (CSF) change across 24 h and show high values at the end of the dark period and low values at the end of the light period (Fujiki et al., 2001; Yoshida et al., 2001). It is unknown, however, whether the observed daily rhythms were endogenous or were driven by the daily light cycle. We performed experiments in rats kept in constant dim light intensities and measured CSF hypocretin-1 levels both in control and SCN-lesioned animals to investigate the circadian regulation of hypocretin-1.

Sleep homeostatic regulation of hypocretin-1 was investigated in both rhythmic and SCN-lesioned rats by sleep deprivation (SD) experiments. Sleep homeostasis is reflected by the amount of slow-wave activity during non-rapid eye-movement sleep, which increases as a function of prior waking duration (Tobler and Borbély, 1986; Dijk et al., 1987; Lancel et al., 1991; Strijkstra and Daan, 1998; Borbély and Achermann, 2000; Huber et al., 2000; Deboer and Tobler, 2003). SD induces an increase in hypocretin-1 suggesting that hypocretin is closely associated with homeostatic sleep regulation (Yoshida et al., 2001). To investigate the role of the SCN in homeostatic regulation of hypocretin-1, we exposed control and SCN-lesioned animals to SD and determined the response in hypocretin levels.

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Abbreviations: ANOVA, analysis of variance; CSF, cerebrospinal fluid; CT, circadian time; SCN, suprachiasmatic nucleus; SD, sleep deprivation; vSPZ, ventral subparaventricular zone.

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EXPERIMENTAL PROCEDURES

Animals

All experiments were performed under the approval of the Animal Experiments Ethical Committee of the Leiden University Medical Center. All efforts were made to minimize the number of animals and their suffering. Male Wistar rats (approximately 300 g; Harlan, Zeist, The Netherlands) were subjected to an SCN or sham lesion. Lesions of the SCN were made under Hypnorm/Dormicum anesthesia. An electrode (diameter 0.3 mm), insulated with Epoxylite except at the 0.5 mm tip, was stereotaxically placed 1.4 mm anterior to bregma, 0.3 mm lateral to midline and at a depth of 9.4 mm. Lesions were made with a Grass DC constant current lesion maker (1.2 mA for 10 s) in the left and right SCN separately, while in the control group no current was applied. The animals were housed individually under continuous dim red light (<1 lux) with food and water *ad libitum*. Activity patterns were determined by recording drink nipple contacts in 1-min intervals. To diminish entrainment by external disturbances, cages were cleaned at random times of the day, once every week. During the last 10 days before the start of CSF collection (approximately 3 weeks post-lesion) the presence of a circadian rhythm was verified by a standard deviation based periodogram analysis (Dörrscheid and Beck, 1975). Peaks in the periodogram were considered statistically significant if they exceeded the 99.5% confidence limit.

CSF collection

Arrhythmic ($n=21$) and control animals ($n=16$) were implanted with a CSF cannula in the cisterna magna to allow for repetitive sampling with intervals of 6–8 h. Sampling cannulas were implanted and anchored to the skull as described by Schwartz et al. (1983). The total length of the cannula was 14 mm, with both tips bevelled at a 45° angle. When inserted with the bevel facing caudal, the depth of the cannula was 7.5 mm. In order to characterize the entire circadian cycle with intervals of approximately 2 h, we sampled over a 3-day period. Per 2-hour time point, we obtained samples of six to 10 different individuals. Approximately 50–100 μl of CSF was collected on ice and stored at −80 °C. Hypocretin-1 levels in the CSF were determined in 40 μl crude samples of CSF using a radioimmunoassay (Phoenix Pharmaceuticals, Belmont, CA, USA) as described previously (Yoshida et al., 2001; Ripley et al., 2001). Reference samples were included to allow correction for intra-assay variability.

Sleep deprivation

For SD experiments, a total number of eight arrhythmic and six rhythmic animals were used. The SD lasted 6 h and was performed under constant dim red light conditions. To allow comparison between control and SCN-lesioned animals, SD episodes were randomly distributed over circadian time (CT) in the rhythmic group. During SD, the animals were continuously observed. As soon as the animals attempted to adopt a sleep posture they were disturbed by acoustic stimuli or by introducing objects into the cage, which is a routine procedure for mild SD (Tobler et al., 1997; Antle and Mistlberger, 2000; Huber et al., 2000; Deboer and Tobler, 2003). As a consequence the animals showed low levels of behavioral activity such as grooming, drinking, and low levels of locomotor activity throughout the SD procedure. During the last hour of the SD, two observers were present.

Histology and statistics

The rats were killed 1 week after CSF sampling under Nembutal anesthesia. Brains were frozen and processed for histological evaluation of the lesion site. Inspection of the lesioned areas indicated that most lesions did not extended beyond 250 μm outside the SCN (Fig. 1).

Analysis of variance (ANOVA) served to determine effects of 'time of day' on hypocretin-1 in CSF baseline samples. Whenever significant effects were present ($P<0.05$) a Duncan-test was used to further evaluate differences among CTs. Two-tailed *t*-tests were used to determine the effects of SD on hypocretin-1 levels in CSF.

RESULTS

Recordings of drinking activity and periodogram analysis in SCN-lesioned animals showed no significant rhythm within the circadian range (Fig. 2A, B), whereas the sham lesioned animals were rhythmic (Fig. 2C, D). In all rhythmic animals, hypocretin-1 reached lowest levels in the middle of the resting phase, and remained low until the start of their active phase. Within 4 h after onset of activity, hypocretin-1 increased and reached maximum levels (Fig. 3). Hypocretin-1 levels during the end of the activity (between CT23 and CT1) differed significantly from levels during the end of the resting phase (CT7–CT11, $P<0.05$, Duncan after significant ANOVA), and mean levels during the active phase differed significantly from mean levels during the rest phase (Rest: 775.5±36.6 pg/ml; Active 914.8±28.3 pg/ml, $P<0.001$ two-tailed paired *t*-test). Mean hypocretin-1 levels in SCN-lesioned animals were significantly lower than mean levels in rhythmic animals (Fig. 3), and equaled hypocretin levels obtained during rest in rhythmic animals ($P<0.0005$, $F=15.465$, ANOVA factor 'group').

SD induced a clear increase in hypocretin-1 in SCN-lesioned as well as in control animals (Fig. 4). After SD the mean hypocretin levels were the same in both groups. The hypocretin-1 response to SD was not different between the two groups (magnitude of the response: Control 336.2±55.4 pg/ml; SCN-lesioned: 362.0±113.8 pg/ml, $P>0.8$, two tailed *t*-test). Also the absolute levels reached after SD did not differ significantly between control and SCN-lesioned animals (Control: 1205.3±51.0; SCN-lesioned: 1205.2±71.5 pg/ml, $P>0.8$, two-tailed *t*-test).

DISCUSSION

Under constant conditions a clear circadian rhythm in hypocretin-1 levels was obtained in control animals providing evidence that the 24-hour fluctuations are not imposed on the animal by the environmental light dark cycle, but are endogenous and circadian in nature. A possible confounding factor in this study may be the indirect effect of motor activity. Studies in narcoleptic and control dogs have shown increased CSF hypocretin levels with exercise (Wu et al., 2002). Hypocretin changes could therefore indirectly reflect changes in the distribution of rest and activity associated with SCN lesions. However this possibility seems unlikely since CSF hypocretin release in rhythmic animals is highest at the end of the active period while motor activity is highest at the beginning. Moreover, the levels of behavioral activity in the SCN-lesioned animals were average as compared with the controls while hypocretin-1 levels in the lesioned animals were low, and significantly below mean hypocretin levels in the controls. Previous research has shown an increase in hypocretin levels (Wu

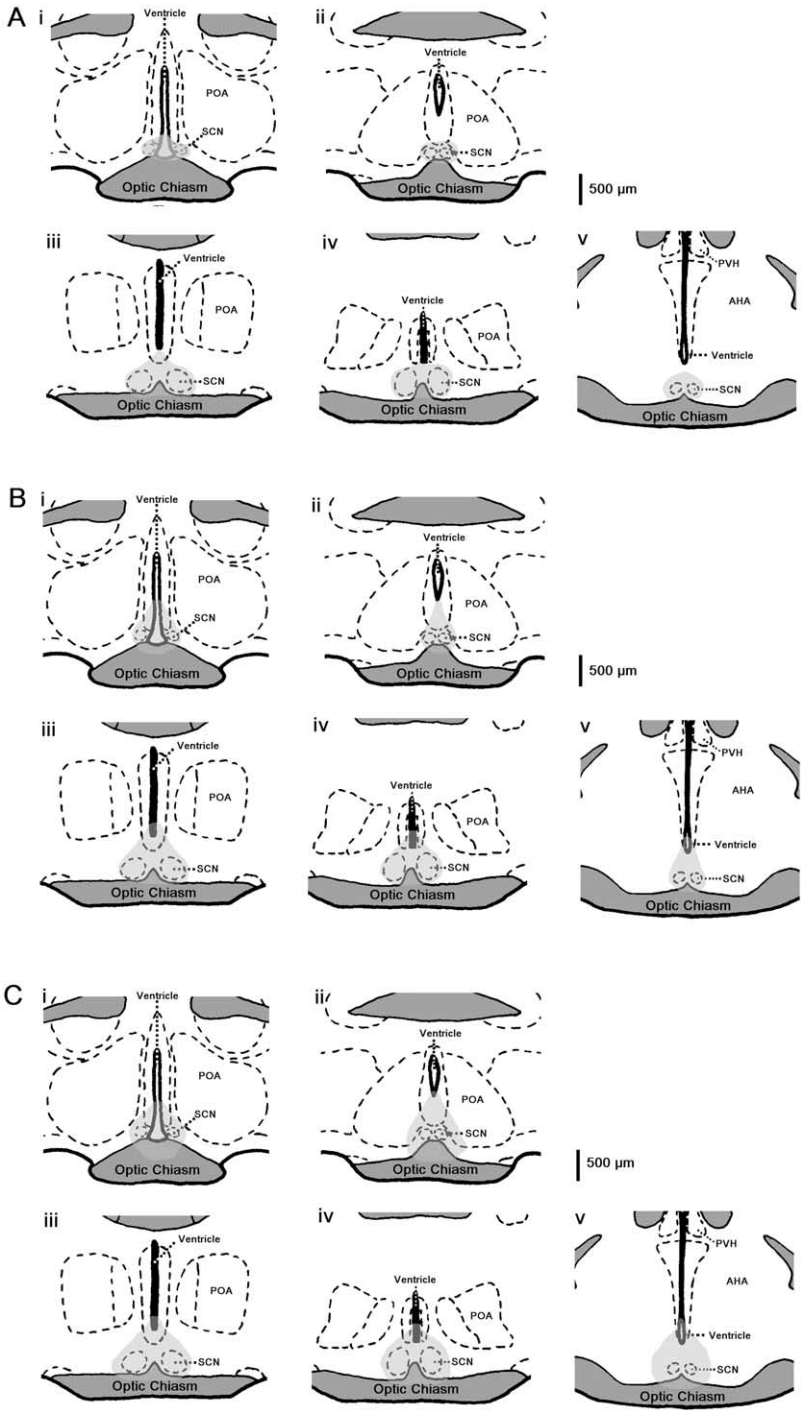

Fig. 1. (A–C) Drawings of SCN lesions. Figures adapted from Pellegrino et al. (1979). The lesioned areas are represented with a light gray hue. Drawings i–v range from 2.0 mm (i) to 1.2 mm (v) rostral to Bregma. Panel A shows typical lesions obtained in 16 of our animals. Those were small lesions, which hardly protruded more than 250 μm outside the borders of the SCN. Panel B shows SCN lesions obtained in four animals, which were a bit larger. Panel C shows the larges lesion we had, obtained in one animal. The absolute hypocretin levels did not differ between the three groups. AHA, anterior hypothalamic area; POA, lateral preoptic area; PVH, paraventricular nucleus of the hypothalamus.

et al., 2002) and c-fos labeling of hypocretin neurons (Tortorolo et al., 2003) after somatosensory activity during waking but only activity levels far above average had this effect. We conclude therefore that circadian rhythmicity of hypocretin-1 is critically dependent on integrity of the SCN.

Mean hypocretin-1 levels in SCN-lesioned animals were significantly lower than mean values in control animals, and equaled hypocretin levels during the subjective day of rhythmic animals. The question arises whether damage to the lateral hypothalamus may have occurred as

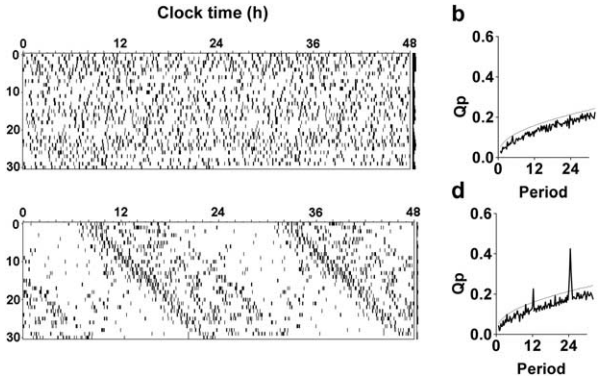

Fig. 2. Examples of drinking activity records of a SCN-lesioned rat (A) and a sham-lesioned control animal (C). Drinking activity is recorded per minute and is indicated in black. The records are double plotted to enable visualization of the activity rhythm. Consecutive days are plotted underneath each other. The occurrence of significant rhythmicity was analyzed by periodogram analysis. Qp values of the SCN-lesioned rat did not reach significance in the circadian range (B), this in contrast with the sham-lesioned animal that showed a significant circadian period of 24.1 h (D).

a consequence of the SCN-lesions and accounts for reduced levels of hypocretin-1. Inspection of the lesioned areas indicated that most lesions were within 250 μm of the outer border of the SCN, and never reached the lateral

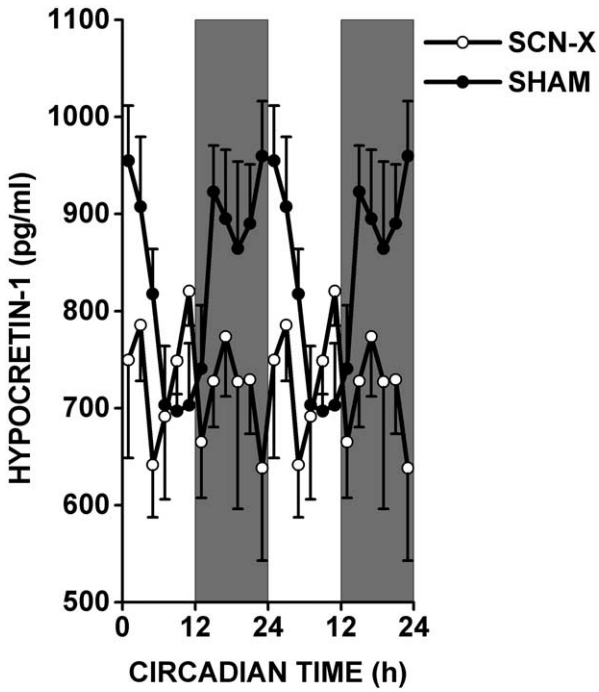

Fig. 3. Combined data of hypocretin-1 levels in CSF of SCN-lesioned (SCN-x) and sham-lesioned control animals under constant dim red light conditions. The data are double plotted for clarity. Data are means±S.E. A gray background indicates subjective night, where the control animals are most active. The fluctuation in CSF hypocretin-1 was significant across the circadian day in the control animals (ANOVA factor time, $P<0.03$, $F=2.191$), but not in the SCN-x animals. In the control group mean hypocretin-1 levels at CT 23-1 differed significantly from levels between CT 7–11 (Duncan $P<0.05$, after significant ANOVA).

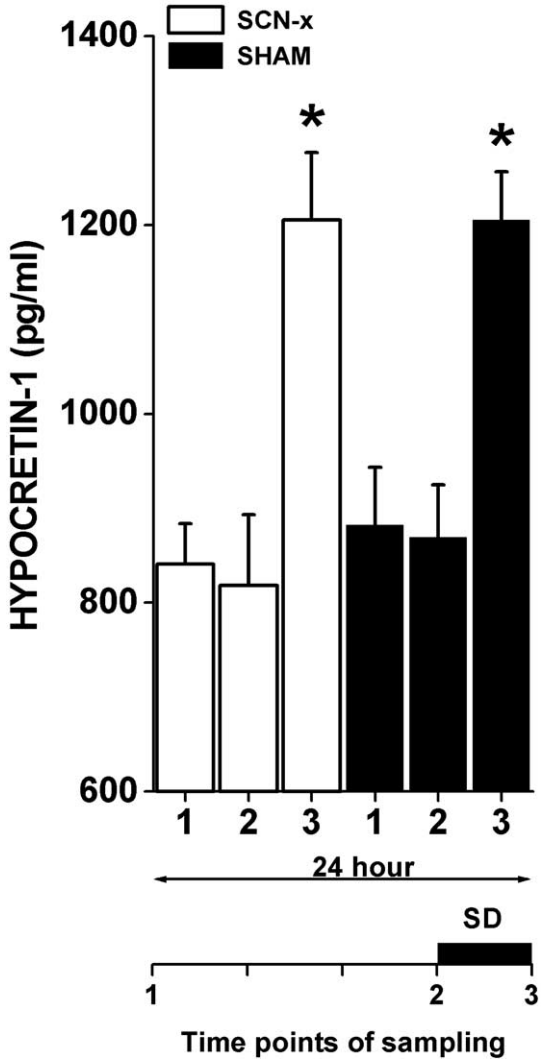

Fig. 4. The effect of SD on hypocretin-1 levels in CSF of SCN-lesioned (SCN-x) and sham-lesioned control animals. Data are means±S.E. (SCN-x $n=8$, Control $n=6$). To allow comparison between control and SCN-x, SD episodes were randomly distributed over CT in the rhythmic group. SD data are in bar number 3. Asterisks indicate a significant increase in hypocretin-1 compared with samples taken before the start of SD (2) or 24 h before the end of SD (1) ($P<0.05$, two-tailed paired *t*-test).

hypothalamic area. We, therefore, conclude that the low mean levels of hypocretin-1 found in SCN-lesioned animals most likely are attributable to lesions of the SCN.

Other areas in the vicinity of the SCN involved in sleep regulation, i.e. the ventral subparaventricular zone (vSPZ) or the dorsomedial nucleus (Lu et al., 2001; Chou et al., 2003) were not damaged, with the exception of the ventral part of the vSPZ. The maximum extend of the lesion beyond the borders of the SCN was 600 μm above the SCN in one animal resulting in 100% damage of the vSPZ. In four animals the lesion was approximately 400 μm above the SCN (damaging approximately 60% of the vSPZ) and in 11 animals the lesion was up to 250 μm above the SCN, damaging approximately 40–50% of the vSPZ. However, since 75–80% of cells of the vSPZ need to be destroyed

before a clear influence on sleep wake distribution is observed (Lu et al., 2001) we consider the damage of the vSPZ too low to account for our results in all but one animal.

Nocturnal animals such as the rat are mostly active during the night. The data suggest that the SCN stimulates hypocretin-1 release during the animal's active phase. This notion is supported by the finding that c-fos expression in hypocretin-synthesizing neurons is increased after spontaneous waking during the night, but not during the day (Espana et al., 2003). The stimulation of hypocretin releasing neurons may occur via a previously established direct projection (Abrahamson et al., 2001), but also synaptic links through other brain regions, like the subparaventricular zone and the dorsomedial nucleus (Lu et al., 2001; Chou et al., 2003) are likely to be involved. The low hypocretin-1 levels observed in arrhythmic animals underscore the importance of the SCN to promote hypocretin up-regulation at a time of day when the animals is awake, active and alert.

This finding may seem to contradict the fact that the SCN, at a neuronal population level, is electrically active during the animal's resting phase and is quiet during the animal's active phase (Inouye and Kawamura, 1979; Meijer et al., 1998; Yamazaki et al., 1998). Recently it was shown that subpopulations in the SCN display out of phase electrical activity rhythms with maximum electrical activity during the animal's activity (Schaap et al., 2003; Saeb-Parsy and Dyball, 2003). These subpopulations may dominate the projection to the hypocretin neurons. It is also possible that structures such as the subparaventricular zone oscillate in anti-phase to the SCN and transmit the information from the SCN to the hypocretin containing neurons.

The question remains whether hypocretin is a wake or a sleep promoting substance. A large body of evidence supports the notion that hypocretin neurons are part of the wake-promoting apparatus of the brain. Fos staining in hypocretin neurons is a function of prior waking duration (Estabrooke et al., 2001), and application of hypocretin in several brain areas increases waking (reviewed in Willie et al., 2001; Taheri et al., 2002). However, the circadian pattern of hypocretin shows a peak at the time of spontaneous sleep onset and after SD the animals initiate sleep at high hypocretin levels. The second possibility is that hypocretin reflects sleep pressure (Yoshida et al., 2001), as it increases during wakefulness as well as during SD. A third possibility is that hypocretin functions predominantly to stabilize wakefulness after it is initiated (Zeitzer et al., 2003). The latter two options may be intimately related since during wakefulness and during SD, an increment in hypocretin may be required to consolidate wakefulness as a consequence of the increment in sleep pressure.

SD of 6 h induced considerable increments in hypocretin-1 levels in rhythmic and arrhythmic animals. As we could not control vigilance state through electroencephalogram recordings the animals were frequently interrupted already before they could assume a sleep posture. Nevertheless, we cannot confirm that we have performed a complete SD, and interpret our experiment as a mild SD procedure (see also Tobler et al., 1997; Antle and Mistlberger, 2000; Huber et al., 2000; Deboer and Tobler, 2003). Our results showed hypocretin-1 increments above the maximum levels obtained in baseline experiments, consistent with previous studies (Yoshida et al., 2001). Increments in hypocretin levels have also been observed following selective rapid eye movement sleep experiments (Pedrazzoli et al., 2004).

In SCN-lesioned animals hypocretin-1 levels also increased following SD, and reached the same level as in control animals. This indicates that the SCN does not play a role in enhancing hypocretin-1 release in response to SD. A number of studies have proposed that sleep is regulated by the interaction of the circadian pacemaker of the SCN and a homeostatic process (for review: Borbély and Achermann, 2000). We recently showed that the SCN is responsive to sleep need and may adjust its fine-tuning to become adaptive with the sleep–wake cycle (Deboer et al., 2003). It has also been shown that SD induces a phase shift in behavioral activity rhythms (Antle and Mistlberger, 2000). While the circadian system appears responsive to sleep, the amount and depth of sleep seems to be regulated separately from the circadian system. In rats, for example, SCN lesions caused a redistribution of sleep and waking, but did not change their total amount over 24 h (Mistlberger et al., 1983). With the accumulating evidence that hypocretin regulates sleep–wakefulness, it becomes important to establish criteria to define its role. As sleep and wakefulness are under both circadian and homeostatic control, hypocretin-1 is expected to be under dual regulation as well. Moreover, as with sleep, homeostatic hypocretin regulation should be independent of the circadian regulation. The present results show that the hypocretin system meets these criteria. Moreover, they indicate that two relatively independent processes converge on one single sleep regulatory substance.

NOTE ADDED IN PROOF

After submission of the manuscript, experiments investigating the endogenous rhythm of hypocretin-1 release and its dependence on the SCN were published by Zhang et al. (2004).

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