Relationship between CSF hypocretin levels and hypocretin neuronal loss

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Abstract

The sleep disorder narcolepsy may now be considered a neurodegenerative disease, as there is a massive reduction in the number of neurons containing the neuropeptide, hypocretin (HCRT). Most narcoleptic patients have low to negligible levels of HCRT in the cerebrospinal fluid (CSF), and such measurements serve as an important diagnostic tool. However, the relationship between HCRT neurons and HCRT levels in CSF in human narcoleptics is not known and cannot be directly assessed. To identify this relationship in the present study, the neurotoxin, hypocretin-2-saporin (HCRT2-SAP), was administered to the lateral hypothalamus (LH) to lesion HCRT neurons. CSF was extracted at circadian times (ZT) 0 (time of lights-on) or ZT8 at various intervals (2, 4, 6, 12, 21, 36, 60 days) after neurotoxin administration. Compared to animals given saline in the LH, rats with an average loss of 73% of HCRT neurons had a 50% decline in CSF HCRT levels on day 60. The decline in HCRT levels was evident by day 6 and there was no recovery or further decrease. The decline in HCRT was correlated with increased REM sleep. Lesioned rats that were kept awake for 6 h were not able to release HCRT to match the output of saline rats. As most human narcoleptics have more than 80% reduction of CSF HCRT, the results from this study lead us to conclude that in these patients, virtually all of the HCRT neurons might be lost. In those narcoleptics where CSF levels are within the normal range, it is possible that not all of the HCRT neurons are lost and that the surviving HCRT neurons might be increasing output of CSF HCRT.

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Introduction

The peptide hypocretin (HCRT), also known as orexin, was recently linked to the human sleep disorder narcolepsy since canines with familial narcolepsy possess a mutation in the HCRT2 receptor (Lin et al., 1999). HCRT/orexin gene knockout mice (Chemelli et al., 1999) or mice with a targeted destruction of the HCRT neurons (Hara et al., 2001) exhibit symptoms of narcolepsy. Subsequently, in a study of a small number of postmortem brains of human narcoleptics, it was discovered that there is a massive reduction in the number of HCRT-containing neurons (Peyron et al., 2000; Thannickal et al., 2000). Neurons containing HCRT are found only in the lateral hypothalamus (LH) from where they innervate widespread regions of the neuroaxis with a propensity to innervate regions known to be involved in sleep/wake control (Peyron et al., 1998; Sakurai et al., 1998).

Narcoleptic patients have reduced cerebrospinal fluid (CSF) levels of HCRT (Dalal et al., 2001; Kanbayashi et al., 2002; Mignot et al., 2002; Nishino et al., 2000, 2001; Ripley et al., 2001), a finding consistent with the loss of HCRT-containing neurons. CSF measurements of HCRT provide a valuable diagnostic tool for narcolepsy, separating narcolepsy from other sleep and neurological disorders (Mignot et al., 2002; Ripley et al., 2001). However, currently in narcolepsy patients, investigators cannot gauge the extent of neuronal loss based on measurements of CSF HCRT levels since the relationship between HCRT neuronal loss and change in levels of the peptide is not known. To provide such data, in the present study, we used the neurotoxin, saporin, conjugated to HCRT2 (HCRT2-SAP)
to lesion HCRT receptor-bearing neurons in the LH. In a previous study, we demonstrated that the conjugate binds with a high affinity to HCRT2 receptor-bearing cells, to a lesser degree to the HCRT1 receptor-bearing cells, and does not bind to substance-P receptor-bearing cells (Gerashchenko et al., 2001). HCRT neurons possess the HCRT receptor and are lesioned by the HCRT2-SAP (Gerashchenko et al., 2001). The lesions in the LH also produce rats with symptoms characteristic of narcolepsy, such as sleep fragmentation, sleep-onset REM sleep periods, increased NREM sleep and REM sleep time during the normally active lights-off period (Gerashchenko et al., 2001). The lesions in the LH also produce

Materials and methods

Animals and surgical preparation

Twenty-one male Sprague–Dawley rats (400–550 g) were housed singly in Plexiglas cages with wood-shavings. In a room with controlled temperature (21 ± 0.5°C) and light–dark cycle (7 AM–7 PM lights-on; 150 lx). Food and water were available ad libitum. The rats were anesthetized (isoflurane) and CSF was collected as described previously. This CSF represented the post-sleep deprivation portion of the study. CSF was collected from the cisterna magna (Isofluorane anesthesia) (Fujiki et al., 2001) with a needle (27G) and immediately frozen on dry ice and maintained at −70°C until analysis. Suction was not applied to extract the CSF but instead the CSF was allowed to flow freely and slowly accumulate in the hub of the syringe needle. On the average, 100 μl of CSF was collected each time. Blood-tinged CSF was discarded.

Since half of the rats were sampled on some days and the other half on other days, it was necessary to combine data so that there would be sufficient sample size for data analysis. Therefore, data collected on days 2, 4 and 6 were combined into one data point, and is represented as day 6. Data collected on days 21 and 36 after the injection and at ZT6 and served as the reference point for the sleep deprivation portion of the study. CSF was collected from the cisterna magna (Isofluorane anesthesia) (Fujiki et al., 2001) with a needle (27G) and immediately frozen on dry ice and maintained at −70°C until analysis. Suction was not applied to extract the CSF but instead the CSF was allowed to flow freely and slowly accumulate in the hub of the syringe needle. On the average, 100 μl of CSF was collected each time. Blood-tinged CSF was discarded.

Sleep deprivation

Fifty days after HCRT2-SAP administration, the rats were kept awake for 6 h (ZT0–ZT6) by lightly tapping the cage whenever they showed EEG and behavioral signs of sleep. Immediately after the sleep deprivation, the rats were anesthetized (Isofluorane) and CSF was collected as described previously. This CSF represented the post-sleep deprivation data point and was compared with CSF collected at the same ZT time point but without any sleep deprivation (CSF collected at day 45).

HCRT radioimmunoassay (RIA)

HCRT1 was measured using commercially available 125I RIA kits (Phoenix Pharmaceuticals, Belmont, CA). Twenty-five microliters of CSF were mixed with 75 μl of RIA buffer and directly applied to the RIA. The detection limits of the assay were 100 pg/ml. All comparative
samples were measured in a single assay and the intra-assay variation was < 5%.

**Immunohistochemistry**

Sixty days after the injection of HCRT2-SAP, the animals were deeply anesthetized with pentobarbital (150 mg/kg i.p.) and perfused transcardially with 0.9% saline (50 ml) followed by 500 ml of phosphate-buffered 4% paraformaldehyde (pH 7.0). The brains were postfixed overnight, equilibrated in 30% sucrose, and stored at 4°C. Five series of coronal sections were cut at 30 µm on a sliding microtome. Each set of coronal brain sections was incubated overnight at room temperature in the primary antibody [rabbit anti-orexin-A (HCRT1)] (1:70,000, Peninsula Laboratories, Inc., San Carlos, CA). After washing, the sections were incubated with the secondary antibody for 1 h (Chemicon; 1:500 dilution) and then reacted with avidin–biotin complex for 1 h (Vector Laboratories, Burlingame, CA). The DAB method was used to visualize the reaction product. Omission of the primary antiserum resulted in no specific staining.

**Analysis of sleep–wake states**

EEG and EMG signals were recorded on a Grass polygraph. The 24-h EEG and EMG recordings obtained on the 11th and 20th day postinjection were analyzed. The other days were not included in the analysis since the CSF was being extracted every other day from days 2–6 post-injection and the effects of the anesthetic (isofluorane) and/or the extraction of the CSF would alter sleep patterns. We also could not record sleep continuously through day 60 because in most of the rats, the EEG screws became disconnected by day 30. The EEG sleep records were scored manually on a computer (Icelsus software, Mark Opp, Ann Arbor, MI, USA) in 12-s epochs for awake, rapid-eye movement (REM) sleep and non-REM (NREM) sleep by staff blind to the type of drug administered to the rats, and using criteria described previously (Gerashchenko et al., 2001). The amount of time spent in wakefulness, NREM and REM sleep was determined for each hour. After the EEG data were scored, the code was broken to reveal the identity of each rat.

**Cell counts**

A person blind to the lesion status of the rats counted clearly stained HCRT-immunoreactive (ir) somata in both hemispheres in 22 sections (2 in 5 series) that encompassed the full extent of HCRT distribution (between − 1.6 and − 3.8 mm from bregma (Paxinos and Watson, 1986)). Cells were counted using Nikon E400 Eclipse microscope equipped with Y-IDT drawing tube that allowed positioning of counting grid. Details of the cell counting procedure were discussed previously (Gerashchenko et al., 2001).

**Statistical analysis**

Analysis of variance and t tests with Bonferroni correction (where appropriate) were used to compare changes in sleep parameters (SYSTAT, Version 8.0, SPSS Inc., 1998). The same statistical methods were also used to compare

![Fig. 1. Photomicrographs of HCRT neurons in the LH of representative rats administered saline (A) or HCRT2-SAP (B). HCRT2-SAP administration to the LH resulted in rats with average loss of 14.4% (range = 5–21.8%) or 72.7% (range = 56.2–86.4%) (B) of HCRT neurons. Abbreviations: f = fornix.](image)

![Fig. 2. The CSF HCRT (pg/ml) levels after administration of HCRT2-SAP or saline into the LH. CSF was extracted at either circadian time (ZT) 0 (time of lights on in a 12:12 h light–dark schedule) or ZT8 (8 h after lights turn on) at progressively longer time periods (6, 21, 60 days) after administration of the neurotoxin. There were no significant changes in HCRT levels across days but in rats with an average loss of 73% of HCRT neurons (closed squares), the HCRT levels were significantly lower compared to saline (open circles) rats at ZT0 and ZT8 (see text for details). Asterisk denotes P < 0.05 compared to closed squares.](image)
counts of HCRT-immunoreactive cells. Statistical significance was evaluated at the $P < 0.05$ level.

**Results**

Previously, we showed that the HCRT2-SAP destroys HCRT receptor-bearing neurons (Gerashchenko et al., 2001) and since HCRT neurons contain the HCRT receptor, there was a loss of HCRT-immunoreactive neurons (see Fig. 1). The rats injected with the neurotoxin were divided into two groups based on the number of HCRT neurons that were present. The first group consisted of rats with average loss of 14.4% of HCRT neurons (range = 5–21.8%). The second group consisted of rats with average loss of 72.7% of HCRT neurons (range = 56.2–86.4%).

Fig. 2 summarizes the change in CSF HCRT levels across postinjection days. A two-way repeated measures ANOVA revealed a significant between group effect in that rats with average loss of 72.7% HCRT neurons displayed a significant decline in CSF HCRT levels compared to saline rats at ZT0 ($F = 20.927; df = 1; P < 0.001$) and at ZT8 ($F = 8.185; df = 1; P < 0.012$) (Fig. 2). However, there was no significant change across days (within group effect; $F = 0.415; df = 2; P < 0.664$), indicating that following the initial decline in CSF HCRT levels on days 2–6, the levels remained low until day 60 when the rats were sacrificed. The interaction between days and lesioned groups was also not significant.

Saline rats had significantly higher HCRT levels at ZT0 ($t = 4.534, P = < 0.001$) compared to ZT8, a finding consistent with recent reports (Fujiki et al., 2001; Yoshida et al., 2001). The rats with a 72.7% HCRT neuronal loss did not show a significant difference between ZT0 and ZT8 ($t = 1.356, P = 0.197$) 60 days after the HCRT2-SAP injection (Fig. 2), indicating a blunting of the diurnal variation in peptide levels. At ZT0, there was a significant correlation between HCRT neurons and HCRT levels ($r = 0.759; P < 0.0000654; n = 21$). If the saline rats are excluded from the correlation analysis, then there is still a significant positive relationship between number of HCRT neurons and CSF HCRT levels ($r = 0.56; n = 13; P < 0.0475$).

Rats with average loss of 14.4% HCRT neurons showed no significant decline in CSF HCRT levels compared to saline rats at ZT0 or at ZT8, nor did they show a significant relationship between CSF levels and HCRT number.

**Fig. 3** summarizes the results of the sleep deprivation portion of the experiment. Six hours of prolonged waking increased HCRT levels in saline (+ 325 pg/ml; $t = 4.051, P < 0.001$) rats and in rats with an average loss of 14.4% HCRT neurons (+ 426 pg/ml; $t = 3.471, P < 0.026$). However, in rats with a greater loss of HCRT neurons, initial decline in CSF HCRT levels on days 2–6, the levels remained low until day 60 when the rats were sacrificed. The interaction between days and lesioned groups was also not significant.

Table 1: Average percent ($\pm$ SEM) of wakefulness, NREM, or REM sleep, in rats with various HCRT cell loss produced by HCRT2-SAP

<table>
<thead>
<tr>
<th>Group</th>
<th>Light-off period</th>
<th>Light-on period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wakefulness</td>
<td>NREM sleep</td>
</tr>
<tr>
<td>Saline ($n = 8$)</td>
<td>73.3 $\pm$ 2.1</td>
<td>22.1 $\pm$ 1.7</td>
</tr>
<tr>
<td>14.4% HCRT neuronal loss ($n = 4$)</td>
<td>75.5 $\pm$ 5.4</td>
<td>19.0 $\pm$ 4.1</td>
</tr>
<tr>
<td>72.7% HCRT neuronal loss ($n = 9$)</td>
<td>73.0 $\pm$ 2.7</td>
<td>21.2 $\pm$ 2.3</td>
</tr>
</tbody>
</table>

The values were calculated during 12 h light-off or 12 h light-on period 11–20 days after the HCRT2-SAP injection. $* P < 0.05$, significant difference compared with the values of the saline group.
increase in HCRT levels was much smaller (+180 pg/ml, \( t = 2.418, P < 0.031 \)).

Table 1 summarizes the percentage of sleep during the lights-on and lights-off periods in the three groups of rats. Consistent with our previous studies (Gerashchenko et al., 2001, 2003), the HCRT2-SAP-induced lesions of the LH produced a significant and long-lasting increase (23%) in REM sleep during the night cycle (\( t = 2.653, df = 15; P < 0.0341 \)). Rats with an average loss of 14.4% HCRT neurons did not have a significant change in sleep. The nighttime REM sleep levels were negatively correlated with CSF HCRT levels measured at ZT8 (Fig. 4) (\( r = -0.635; df = 21; P < 0.00197 \)) and ZT0 (\( r = -0.475; df = 21; P < 0.0341 \)).

**Discussion**

In the only two studies where reductions in the number of HCRT neurons have been determined (Peyron et al., 2000; Thannickal et al., 2000), CSF HCRT levels were not measured. We have now found that there is a 50% decline in CSF HCRT levels when 73% of the HCRT neurons are lost. As the decline in CSF levels does not exactly match the neuronal loss, the 27% surviving neurons appear to compensate for the HCRT neuronal loss by increasing the levels of HCRT. However, human narcoleptics have very low to negligible levels of CSF HCRT, which leads us to conclude that in these patients, virtually all of the HCRT neurons are lost. In those narcoleptics where CSF levels are within the normal range, it is possible that not all of the HCRT neurons are lost and that the surviving HCRT neurons might be increasing output of CSF HCRT.

The HCRT2-SAP kills cells containing the HCRT receptor (Gerashchenko et al., 2001) and it does not bind to non-HCRT receptors (Gerashchenko et al., 2001). The LH contains HCRT receptor 2 and to a lesser extent HCRT-receptor 1 (Marcus et al., 2001). However, it is not known which receptor is found on what neuron in the LH. Previously, we found that HCRT2-SAP also kills other non-HCRT neurons in the LH, such as melanin concentrating hormone (MCH) containing neurons (Gerashchenko et al., 2001). To what extent MCH and other non-HCRT neurons influence release of HCRT is still unclear. HCRT neuronal activity is strongly influenced by glutamate, but it is unclear whether the glutamate is from local neurons or whether glutamate is secreted from the HCRT neuron itself (Li et al., 2002). Nevertheless, through a process of elimination, one can determine which of the LH neuronal populations are relevant to sleep/wakefulness. For instance, one can eliminate the neurons containing MCH since MCH null mice do not show narcoleptic symptoms and narcoleptic patients do not have a loss of MCH neurons (Peyron et al., 2000; Thannickal et al., 2000). To what extent loss of other non-HCRT neurons affects CSF HCRT levels remains to be determined.

Once the HCRT neurons died during the initial phase of the neurotoxin-induced cell death, the CSF HCRT levels also declined and there was no further decline (Fig. 2). In a previous report (Gerashchenko et al., 2001), we showed that the death of most HCRT neurons occurred between 4 and 12 days after drug administration. The present study indicates that HCRT levels also decline during this initial phase and that there is no further change in HCRT levels. Moreover, 6 h of prolonged wakefulness also did not produce as great an increase in HCRT levels in lesioned rats as in saline-treated animals (Fig. 3). The attenuated levels of HCRT in response to prolonged wakefulness may explain one of the primary symptoms of narcolepsy, that is, difficulty in maintaining wakefulness even with alerting stimulation. Other neurotransmitters and/or peptides may also regulate wakefulness but only loss of HCRT neurons has been associated with narcolepsy.

CSF levels were measured at ZT0 (the time when lights turn on) to coincide with the peak of the HCRT levels in non-lesioned rats (Fujiki et al., 2001; Yoshida et al., 2001). We also measured at ZT8 since at this time point, CSF HCRT levels are declining. We reasoned that differences between lesioned and non-lesioned rats would be clearly evident at ZT0 when HCRT levels are at their peak (Fujiki et al., 2001; Yoshida et al., 2001). On the other hand, at ZT8 when HCRT levels decline normally, there might not be a difference between lesioned and non-lesioned rats because of a “floor-effect”. In the present study, the difference in HCRT levels was clearly evident at ZT0, indicating that lesioned rats are unable to match the output of control rats. At ZT8, HCRT levels were 35% lower in HCRT lesioned rats compared to saline rats (Fig. 2). This indicates that the normal decline in CSF HCRT levels can still be detected.

The higher CSF levels at ZT0 in saline rats are most likely due to the increased waking behavior during the lights-off active phase (Wu et al., 2002; Yoshida et al., 2001). In the present study, the rats were kept awake for 6 h during the rat’s normal sleep period to determine whether surviving HCRT neurons were able to fully compensate for the neuronal loss. With an average 27% of the HCRT neurons surviving, the rats were not able to release sufficient amount of HCRT in response to prolonged wakefulness compared to saline-treated rats or rats with 86% of HCRT neurons intact (see Fig. 3). Short-term prolonged waking does not affect HCRT mRNA levels (Terao et al., 2000) yet levels of the peptide are increased, suggesting a different time-course between transcription of HCRT mRNA and HCRT peptide synthesis. We suggest that conducting a forced activity paradigm in narcoleptics could be useful in assessing the extent of the HCRT neuronal loss in narcolepsy. Based on the present findings, we hypothesize that there will not be an increase in HCRT levels in narcoleptics with very low levels of HCRT.

In lesioned rats at day 60, there was no significant difference in CSF HCRT levels between ZT0 and ZT8, indicating a blunting of the diurnal rhythm of peptide levels.
Previously, we found (Gerashchenko et al., 2001, 2003) that when 90% of HCRT neurons were lost, there was a blunting of the diurnal variation in waking, non-REM sleep time and in core temperature rhythm. In the present study, in the lesioned rats, on average 73% of HCRT neurons were lost and this produced a 50% decline in peptide levels, but no blunting of the day versus night variation in waking or non-REM sleep. There may be compensation at the receptor or second messenger level, which may have prevented blunting of the diurnal variation in waking and sleep. We suggest that with greater neuronal loss, there would be less circulating peptide and this could then more adversely effect the diurnal variation in waking, sleep and core temperature. Greater HCRT neuronal loss would also produce more hypersomnia.

The lesion of HCRT neurons and the corresponding 50% decline in peptide levels produced a long-lasting increase in REM sleep at night (Table 1). This is consistent with our previous reports (Gerashchenko et al., 2001, 2003) and findings from the murine knockout models (Chemelli et al., 1999; Hara et al., 2001) where nighttime levels of REM sleep were higher than controls. More importantly, there was a significant negative relationship between HCRT levels and REM sleep amounts (Fig. 4). Such a relationship has not been documented in human narcoleptics or in the canine or murine models of narcolepsy. The decline in HCRT levels would promote REM sleep at night in the lesioned rats and is consistent with the accumulating data that the absence of HCRT is permissive to REM sleep at night (Chemelli et al., 1999; Gerashchenko et al., 2001, 2003; Hara et al., 2001). During the day, REM sleep amounts were lower in HCRT-deficient rats than in control rats in the present study, in our previous studies (Gerashchenko et al., 2001, 2003) and also in murine knockout models (Chemelli et al., 1999; Hara et al., 2001). Why a decline in HCRT produces an imbalance in REM sleep between the day and night is not known, but may involve the effects of light on REM sleep triggering in pigmented versus albino rats (Miller et al., 1999).

In most narcoleptic patients, CSF HCRT levels are low to negligible (Dalal et al., 2001; Kanbayashi et al., 2002; Mignot et al., 2002; Nishino et al., 2000, 2001; Ripley et al., 2001). From the present findings, this would suggest that at these levels, virtually all of the HCRT neurons might be absent. This is consistent with the two published reports (Peyron et al., 2000; Thannickal et al., 2000) that also found few HCRT-containing neurons in narcoleptic patients. However, in some narcoleptic patients, the levels are normal (Kanbayashi et al., 2002; Nishino et al., 2001), suggesting that in these patients, the majority of the HCRT neurons might still be intact. Since the actual HCRT neuronal counts can be determined only upon autopsy, the present study provides data regarding extent of HCRT-neuronal loss and selection of treatment choices for narcolepsy.

Unlike Parkinson’s disease where the primary defect is in the nigrostriatal dopamine pathway (Fearnley and Lees, 1991; Hornykiewicz, 1975; Kish et al., 1988), in narcolepsy it is not known which HCRT innervation to which target site is responsible for each individual aspect of the disease phenotype. HCRT neurons heavily innervate neuronal populations implicated in arousal (Peyron et al., 1998). Projections to the dopamine system might also influence arousal and motor control. In future studies, it will be important to monitor levels of HCRT at all major target sites to determine whether HCRT levels are preserved in some target sites relative to other targets.

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