

## Localized Loss of Hypocretin (Orexin) Cells in Narcolepsy Without Cataplexy

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**Study Objectives:** Narcolepsy with cataplexy is characterized by a loss of approximately 90% of hypocretin (Hcrt) neurons. However, more than a quarter of narcoleptics do not have cataplexy and have normal levels of hypocretin in their cerebrospinal fluid, raising the possibility that their disease is caused by unrelated abnormalities. In this study we examined hypocretin pathology in narcolepsy without cataplexy.

**Design:** We examined postmortem brain samples, including the hypothalamus of 5 narcolepsy with cataplexy patients; one narcolepsy without cataplexy patient whose complete hypothalamus was available (patient 1); one narcolepsy without cataplexy patient with anterior hypothalamus available (patient 2); and 6 normal brains. The hypothalamic tissue was immunostained for Hcrt-1, melanin-concentrating hormone (MCH), and glial fibrillary acidic protein (GFAP).

**Measurements and Results:** Neither of the narcolepsy without cataplexy patients had a loss of Hcrt axons in the anterior hypothalamus.

The narcolepsy without cataplexy patient whose entire brain was available for study had an overall loss of 33% of hypocretin cells compared to normals, with maximal cell loss in the posterior hypothalamus. We found elevated levels of gliosis with GFAP staining, with levels increased in the posterior hypothalamic nucleus by (295%), paraventricular (211%), periventricular (123%), arcuate (126%), and lateral (72%) hypothalamic nuclei, but not in the anterior, dorsomedial, or dorsal hypothalamus. There was no reduction in the number of MCH neurons in either patient.

**Conclusions:** Narcolepsy without cataplexy can be caused by a partial loss of hypocretin cells.

**Keywords:** Hypocretin, orexin, narcolepsy, cataplexy

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STANDARD NOSOLOGY CLASSIFIES PATIENTS WHO HAVE SHORT SLEEP LATENCY AND  $\geq 2$  SLEEP ONSET REM SLEEP PERIODS ON THE MULTIPLE SLEEP LATENCY test as narcoleptic.<sup>1</sup> Under this definition, more than a quarter of those classified as narcoleptic do not have cataplexy.<sup>2,3</sup> The intensity of cataplexy in patients classified as having narcolepsy with cataplexy varies greatly: it is the most debilitating symptom in a few patients, causing total loss of muscle tone and consequent collapse several times a day; it occurs rarely and causes only transient weakness of the facial musculature in others.<sup>4</sup>

Narcolepsy with cataplexy has been shown to be characterized by a loss of the hypocretin (orexin) peptide and of the cells generating this peptide.<sup>5-8</sup> All human narcoleptic brains we have examined have some surviving hypocretin cells with approximately normal morphology.<sup>9</sup> Unfortunately, collection of the brains of human narcoleptics has been largely limited to patients with cataplexy. We now present data from an analysis of the complete hypothalamus of one patient with narcolepsy without cataplexy (patient 1), data from a second narcolepsy without cataplexy patient from which we received only anterior hypothalamic tissue (patient 2), 5 narcolepsy with cataplexy patients, and 6 normal individuals.

## MATERIALS AND METHODS

Our subjects were 5 narcolepsy with cataplexy patients (3 patients previously reported in 2000,<sup>8</sup> mean age  $61 \pm 4$  years; 3 males and 2 females), 2 narcolepsy without cataplexy patients (2 males: patient 1, age 51 [whose entire hypothalamus was preserved], and patient 2, age 86 [in whom only the anterior hypothalamus was available, previously reported<sup>8</sup>]), and 6 normal individuals (mean age  $65 \pm 8$  years; 2 males and 4 females). Brains were fixed in 10% buffered formalin containing 0.1M phosphate buffer (pH 7.4). The hypothalamic tissue was cut into 40- $\mu$ m sections. Sections were immunostained for hypocretin-1, melanin concentrating hormone (MCH) and glial fibrillary acidic protein (GFAP). Cell number, distribution, and size were determined with stereological techniques on a 1 in 6 series of sections. All values are reported as mean and S.E.M. Comparisons were made using the *t*-test. The densities of hypocretin and GFAP cells were calculated as the number of cells per unit area ( $\text{mm}^2$ ). We used the density of hypocretin axons in the anterior hypothalamus as an additional indicator of overall hypocretin cell integrity.

## Hypocretin and MCH Immunohistochemistry

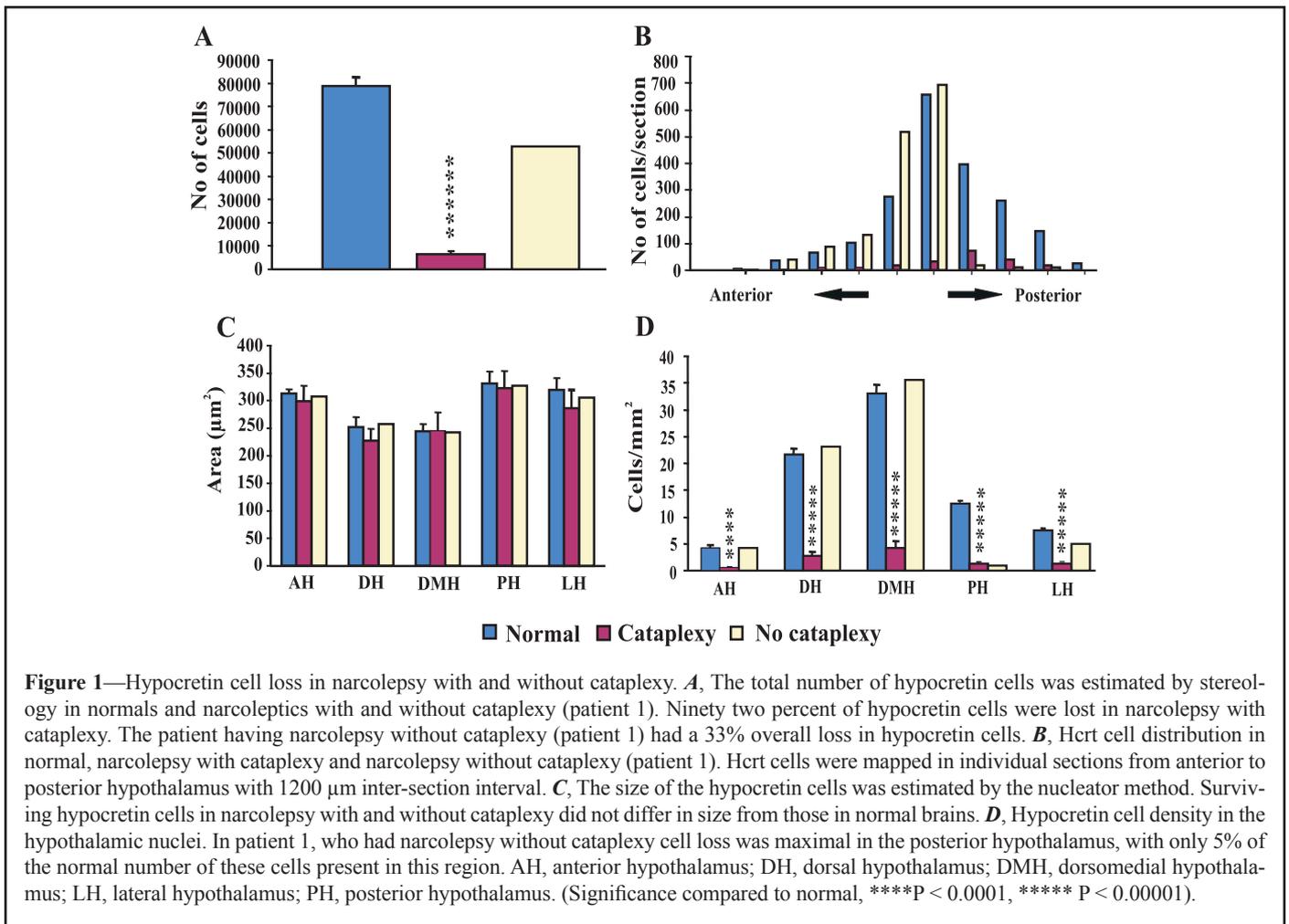
The sections were treated with 0.5% sodium borohydride in phosphate buffered saline (PBS) for 30 min and washed with PBS, and then incubated for 30 min in 0.5%  $\text{H}_2\text{O}_2$  for the blocking of endogenous peroxidase activity. For antigen retrieval, sections were heated for 30 min at 80°C in 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

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**Figure 1**—Hypocretin cell loss in narcolepsy with and without cataplexy. **A**, The total number of hypocretin cells was estimated by stereology in normals and narcoleptics with and without cataplexy (patient 1). Ninety two percent of hypocretin cells were lost in narcolepsy with cataplexy. The patient having narcolepsy without cataplexy (patient 1) had a 33% overall loss in hypocretin cells. **B**, Hert cell distribution in normal, narcolepsy with cataplexy and narcolepsy without cataplexy (patient 1). Hert cells were mapped in individual sections from anterior to posterior hypothalamus with 1200  $\mu\text{m}$  inter-section interval. **C**, The size of the hypocretin cells was estimated by the nucleolar method. Surviving hypocretin cells in narcolepsy with and without cataplexy did not differ in size from those in normal brains. **D**, Hypocretin cell density in the hypothalamic nuclei. In patient 1, who had narcolepsy without cataplexy cell loss was maximal in the posterior hypothalamus, with only 5% of the normal number of these cells present in this region. AH, anterior hypothalamus; DH, dorsal hypothalamus; DMH, dorsomedial hypothalamus; LH, lateral hypothalamus; PH, posterior hypothalamus. (Significance compared to normal, \*\*\*\*P < 0.0001, \*\*\*\*\* P < 0.00001).

uniform antigen retrieval than other heating techniques.<sup>10</sup> After thorough washing with PBS, the sections were placed for 2 h in 1.5% normal goat serum in PBS and incubated for 72 h at 4°C with a 1:2,000 dilution of hypocretin-1 (Orexin-A, Calbiochem, San Diego, CA). Sections were then incubated in a secondary antibody (biotinylated goat anti-rabbit IgG; Vector Laboratories, Burlingame, CA) followed by avidin-biotin peroxidase (ABC Elite Kit; Vector laboratories), for 2 h each at room temperature. The tissue-bound peroxidase was visualized by a diaminobenzidine reaction (Vector laboratories). Adjacent series of sections were immunostained for MCH (1:20,000, polyclonal rabbit anti-melanin concentrating hormone, Phoenix Pharmaceuticals, Inc., Belmont, CA). Pretreatment and staining were carried out as described for hypocretin staining.

### GFAP Immunohistochemistry

For GFAP staining, sections were immunostained with a 1:2,000 dilution of primary polyclonal rabbit anti-GFAP antibody (Dako, Carpinteria, CA). Antigen retrieval was not required for GFAP staining. After a hydrogen peroxide treatment and blocking serum, the sections were immunostained with GFAP antibody followed by biotinylated goat anti-rabbit secondary antibody, and an avidin-biotin-HRP complex (Vectastain ABC kit, Vector Laboratories). Incubation times were 24 h (at 4°C) for the primary antibody, 30 min (at room temperature) for the secondary antibody, and 1 h (at room temperature) for

the avidin-biotin-HRP complex. Sections were treated with the DAB reaction (Vector Laboratories).

### Quantitative Analysis

Hypocretin and MCH cell number, distribution, and size were determined with stereological techniques on a 1 in 6 series of sections through the complete hypothalamus. We used a Nikon E600 microscope with 3 axis motorized stage, video camera, Neurolucida interface and Stereoinvestigator software (MicroBrightfield Corp., Colchester, VT). The density of GFAP cells was calculated as the number of cells per unit area ( $\text{mm}^2$ ). Hypocretin axon density was calculated as the number of axons per unit area ( $\text{mm}^2$ ). After delineating the nucleus, we used a  $250 \times 250\text{-}\mu\text{m}$  counting frame for random sampling with stereological procedures. In patient 2, we received only the anterior hypothalamus. Therefore our estimate of the number of remaining cells was based on a comparison of the number of hypocretin cells in the anterior hypothalamic regions remaining, with the corresponding hypothalamic region of tissue from normal brains. In an earlier publication,<sup>8</sup> we had used the number of hypocretin somas in this tissue to estimate total hypocretin cell population. In the current paper, we used the axonal count in the anterior hypothalamus, which, because it contains ascending axons from throughout the hypothalamus, is likely to give a more accurate estimate of total hypocretin cell processes, including axons from cells in the tissue that was lost.

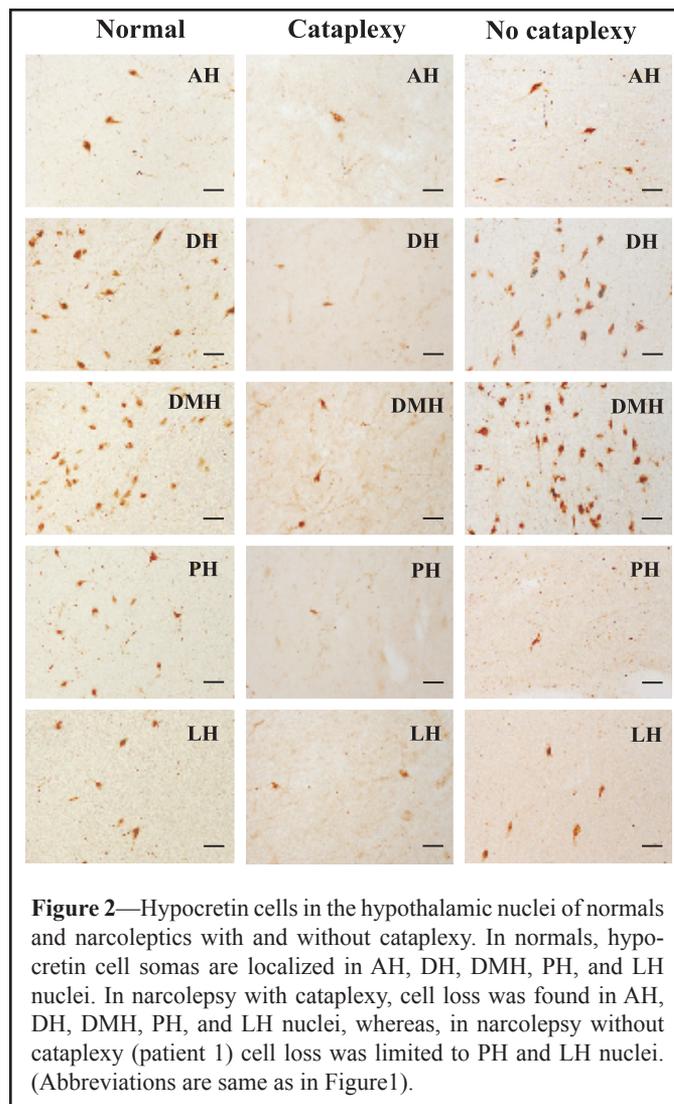
This approach allowed a direct comparison with patient 1. In our earlier study, we calculated an average loss of 90% of the Hcrt cells in narcolepsy with cataplexy patients, a value that is consistent with that we have calculated from the more than 10 additional brains of narcolepsy with cataplexy patients we have subsequently acquired. Values of each nucleus were calculated for each subject. These were pooled to give means and S.E.M. for each region and each group. Brain regions and nuclei were identified by Nissl staining of adjacent sections and using the Atlas of the Human Brain.<sup>11</sup> The “nucleator probe” in the MicroBrightfield stereology program was used to estimate the mean cross-sectional area of the hypocretin cells. Neurons with a clearly visible nucleus were chosen for analysis. The nucleator probe was used with the optical fractionator and stereology procedures for systematic random sampling to identify cells.<sup>12</sup> In the sampling results, the volume estimate associated with each cell was calculated, along with the average volume for the group of cells measured. All data were expressed as mean  $\pm$  SEM, except for the samples from the 2 patients with narcolepsy without cataplexy.

## RESULTS

Narcoleptics with cataplexy had a mean loss of 92% of hypocretin neurons compared to normals. The complete hypothalamus of patient one had 33% fewer hypocretin cells than normals (Figure 1A, B, and D). The maximum percentage reduction occurred in the posterior hypothalamic nucleus (95% loss). A stereological analysis of the size of surviving hypocretin cells showed no significant difference between the size of surviving cells in narcolepsy with or without cataplexy patients and cells in the same hypothalamic subregions in normal individuals (Figure 1C). There was no hypocretin cell loss in anterior, dorsal and dorsomedial nuclei of the narcolepsy without cataplexy patients (Figure 2). Figure 3 shows the distribution of hypocretin cells in 3 typical normal individuals, 3 narcolepsy with cataplexy patients, and the narcolepsy without cataplexy patient whose entire hypothalamus was available (patient 1).

Patient 1 was a 51-year-old male who died of colon cancer. Onset of symptoms was at 16 years and he was treated with methylphenidate and modafinil. He had 3 MSLT tests performed over a 9-year period at 2 different sleep centers. The tests were performed at least 2 weeks after drug withdrawal. Mean sleep latency was 2.8, 1.4, and 4 minutes in these 3 tests. Two sleep onset REM sleep periods were recorded in each of these tests. There was no indication of hypnagogic hallucinations or sleep paralysis in his records. The clinical records and postmortem interviews with 2 of his friends agreed on the complete absence of cataplexy. Patient 2 (in whom narcolepsy without cataplexy was diagnosed by Dr. Michael Aldrich), was an 86-year-old male who died of pneumonia. Onset of symptoms was at 35 years of age, and he was treated with methylphenidate. Premortem HLA typing was not done and was not possible on the fixed brain tissue in either patient 1 or 2.

There was a mean loss of 65% of hypocretin axons in the anterior hypothalamus on average in patients having narcolepsy with cataplexy (Figure 4 A), considerably less than the 90% loss of cell somas; this suggests that some axonal sprouting may have occurred in surviving cells, or that rostrally project-



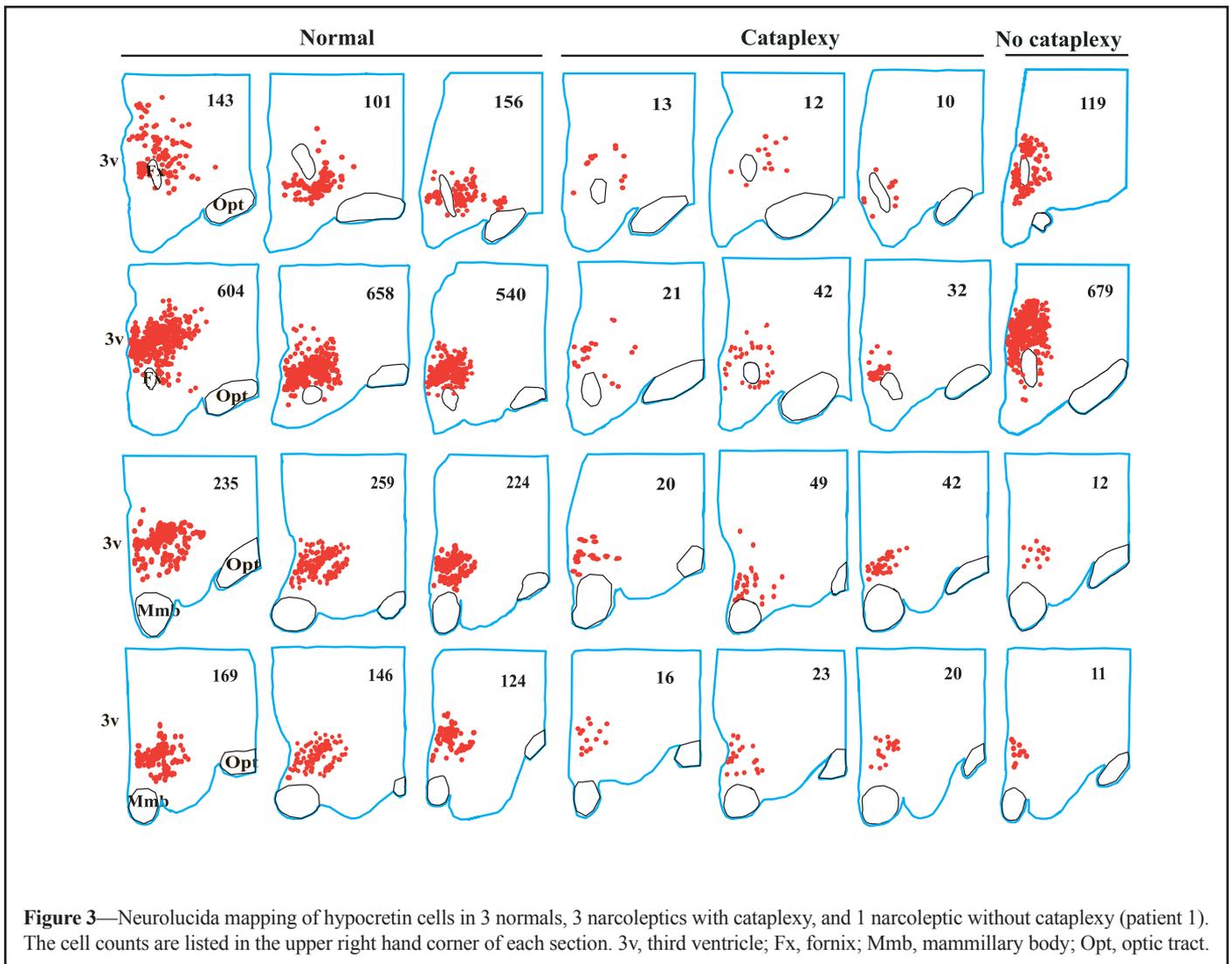
**Figure 2**—Hypocretin cells in the hypothalamic nuclei of normals and narcoleptics with and without cataplexy. In normals, hypocretin cell somas are localized in AH, DH, DMH, PH, and LH nuclei. In narcolepsy with cataplexy, cell loss was found in AH, DH, DMH, PH, and LH nuclei, whereas, in narcolepsy without cataplexy (patient 1) cell loss was limited to PH and LH nuclei. (Abbreviations are same as in Figure 1).

ing hypocretin cells may be less likely to die than caudally projecting cells in narcolepsy with cataplexy. Patients 1 and 2 had no reduction in the number of hypocretin axons in the anterior hypothalamic region. There was no reduction in the number of MCH neurons in either type of narcolepsy (Figure 4 B).

We found increased gliosis concentrated in the posterior hypothalamic nucleus of patient 1 (Figure 4C). Patient 1 had a 295% increase in GFAP staining in the posterior hypothalamic nucleus compared to controls. Increased GFAP density was also found in this patient in the paraventricular (211%), periventricular (123%), arcuate (126%), and lateral (72%) hypothalamus, but not in the anterior, dorsomedial or dorsal hypothalamus (Figure 4D). Narcoleptics with cataplexy had increased GFAP staining throughout the hypothalamus, with a maximum percentage GFAP increase in the posterior hypothalamic nucleus (340%, Figure 4D).

## DISCUSSION

The number of hypocretin axons in the anterior hypothalamus was normal in both patients with narcolepsy without cataplexy. However, there was a depletion of hypocretin cells and an increased gliosis in the posterior hypothalamic nucleus of the patient whose entire hypothalamus was available. This find-



ing suggests, for the first time, that a localized loss of hypocretin cells can cause narcolepsy without cataplexy. Work in animals<sup>13</sup> has shown that cerebrospinal hypocretin levels can be normal even when there is a substantial loss of hypocretin cells. CSF levels may be a function not only of the percentage of hypocretin cells lost, but also the activity and the mean distance of surviving hypocretin cells from the ventricles. Thus, patients with loss of posterior hypothalamic hypocretin cells may still have normal CSF levels of the peptide, even though it is not being synaptically delivered to the cells normally receiving hypocretin axonal projections.<sup>14</sup> More patients need to be studied to determine if the pattern we observe here is typical of patients with narcolepsy without cataplexy.

It has recently been observed that patients with Parkinson disease have a loss of hypocretin neurons, although to a lesser extent than in narcolepsy with cataplexy.<sup>15,16</sup> These patients have many of the symptoms of narcolepsy; however, distinct episodes of cataplexy have not been reported. This is consistent with the current observations of narcolepsy with partial loss of hypocretin neurons. Both patients with Parkinson disease and narcolepsy exhibit impaired olfactory discrimination, which in narcoleptics can be corrected by intranasally administered orexin.<sup>17,18</sup> Since many cell groups are lost in Parkinson disease, the relative contribution of hypocretin cell loss remains to be determined.

Prior animal work indicates that partial loss of hypocretin cells can cause symptoms of narcolepsy, but that cataplexy is not seen after such lesions.<sup>13,19</sup> Intranasal hypocretin administration has been shown to be effective in reversing sleepiness in sleep deprived monkeys.<sup>20</sup> The current results suggest that syndromes of partial hypocretin cell loss may contribute to a variety of neurodegenerative or stroke disorders and may respond to treatments that increase hypocretin levels.

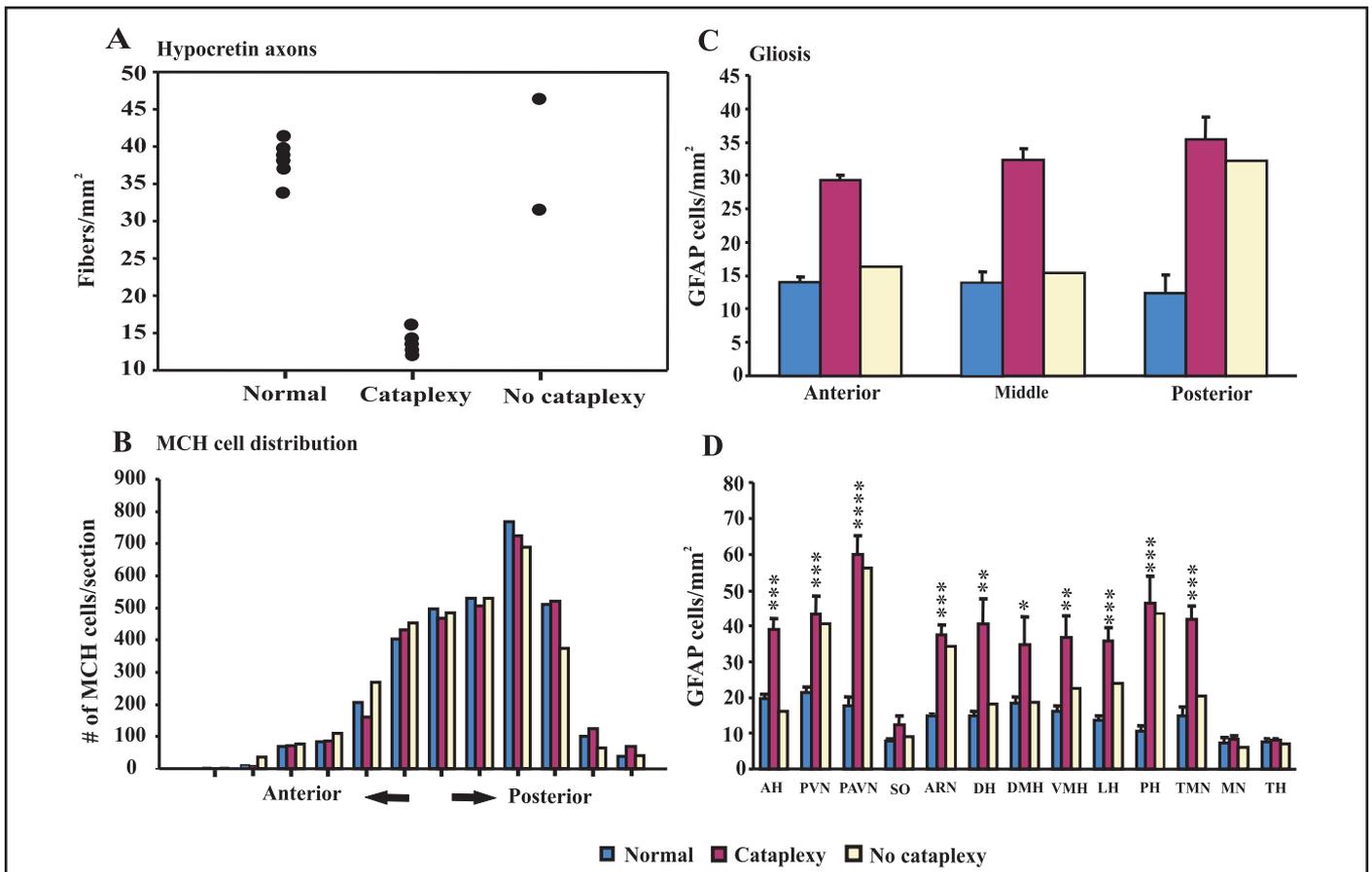
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The work was performed at Veterans Administration Greater Los Angeles Healthcare System, North Hills and U.C.L.A.

#### DISCLOSURE STATEMENT

This was not an industry supported study. The authors have indicated no financial conflicts of interest.



**Figure 4**—Hypocretin axon density, MCH distribution and gliosis. **A**, Hypocretin fiber density (fibers/mm<sup>2</sup>) in the anterior hypothalamus of normals, narcolepsy with cataplexy patients and narcolepsy without cataplexy patients (patients 1 and 2). Hypocretin axon density in the anterior hypothalamus is within the normal range in both of the narcolepsy without cataplexy patients (patients 1 and 2). **B**, MCH cell distribution in normals, narcolepsy with cataplexy and narcolepsy without cataplexy (patient 1). MCH cells were mapped in individual sections from anterior to posterior hypothalamus with 1200  $\mu$ m inter-section interval. MCH cell number is normal in narcolepsy with and without cataplexy. **C**, GFAP density (cells/mm<sup>2</sup>) in the hypothalamus of normal and narcoleptic brains. The data are grouped into anterior, middle, and posterior regions of the hypothalamus. In narcolepsy without cataplexy (patient 1), increased number of GFAP cells were found only in the posterior hypothalamus. **D**, GFAP density in different nuclei of the hypothalamus of normal and narcoleptic brains (significance compared to normal, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ ). AH, anterior hypothalamus; PVN, periventricular nucleus; PAVN, paraventricular nucleus; SO, supraoptic; ARN, arcuate nucleus; DH, dorsal hypothalamus; DMH, dorsomedial hypothalamus; VMH, ventromedial hypothalamus; LH, lateral hypothalamus; PH, posterior hypothalamus; TMN, tuberomammillary nucleus; MN, mammillary nucleus; TH, thalamus.

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