

Greatly Increased Numbers of Histamine Cells in Human Narcolepsy with Cataplexy

Joshi John, PhD,¹ Thomas C. Thannickal, PhD,¹ Ronald McGregor, MS,¹
Lalini Ramanathan, PhD,¹ Hiroshi Ohtsu, MD, PhD,² Seiji Nishino, MD, PhD,³
Noriaki Sakai, DVM, PhD,³ Akhiro Yamanaka, PhD,⁴ Carly Stone, BS,¹
Marcia Cornford, MD, PhD,⁵ and Jerome M. Siegel, PhD¹

Objective: To determine whether histamine cells are altered in human narcolepsy with cataplexy and in animal models of this disease.

Methods: Immunohistochemistry for histidine decarboxylase (HDC) and quantitative microscopy were used to detect histamine cells in human narcoleptics, hypocretin (Hcrt) receptor-2 mutant dogs, and 3 mouse narcolepsy models: Hcrt (orexin) knockouts, ataxin-3-orexin, and doxycycline-controlled-diphtheria-toxin-A-orexin.

Results: We found an average 64% increase in the number of histamine neurons in human narcolepsy with cataplexy, with no overlap between narcoleptics and controls. However, we did not see altered numbers of HDC cells in any of the animal models of narcolepsy.

Interpretation: Changes in histamine cell numbers are not required for the major symptoms of narcolepsy, because all animal models have these symptoms. The histamine cell changes we saw in humans did not occur in the 4 animal models of Hcrt dysfunction we examined. Therefore, the loss of Hcrt receptor-2, of the Hcrt peptide, or of Hcrt cells is not sufficient to produce these changes. We speculate that the increased histamine cell numbers we see in human narcolepsy may instead be related to the process causing the human disorder. Although research has focused on possible antigens within the Hcrt cells that might trigger their autoimmune destruction, the present findings suggest that the triggering events of human narcolepsy may involve a proliferation of histamine-containing cells. We discuss this and other explanations of the difference between human narcoleptics and animal models of narcolepsy, including therapeutic drug use and species differences.

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Human narcolepsy with cataplexy is linked to the loss of approximately 90% of hypocretin (Hcrt; orexin) neurons,¹ a cell group whose somas are localized to the hypothalamus.² Intermixed hypothalamic cells containing melanin-concentrating hormone are normal in number, size, and distribution, indicating that the loss of Hcrt cells is selective.³ One major symptom of narcolepsy is excessive daytime sleepiness. Another is cataplexy, a loss of muscle tone in waking linked most strongly to laughter and certain other sudden onset emotions.⁴

A canine genetic model of narcolepsy is linked to a mutation in the Hcrt receptor-2 (Hcrt-R2) gene.⁵ Mouse models of narcolepsy include the orexin knockout (KO) mouse, in which a null mutation is induced by targeted disruption of the prepro-orexin gene.⁶ The neurons that would have contained Hcrt appear to be intact in these mice, as indicated by staining for Narp and dynorphin.^{7,8} Another murine model that has been extensively studied is the ataxin-3-orexin mouse, in which the Hcrt-containing neurons degenerate postnatally, with extensive

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Address correspondence to Dr John, Dr Thannickal, or Dr Siegel, Neurobiology Research 151A3, VA GLAHS, UCLA, 16111 Plummer St, North Hills, CA 91343. E-mail: jjohn@ucla.edu, thomastc@ucla.edu, JSiegel@ucla.edu

From the ¹Neurobiology Research, Veterans Administration Greater Los Angeles Healthcare System, Neuropsychiatric Institute and Brain Research Institute, University of California, Los Angeles, Los Angeles, CA; ²Graduate School of Engineering, Tohoku University, Sendai, Japan; ³Stanford Sleep Research Center, Stanford University School of Medicine, Palo Alto, CA; ⁴Department of Neuroscience II, Research Institute of Environmental Medicine, Nagoya University, Nagoya, Japan; and ⁵Department of Pathology, Harbor University of California, Los Angeles Medical Center, Torrance, CA.

TABLE . Characteristics of Narcoleptic and Normal Subjects

Subject	Sex	Age, yr	Cause of Death	Disease Onset Age, yr	Cataplexy	Medications	Time in Fixative, yr
Normal subjects							
1	F	81	Cerebrovascular				2.3
2	M	32	MI, aneurism				8.0
3	F	44	Breast cancer				8.0
4	M	82	Aortic aneurism				10.9
5	M	61	Pneumonia, testicular tumor				8.0
6	F	57	Septic shock				5.2
7	F	50	Pulmonary embolism				5.0
Narcoleptics							
8	F	91	^a	16	+	Provigil	4.9
9	F	61	Pneumonia	^a	+	Desoxyn	8.1
10	M	72	Cerebral hemorrhage	20	+	Ritalin	7.9
11	M	79	Lung cancer	21	+	Methylphenidate, simvastatin, donepezil	^a
12	F	88	^a	13	+	Dextroamphetamine	5.1

MI = myocardial infarct.
^aInformation not available.

degeneration within 2 weeks of birth.⁹ A recently developed model is the doxycycline-controlled-diphtheria-toxin-A-orexin mouse (DTA-orexin), in which withdrawal of doxycycline at any age induces expression of diphtheria toxin fragment A in Hcrt cells, causing their death. This model allows a closer simulation of narcolepsy onset in humans, which typically begins in adolescence. All these models show the major symptoms of human narcolepsy, and those that have been tested respond to drugs that reduce or increase symptoms in a similar manner as do human narcoleptics.¹⁰

The Hcrt system is thought to help maintain wakefulness and prevent loss of muscle tone (cataplexy) through its projections to spinal, brainstem, and fore-brain regions, with a particularly dense projection to histamine neurons in the tuberomammillary region^{1,11} and to locus coeruleus. Activity of the histamine cell group is linked to waking, with greatly decreased activity in both non-rapid eye movement (NREM) and REM sleep.¹² Antihistamines increase sleepiness, and a histamine receptor-3 (H3) inverse agonist (autoreceptor antagonist) decreases sleepiness in both orexin KO mice and human narcoleptics.¹³ The alerting effects of intraventricularly

administered Hcrt are dependent on the integrity of the H1 receptor.¹⁴ In narcoleptic dogs, we have shown that whereas locus coeruleus noradrenergic cells ceased discharge immediately before and during cataplexy, histamine cells increased firing during cataplexy, suggesting that they are related to waking maintenance, rather than muscle tone regulation.¹⁵ These observations suggested that histamine cells might be altered in human narcoleptics.

In the current work, we find a large increase in the number of histamine cells in the tuberomammillary region of human narcoleptics. We do not see any such change in Hcrt-R2 mutant narcoleptic dogs or in any of the 3 mouse models of narcolepsy studied.

Subjects and Methods

Patients

Five narcoleptic and 7 non-narcoleptic (control) human brains were used. All the narcoleptic patients were diagnosed by a sleep disorder center as having narcolepsy with cataplexy. The characteristics of the patients are presented in the Table. All patients and controls were Caucasian. Control brains had been in formalin for 2 to 11 years, narcoleptics for 5 to 8 years.

Histidine decarboxylase (HDC) staining intensity and cell number were not correlated with time in fixation.

Study Design

We first investigated the number and size of histamine neurons in humans having narcolepsy with cataplexy and in control humans, using immunohistochemical, stereological,¹⁶ and quantitative microscopy techniques. We conducted parallel investigations in narcoleptic dogs and controls. We also counted and measured histamine neurons in age-matched and littermate orexin KO mice, ataxin-3-orexin mice, and DTA-orexin mice.

Human Brains

Human brains were fixed by formalin immersion from 4 to 48 hours after death. Formalin-fixed normal and narcoleptic human brains were equilibrated in 20% and then in 30% sucrose, and 40 μ m coronal sections of the hypothalamus were cut on a freezing microtome. Immunostaining was done on a 1-in-24 series of the hypothalamic sections. Free-floating sections from both normal and narcoleptic patients were subjected to antigen retrieval prior to immunostaining.

Antigen Retrieval

Forty-micrometer sections of hypothalamic tissue were placed in 0.5% sodium borohydride for 30 minutes, and after 3 washes in phosphate-buffered saline (PBS), sections were transferred to 0.5% H₂O₂ for 30 minutes, washed again, and then heated at 80°C for 30 minutes in sodium citrate solution (10mM, pH 9.0).

Doberman Dogs

Narcoleptic (Hcrt-R2 mutant) Dobermans pincers and breed-matched controls were studied. The controls were two 7-month-old females and one 6-month-old male. The narcoleptics were two 7-month-old females and one 5-month-old male. All had been perfused with saline and formalin, and the brains removed and stored in formalin for 17 years prior to sectioning and staining. Formalin-fixed brains were equilibrated in 20% and then in 30% sucrose, and 40 μ m coronal sections of the hypothalamus were cut on a freezing microtome. Antigen retrieval and immunohistochemical staining for HDC was done as in the human studies. A 1-in-6 series was analyzed.

Four Mouse Models

HDC KO. We tested the specificity of our HDC antibody by staining 4 HDC KO mice and 4 controls¹⁷ (2 male and 2 female C57B6/CrSlc KO mice and 2 male and 2 female C57B6/CrSlc controls, all between 127 and 148 days old).

Orexin KO. Age-matched, littermate male C57BL/6J-129/SvEv orexin KO and wild-type (WT) mice (aged 4–10 months) were sacrificed and perfused with saline and formalin (n = 12).

Ataxin-3-Orexin Transgenic. C57BL/6-DBA1 ataxin-mice and control, age-matched littermates (sets at ages in days: 76 [2 males], 96 [6 males], 142 [4 males], 145 [2 males], 160 [2

males], 206 [2 females], 211 [2 males], 246 [2 females], and 338 days [2 females]) were sacrificed (n = 24, 9 male pairs and 3 female pairs). These animals were also separated into 3 age groups for data analysis: group 1, aged 2.5 to 3.2 months; group 2, aged 4.7 to 5.3 months; and group 3, aged 7 to 11.3 months.

tTA/Tet-O Diphtheria-Toxin-Orexin Mice. These were C57BL/6 mice with postnatal ablation of Hcrt neurons, induced by doxycycline withdrawal (tTA/Tet-O diphtheria-toxin-fragment-A-orexin or DTA-orexin mice). Experimental mice had doxycycline removed at 42 days of age and were sacrificed at 84 days. Controls were administered doxycycline throughout and sacrificed at 84 days (5 experimental mice [all males] and 5 controls [3 males, 2 females], total n = 10).¹⁸

The brains of all 3 mouse models of narcolepsy and of the HDC KO mice and controls were perfused, removed, and post-fixed, then transferred to 20% sucrose and then 30% sucrose. No antigen retrieval was required for the mouse sections. Brains were sectioned at 40 μ m, and a 1-in-3 series was analyzed.

Histamine Neuron Immunostaining

Sections were treated with 0.5% hydrogen peroxide for 30 minutes at room temperature. Sections from all species were incubated for 2 hours in 1.5% normal goat serum (Vector Laboratories, Burlingame, CA) and 0.3% Triton X-100 containing PBS, followed by 72 hours at 4°C in a solution containing rabbit polyclonal antibody to HDC (catalog # EUD2601; lot #s MN2078, MN2067, ON2020; Acris, Hiddenhausen, Germany) 1:3,000 in PBS containing 0.3% Triton X-100 and 1.5% normal goat serum, rinsed in PBS, and incubated with biotinylated antirabbit immunoglobulin G (1:300; Vector) for 2 h at room temperature followed by incubation with avidin–biotin complex (1:200; Vector) for 2 h at room temperature. After rinsing, sections were incubated for 5 to 10 minutes in 0.05% diaminobenzidine and 0.001% H₂O₂, yielding a brown precipitate in histidine decarboxylase immunoreactive neurons.

Data Analysis

Mapping of cell distribution was done with a NeuroLucida Imaging System (MBF Bioscience, Williston, VT), using the optical fractionator method, assisted by a computerized stereology system with $\times 40$ magnification. Cell size was measured using the NeuroLucida Nucleator program. HDC cell staining intensity in the human subjects was statistically assessed using the nonparametric Mann–Whitney *U* test. Analysis of cell count and cell size data was done using paired or unpaired 2-tailed Student *t* tests as appropriate. In the 3 mouse models of narcolepsy, data were compared to those of respective WT littermates or age-, strain- and sex-matched controls. Controls and experimental animals were always processed simultaneously for staining to avoid any batch-to-batch variation.

All staining, counting, and cell measurements were done on coded tissue, with the researchers doing this work blind to

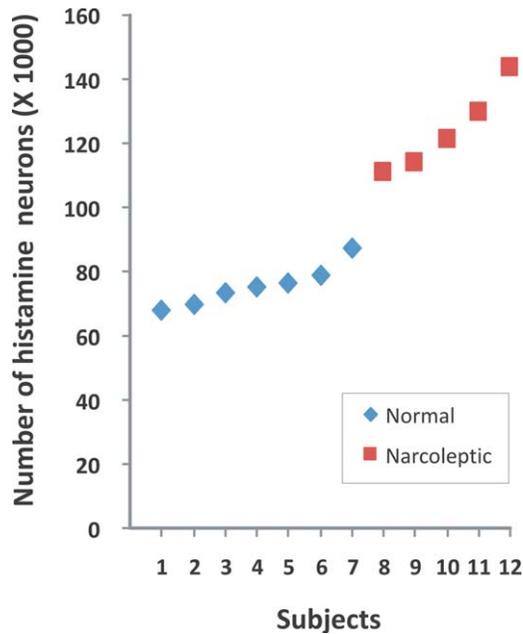


FIGURE 1: Number of histamine neurons in human controls and in narcoleptics with cataplexy, arranged as in the Table. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the nature of the tissue. In every case, the same individual counted both experimental and control tissues in randomized order.

Results

Human Subjects

HDC-labeled cells are localized to the tuberomammillary regions of the human brain.¹⁹ The 7 human control subjects had an average projected total of $75,469 \pm 2,409$ (standard error) HDC cells, a number similar to that previously reported in normal humans using a different antibody.^{20,21} In contrast, the 5 narcoleptics, all of whom had been symptomatic for at least 50 years, had an average projected total of $123,998 \pm 5,898$, a 64% increase (range = 47–90%; $p < 0.0001$, $df = 10$, $t = 8.5$; Figs 1–3). The increase was greatest in the middle of the tuberomammillary field. The number of HDC cells was not correlated with the age or sex of the individual, the duration of narcolepsy, or the cause of death (see Table). The HDC-positive cells were not significantly different in size between the controls and narcoleptics ($338 \pm 9.7\mu\text{m}^2$ in controls vs $370 \pm 4.7\mu\text{m}^2$ in narcoleptics). HDC cell staining had a higher signal-to-background ratio in the narcoleptics compared to controls, as assessed by the rankings of 3 raters blind to the condition of the samples (Mann–Whitney $U = 0$, $n_1 = n_2 = 5$, $Z = -2.61$, $p < 0.01$). This is consistent with the increased number of HDC cells, suggesting a widespread enhancement of HDC expression in narcoleptics.

Hcrt-R2 Mutant Dobermans and Age-Matched Control Dobermans

We next compared the HDC cells in the tuberomammillary and adjacent regions in narcoleptic dogs with normal dogs matched for breed, age, and time in fixation. Total HDC cell number was not significantly different between groups ($2,572 \pm 321$ in normal animals vs $2,507 \pm 151$ in narcoleptics; $t = 0.183$, $df = 4$, $p = 0.86$). HDC cell size did not differ between controls and narcoleptics ($277 \pm 20.6\mu\text{m}^2$ in normal animals and $280 \pm 27.9\mu\text{m}^2$ in narcoleptics; $t = 0.09$, $df = 4$, $p = 0.93$). The anatomical distribution of the HDC cells throughout the tuberomammillary nucleus was similar between control and narcoleptic dogs.

HDC KO Mice (Used to Test for Antibody Specificity)

Coded HDC KO and control mice were stained with the same Acris anti-HDC antibody used in our human, dog, and mouse studies. No HDC staining was seen in the KO mice, and normal staining was seen in the identically processed control animals,¹⁷ validating the specificity of the antibody (Fig 4).

Orexin KO Mice and Controls

We studied HDC cells in orexin KO mice and controls. Stained sections (6 controls and 6 orexin KO mice) were analyzed with the NeuroLucida System. We found no significant differences in HDC cell number or distribution between KO mice and their age-matched or littermate controls (718 ± 58 in WT mice vs 678 ± 47 in KO mice; $t = 0.98$, $df = 5$, $p = 0.4$). Similarly, there was no significant difference in HDC cell size between controls and orexin KO mice ($160 \pm 7\mu\text{m}^2$ vs $151 \pm 6\mu\text{m}^2$, $t = 2.10$, $df = 5$, $p = 0.09$).

Ataxin-3-Orexin Mice and Controls

The number of HDC neurons in the ataxin group was not different from the controls (356 ± 19.5 in control vs 346 ± 30.7 in ataxin, $t = 3.18$, $df = 11$, $p = 0.7$). If we restricted our analysis to the males (362.0 ± 21.1 in control vs 359.7 ± 39.2 in ataxin, $t = 0.06$, $df = 8$, $p = 0.95$) or to the females (339.7 ± 53.5 in control vs 304.33 ± 33.3 in ataxin, $t = 0.42$, $df = 2$, $p = 0.7$), there was also no difference. Moreover, there was no significant difference in HDC cell numbers when the ataxin animals were separated into different age categories. In group 1 animals (aged 2.5–3.2 months), HDC cell numbers in control versus ataxin were 316 ± 25.3 in control versus 296 ± 36.1 in ataxin ($t = 0.5$, $df = 3$, $p = 0.67$); in group 2 (aged 4.7–5.3 months), cell numbers were 383 ± 19.0 in control versus 417 ± 73.5 in ataxin ($t = 0.44$, $df = 3$, $p = 0.68$); in group 3 (aged 7–11.3

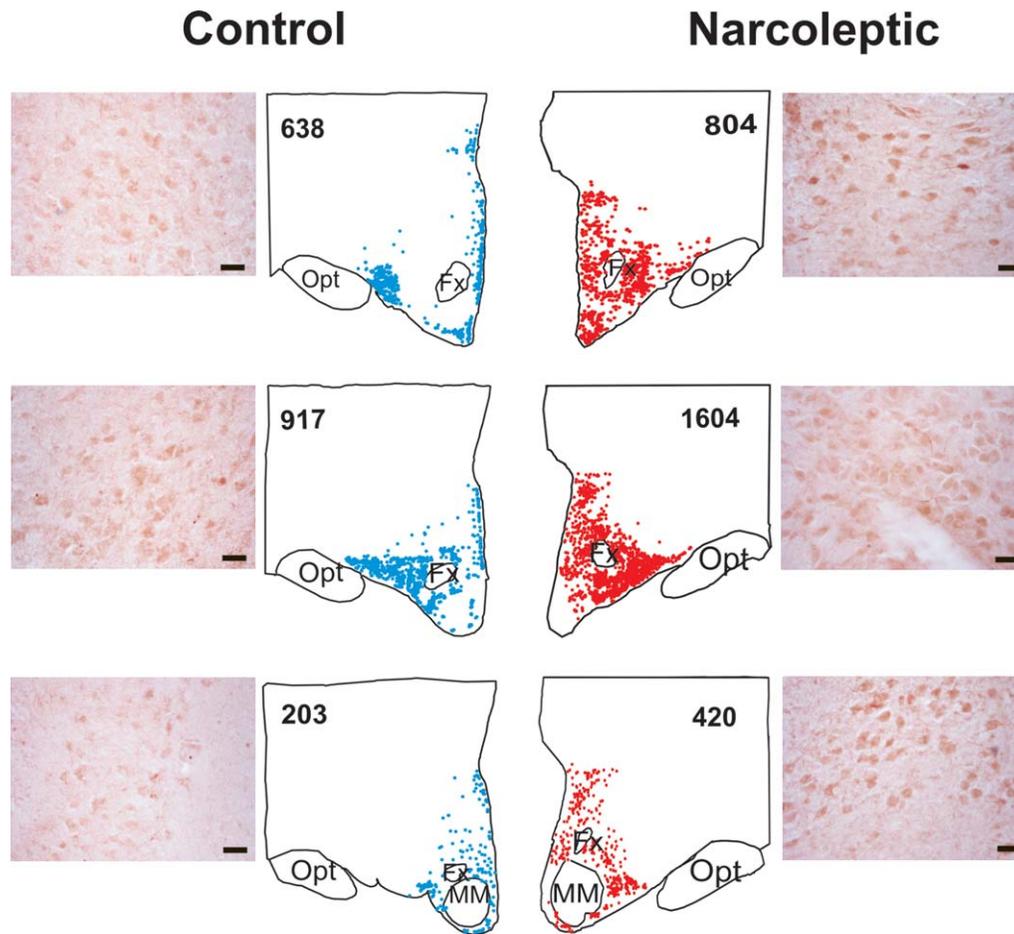


FIGURE 2: Distribution of histamine cells in narcoleptic and control. The number of cells at each level is indicated in a representative control (Patient 6) and narcoleptic (Patient 11). Fx = fornix; MM = mammillary body; Opt = optic tract. Scale bars = $50\mu\text{m}$. Note the increased intensity of histidine decarboxylase staining seen in the narcoleptic brain processed identically to the normal control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

months), cell numbers were 370 ± 48.4 in control versus 326 ± 31.6 in ataxin ($t = 0.7$, $df = 3$, $p = 0.5$). The cell size of HDC neurons was not significantly different between the control mice ($105 \pm 9.3\mu\text{m}^2$) and the ataxin mice ($100 \pm 3.2\mu\text{m}^2$; $t = 2.0$, $df = 11$, $p = 0.07$). If we restricted cell size analysis to the males ($102.9 \pm 3.0\mu\text{m}^2$ in control vs $97.0 \pm 3.7\mu\text{m}^2$ in ataxin, $t = 2$, $df = 8$, $p = 0.08$) or to the females ($112.5 \pm 4.1\mu\text{m}^2$ in control vs $109.1 \pm 4.1\mu\text{m}^2$ in ataxin, $t = 0.5$, $df = 2$, $p = 0.68$), there was also no difference.

DTA-Orexin Mice

We observed a >90% total loss of Hcrt cells in the experimental DTA animals. The number of HDC neurons in experimental DTA-orexin mice was not different from age-matched control mice (453 ± 63.3 in control vs 475 ± 101.5 in experimental; $t = 0.18$, $df = 8$, $p = 0.86$). HDC cell size in experimental DTA animals was not significantly different between groups ($113.7 \pm$

$2.6\mu^2$ in control vs $105.5 \pm 3.7\mu^2$ in experimental; $t = 1.8$, $df = 8$, $p < 0.1$).

Discussion

We found a substantially elevated number of HDC-labeled cells in narcoleptic patients, but not in Hcrt-R2 mutant narcoleptic dogs, orexin KO mice, ataxin-3-orexin mice, or DTA-orexin mice compared to their respective controls. Because the principal symptoms of narcolepsy, excessive daytime sleepiness and cataplexy, appear in all 4 animal models of narcolepsy studied, the increase in HDC cells is not necessary for these symptoms and is not an inevitable consequence of Hcrt dysfunction.

The increased number of histamine cells in human narcoleptics can be seen as a homeostatic compensation for the loss of Hcrt cells. However, it does not appear to be an effective compensation. Measurements of histamine in the cerebrospinal fluid of human narcoleptics with cataplexy have indicated that the histamine level is below

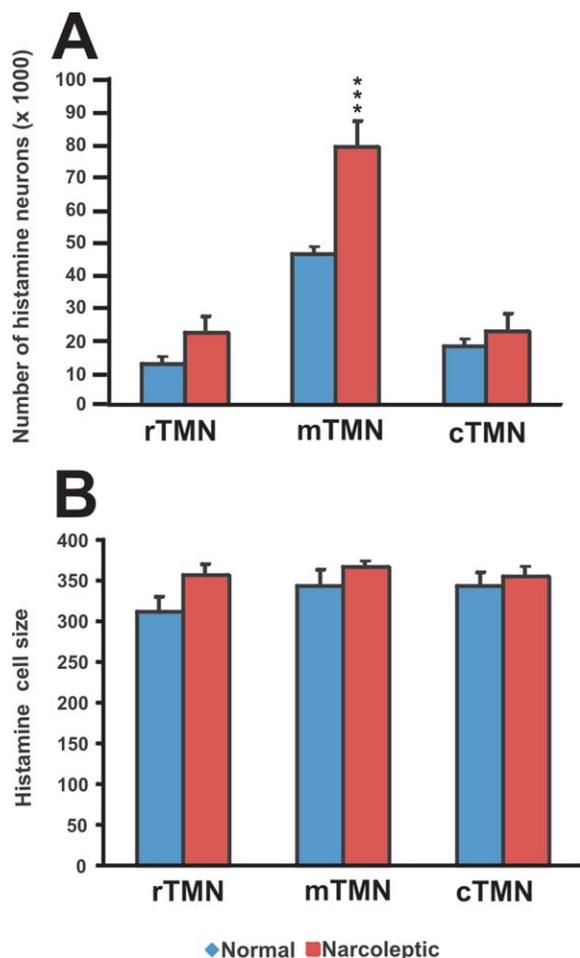


FIGURE 3: The (A) cell number and (B) size of histidine decarboxylase neurons in the tuberomammillary nucleus (TMN). The total number of histamine neurons is significantly increased in the mTMN subregion (** $p = 0.006$). There is no difference in the cell size in all subregions. cTMN = caudal part of TMN; mTMN = middle part of TMN; rTMN = rostral part of TMN. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

normal, or perhaps approaching normal.^{22–24} No study shows that histamine levels are above normal, as might be expected for an effective compensation. Histamine levels are also lower in narcoleptic dogs than in controls,²⁵ suggesting that these levels may be lowered as a correlate of sleepiness, independent of the number of HDC cells.

HDC staining has been shown to selectively label histamine cells in the tuberomammillary and adjacent regions.¹⁹ It remains to be determined whether all the neurons in the enlarged HDC population that we see in human narcoleptics are producing and releasing histamine at normal levels. We also do not know whether the cells of the enlarged neuronal population staining for HDC receive the same inputs or project to the same targets as the HDC population in normal individuals.

We are unaware of any prior reports of an increased number of neurons staining for HDC in any neurological

disease or in response to any other condition. A prior study reported increased levels of HDC mRNA in Huntington disease, with no change in cell number.²⁶ It has previously been reported that Hcrt neurons are lost in Parkinson disease^{27–29}; however, overall cerebrospinal fluid histamine level was reported to be at normal levels in this disorder.^{30,31} In the current study, we have evidence for both increased number and increased intensity of staining of HDC cells in human narcoleptics.

None of the 4 narcolepsy models we examined (mutation of the Hcrt-R2 receptor [in the dog], loss of the Hcrt peptide [in the KO mouse], or loss of the Hcrt neurons [in the ataxin and in the DTA models]) exhibited an elevation of HDC cell number. In all of these models, narcolepsy is caused by genetic factors. The cause of Hcrt cell loss in human narcolepsy is unclear. However, extensive evidence suggests an autoimmune etiology. More than 90% of narcoleptics have a human leukocyte antigen (HLA) haplotype that is present in <30% of the normal population.^{32,33} HLA molecules present antigens to the immune system. Evidence of inflammation has been seen in the regions of Hcrt cell loss in

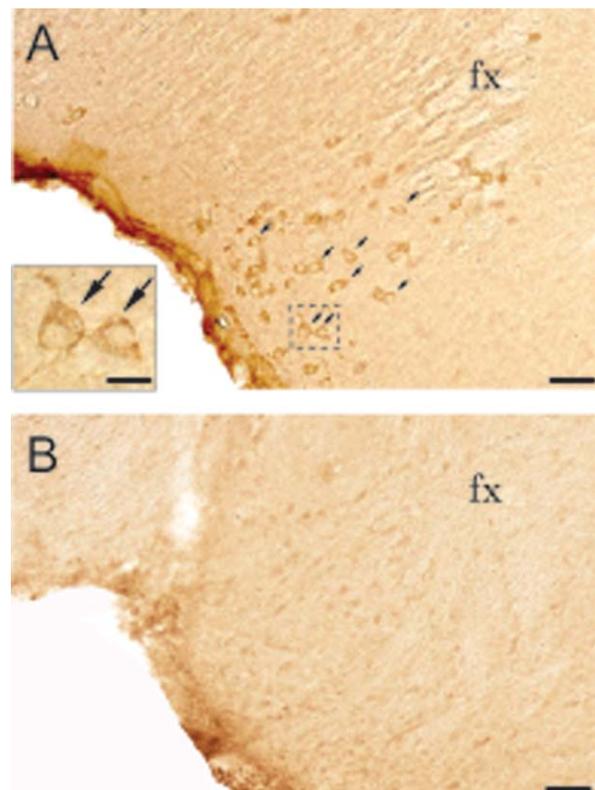


FIGURE 4: Histidine decarboxylase (HDC) staining (A) in a wild-type mouse and (B) in an HDC knockout (KO) mouse. Arrows indicate HDC-labeled cells. Lack of staining in KO demonstrates the specificity of the antibody and staining procedures we used. Scale bars = 50 μ m (A and B) and 12.5 μ m (inset). fx = fornix. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

human narcoleptics.^{1,3,34} The incidence of human narcolepsy has been shown to be increased by vaccination against H1N1 influenza and H1N1 infection without vaccination.^{35,36} Streptococcus infection has also been linked to narcolepsy.³⁷ However, at the cellular level, the process responsible for Hcrt loss in human narcoleptics remains unclear. Histamine is known to trigger immune-mediated inflammatory responses in the periphery. Prior work has implicated histamine release in the etiology of Parkinson disease, such that increased levels of histamine are hypothesized to cause the death of neurons in the substantia nigra.^{20,21,30}

The increased numbers of HDC cells in human narcoleptics, combined with the lack of changes in HDC labeling in animal models of narcolepsy, suggest that histamine may be linked to the factor causing Hcrt cell loss in humans. The greatly increased number of HDC cells that we see in human narcoleptics may be a residual effect of an increased histamine expression causing Hcrt cell loss, perhaps induced by immune activation of HDC synthesis. Histamine and Hcrt cells are tightly interconnected.^{38–42} A study in the zebrafish has shown a tight linkage between the development of HDC and Hcrt cells.⁴³ If histamine cell activity is involved in the etiology of narcolepsy, pharmacologic blockade of histamine cell activity or of the action of histamine on Hcrt cells early in the disorder might restrict symptom progression in humans.

An alternate explanation for the difference between the human narcoleptics and all animal models we have studied is the use of stimulant and antidepressant drugs by human narcolepsy patients. We know of no evidence that such drugs can alter neuronal numbers, but appropriate animal studies have not been done. Conversely, none of the human narcoleptic brains we acquired is from an individual who has never taken stimulant or antidepressant medication. However, the reported medication usage by the patients in the current study varied quite broadly, yet the HDC elevation occurred in all of these patients.

Because all of our patients had been symptomatic for >50 years, an additional hypothesis is that the HDC cell increase may be a response to Hcrt cell loss, but the change requires such a long duration that it is not observable in mouse or dog models, despite their higher metabolic rate and shorter lifespan. However, if this were the case, one might expect a smaller change in HDC expression or a trend in that direction in these animal models. We did not see convincing evidence of such a change. Species differences in response to Hcrt cell loss are also a possibility.

Whatever the trigger, there are 2 principal hypotheses for how the increased numbers of HDC cells might be generated. One is that transmitter reassignment^{43,44} might cause non-HDC hypothalamic cells adjacent to the normal popula-

tion of histamine cells to begin to synthesize HDC or increase their synthesis of HDC to detectable levels. A second is that neurogenesis might be responsible for the increased number of HDC cells. There is evidence for hypothalamic neurogenesis under other conditions.⁴⁵ This hypothesis cannot be tested in the current animal models of narcolepsy, because they do not show any change in HDC number.

Since Dahlstrom and Fuxe's⁴⁶ discovery that monoaminergic cells are segregated in discrete populations, it has been assumed that brains contain a fixed number of each type of monoaminergic neuron. These populations can only be diminished, for example by pathology, as in narcolepsy,^{1,2} in aging,⁴⁷ or by hormone treatments.⁴⁸ The current work shows that the HDC cell population can increase drastically. Future work will determine whether similar phenomena in this and other neurotransmitter systems might occur as a normal component of neuronal plasticity or occurs only in disease conditions.

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Authorship

J.J. and T.C.T. contributed equally.

Potential Conflicts of Interest

Nothing to report.

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