Reduced Number of Hypocretin Clinical Study
Neurons in Human Narcolepsy

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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.

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. 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.

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...
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 μm) 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. = 25μm) 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.
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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 (Guillemainault, 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 (Erlich 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

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

**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 µm.
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

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age</th>
<th>Cause of Death</th>
<th>Antibody</th>
<th>Time in Fix (Years)</th>
<th>Hypothalamic Gliosis (Cells/mm²)</th>
<th>Thalamic Gliosis (Cells/mm²)</th>
<th>Number of Hcrt Cells</th>
<th>Disease Onset Age</th>
<th>Medications</th>
<th>Cataplexy</th>
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<tr>
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<td>63</td>
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<td>3</td>
<td>5</td>
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<td>49</td>
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<td>4.5</td>
<td>27.2</td>
<td>5.9</td>
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<td>Dexamethasone, Vivactil, GHB</td>
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<td>4.2</td>
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<td>6,795</td>
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<td>12</td>
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<td>5.1</td>
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<tr>
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<td>nonneurol</td>
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<td></td>
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</table>

cv, cardiovascular; AML, acute myelocytic leukemia; DIC, disseminated intravascular coagulation. “nonneurol” 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.
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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 μm 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% H2O2 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 primary antibody and 1:10000 dilution of primary antibody (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 diamino-benzidine 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, proprohocretin. 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 μm 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 × 500 × 20 μm 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).

Acknowledgments

This work was dedicated to the memory of Dr. Michael Aldrich who had the vision to found a brain bank for the acquisition and distribution of narcoleptic brains and contributed enormously to our understanding of narcolepsy. Supported by NS14610, HL41370, HL6029C, and the Medical Research Service of the Department of Veterans Affairs. We thank Kristi Alderman and Melanie Patton for help with data analysis, Dr. Per E. Hesla for providing one of the narcoleptic brains, Dr. Roger Albin for maintaining the brain bank begun by Dr. Aldrich, and the patients and families who thought of others at the time of their loss and donated these precious tissues. A preliminary version of these findings was submitted on April 24, 2000 for presentation at the Society for Neuroscience Meeting.

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