

# Reduced Number of Hypocretin Neurons in Human Narcolepsy

# Clinical Study

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## Summary

Murine and canine narcolepsy can be caused by mutations of the hypocretin (Hcrt) (orexin) precursor or Hcrt receptor genes. In contrast to these animal models, most human narcolepsy is not familial, is discordant in identical twins, and has not been linked to mutations of the Hcrt system. Thus, the cause of human narcolepsy remains unknown. Here we show that human narcoleptics have an 85%–95% reduction in the number of Hcrt neurons. Melanin-concentrating hormone (MCH) neurons, which are intermixed with Hcrt cells in the normal brain, are not reduced in number, indicating that cell loss is relatively specific for Hcrt neurons. The presence of gliosis in the hypocretin cell region is consistent with a degenerative process being the cause of the Hcrt cell loss in narcolepsy.

## Introduction

Narcolepsy is an incurable, debilitating, neurological disease characterized by sleep attacks, episodic loss of muscle tone (cataplexy), hypnagogic hallucinations, and abnormalities of the sleep–wake cycle. It was first described more than 120 years ago (Westphal, 1877; Fisher, 1878; Gelineau, 1880), but its cause is unknown. Recently, it was found that genetically narcoleptic dogs have a mutation in the Hcrt receptor 2 gene (Lin et al., 1999). At about the same time, it was reported that mice with a null mutation of the preprohypocretin gene, which produces Hcrt-1 and Hcrt-2, also show symptoms of narcolepsy (Chemelli et al., 1999). These findings suggest that malfunction of the Hcrt system may underlie the syndrome of human narcolepsy.

However, the genetics of human narcolepsy differ significantly from that in these animal models. Most human narcoleptics have no first degree narcoleptic relatives (Guilleminault, 1994), and most pairs of monozygotic twins are discordant for narcolepsy (Hublin et al., 1994). Nearly all narcoleptics examined so far do not have mutations of the Hcrt peptide precursor or receptor genes (Faraco et al., 2000). Because of these findings and the relatively late onset of the disorder in most narcoleptics, usually in the second, third, or fourth decade, it has been speculated that narcolepsy may be linked to postnatal changes in brain function.

Genetically narcoleptic dogs have been shown to have normal numbers of Hcrt cells (Thannickal et al., 2000). However, we hypothesized that whereas mutations in the peptide precursor and receptor genes can trigger the disease in mice and dogs, loss of the Hcrt neurons themselves could be responsible for most cases of human narcolepsy (Siegel, 1999).

## Results and Discussion

The hypothalamus of 16 human brains, including four narcoleptics and 12 neurologically normal individuals, were immunostained for Hcrt. Three of the narcoleptic brains had been in 10% formalin fixative for 4–5 years. A fourth was moved from formalin to a sodium azide solution shortly after fixation and remained refrigerated in this solution at 4° C for 12 years. We found that it was necessary to use antigen retrieval techniques (Jiao et al., 1999) on all the narcoleptic brains to stain the Hcrt neurons.

To control for the time spent in fix, we processed three neurologically normal brains that, like the narcoleptic brains, had been held in fix in our laboratory for five or more years and stained them with the same antibodies (Table 1). As in the narcoleptic brains, no Hcrt staining was apparent in these normal brains unless antigen retrieval techniques were used. An additional nine nonnarcoleptic human brains that had been in fix for 2 months to 2 years were stained without the use of antigen retrieval.

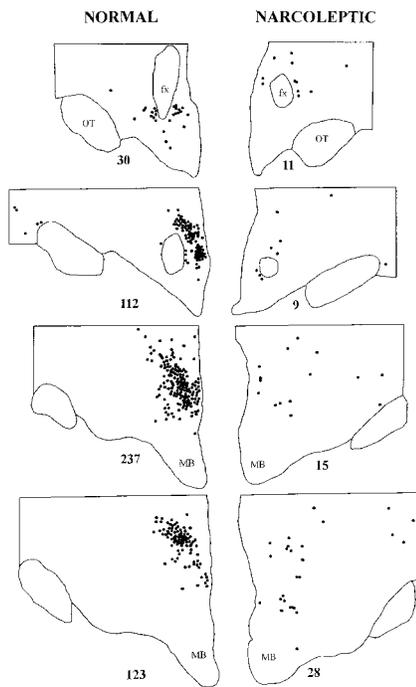
Hypothalamic tissue from the brains of neurologically normal individuals held in fix for up to 16 years and treated for antigen retrieval, and relatively fresh neurologically normal tissue held in fix for less than 2 months and stained without antigen retrieval techniques showed the same pattern of Hcrt staining. There was no significant relation between time in fix and number of Hcrt cells stained ( $p > 0.5$ ,  $r = 0.25$ ,  $df = 7$ ). There was also no significant correlation with the age ( $p > 0.2$ ,  $r = -0.60$ ,  $t = -1.29$ ,  $df = 3$ ), or sex ( $p > 0.25$ ,  $t = 1.2$ ,  $df = 7$ ) of the subjects (see Table 1).

We found that the narcoleptic brains had a dramatic loss of Hcrt cells. The number of Hcrt neurons in narcoleptics was reduced by between 85% and 95% percent from the number seen in controls ( $p < 0.000001$ ,  $t = 10.9$ ,  $df = 11$ ; Figures 1, 2, and 3).

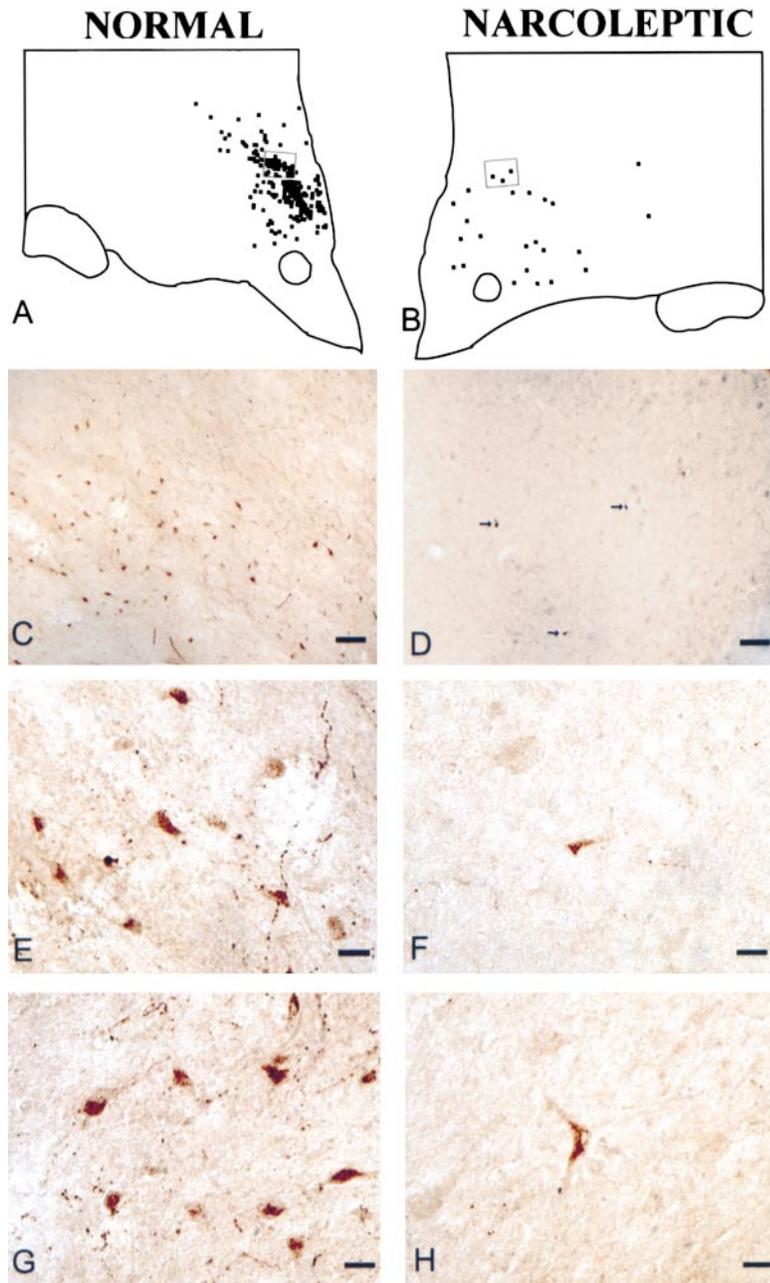
Despite the difference in the number of Hcrt cells, there was no marked difference in the number of melanin-concentrating hormone (MCH) neurons (Figure 4), which are intermixed with the Hcrt neurons in the normal

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# Deceased.



**Figure 1. Distribution of Hypocretin-Labeled Somas in Normal and Narcoleptic Subjects**  
Normal is subject CG, and narcoleptic is subject NA. Cell counts are listed below each section. On average, the narcoleptics had only 7% of the Hcrt cells seen in normals.



**Figure 2. Distribution of Cells in Perifornical and Dorsomedial Hypothalamic Regions of Normal and Narcoleptic Humans Plotted in Figure 1**

All cells in figure are from the levels indicated in (A) and (B). (C and D) Low power photomicrographs (calibration [Cal.] = 100  $\mu$ m) covering regions outlined in gray at the top shows many Hcrt somas and axons in the normal and few in the narcoleptic (arrows in narcoleptic indicate Hcrt somas verified as immunolabeled at higher magnification). Higher power photomicrographs (Cal. = 25  $\mu$ m) show Hcrt cells and axons in normal (E and G) and narcoleptic (F and H) subjects. Note the reduced density of axonal staining in the background of the narcoleptic as compared to that seen in the normal.

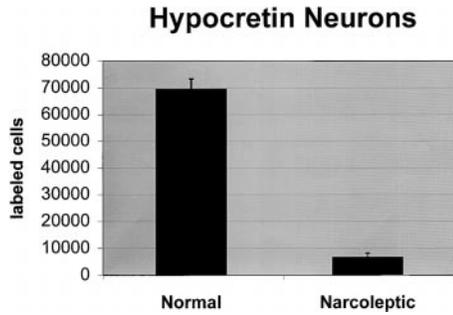


Figure 3. The Number of Hcrt Cells Is Decreased in Narcoleptics

brain (Broberger et al., 1998). We counted MCH cells at their level of highest density (anterior mammillary nucleus) in matched sections from four narcoleptics and four controls (Table 1). The number of MCH cells in the narcoleptics ( $731 \pm 157$ ) did not differ from the number in controls ( $734 \pm 119$ ) ( $p > 0.9$ ,  $t = 1.1$ ,  $df = 7$ ). Thus, the reduction in numbers of Hcrt neurons in narcoleptics was not due to a nonspecific loss of hypothalamic cells or a generalized loss of antigenicity in the tissue we tested. The significantly increased glial fibrillary acidic protein (GFAP) labeling seen in the narcoleptic brains in comparison to controls (see below) is additional evidence of the preservation of antigenicity.

The antigen retrieval techniques that we have employed also do not contribute to the observed loss of Hcrt cells. We were able to apply the same antigen retrieval techniques to human brains that we had stored in the laboratory under identical conditions for the same or greater amounts of time. Antigen retrieval is thought to act by reversing the protein cross-linking and protein denaturation that occurs with aldehyde-based fixation. Antigen retrieval by this technique produces a progressive increase in the intensity of staining in labeled cells from below detectable levels in "overfixed" tissue to readily observable levels. More extended treatment does not alter the number of cells labeled but will cause disintegration of the tissue (Jiao et al., 1999). As was the case with the narcoleptic brains, Hcrt staining was not visible in the untreated normal brains. However, with antigen retrieval treatment under conditions identical to those applied to the narcoleptic tissue, these brains had normal numbers of labeled Hcrt neurons, whereas the narcoleptics did not.

Hcrt neurons in humans have dense projections to locus coeruleus, raphe nuclei, and basal forebrain

(Moore et al., 2000). It has been hypothesized that a reduction of Hcrt activation of the monoaminergic nuclei of the brainstem may be responsible for the occurrence of cataplexy and that a reduction in Hcrt activation of cholinergic brainstem and basal forebrain neurons may cause the sleepiness of narcolepsy (Siegel, 1999; Wu et al., 1999). The loss of Hcrt neurons seen in the present study is consistent with these hypotheses. The greatly reduced numbers of Hcrt neurons can also explain the reduced levels of Hcrt reported in the cerebrospinal fluid of narcoleptics (Nishino et al., 2000). Hcrt neurons have a dense projection to the suprachiasmatic nucleus (Moore et al., 2000). The loss of this projection can explain the reduced amplitude of the circadian sleep rhythm in narcoleptics, resulting in increased sleepiness during the day and interrupted sleep at night (Guilleminault, 1994).

The reduced numbers of Hcrt cells that we see in the narcoleptics could result from a failure of the normal developmental processes that produces these cells. However, this hypothesis would not explain the relatively late and variable age of onset of the disorder. An alternative hypothesis is that neuronal degeneration causes the cell loss. Recent studies in narcoleptic dogs reported neuronal and axonal degeneration at the time of symptom onset (Siegel et al., 1999), although this degeneration does not cause a significant loss of Hcrt neurons (Thannickal et al., 2000). We stained the hypothalamus at the level of the mammillary bodies in the human narcoleptic and control brains for GFAP and counted the number of astrocytes per square millimeter in the center of the hypothalamic Hcrt cell region, 2.3 mm lateral to the ventricular surface, blind to the condition of the subjects (Figures 5 and 6). The narcoleptics showed a significantly higher number of labeled astrocytes compared to controls ( $p < 0.001$ ,  $t = 6.4$ ,  $df = 7$ ) (Figure 6). The same comparison was made on the anterior thalamic nucleus, just dorsal to the Hcrt region, 2.5 cm dorsal to the base of the brain, and 2.3 mm from the lateral ventricle. The thalamus has no Hcrt cells (Peyron et al., 1998). There was no significant difference between the level of gliosis in the narcoleptics and controls in the thalamus ( $p > 0.2$ ,  $t = 1.5$ ,  $df = 7$ ) (Figure 6). These observations and a prior case report of hypothalamic gliosis in an idiopathic narcoleptic (Erich and Itabashi, 1986) support the neuronal degeneration hypothesis.

Human narcolepsy has been shown to be linked to the presence of the HLA-DR2 or HLA-DQB1\*0602, with 85%–95% of narcoleptics possessing this HLA haplotype (Honda et al., 1984; Mignot, 1998). However, more

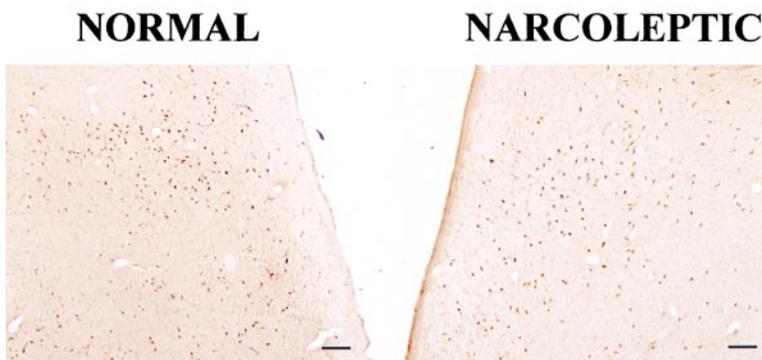


Figure 4. Melanin-Concentrating Hormone Neurons in Normal and Narcoleptic Subjects. Normal is subject CK, and narcoleptic is subject NA. Narcoleptics have normal numbers of MCH neurons despite the loss of 93% of Hcrt cells in the same region. Cal. = 250  $\mu\text{m}$ .

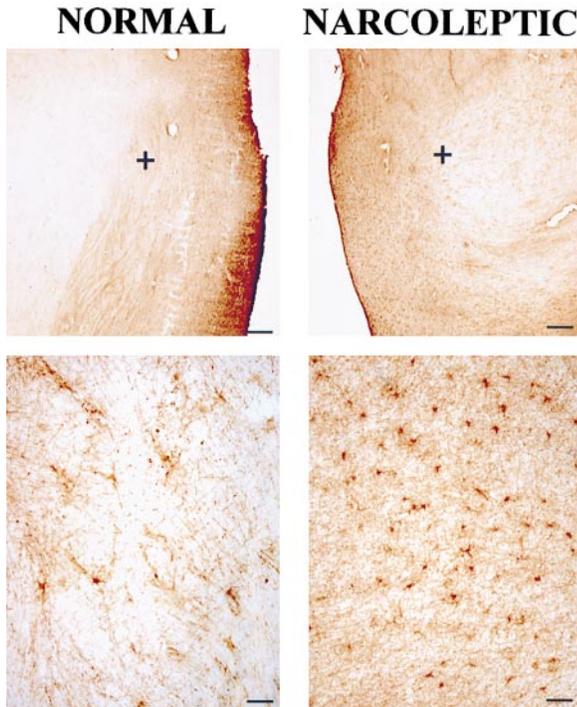


Figure 5. Glial Fibrillary Acidic Protein–Labeled Astrocytes in the Periventricular/Perifornical Region of a Normal and Narcoleptic Brain  
Low power photomicrograph is at the top, and a higher magnification of area marked with plus signs is at the bottom. Elevated GFAP staining in the hypothalamus of narcoleptics is consistent with a degenerative cause of cell loss. Top, Cal. = 500  $\mu$ m; bottom, Cal. = 50  $\mu$ m.

than 30% of the general population has this same genetic marker. DNA sequencing has not revealed any mutations in the HLA region in narcoleptics (Strohmaier

### Gliosis in Narcolepsy

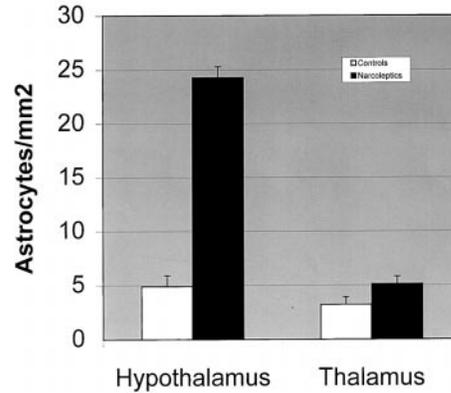


Figure 6. Pattern of Gliosis in the Hypothalamus and Thalamus of Narcoleptics and Controls

The number of GFAP labeled astrocytes is significantly increased in the hypothalamus of narcoleptics, the region containing Hcrt cells, but not in the thalamus.

et al., 1988; Olerup et al., 1990). It is known that HLA haplotypes are linked to a number of autoimmune diseases including multiple sclerosis, insulin-dependent diabetes, rheumatoid arthritis, Graves' disease, myasthenia gravis, and systemic lupus erythematosus (Strohmaier et al., 1988; Olerup et al., 1990). This has led to much speculation that narcolepsy is an autoimmune disorder. However, no clear evidence for immune activation or for a target of immune activity has yet been found in several studies of human narcoleptics (Fogdell et al., 1995; Hinze-Selch et al., 1998). The cause of the Hcrt cell loss we observe remains to be determined. An as yet uncharacterized autoimmune attack on Hcrt neurons or a sensitivity of Hcrt cells to specific environmental or biological toxins are reasonable possibilities.

Table 1. Characteristics of the Subjects, Treatment of Hypothalamic Tissue, and Characteristics of Hcrt Cells

Subject	Sex	Age	Cause of Death	Antibody	Time in Fix (Years)	Hypothalamic Gliosis (Cells/mm <sup>2</sup> )	Thalamic Gliosis (Cells/mm <sup>2</sup> )	Number of Hcrt Cells	Disease Onset Age	Medications	Cataplexy
<b>Narcoleptics</b>											
NA	F	63	adenocarcinoma	3	5	25.5	3.4	4,272	20	clomipramine Vivalan	+
NB	M	49	cv	2	4.5	27.2	5.9	3,073	18	Dexedrine, Vivactil, GHB	+
NC	M	60	sepsis	1	4.2	27.2	5.9	6,795	23	Ritalin Desoxyn	+
ND	M	86	pulmonary	2	12	17.0	5.1	9,698	35	Ritalin	-
<b>Controls</b>											
CA	M	41	cv, HIV	1	1			79,575			
CB	F	54	liver failure	1	0.5			61,050			
CC	F	42	AML/DIC	1	1	8.5	0	73,424			
CD	M	n/a	nonneuro	1	2	0.0	3.4	83,121			
CE	M	73	pulmonary, cv	1	5	6.8	4.3	n/a			
CF	M	n/a	nonneuro	1	16			72,950			
CG	M	n/a	nonneuro	3	16			77,727			
CH	M	38	lung cancer	1	2	8.5	2.6	n/a			
CI	F	29	nonneuro	1	2	0.9	5.9	n/a			
CJ	M	n/a	nonneuro	3	2			72,457			
CK	F	83	Alzheimer's	3	0.2			56,000			
CL	M	50	sepsis	3	4			50,576			

cv, cardiovascular; AML, acute myelocytic leukemia; DIC, disseminated intravascular coagulation. "nonneuro" indicates no history of neurological illness and no specified cause of death. Note that the elderly nonnarcoleptic Alzheimer's patient whose brain we analyzed (subject CK) had a relatively preserved Hcrt neuronal population, in contrast to the 85%–95% loss of Hcrt neurons seen in all of the narcoleptics, including one as young as 49 (subject NB). The hypothalamus was not completely available in CE, CH, and CI, so the total numbers of Hcrt neurons could not be determined. Antibodies 1 and 2 indicate Hcrt-1 and -2 antibodies from Oncogene Research Products; 3 refers to a polyclonal antiserum raised against a synthetic hypocretin-2.

The loss of Hcrt cells in human narcoleptics suggests that the replacement of the missing hypocretins may reverse some of the symptoms of this disease. A recent study in narcoleptic dogs demonstrated that intravenous administration of Hcrt-1 produces a rapid reversal of narcoleptic symptomatology, with increased activity levels, reduction or elimination of cataplexy, reduction of sleepiness during the daytime, and consolidation of sleep periods at night (John et al., 2000). Administration of hypocretins or their agonists to human narcoleptics may be an effective treatment for this disorder.

#### Experimental Procedures

##### Diagnosis and Tissue

Narcolepsy was diagnosed by members of the American Academy of Sleep Medicine according to standard criteria (Guilleminault, 1994). Three of the control brains had been subjected to an autopsy that removed parts of the hypothalamus. We used the remaining region of the hypothalamus at the level of the mammillary bodies for GFAP staining, but we could not use these control brains for total Hcrt number counts (see Table 1).

##### Immunohistochemistry and Antigen Retrieval

Brains were cut into 40  $\mu\text{m}$  thick coronal sections. The sections were treated with 0.5% sodium borohydride in PBS for 30 min and washed with PBS, and then incubated for 30 min in 0.5%  $\text{H}_2\text{O}_2$  for blocking of endogenous peroxidase activity.

For antigen retrieval, sections were heated for 30 min at 80°C in a water bath with 10 mM sodium citrate (pH 8.5) solution. The sections were cooled to room temperature in sodium citrate and washed with PBS. Water bath heating produces less tissue damage and more uniform antigen retrieval than other heating techniques (Jiao et al., 1999).

After thorough washing with PBS the sections were placed for 2 hr in 1.5% normal goat serum in PBS and incubated for 72 hr at 4°C with a 1:2000 dilution of Orexin-B or a 1:2000 dilution of Orexin-A (Oncogene Research Products (Ab-1). In some cases, a polyclonal antiserum raised in rabbit against a synthetic Hcrt-2, a kind gift of A. N. van den Pol, was used (Table 1). Sections were then incubated in a secondary antibody (biotinylated goat anti-rabbit IgG; Vector Laboratories) followed by avidin-biotin peroxidase (ABC Elite Kit; Vector Laboratories), for 2 hr each at room temperature. The tissue-bound peroxidase was visualized by a diaminobenzidine reaction (Vector Laboratories). We used a 15 min diaminobenzidine incubation on all tissues, having determined in pilot studies on normal and narcoleptic human tissue that this duration produced optimal staining with cell boundaries clearly visible and minimal background artifact. Narcoleptic and normal tissue were always stained in pairs along with a no-antibody control. Placing the sections in diaminobenzidine for longer time periods (e.g., 20 min) produced higher background staining but no further cellular staining in either the narcoleptic or normal brains. For GFAP staining, sections from subjects NA-ND and CC-CE, CH, and CI were immunostained with a 1:2000 dilution of primary polyclonal rabbit anti-cow GFAP antibody (DAKO, Carpinteria, CA). The four narcoleptic (subjects NA-ND) and five control brains (subjects CC-CE, CH, and CI) were also stained for MCH. The antibody was a kind gift of Drs. P. E. Sawchenko and W. W. Vale. Pretreatment was carried out as described for Hcrt staining, and sections were immunostained in rabbit anti-MCH diluted at 1:10000. Control sections from each brain were processed without the primary antibody and did not show staining.

Hcrt-1 and Hcrt-2 are both cleaved from the same precursor peptide, preprohypocretin. The distribution of cells labeled for Hcrt-1 and -2 has been reported to be very similar, perhaps identical (Mondal et al., 1999; Thannickal et al., 2000). In the current studies, we saw no significant difference in the number or distribution of cells labeled with the Hcrt-1 antibody or either of two Hcrt-2 antibodies (Table 1) and therefore considered the data from the two antibody stains together in our statistical analyses.

Cell number and distribution were determined with stereological techniques on a one in six series of 40  $\mu\text{m}$  sections through the

Hcrt containing regions of the hypothalamus. We used a Nikon E600 microscope with three axis motorized stage, video camera, Neurolucida interface, and Stereoinvestigator software (MicroBrightfield Corp., Colchester, Vermont). The optical fractionator method was used. A "counting brick" of 600  $\times$  500  $\times$  20  $\mu\text{m}$  was employed. Cells in 30 randomly selected sites within the outlined hypothalamic area of interest were counted within each slide. Only cells whose tops came into focus as the microscope objective was lowered through the brick were counted. Cells that extended across the left or bottom boundary of the counting brick were excluded. Pilot studies of a normal and a narcoleptic human brain determined that these parameters would produce a total count of at least 300 cells per brain and that the coefficient of error would be less than 0.08, well within the parameters required for accurate stereology (Howard and Reed, 1998).

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