The Wake-Promoting Hypocretin/Orexin Neurons Change Their Response to Noradrenaline after Sleep Deprivation

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Sleep deprivation is accompanied by the progressive development of an irresistible need to sleep, a phenomenon whose mechanism has remained elusive. Here, we identified for the first time a reflection of that phenomenon in vitro by showing that, after a short 2 h period of total sleep deprivation, the action of noradrenaline on the wake-promoting hypocretin/orexin neurons changes from an excitation to an inhibition. We propose that such a conspicuous modification of responsiveness should contribute to the growing sleepiness that accompanies sleep deprivation.

Key words: arousal; hypothalamus; sleep; waking; noradrenaline; orexin

Introduction

Sleep-deprivation studies in humans and many other mammals have repeatedly shown a homeostatic regulation of sleep, consisting of an increase in electroencephalogram (EEG) slow-wave activity during recovery sleep (for review, see Borbely and Achermann, 2000; Tobler, 2000). Slow-wave activity is considered an intensity parameter of sleep that reflects the homeostatic process. The increasing sleep pressure and subjective sleepiness during the deprivation is accompanied by an increase in β activity in the waking EEG (Finelli et al., 2000) and correlates with the subsequent increase in slow-wave activity during sleep. However, in vitro cellular correlates of the sleep-deprived (SD) state, which would be relevant to sleepiness, have not yet been uncovered.

The neurons of the lateral hypothalamic and perifornical areas (LHA/PF) that express hypocretin/orexin (hcrt/orx) (de Lecea et al., 1998; Sakurai et al., 1998) are thought to play a major role in promoting wakefulness (for review, see Siegel et al., 2001; Beuckmann and Yanagisawa, 2002; Sutcliffe and de Lecea, 2002; Taheri et al., 2002). Alteration of the hcrt/orx system is associated with narcolepsy in dogs, mice, and humans (Chemelli et al., 1999; Lin et al., 1999; Nishino et al., 2000; Peyron et al., 2000; Thannickal et al., 2000; Hara et al., 2001), and one dominating feature of that pathology is an abnormal sleepiness.

We thus hypothesized that sleepiness associated with sleep deprivation might be reflected on hcrt/orx neurons. Because preliminary studies have indicated that rat hcrt/orx neurons are consistently excited by noradrenaline (Bayer et al., 2005), a major transmitter of arousal, we examined in vitro whether this response was modified after a short-lasting period of sleep deprivation.

Parts of this work have been published previously in abstract form (Grivel et al., 2004).

Materials and Methods

Slice preparation and electrophysiological recordings. Young Sprague-Dawley rats (from the animal facility of the Geneva Medical Center or Charles River Laboratories, Saint-Aubin les Elbeuf, France) were maintained in a 12 h light/dark cycle (lights on from 8:00 A.M. to 8:00 P.M.) and treated according to the regulations of the Swiss Federal Veterinary Office. At the end of either the sleep deprivation or the control period (see Results), hypothalamic coronal brain slices (thickness, 250–300 μm) containing the hcrt/orx neurons were obtained. They were left to recover for 1 h (until 11:00 A.M.) at room temperature in artificial CSF (ACSF) containing the following (in mM): 130 NaCl, 5 KCl, 1.25 KH2PO4, 1.3 MgSO4, 20 NaHCO3, 10 glucose, and 2.4 CaCl2 (bubbled with 95% O2 and 5% CO2). Individual slices were then transferred to a thermoregulated (32°C) recording chamber, in which they were immersed and continuously superfused at 4–5 ml/min with ACSF. The chamber was mounted on an upright microscope (Axioskop; Zeiss, Oberkochen, Germany) equipped with an infrared camera.

Electrophysiological experiments were then started and, in both conditions, were performed for only 2 h (11:00 A.M. to 1:00 P.M.; see Results). Whole-cell recordings were obtained with patch pipettes that were pulled on a DMZ universal puller (Zeitz-Instrumente, Munich, Germany) from borosilicate glass capillaries (GC150F-10; Clark Electro-medical Instruments, Edenbridge, UK). The pipettes were filled with a solution [8 μl when performing single-cell reverse transcription (RT)-PCR] containing the following (in mM): 126 KMeSO4, 4 KCl, 5 MgCl2, 0.1 BAPTA, 10 HEPES, 8 phosphocreatine, 3 ATP, and 0.1 GTP, pH 7.3 (285–300 mOsm). Whole-cell recordings were performed in the current-clamp mode using an Axopatch 200B amplifier (Axon Instruments, Union City, CA). The membrane potential values were not compensated for junction potential (estimated at ~9.6 mV). Noradrenaline (Sigma, Schnelldorf, Germany) and tetrodotoxin (Latoxan, Rosans, France) were applied at known concentrations in the bath. Synaptic blockade was
achieved by lowering calcium and increasing magnesium (0.1 mM Ca²⁺; 10 mM Mg²⁺).

**Single-cell RT-PCR.** At the end of the recording, the content of the cell was aspirated under visual control into the recording pipette (3–5 μM) and expelled into a test tube in which reverse transcription was performed. hcrt/orx cDNAs were synthesized overnight at 37°C in a total reaction volume of 10 μl containing random hexamer primers (25 μM; Roche Diagnostics, Mannheim, Germany), the four deoxyribonucleotide triphosphates (2.5 mM; Invitrogen, Basel, Switzerland), dithiothreitol (10 μM; Sigma, Buchs, Switzerland), RNase inhibitor (20 U; Promega, Wallisellen, Switzerland), and reverse transcriptase (Superscript II; 200 U; Invitrogen). hcrt/orx cDNAs were then amplified by PCR using the following primers: sense, 5'-TGC CTT CAC TAC GAA CTG TTG CAC CG-3' and antisense, 5'-AGG GAT ATG CCTA GCT CTG CGC C-3'. RT reaction (10 μl), Taq polymerase (Herculase Hotstart DNA polymerase; 2.5 U; Stratagene, Amsterdam, The Netherlands), deoxyribonucleotide triphosphates (0.25 mM; Invitrogen), and a 0.1 μM concentration of each primer were added to a buffer solution (final volume, 50 μl). After 5 min at 94°C, 22 cycles (94°C for 30 s; 72°C for 2 min) of PCR were run, followed by a final elongation period of 10 min at 72°C. A second round of PCR was performed with 5 μl of the first PCR product under similar conditions for 35 cycles. PCR products were separated and visualized in ethidium bromide-stained agarose gel (1%) by electrophoresis. The predicted size of the PCR fragment was 189 bp. In a few experiments, the pipette content was expelled first into tubes containing 20 U of RNase inhibitor (Stratagene) for blocking RNase activity and 20 U of DNase I (Stratagene) for digestion of genomic DNA. Samples were then incubated at 37°C for 30 min, followed by incubation at 75°C for 5 min to inactivate the DNase. The rest of the RT-PCR procedure was as described above.

**Statistics.** To evaluate the degree of independence between the two experimental conditions [control condition (CC) and SD] and the two types of effects observed (regrouped into pure excitatory vs inhibitory component), we tested whether the variation in the distributions arises from a systematic factor or from a random fluctuation using the χ² independence test (S-PLUS 6 software; Insightful Corporation, Reinach, Switzerland).

**Results**

Identification of hcrt/orx cells was done according to criteria devised previously and detailed here. In two consecutive published studies using neurobiotin injection and hcrt/orx immunohistochemistry (Eggermann et al., 2003; Bayer et al., 2005), implying a total of 21 cells, we were indeed able to demonstrate that every single cell in the LHA/PF that had a low-threshold spike (LTS) followed by a depolarizing afterpotential (DAP) and an Iᵥ-dependent rectification was an hcrt/orx cell. An additional five cells, of which one is shown here to demonstrate these basic electrophysiological criteria [Fig. 1B, arrow (LTS) and asterisk (DAP)], were also all hcrt/orx positive [using the same protocols as those used by Bayer et al. (2005)]. Our basic identification criteria are also supported by single-cell RT-PCR studies. Indeed, of 21 cells that had the electrophysiological properties of hcrt/orx neurons, 19 expressed hcrt/orx (data not shown). In contrast, none of the cells (n = 10) that were deemed electrophysiologically to be melanin-concentrating hormone neurons (Eggermann et al., 2003) expressed hcrt/orx. It is noteworthy that, in addition, although care was taken to avoid harvesting the nucleus for the reported RT-PCR studies, an additional six electrophysiologically characterized hcrt/orx cells were analyzed in the presence of DNase to avoid genomic DNA contamination (see Materials and Methods). All six of them expressed hcrt/orx (one is shown in Fig. 1F). The following study was thus done exclusively with cells that had the electrophysiological criteria reported above. In detail, of a total of 125 recorded neurons in the LHA/PF, 48 were hcrt/orx according to these criteria. Of those 48 cells, 34 were found to be stable enough (0.5 h of recording) for a full pharmacological appraisal and thus were used in the present study.

To study hcrt/orx neurons in a CC and an SD condition, avoiding circadian influences, the following experimental protocol was used (Fig. 2). After a normal cycle of sleep and waking, CC rats (n = 13 animals) were allowed to sleep for 2 h (8:00–10:00 A.M.), starting at the onset of the physiological “rest” period (8:00 A.M.). In that condition, when their behavior was continuously monitored by video, rats slept >70% of the time (80.3 ± 1.63%; n = 36). In contrast, rats in the SD group (n = 10) were kept fully awake, being gently sleep deprived (V. V. Vyazovskiy et al., 2004) for the same 2 h interval. The well known homeostatic changes in the sleep EEG after sleep deprivation have been shown to change in proportion to the duration of previous wakefulness and are consistent with the changes after spontaneous episodes of waking (Tobler and Borbely, 1986; Huber et al., 2000). In both control and SD conditions, hypothalamic coronal brain slices containing the hcrt/orx cells were obtained at the same hour (10:00 A.M.) and left to recover for 1 h (until 11:00 A.M.). Electrophysiological experiments were then started and, in both conditions, were performed for only 2 h (11:00 A.M. to 1:00 P.M.). Based on our studies in which the EEG was measured, we hypothesized that the consequences of the sleep deprivation would be manifested during that period (Alfoldi et al., 1990; Tobler et al., 1996; V. V. Vyazovskiy et al., 2004).

When comparing first the mean resting potentials of hcrt/orx neurons in the CC (n = 16) and SD condition (n = 18), we found no difference between the two conditions (mean ± SEM, −46.99 ± 1.07 mV for CC vs −45.36 ± 0.91 mV for SD; Student’s t test; p > 0.05). Similarly, we found no difference in the resting firing rates of hcrt/orx neurons between CC and the SD condition (1.23 ± 0.25 Hz for CC vs 1.17 ± 0.25 Hz for SD; p > 0.05).

We then tested the effect of noradrenaline, briefly bath applied at 100 μM, in both conditions. In the CC, we found that most hcrt/orx cells (12 of 16; 75.0%) were exclusively excited by noradrenaline (Fig. 3A₁), whereas a minority (4 of 16; 25.0%; data not shown) displayed an inhibitory component to the response [pure inhibitory (2 of 16) or biphasic inhibitory/excitatory (2 of
The results for the CC are summarized in Figure 3A. In a striking contrast, after sleep deprivation, almost all cells (16 of 18; 88.9%) displayed an inhibitory component in the response to noradrenaline (asterisk) after sleep deprivation. B, Summary of noradrenaline (asterisk) effects after sleep deprivation. C, D, Comparison of noradrenaline effects on membrane potential and firing rate in the CC (C) and the SD condition (D). Calibration: A<sub>1</sub>, B<sub>1</sub>, 20 mV, 60 s. E, Excitation; I, inhibition; I-E, inhibition/excitation.

To further quantify the results, the variation in membrane potential and firing frequency in response to noradrenaline in both conditions was compared. It was found that the variation in membrane potential in response to noradrenaline differed in a highly significant manner (Student’s t test; p < 0.001) (Fig. 3C) between the CC (ΔV<sub>m</sub> = +1.78 ± 0.79 mV; n = 16) and the SD condition (ΔV<sub>m</sub> = −2.59 ± 0.63 mV; n = 18). Similarly, the variation in firing frequency in response to noradrenaline also differed in a highly significant manner (p < 0.001) (Fig. 3D) between the CC (Δf = +0.92 ± 0.22 Hz; n = 16) and the SD condition (Δf = −0.5 ± 0.11 Hz; n = 17).

We finally tested whether the site of noradrenaline action on hcrt/orx cells is presynaptic or postsynaptic in either condition. We first found that the depolarizing effect characteristic of the CC was present in a high-magnesium/low-calcium solution (n = 3), thus indicating that it is postsynaptic. We then found similar results for the SD condition, because both the pure inhibitory (n = 3) type of response and the mixed inhibitory/excitatory response persisted in either tetrodotoxin (n = 3) or a high-magnesium/low-calcium solution (n = 3).

Discussion

The present study shows that 2 h of total sleep deprivation is sufficient to change the responses of hcrt/orx neurons to noradrenaline from being mostly excitatory (75% of cells) in control conditions to mostly inhibitory (nearly 90% of cells) after sleep deprivation. Both response types are postsynaptic.

Although the mechanism underlying this change was not investigated in the present report, it is noteworthy that it does not involve a modification of the resting membrane potential or the spontaneous firing rate of the hcrt/orx cells. Also of importance is the observation that inhibitory and excitatory responses to noradrenaline are still present when synaptic transmission is blocked. This latter result shows that the change induced by sleep deprivation is expressed postsynaptically. It could depend on the noradrenergic receptors themselves or the pathways linking them to channels modulated by their activation. Additional studies will be required to address these issues. Interestingly, in that respect, immunohistochemical studies of hcrt/orx neurons have identified recently that, in rats, both α<sub>1</sub>- and α<sub>2</sub>-noradrenergic receptors can be present on these cells (B. E. Jones, personal communication).

As discussed in our recent report of the excitatory effects of noradrenaline on hcrt/orx neurons in rats (Bayer et al., 2005), we do not have a firm explanation for the hyperpolarizing effect reported for noradrenaline on mice hcrt/orx neurons in the absence of sleep deprivation (Li et al., 2002; Yamanaka et al., 2003; Li and van den Pol, 2005). One is left with the speculation that it could be attributable to a species difference. Additional studies in mice in different circadian and homeostatic conditions might eventually shed light on this intriguing discrepancy.

Most recent sleep-deprivation studies have dealt with the molecular aspects of sleep homeostasis. Gene expression studies during the sleep–waking cycle and after sleep deprivation have thus indicated that different processes might be favored in one or the other state (Cirelli et al., 2004). During wakefulness, genes related to excitatory synaptic transmission and synaptic potentiation are expressed preferentially, whereas during sleep, genes implicated in synaptic inhibition and depression as well as protein synthesis are favored. These evidences support the notion that one important role of sleep might be related to synaptic plasticity (Tononi and Cirelli, 2003; Huber et al., 2004). However, these studies,
which deal mainly with the homeostatic function of sleep, do not provide an insight into a cellular mechanism of sleepiness, which might or might not be directly related to the homeostatic process. To our knowledge, the findings reported here represent the first neurobiological trace of sleep deprivation discernible \textit{in vitro} that is directly relevant to the phenomenon of sleepiness. Indeed, the emergence of inhibition by noradrenaline after sleep deprivation is functionally equivalent to a decreased efficacy of the hcrt/orx system, which is directly relevant to the phenomenon of sleepiness. Indeed, the striking modification of responsiveness of hcrt/orx neurons to noradrenaline observed in the brain (Peyron et al., 1998) that are involved in maintaining the waking state (Jones, 2003). Therefore, the striking modification of responsiveness of hcrt/orx neurons to noradrenaline observed in the present study might contribute to the increasing sleepiness that accompanies sleep deprivation.

References


