RESEARCH ARTICLE

Pattern of Hypocretin (Orexin) Soma and Axon Loss, and Gliosis, in Human Narcolepsy

Thomas C Thannickal[1*]; Jerome M. Siegel[1*]; Robert Nienhuis[1]; Robert Y. Moore[2]

[1] Department of Psychiatry and Brain Research Institute, University of California, Los Angeles and Neurobiology Research, VA Greater Los Angeles Healthcare System, North Hills, Calif.
[2] Department of Neurology, University of Pittsburgh, Pa.
[*] Authors contributed equally to this work.

Human narcolepsy is correlated with a greatly reduced number of hypocretin (orexin) containing neurons and axons, and an elevated level of hypothalamic gliosis. We now report that the percentage loss of Hcrt cells and percentage elevation of GFAP staining are variable across forebrain and brainstem nuclei, and are maximal in the posterior and tuberomammillary hypothalamic region. Regional gliosis and percent loss of hypocretin axons in narcoleptics are not correlated with regional hypocretin cell soma density in normals or with regional percent soma loss in narcoleptics. Rather they are independently and strongly correlated with the regional density of hypocretin axons and the message density for hypocretin receptor 2, as quantified in the rat. These results are consistent with the hypotheses that the loss of hypocretin function in narcolepsy results from a cytotoxic or immunologically mediated attack focused on hypocretin receptor 2 or an antigen anatomically linked to hypocretin receptor 2, and that this process is intensified in regions of high axonal density.

Brain Pathol 2003;13:340-351.

Introduction

We previously reported that human narcolepsy patients had an 85 to 95% reduction in the number of hypocretin (Hcrt) neurons (45, 46). At the same time, Peyron et al (39) reported that the level of preprohypocretin mRNA in narcoleptics was below their detection threshold. In our prior work, we also noted the presence of gliosis in the Hcrt cell region.

The cause of Hcrt cell loss in narcolepsy remains unclear. Narcolepsy is associated with the presence of a particular human leukocyte antigen, DQB1*0602, with more than 90% of narcoleptics having this haplotype, compared to 20 to 30% of the overall population (12, 15, 26, 31, 33). This association and the presence of gliosis within Hcrt cell regions of narcoleptics (46) suggests that Hcrt cell loss may be caused by an inflammatory or immune mediated process rather than by a developmental abnormality (11, 43). A better understanding of the pattern of damage to Hcrt containing somas and axons and of the gliosis occurring in narcolepsy should clarify the nature of the pathological process responsible for this disorder.

In the current study we have quantified the anatomical distribution of Hcrt cell loss, the reduction in numbers of Hcrt containing axons and the increased numbers of astrocytes in narcoleptics. We have examined the correlations between these measures and also compared the extent of cell loss and gliosis to the reported density of the 2 known Hcrt receptor mRNA's, quantified in the rat (30, 49). These data have been reported in preliminary form (48).

Materials and Methods

Narcolepsy was diagnosed according to standard criteria (10). In the current study we quantified the histological changes in 5 narcoleptic patients, including 4 used in our prior study that found Hcrt cell loss and gliosis in narcolepsy, and 6 matched normals. We did not receive brain stem tissue from patient ND (Table 1). Immunohistochemistry and antigen retrieval for Hcrt staining on the brains that had been in fixative for an extended period of time was done as previously described (19, 46). The hypothalamic and brainstem tissue blocks were cut into 40-μm thick coronal sections with a microtome. The sections were treated with 0.5% sodium borohydride in PBS for 30 minutes and washed with PBS, and then incubated for 30 minutes in 0.5% H_2O_2 for blocking of endogenous peroxidase activity. For antigen retrieval, sections were heated for 30 minutes 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. Antigen retrieval was not required on narcoleptic brain NE, which had been in fixative for only 3 months or for GFAP staining in any of the brains. After thorough washing with PBS, the sections were placed for 2 hours in 1.5% normal goat serum in PBS and incubated for 72 hours at 4°C with a 1:2000 dilution of rabbit anti-Hcrt-

Corresponding author:
Jerome M. Siegel, Department of Psychiatry and Brain Research Institute, University of California, Los Angeles and Neurobiology Research 151A3, VA Greater Los Angeles Healthcare System, North Hills, California 91343 (e-mail: jsiegel@ucla.edu)

Subject	Sex	Age	Cause of death	Time in Fix years	Disease Onset age	Medications	Cataplexy
Narcoleptics							
NA	F	63	adenocarcinoma	5.0	20	Clomipramine, Vivactil	+
NB	M	49	cardiovascular	4.5	18	Dexedrine, Vivactil, GHB	+
NC	M	60	sepsis	4.2	23	Ritalin, Dexedrine	+
ND	M	86	pulmonary	12.0	35	Ritalin	-
NE	M	81	cardiovascular	0.3	13	Dexedrine	+
Normals							
CA	F	54	liver failure	0.5			
CB	F	42	AML/DIC	1.0			
CC	M	n/a	non-neurological	1.0			
CD	M	73	pulmonary, cv	5.0			
CE	M	n/a	non-neurological	16.0			
CF	M	n/a	non-neurological	16.0			

cv, cardiovascular; AML, acute myelocytic leukemia; DIC, disseminated intravascular coagulation; GHB, gamma-hydroxy butyrate; "non-neurological" indicates no history of neurological illness and no specified cause of death.

Table 1. Characteristics of narcoleptic and normal subjects.

1 or rabbit anti-Hcrt-2 (Orexin A and B, Oncogene Research Products). 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 hours each at room temperature. The tissue-bound peroxidase was visualized by a diaminobenzidine reaction (Vector laboratories). We used a 15-minute 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. For identification of activated astrocytes, glial fibrillary acidic protein (GFAP) was immunostained with a 1:2000 dilution of primary polyclonal rabbit anti-cow GFAP antibody (DAKO, Carpinteria, Calif). Counterstaining of adjacent sections with cresyl violet aided in the localization of immunoreactive elements in relation to nuclei.

Brain regions and nuclei were identified using the atlas *Structure of the Human Brain* by DeArmond et al, 1989 (7). The following nuclei were examined: anterior hypothalamus (AH), periventricular nucleus (PVN), paraventricular nucleus (PAVN), supraoptic (SO), arcuate nucleus (ARN), dorsal hypothalamus (DH), dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), lateral hypothalamus (LH), posterior hypothalamus (PH), tuberomammillary nucleus (TMN), mammillary nucleus (MN), thalamus (dorsomedial nucleus, TH), locus coeruleus (LC), raphe dorsalis (RAD) and raphe centralis (RAC). Hcrt labeled

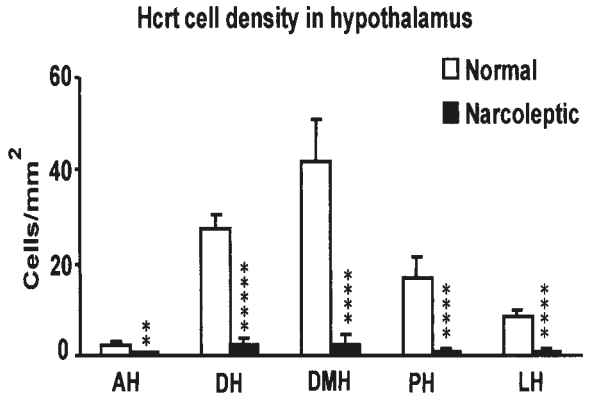


Figure 1. Hypocretin (Hcrt) soma density is greatly reduced in human narcolepsy, with maximal percent loss in posterior hypothalamus. Density of Hcrt cells in the hypothalamus of normal and narcoleptic brains: anterior hypothalamus (AH), dorsal hypothalamus (DH), dorsomedial hypothalamus (DMH), posterior hypothalamus (PH), lateral hypothalamus (LH). (** p<0.01, ****p<0.0001, *****p<0.00001, Student's *t* test, df = 6).

cells and axons, and GFAP labeled cells were counted "blind" on a Neurolucida computerized microscope interface. We used a Nikon E600 microscope with three axis motorized stage, video camera, and Stereoinvestigator software (MicroBrightfield Corp., Colchester, Vt). Digital image acquisition was carried out with a Spot camera (Diagnostic Instruments, Sterling Heights, Mich) and imported to the Corel Draw program. Contrast and brightness were corrected. Hcrt soma and axon loss and observed gliosis were compared with each other and with the relative density of Hcrt receptor mRNA expression reported for the rat (30).

Data analysis. Hypothalamic tissue from all 5 narcoleptic brains (NA-NE) and 6 matched controls (CA-CF) was used for quantification of Hcrt cells, axon and GFAP stained astrocytes. Brainstem tissue from 4 narcoleptic (NA-NC and NE) and 4 neurologically normal individuals (CC-CF) was used for Hcrt and GFAP staining. To obtain unbiased number and size estimates from a sample of axons in each nucleus, axon profiles of any shape, size and orientation and in all locations must have an equal probability of being sampled. The unbiased counting frame provides rules for defining which profiles belong to the area of the counting frame. In a complete tessellation of unbiased counting frames, all axon profiles were sampled once and only once. Systematic, uniform, random sampling with counting frames across the entire nucleus ensures that all locations within the nucleus are equally represented and that all axon profiles are sampled with equal probability. Hcrt and GFAP cell number were determined with stereological techniques on a one in 6 series of 40-μm sections through the Hcrt containing regions of the hypothalamus (16, 46) and brain stem nuclei. The numerical densities of the cells were calculated as number of cells per unit area (mm^2). The number of axons counted with the unbiased counting frames yields an unbiased estimate of the total number of axons (25). We used 250×250 μm as the counting frame size. By identifying the axon hillock or by excluding tapering processes each axon could be distinguished from dendritic branches in the regions containing Hcrt soma. Each axon and its branches was counted as a single item. The numerical density of Hcrt axons were calculated as number of axons per unit area (mm^2). All values for the each nucleus were calculated for each subject. These were pooled to give means and SD for each region and each group. Once the numerical densities were obtained, the density and percentage change of axons, somas and GFAP labeled cells was calculated. The data were analyzed using a one-way analysis of variance (ANOVA). All "t" values reported are Bonferroni adjusted. Statistical significance was set at $P<0.05$.

Partial correlation analysis. In order to statistically separate the effects of cell loss, fiber loss, Hcrt receptor density and gliosis, we performed a series of partial correlation analyses. The relative message density for receptor 1 or 2 was taken from Marcus et al (30). The message density was rated on a scale from 0 to 3: 0 applied to areas nearly entirely devoid of receptor; 1 applied to areas with low density; 2 applied to areas with moderate density; and 3 applied to areas with highest density. For the case of the partial correlation of Z (eg, message density for type 2 receptor) on Y (eg, % of fiber loss) controlling X (eg, gliosis), the bivariate regression of Z on X was performed, so that the residuals were the variance in Z after the effect of X was considered. The bivariate regression of Y on X was performed, so that the residuals were the variance in Y after the effect of X is considered.

Results

All 5 narcoleptic brains, including one in which antigen retrieval had not been performed, showed the same pattern of Hcrt cell and axonal loss, and gliosis. To control for the time spent in fix, we processed 3 neurologically normal brains that, like the narcoleptic brains, had been held in fix in our laboratory for 5 or more years and stained them with the same antibodies. As in the narcoleptic brains, no Hcrt staining was apparent in these normal brains unless our antigen retrieval procedure was followed. All normal brains, including the three in which antigen retrieval had been performed showed the same pattern of Hcrt cell and axon staining.

Hcrt cell loss in the narcoleptic hypothalamus. Hcrt immunoreactive cells were found in anterior (AH), dorsal (DH), dorsomedial (DMH), posterior (PH) and lateral (LH) hypothalamic nuclei of both normal and narcoleptic brains as previously reported (32, 45, 46) (Figure 1). Cell number was significantly reduced in narcoleptics compared to normals ($p<0.00001$, $F=238.35$, $df=1, 30$) and there was a significant interaction between hypothalamic nucleus and the percentage of Hcrt cells lost ($p<0.00001$, $F=33.1$, $df=4, 30$), indicating that percentage cell loss was significantly more severe in certain nuclei. There was no significant correlation between Hcrt cell density in normals and percent of Hcrt cell loss in narcoleptics ($p<0.15$, $r=0.54$, $t=1.91$, $n=10$). In the normal brain, Hcrt cell density was highest in DMH (41 cells/mm^2) and lowest in AH (2 cells/mm^2). In narcoleptics, the maximum percentage loss of Hcrt cells occurred in the posterior hypothalamus (97%, $p<0.0004$, $t=7.9$, $df=6$; Figure 2). The minimum percent loss of Hcrt cells was seen in AH (74%, $p<0.022$, $t=3.6$, $df=6$). Overall, narcoleptics had a mean 89% reduction in Hcrt cell number (range 74-97% between subjects) compared to the average number seen in normals.

Hcrt axon loss in the narcoleptic brain. Hcrt immunoreactive axons were found throughout the hypothalamic and brainstem nuclei of both normal and

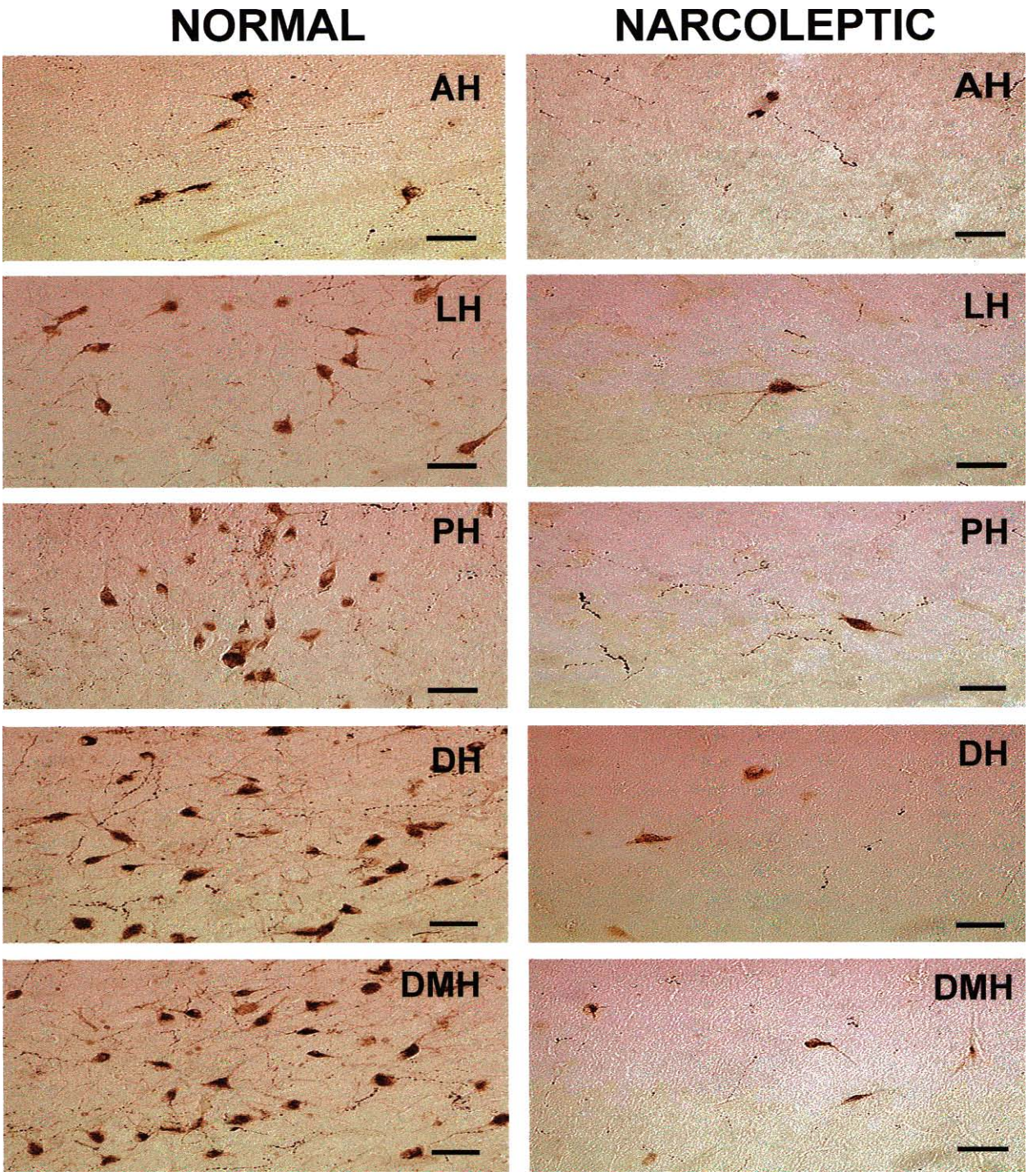


Figure 2. Hcrt cells in the hypothalamic nuclei of normal and narcoleptic humans. The abbreviations are same as in Figure 1. Scale bar = 50 μm.

narcoleptic brains (Figures 3A, 4; Table 2). In the hypothalamus of normal brains, a high density of Hcrt axons was found in paraventricular (69.7 axons/mm^2), periventricular (59.0 axons/mm^2), posterior (48.7 axons/mm^2), dorsomedial (48.5 axons/mm^2) and tuberomammillary nuclei (47.5 axons/mm^2). A low axon den-

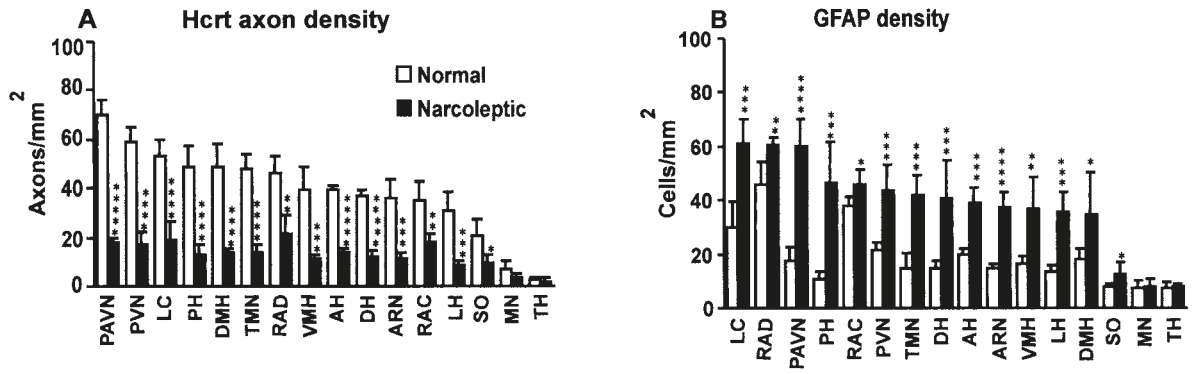


Figure 3. Density of Hcrt axons and GFAP labeled cells varies across nuclei between normal and narcoleptic brains. **A.** Hcrt axon density (axons/mm^2) in normal and narcoleptic brain arranged according to descending magnitude in normal brain. **B.** GFAP density (cells/mm^2) in normal and narcoleptic brain arranged according to descending magnitude in narcoleptic brain. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, *****p<0.00001, Student's *t* test, df = 6). Paraventricular nucleus (PAVN), periventricular nucleus (PVN), locus coeruleus (LC), posterior hypothalamus (PH), dorsomedial hypothalamus (DMH), tuberomammillary nucleus (TMN), raphe dorsalis (RAD), ventromedial hypothalamus (VMH), anterior hypothalamus (AH), dorsal hypothalamus (DH), arcuate nucleus (ARN), raphe centralis (RAC), lateral hypothalamus (LH), supraoptic (SO), mammillary nucleus (MN), thalamus (TH).

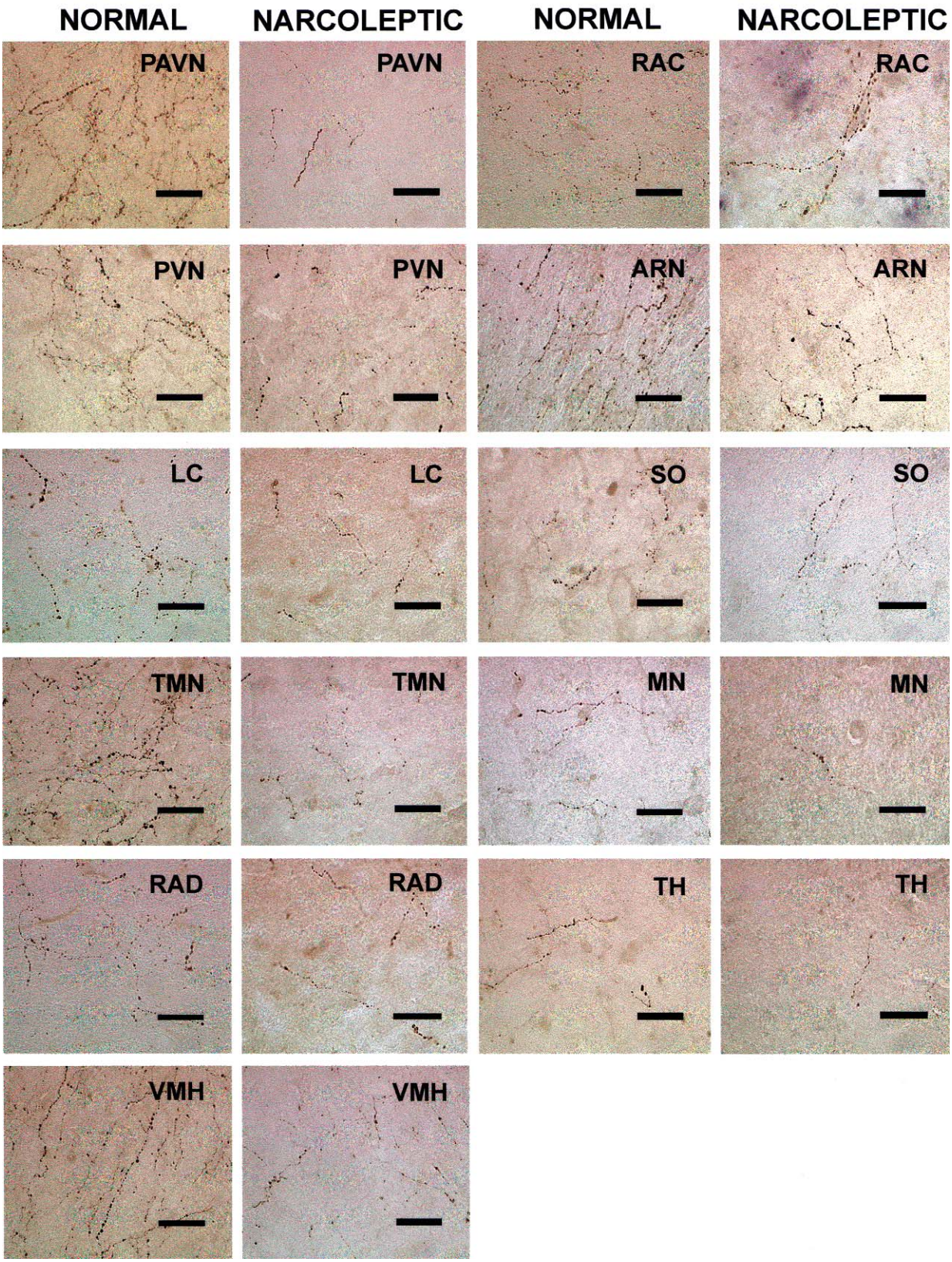
Nucleus	Axon Density Normals (axons/mm^2)	% Loss in Narcoleptics	Hcrtr-2 message density	GFAP (cells/mm^2) Narcoleptics	Normals	GFAP % increase
AH	39.0 ± 1.7	65.02	1	39.0 ± 5.7	19.6 ± 2.2	98.38
PVN	59.0 ± 5.6	71.69	2	43.3 ± 9.7	21.3 ± 3.1	103.05
PAVN	69.6 ± 6.7	76.89	3	59.9 ± 10.3	17.7 ± 4.8	238.61
SO	20.7 ± 6.6	54.12	1	12.4 ± 4.6	7.8 ± 1.2	57.84
ARN	35.7 ± 8.1	69.49	3	37.3 ± 5.8	14.9 ± 1.2	150.10
DH	36.8 ± 2.2	67.99	1	40.5 ± 14.1	14.7 ± 2.6	174.50
DMH	48.4 ± 9.8	72.53	2	34.6 ± 15.5	18.3 ± 3.5	89.05
VMH	39.1 ± 9.5	72.58	2	36.8 ± 11.8	16.2 ± 3.0	127.29
LH	30.5 ± 8.2	71.38	3	35.8 ± 7.3	13.6 ± 2.1	161.70
PH	48.7 ± 6.4	74.04	2	46.3 ± 15.1	10.5 ± 3.3	340.74
TMN	47.4 ± 6.4	71.44	3	41.7 ± 7.3	14.7 ± 5.3	183.54
MN	6.9 ± 3.5	52.46	1	8.1 ± 2.4	7.2 ± 2.9	12.96
TH	2.5 ± 0.6	22.20	0	7.9 ± 1.2	7.5 ± 2.2	5.61
RAC	35.3 ± 7.1	49.64	0	45.8 ± 5.5	38.12 ± 3.0	20.21
RAD	45.9 ± 7.0	53.15	1	60.1 ± 3.0	50.48 ± 8.7	19.24
LC	53.2 ± 6.2	64.32	0	61.2 ± 9.1	30.21 ± 9.5	102.71
Mean	38.70 ± 2.7	63.06	1.56	38.20 ± 4.4	18.95 ± 2.3	117.86

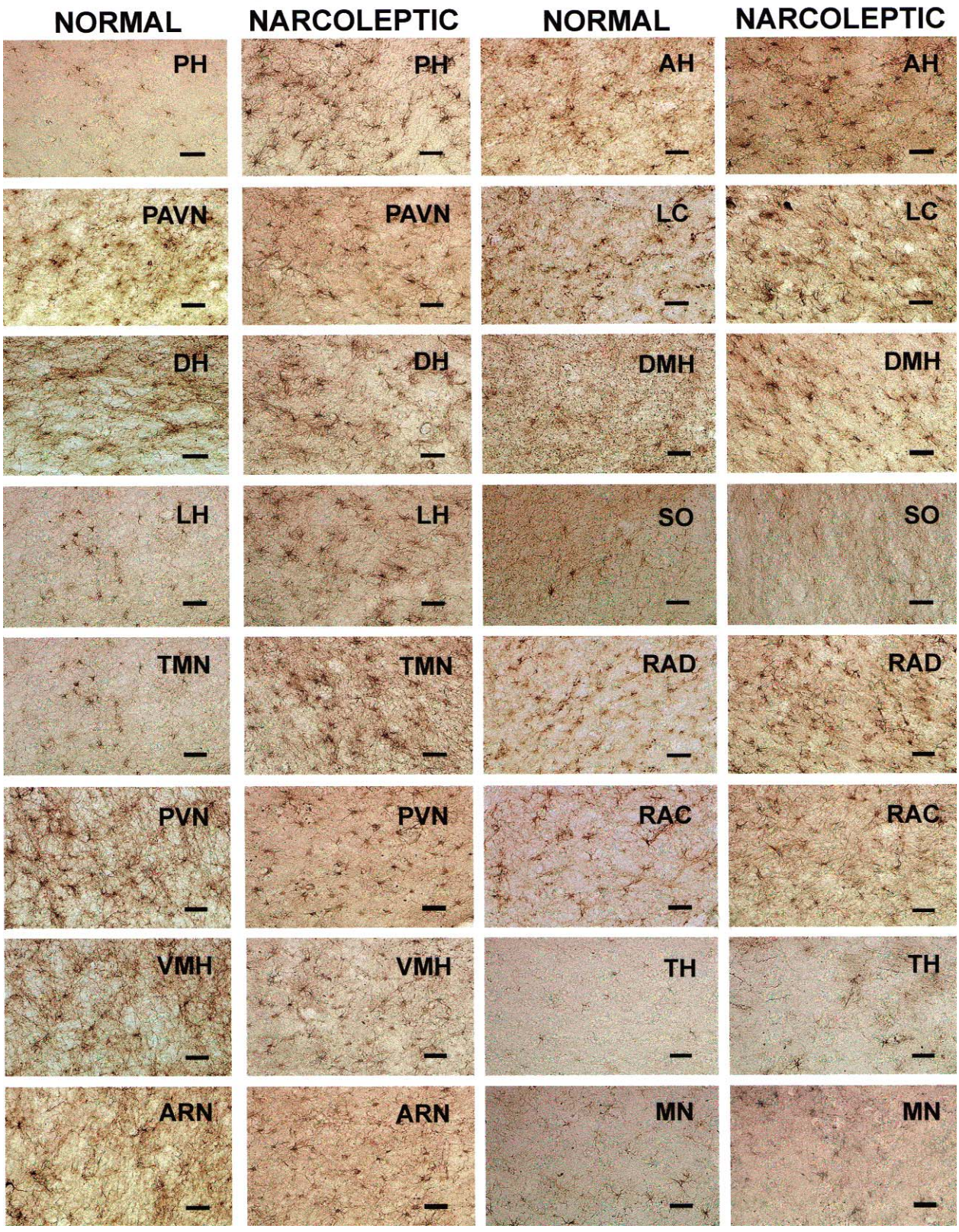
Data expressed as mean ± SD.

Table 2. Axon density in normals, percent loss in narcoleptics, message density for Hcrt receptor (Marcus et al, 2001) and GFAP percent increase in narcoleptics in surveyed nuclei.

sity was observed in mammillary nucleus (6.9 axons/mm^2) and thalamus (dorsomedial nucleus, 2.5 axons/mm^2). In the brainstem, locus coeruleus had the highest density of Hcrt axons (53.3 axons/mm^2). Raphe centralis and raphe dorsalis had 46.0 and 35.4 axons/mm^2 respectively. Compared to normals, narcoleptics had a significant reduction in the number of Hcrt axons in brainstem nuclei and hypothalamic nuclei ($p<0.00001, F = 720.42, df = 1, 96$) and there was a significant interaction between nucleus and percent axon loss ($p<0.00001, F = 12.02, df = 15, 96$), indicating that percent axonal loss was significantly greater in certain

Figure 4. (Opposing page) Hcrt axons in hypothalamus and brainstem nuclei of normal and narcoleptic brains: paraventricular (PAVN), periventricular (PVN), locus coeruleus (LC), tuberomammillary (TMN), raphe dorsalis (RAD) ventromedial hypothalamus (VMH), raphe centralis (RAC), arcuate nucleus (ARN), supraoptic (SO), mammillary (MN), thalamus (TH). Scale bar = 50 μm.


NORMAL
NARCOLEPTIC
NORMAL
NARCOLEPTIC
PAVN
PAVN
RAC
RAC
PVN
PVN
ARN
ARN
LC
LC
SO
SO
TMN
TMN
MN
MN
RAD
RAD
TH
TH
VMH
VMH


NORMAL
NARCOLEPTIC
NORMAL
NARCOLEPTIC
PH
PH
AH
AH
PAVN
PAVN
LC
LC
DH
DH
DMH
DMH
LH
LH
SO
SO
TMN
TMN
RAD
RAD
PVN
PVN
RAC
RAC
VMH
VMH
TH
TH
ARN
ARN
MN
MN

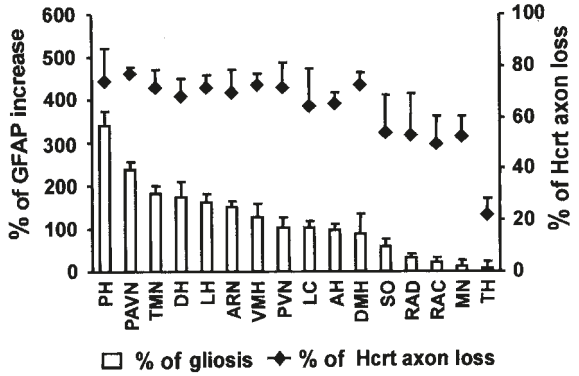


Figure 6. Pattern of gliosis and percentage of Hcrt axon loss in the hypothalamus and brain stem nuclei of normal and narcoleptic brain. Number of GFAP labeled cells is correlated with percent Hcrt axon loss. The abbreviations are same as in Figure 3.

nuclei. Among the hypothalamic nuclei, PAVN (77%, $p<0.00044$, $t=14.68$, $df=6$), PH (74%, $p<0.0023$, $t=7.3$, $df=6$), VMH (73%, $p<0.0075$, $t=5.86$, $df=6$), DMH (73%, $p<0.0031$, $t=6.9$, $df=6$), TMN (71%, $p<0.00059$, $t=9.3$, $df=6$), PVN (72%, $p<0.00029$, $t=10.57$, $df=6$) and LH (71%, $p<0.014$, $t=5.19$, $df=6$) had a high percentage reduction of Hcrt axons (Figure 3A). There was a significant, but lesser percent reduction of Hcrt axons in LC (64%, $p<0.0014$, $t=6.89$, $df=6$), RAD (53%, $p<0.008$, $t=4.8$, $df=6$) and RAC (50%, $p<0.014$, $t=4.36$, $df=6$). The percent axon density reduction in Hcrt innervated nuclei in narcoleptics was positively correlated with the density of Hcrt axons in normal humans ($r=0.75$, $t=3.37$, $p<0.0008$, $n=11$). The total number of Hcrt axons in all the structures analyzed was reduced by 67% compared to an 89% reduction of Hcrt soma count in the same brains, suggesting that either Hcrt cells with smaller axonal fields are lost to a greater extent in narcolepsy or that some sprouting of axons of surviving Hcrt cells may have occurred in narcoleptics.

GFAP labeled astrocytes. GFAP labeled astrocytes were found throughout the hypothalamic and brainstem nuclei of normal and narcoleptic brains (Figures 3B, 5). In the normal brain, compared with hypothalamic nuclei, locus coeruleus, raphe dorsalis and centralis had a higher density of GFAP stained cells (Figure 3B; Table 2). There was a significant increase in gliosis indicated by GFAP staining ($p<0.00001$, $F=243.86$, df

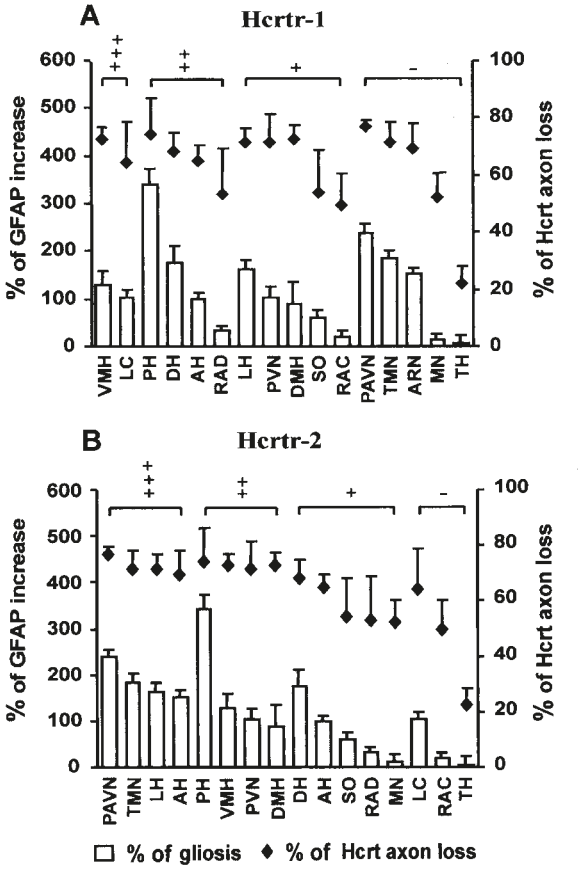


Figure 7. The pattern of gliosis and Hcrt axon loss ranked in relation to the message density for Hcrt receptors. **A.** The percent increase in gliosis and axon loss was not correlated with the message density for Hcrt receptor 1. **B.** The percent increase in gliosis and percent axon loss was correlated with the message density for Hcrt receptor 2. The abbreviations are same as in Figure 3. The message density for receptor data from previously reported data (Marcus et al, 2001, +++ highest density, ++ moderate density, + low density, - absent).

$=1, 102$) and a significant interaction between nucleus and amount of gliosis in narcoleptics ($p<0.00001$, $F=5.65$, $df=15, 102$) indicating that the increase in GFAP staining was significantly greater in certain nuclei. The size of GFAP labeled astrocytes did not differ between narcoleptics and controls. In narcoleptics, GFAP density was high in paraventricular (60.0 cells/mm^2), posterior (46.3 cells/mm^2), periventricular (43.3 cells/mm^2), tuberomammillary (41.8 cells/mm^2), and dorsal nuclei of hypothalamus (40.5 cells/mm^2). Compared to normals,

Figure 5. (Opposing page) GFAP labeled astrocytes in the hypothalamus and brainstem nuclei of normal and narcoleptic brain. The abbreviations are same as in Figure 3. Scale bar = 50 μm.

A

X = Hcrt axon density normals
Y = % axon loss narcoleptics
Z = message density for Hcrt type 2 receptor

Simple	r	r^2	P	Partials	r	r^2	t	P
rxy	0.752	0.565	0.0008	rxy.z	0.682	0.466	3.37	0.0050
rxz	0.426	0.181	0.0995	rxz.y	-0.181	0.033	-0.66	0.5207
ryz	0.724	0.523	0.0015	ryz.x	0.644	0.415	3.04	0.0094

Conclusions:
1. Hcrt axon loss is correlated with normal fiber density
2. Hcrt axon loss is correlated with the message density for Hcrt type 2 receptor
3. These correlations are independent

B

X = gliosis (GFAP) percent increase in narcoleptics
Y = axon density normals
Z = message density for Hcrt type 2 receptor

Simple	r	r^2	P	Partials	r	r^2	t	P
rxy	0.559	0.312	0.0244	rxy.z	0.413	0.171	1.64	0.1249
rxz	0.632	0.399	0.0086	rxz.y	0.525	0.276	2.22	0.0448
ryz	0.426	0.181	0.0995	ryz.x	0.113	0.013	0.41	0.6884

Conclusions:
1. gliosis is correlated with the message density for Hcrt type 2 receptor
2. This correlation is independent of axon density in normals

r = correlation coefficient (xy, xz, yz). rxy.z, rxz.y and ryz.x are correlations of variable x with y with the statistical effect of z (or y or x) removed.

Table 3. Partial correlation analysis of axonal loss, axonal density and GFAP staining.

narcoleptics had a very high percentage increase of GFAP in posterior hypothalamus (341%, $p<0.017$, $t=4.65$, $df=6$), paraventricular (239%, $p<0.025$, $t=4.28$, $df=6$), tuberomammillary (184%, $p<0.0049$, $t=5.9$, $df=6$), dorsal hypothalamus (175%, $p<0.056$, $t=3.56$, $df=6$), and arcuate nucleus (150%, $p<0.0014$, $t=7.4$, $df=6$). In supraoptic, mammillary and thalamic nuclei we found a very low density of GFAP in both normal and narcoleptic brains (Figure 3B). Locus coeruleus had a higher percentage of GFAP increase (103%, $p<0.0026$, $t=5.14$, $df=8$) than raphe dorsalis (32%, $p<0.59$, $t=1.4$, $df=8$) and raphe centralis (20%, $p<0.061$, $t=2.8$, $df=8$) (Figure 6), and brainstem nuclei in general showed smaller increases than forebrain nuclei. Regional gliosis in narcoleptics was highly correlated with the density of Hcrt axons in the normal brain ($r=0.87$, $p<0.0001$, $n=11$).

Hcrt soma and axon loss and message density for Hcrt receptors. The reported anatomical distribution of Hcrt receptor 1 and 2 in the rat are largely distinct (Figure 7 A, B). Hcrt soma loss was not correlated with the message density reported for Hcrt receptor 1 ($p<0.66$, $r=0.26$, $t=0.47$, $n=10$) or with that reported for Hcrt receptor 2, as quantified in the rat (30) ($p<0.53$, $r=0.37$, $t=0.69$, $n=10$), across the hypothalamic nuclei. The percentage of Hcrt axon loss in narcoleptics was compared with message density for Hcrt receptors. Hcrt axon loss was positively correlated with the message density for Hcrt receptor 2 ($r=0.72$, $t=10.95$, $p<0.002$). Axon loss was not correlated with the message density for Hcrt receptor 1 ($r=0.05$, $t=10.55$, $p<0.84$).

Partial correlation analysis. In order to statistically separate the effects of cell loss, fiber loss, message density for receptor and gliosis, we performed a series of partial correlation analyses (Table 3A, B). These analyses showed that Hcrt axonal density in normals correlated positively with percent axonal loss in narcoleptics over all examined nuclei. They also showed that the message density for Hcrt receptor 2, as measured in the rodent, correlated positively with the percent axonal loss in human narcoleptics. These 2 correlations were found to be independent. The increase in gliosis in narcoleptics was also shown to be correlated independently with message density for type 2 receptor independent of normal axon density. In summary, we identify 2 independent correlates of axonal loss and gliosis in nar-

coleptics, the density of axonal fibers and the message density for Hcrt type 2 receptor.

Discussion

We find the highest percentage depletion of Hcrt cells and GFAP staining in the posterior and tuberomammillary hypothalamic regions. This region contains all of the hypothalamic histamine cells (1, 27, 37, 44). Hypocretin potently excites histamine neurons (3, 9, 54). A reduction of histamine has been seen in the cerebrospinal fluid of genetically narcoleptic dogs (35) and narcoleptic humans (36). It is well known that antihistamines cause sleepiness and that stimulation of the histamine cells produces arousal (28). The arousing effect of Hcrt has been shown to be heavily dependent on activation of histamine containing cells (18). The loss of Hcrt cells in human narcoleptics suggests that reduction in Hcrt activation of histamine cells may mediate some of the symptoms of sleepiness in narcolepsy. The increased percentage loss of Hcrt cells, axons and gliosis in the posterior hypothalamus, suggests that this region may be a focus of inflammation and damage in narcolepsy.

In contrast to our prior report (46) and current findings, a prior study (Peyron et al, 2000) did not detect gliosis in the hypothalamus of the 2 narcoleptic brains inspected or in any control tissue. The relative sensitivities of the staining techniques and procedures used may account for this difference between the Peyron et al study and the current work.

The distribution of Hcrt cell somas and axonal projections is quite similar in mice (8, 41), rats (34, 40), cats (52), dogs (47) and humans (46). In the current study, we correlated the levels of cell and axonal loss with prior published work on Hcrt mRNA receptor 1 and 2 message density in the rat (30, 49). Comparable receptor data are not available for humans. In general, receptor type distributions, like soma and axonal distributions, are preserved across species (13, 14, 17, 20, 42, 51), so it is likely that the reported Hcrt receptor data in the rat approximate that in the human. The very high correlations seen here would be less likely if the rodent receptor density was not linked to a meaningful neurological parameter in humans, most likely receptor density. However, we cannot rule out the possibility that the underlying determinant of the relationships we see here is a function of some other correlate of rodent Hcrt receptor 2 mRNA concentration.

Our partial correlation analyses lead to the following conclusions: *i)* percentage Hcrt axon loss/increase in gliosis is not correlated with the density of Hcrt cell somas; *ii)* percentage Hcrt axon loss is correlated with Hcrt axon density; *iii)* percentage Hcrt axon loss is also correlated with the message density for Hcrt receptor 2; *iv)* percentage increase in gliosis is correlated with the message density for Hcrt receptor 2; and *v)* these correlations are independent.

The correlation between percentage loss of axons and percentage increase in gliosis, and the message density for Hcrt receptor 2 suggests that this receptor or antigens linked to it may be associated with processes that intensify the pathological process in narcolepsy.

Canine genetic narcolepsy is caused by one of 2 distinct mutations of the Hcrt receptor 2, but not by mutations of Hcrt receptor 1 (29). Similarly, knockouts of the Hcrt 2 receptor, but not the Hcrt 1 receptor produce symptoms of narcolepsy in mice (6, 21). Our current finding that damage in human narcolepsy is correlated with the best available estimate of Hcrt receptor 2 message density suggests that damage in the region of this receptor is a determining factor in human narcolepsy. Both cell loss in humans (46) and animals (11) and receptor mutation (29, 39) lead to the similar syndromes of excessive sleepiness during the active period of the circadian cycle and disrupted sleep during the inactive phase, cataplexy, and, as studied in dogs and humans, therapeutic response to anticholinergic drugs and to drugs that enhance monoamine levels, and symptom exacerbation by cholinesterase inhibitors and the alpha-1 antagonist prazosin (2, 5, 43, 53). The striking similarity of the symptoms and pharmacology of narcolepsy in Hcrt receptor 2 mutant animals to those in hypocretin deficient humans (46) suggests that normal receptor 2 operation is necessary for adequate regulation of Hcrt release at terminal sites.

In genetically narcoleptic dogs, we have seen an abrupt termination of activity in locus coeruleus in conjunction with cataplexy (53). In normal animals, locus coeruleus cells never have a comparable cessation of activity in waking, and have an important role in muscle tone facilitation (24). Although the locus coeruleus has the densest extrahypothalamic concentration of Hcrt axons (40), the predominant receptor type in this region is Hcrt receptor 1 (30, 49). Therefore the Hcrt receptor 2 mutation effect in genetically narcoleptic dogs must be indirect. Thus, we hypothesize that the Hcrt receptor 2 dysfunction disrupts the normal pattern of Hcrt release into locus coeruleus and other sites during waking. In human narcolepsy the loss of Hcrt projections to the locus coeruleus would have a similar effect. We have found that Hcrt -1 release in hypothalamic regions is high during REM sleep, equally high in waking, but significantly reduced in nonREM sleep (22, 23). It is like-

ly that Hcrt release interacts with GABA and glutamate to produce differing state specific postsynaptic effects (4, 38, 50) thereby modulating arousal and motor control.

Acknowledgments

This work was supported by the Medical Research Services of the Dept. of Veterans Affairs and NIH grants NS14610, HL41370 and MH64109 to JM Siegel.

References

1. Airaksinen MS, Panula P (1988) The histaminergic system in the guinea pig central nervous system: an immunocytochemical mapping study using an antiserum against histamine. *J Comp Neurol* 273:163-186.
2. Aldrich MS (1998) Diagnostic aspects of narcolepsy. *Neurology* 50:S2-7.
3. Bayer L, Eggermann E, Serafin M, Saint-Mleux B, Machard D, Jones B, Muhlethaler M (2001) Orexins (hypocretins) directly excite tuberomammillary neurons. Eur *J Neurosci* 14:1571-1575.
4. Burlet S, Tyler CJ, Leonard CS (2002) Direct and indirect excitation of laterodorsal tegmental neurons by Hypocretin/Orexin peptides: implications for wakefulness and narcolepsy. *J Neurosci* 22:2862-2872.
5. Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, Richardson JA, Williams SC, Xiong Y, Kisanuki Y, Fitch TE, Nakazato M, Hammer RE, Saper CB, Yanagisawa M (1999) Narcolepsy in orexin knockout mice: Molecular genetics of sleep regulation. *Cell* 98:437-451.
6. Chemelli RM, Sinton CM, Yanagisawa M (2000) Polysomnographic characterization of orexin-2 receptor knockout mice. *Sleep* 23:A296-A297.
7. DeArmond SJ, Fusco MM, Dewey (1989) *Structure of the human brain a photographic atlas*, third edition, Oxford University Press.
8. de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FS 2nd, Frankel WN, van den Pol AN, Bloom FE, Gautvik KM, Sutcliffe JG. (1998) The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci USA* 95:322–327.
9. Eriksson KS, Sergeeva O, Brown RE (2001) Orexin/hypocretin excites the histaminergic neurons of the tuberomammillary nucleus. *J Neurosci* 21:9273-9279.
10. Guilleminault C. Narcolepsy syndrome (1994) In: *Principles and practice of sleep medicine*, M.H. Kryger, T. Roth, and W.C. Dement, eds. (Philadelphia: W.B. Saunders Company).
11. Hara J, Beuckmann CT, Nambu T, Willie JT, Chemelli RM, Sinton CM, Sugiyama F, Yagami K, Goto K, Yanagisawa M, Sakurai T (2001) Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. *Neuron* 30:345-354.
12. Harvald B (1989) Genetic epidemiology of Greenland. *Clin Genet* 36:364-367.
13. Hebebrand J, Friedl W (1987) Phylogenetic receptor research: implications in studying psychiatric and neurological disease. *J Psychiatr Res* 21:531-537.
14. Hebebrand J, Friedl W, Reichelt R, Schmitz E, Moller P, Propping P (1988) The shark GABA-benzodiazepine receptor: further evidence for a not so late phylogenetic appearance of the benzodiazepine receptor. *Brain Res* 446:251-261.
15. Honda Y, Doi Y, Juji T, Satake M (1984) Narcolepsy and HLA: Positive DR2 as a prerequisite for the development of narcolepsy. *Folia Psychiatr Neurol Jpn* 38:360.
16. Howard CV, Reed MG (1998) *Unbiased stereology: three dimensional measurement in microscopy* (New York: Springer-Verg).
17. Hoyer D, Schoeffter P, Waeber C, Palacios JM (1990) Serotonin 5-HT1D receptors. *Ann NY Acad Sci* 600:168-181.
18. Huang ZL, Qu WM, Li WD, Mochizuki T, Eguchi N, Watanabe T, Urade Y, Hayaishi O (2001) Arousal effect of orexin A depends on activation of the histaminergic system. *Proc Natl Acad Sci USA* 98:9965-9970.
19. Jiao Y, Sun Z, Lee T, Fusco FR, Kimble TD, Meade CA, Cuthbertson S, Reiner A (1999) A simple and sensitive antigen retrieval method for free-floating and slide-mounted tissue sections. *J Neurosci Methods* 93:149–162.
20. Kapsimali M, Dumond H, Le Crom S, Coudouel S, Vincent JD, Vernier P (2000) Evolution and development of dopaminergic neurotransmitter systems in vertebrates. *J Soc Bio*l 194:87-93.
21. Kisanuki Y, Chemelli RM, Sinton CM, Williams SC, Richardson JA, Hammer RE, Yanagisawa M (2000) The role of orexin receptor type-1 (OX1R) in the regulation of sleep. *Sleep* 23:A91.
22. Kiyashchenko LI, Mileykovskiy BY, Lai,YY, Siegel JM (2001) Increased and decreased muscle tone with orexin (hypocretin) microinjections in the locus coeruleus and pontine inhibitory area. *J Neurophysiol* 85:2008-2016.
23. Kiyashchenko LI, Mileykovskiy BY, Maidment N, Lam HA, Wu MF, John J, Peever J, Siegel JM (2002) Release of hypocretin (orexin) during waking and sleep states. *J Neurosci* 22:5282-5286.
24. Lai YY, Kodama T, Siegel JM (2001) Changes in monoamine release in the ventral horn and hypoglossal nucleus linked to pontine inhibition of muscle tone: an in vivo microdialysis study. *J Neurosci* 21:7384-91.
25. Larsen JO (1998) Stereology of nerve cross sections. *J Neurosci Methods* 85:107-18.
26. Leslie RD, Hawa M (1994) Twin studies in auto-immune disease. *Acta Genet Med Gemellol (Roma)* 43:71-81.
27. Lin JS, Luppi PH, Salvert D, Sakai K, Jouvet M (1986) Histamine-immunoreactive neurons in the hypothalamus of cats. *C R Acad Sc Paris* 303:371-376.
28. Lin JS, Sakai K, Vanni-Mercier G, Jouvet M (1989) A critical role of the posterior hypothalamus in the mechanisms of wakefulness determined by microinjection of muscimol in freely moving cats. *Brain Res* 479:225-240.

29. Lin L, Faraco J, Li R, Kadotani H, Rogers W, Lin X, Qiu X, de Jong PJ, Nishino S, Mignot E (1999) The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. *Cell* 98:365-376.

30. Marcus JN, Aschkenasi CJ, Lee CE, Chemelli RM, Saper CB, Yanagisawa M, Elmquist JK(2001) Differential expression of orexin receptors 1 and 2 in the rat brain. *J Comp Neurol* 435:6-25.

31. Mignot E, Lin L, Rogers W (2001) Complex HLA-DR and -DQ interactions confer risk of narcolepsy-cataplexy in three ethnic groups. *Am J Hum Genet* 68:686-699.

32. Moore RY, Abrahamson EA, van den Pol A (2001). The hypocretin neuron system: an arousal system in the human brain. *Arch Ital de Biol* 139:195-206.

33. Naito S (1986) The association of HLA with diseases in Japanese. *Jinrui Idengaku Zasshi* 31:323-329.

34. Nambu T, Sakurai T, Mizukami K, Hosoya Y, Yanagisawa M, Goto K (1999) Distribution of orexin neurons in the adult rat brain. *Brain Res* 827:243-60.

35. Nishino S, Fujiki N, Ripley B, Sakurai E, Kato M, Watanabe T, Mignot E, Yanai K (2001) Decreased brain histamine content in hypocretin/orexin receptor-2 mutated narcoleptic dogs. *Neurosci Lett* 313:125-128.

36. Nishino S, Sakurai E, Nvsimalova S, Vankova J, Yoshida Y, Watanabe T, Yanai K, Mignot E (2002) CSF histamine content is decreased in hypocretin-deficient human narcolepsy. *Sleep* 25:A476.

37. Panula P, Yang HY, Costa E (1984) Histamine-containing neurons in the rat hypothalamus. *Proc Natl Acad Sci U S A* 81:2572-2576.

38. Peever JH, Lai YY, Siegel JM (2002) Excitatory effects of hypocretin in the trigeminal nucleus are reversed by NMDA antagonism. *J Neurophysiol* (in press).

39. Peyron C, Faraco J, Rogers W, Ripley B, Overeem S, Charnay Y, Nevsimalova S, Aldrich M, Reynolds D, Albin R, Li R, Hungs M, Pedrazzoli M, Padigaru M, Kucherlapati M, Fan J, Maki R, Lammers GJ, Bouras C, Kucherlapati R, Nishino S, Mignot E (2000) A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. *Nat Med* 6:991-997.

40. Peyron C, Tighe DK, van den Pol AN, de Lecea L, Heller HC, Sutcliffe JG, Kilduff TS (1998) Neurons containing hypocretin (orexin) project to multiple neuronal systems. *J Neurosci* 18:9996-10015.

41. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR et al (1998) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 92:573-85.

42. Schmitz E, Reichelt R, Walkowiak W, Richards JG, Hebebrand J (1988) A comparative phylogenetic study of the distribution of cerebellar ABAA/benzodiazepine receptors using radioligands and monoclonal antibodies. *Brain Res* 473: 314-320.

43. Siegel JM (1999) Narcolepsy: A key role for hypocretins (orexins). *Cell* 98: 409-412.

44. Steininger TL, Alam MN, Gong H, Szymusiak R, McGinty D (1999) Sleep-waking discharge of neurons in the posterior lateral hypothalamus of the albino rat. *Brain Res* 840:138-147.

45. Thannickal TC, Moore RY, Aldrich M, Albin R, Cornford M, Siegel JM (2000) Human narcolepsy is linked to reduced number, size and synaptic bouton density in hypocretin-2 labeled neurons. *Abstr Soc Neurosci* 26:2061-2061.

46. Thannickal TC, Moore RY, Nienhuis R, Ramanathan L, Gulyani S, Aldrich M, Cornford M, Siegel JM (2000) Reduced number of hypocretin neurons in human narcolepsy. *Neuron* 27:469-474.

47. Thannickal TC, Nienhuis R, Ramanathan L, Gulyani S, Turner K, Chestnut B, Siegel JM (2000) Preservation of hypocretin neurons in genetically narcoleptic dogs. *Sleep* 23 A296.

48. Thannickal TC, Moore RY, Nienhuis R, Siegel JM (2002) Increased number of astrocytes and reduced density of hypocretin/orexin fibers in human narcolepsy. *Sleep* 25A:354-355.

49. Trivedi P, Yu H, MacNeil DJ, Van der Ploeg LH, Guan XM (1998) Distribution of orexin receptor mRNA in the rat brain. *FEBS Lett* 438:71-75.

50. van den Pol AN, Gao XB, Obrietan K, Kilduff TS, Belousov AB (1998) Presynaptic and postsynaptic actions and modulation of neuroendocrine neurons by a new hypothalamic peptide, hypocretin/orexin. *J Neurosci* 18:7962-7971.

51. Verge D, Calas A (2000) Serotoninergic neurons and serotonin receptors: gains from cytochemical approaches. *J Chem Neuroanat* 18:41-56.

52. Wagner D, Salin-Pascual R, Greco MA, Shiromani PJ (2000) Distribution of hypocretin-containing neurons in the lateral hypothalamus and C-fos-immunoreactive neurons in the VLPO. *Sleep Res Online* 3:35-42.

53. Wu MF, Gulyani SA, Yau E, Mignot E, Phan B, Siegel JM (1999) Locus coeruleus neurons: cessation of activity during cataplexy. *Neuroscience* 91:1389-1399.

54. Yamanaka A, Tsujino N, Funahashi H, Honda K, Guan JL, Wang QP, Tominaga M, Goto K, Shioda S, Sakurai T (2002) Orexins activate histaminergic neurons via the orexin 2 receptor. *Biochem Biophys Res Commun* 290:1237-1245.