# A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains

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**We explored the role of hypocretins in human narcolepsy through histopathology of six narcolepsy brains and mutation screening of *Hcrt*, *Hcrtr1* and *Hcrtr2* in 74 patients of various human leukocyte antigen and family history status. One *Hcrt* mutation, impairing peptide trafficking and processing, was found in a single case with early onset narcolepsy. *In situ* hybridization of the perifornical area and peptide radioimmunoassays indicated global loss of hypocretins, without gliosis or signs of inflammation in all human cases examined. Although hypocretin loci do not contribute significantly to genetic predisposition, most cases of human narcolepsy are associated with a deficient hypocretin system.**



Narcolepsy is a disabling sleep disorder characterized by excessive daytime sleepiness and abnormal manifestations of rapid eye movement (REM) sleep, including cataplexy, sleep paralysis and hypnagogic hallucinations[1]. Human narcolepsy, affecting approximately 1 in 2000 individuals, is genetically complex[2]. Most cases are sporadic, and twin studies indicate a role for environmental triggers: only 25–31% of monozygotic twin pairs are concordant for the disorder[2]. Predisposition to narcolepsy is influenced by human leukocyte antigen (HLA) subtypes; up to 95% of patients with typical and severe cataplexy carry DQB1*0602 (ref. 3). These findings suggest an autoimmune etiology, although one has never been established[4,5]. In contrast, rare families with apparently autosomal dominant narcolepsy exist, and one third of these probands are DQB1*0602 negative[2]. These cases are more likely to have a different etiology, potentially involving single gene defects.

Discovered only 2 years ago, the hypocretin peptides (Hcrt-1 and Hcrt-2), also known as orexins, are neurotransmitters processed from a common precursor, preprohypocretin[6,7]. Two hypocretin receptors (Hcrtr1 and Hcrtr2) have been identified to date[7]. The exclusive localization of Hcrt cells in the tuberal region of the hypothalamus in rodents initially suggested a primary role in appetite regulation, which was substantiated by some functional studies[7]. However, the widespread distribution of Hcrt projection fields, with dense localization in monoaminergic cell groups, suggested other physiological functions[8].

Recent publications have implicated the hypocretin system in sleep regulation and narcolepsy[9–12]. Intracerebroventricular administration of Hcrt-1 induces wakefulness in rats[12]. Genetically induced Hcrt deficiencies produce narcolepsy in animals: canine narcolepsy results from mutations in *Hcrtr2* (ref. 9) and prepro-hypocretin knockout mice have abnormalities reminiscent of the disease[10]. The preliminary observation that human narcolepsy is associated with low cerebrospinal fluid (CSF) concentrations of Hcrt-1 (ref. 11) also suggests an absence of hypocretins in the central nervous system (CNS) of human patients. Here we investigated the role of the hypocretin system in the pathophysiology of human narcolepsy through mutation screening of the three hypocretin loci and histopathological experiments in human brains.

## *Hcrt*, *Hcrtr1* and *Hcrtr2* mutation screening

We identified the exon–intron boundaries of the *Hcrtr1* and *Hcrtr2* genes. Each receptor gene has seven coding exons with conserved positions of the splice junctions across species and receptor subtypes. The genomic sequence of the human *Hcrt* gene

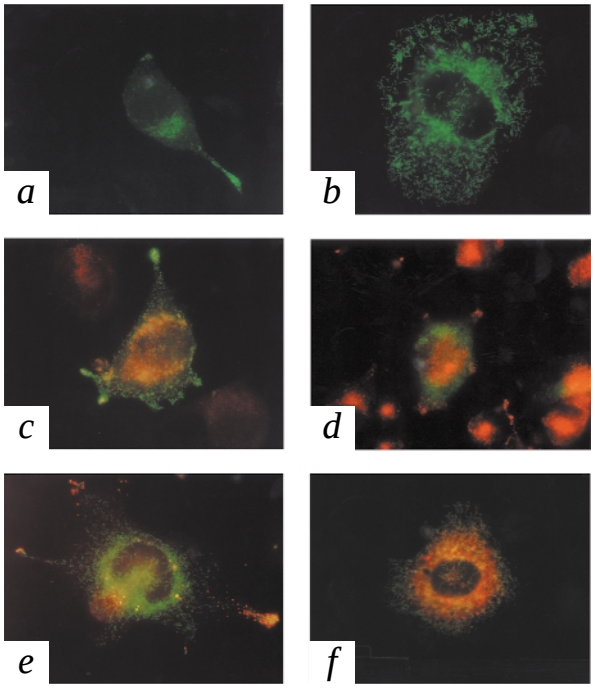


**Fig. 1** Control and mutant Hcrt trafficking in Neuro-2A cells**.** Wild-type protein fused to GFP is processed into mature secretory vesicles and is not affected by the presence of mutant-induced tubules. Mutant protein fused to GFP is retained in a tubular network overlapping with syntaxin-17, and is not processed into secretory vessicles. ***a,*** Typical phenotype of cell expressing wild type-GFP construct displaying Golgi and vesicular localization. ***b,*** Tubular network induced by expression of mutant-GFP construct. ***c,*** An orange-yellow color is the result of co-localized signals of wild-type GFP protein (green) and secretory vessicles (red). ***d,*** Lack of co-localization of mutant-GFP protein (green) with S V2-containing secretory vessicles (red). ***e,*** Differential trafficking of mutant-GFP protein (green) versus wild-type V5 protein (red). ***f,*** Overlapping localization of mutant-GFP protein (green) co-transfected with syntaxin-17-myc (red). All images are presented at the same magnification and relative size, neuro 2A cells are heterogeneous with significant variation in size and morphology.

has been previously reported[13]. We amplified and sequenced at least 50 bp flanking each exon to identify coding alterations and mutations affecting mRNA splicing. We included all patients with a family history or lacking HLA-DQB1*0602 from our database of more than 500 narcoleptic patients. Fourteen polymorphisms were found among 74 Caucasian patients and 118 ethnically matched controls (Table 1). Sequencing in control samples and family members indicated that 14 of these polymorphisms were not linked or associated with narcolepsy (Table 1). In addition, the 5 informative *Hcrtr1* and *Hcrtr2* polymorphisms indicated no significant statistical association with narcolepsy. We did not identify common polymorphisms of *Hcrt* in these subjects.

### An *Hcrt* mutation in early onset narcolepsy

One narcoleptic subject had a G->T transversion introducing a highly charged arginine into the poly-leucine hydrophobic core of the Hcrt signal peptide. This allele was not present in 212 control chromosomes, including the patient's unaffected mother. DNA from the unaffected father was unavailable to establish this allele as a *de novo* mutation.

This patient has severe cataplexy (5–20 attacks per day when untreated), daytime sleepiness, sleep paralysis and hypnagogic hallucinations. Notably, he first demonstrated cataplexy (expressed as head dropping when laughing) at age 6 months. Sudden episodes of imperative sleep (a few minutes to 1hour) were also noted at this early age. Sleep onset REM periods (SOREMPs) were first documented at 2.5 years of age. Multiple sleep latency testing per-

**Table 1** Allelic variance of the HCRT, HCRTR-1, and HCRTR-2 loci in narcoleptic and control caucasian subjects

| DNA change | Amino acid change | Domain | Narcolepsy F+ | F- | S+ | S- | Control | Notes |
|---|---|---|---|---|---|---|---|---|
| Preprohypocretin (Hcrt) | | | | | | | | |
| -20C A | non-coding | 5' UTR | 0.00 (17) | 0.00 (10) | 0.00 (26) | 0.053 (19) | 0.00 (24) | Presumed benign polymorphism |
| 47T G | Leu16Arg | signal peptide | 0.00 (17) | 0.00 (10) | 0.00 (26) | 0.026 (19) | 0.00 (106) | Dominant mutation |
| Hypocretin receptor 1 (Hcrtr1) | | | | | | | | |
| 111T C | synonymous | N-terminus | 0.32 (17) | 0.56 (8) | 0.35 (27) | 0.44 (16) | 0.36 (40) | Benign polymorphism |
| 793C A | Leu265Met | I 3 | 0.00 (18) | 0.00 (9) | 0.02 (27) | 0.00 (16) | 0.00 (45) | Presumed benign polymorphism |
| 842G A | Arg281His | I 3 | 0.00 (18) | 0.00 (9) | 0.00 (27) | 0.00 (16) | 0.01 (45) | Benign polymorphism |
| IVS6(+6C T) | non-coding | intron | 0.06 (18) | 0.06 (9) | 0.02 (27) | 0.00 (16) | 0.06 (40) | Benign polymorphism |
| 1222G A | Val408Ile | C-terminus | 0.28 (18) | 0.50 (9) | 0.33 (27) | 0.44 (16) | 0.34 (46) | Benign polymorphism |
| Hypocretin receptor 2 (Hcrtr2) | | | | | | | | |
| 28C T | Pro10Ser | N-terminus | 0.00 (17) | 0.00 (10) | 0.02 (25) | 0.00 (18) | 0.000 (90) | Presumed benign polymorphism |
| 31C A | Pro11Thr | N-terminus | 0.00 (17) | 0.10 (10) | 0.00 (25) | 0.00 (18) | 0.006 (90) | Unlinked with phenotype |
| IVS1(-25A C) | non-coding | intron | 0.15 (17) | 0.06 (9) | 0.17 (24) | 0.18 (17) | 0.16 (58) | Benign polymorphism |
| IVS2(+49C T) | non-coding | intron | 0.29 (17) | 0.35 (10) | 0.18 (25) | 0.26 (17) | 0.17 (61) | Benign polymorphism |
| 577T A | Cys193Ser | TM IV | 0.00 (16) | 0.00 (10) | 0.00 (25) | 0.00 (17) | 0.01 (39) | Benign polymorphism |
| 922G A | Val308Ile | TM VI | 0.12 (17) | 0.05 (10) | 0.18 (25) | 0.24 (17) | 0.19 (35) | Benign polymorphism |
| 942A G | synonymous | TM VI | 0.06 (17) | 0.00 (10) | 0.00 (25) | 0.00 (17) | 0.01 (35) | Benign polymorphism |
| 1202C T | Thr401Ile | C-terminus | 0.03 (17) | 0.00 (10) | 0.00 (25) | 0.00 (16) | 0.00 (99) | Possible weakly penetrant allele in combination with DQB1*0602 |

Data reported are allele frequencies. The number of subjects studied in each group for each polymorphism is indicated in parentheses. F+, familial, DQB1*0602 positive F- , familial, DQB1*0602 negative S+, sporadic, DQB1*0602 positive. S– , sporadic, DQB1*0602 negative. DNA and amino acid changes are counted from the ATG-codon and Met-residue respectively. Cosegregation in multiplex families was tested whenever possible (see Notes). IVS, intervening sequence (intron), position relative to adjacent exon. 5′ UTR, 5′ untranslated region; TM, transmembrane domain; I, intracellular loop.

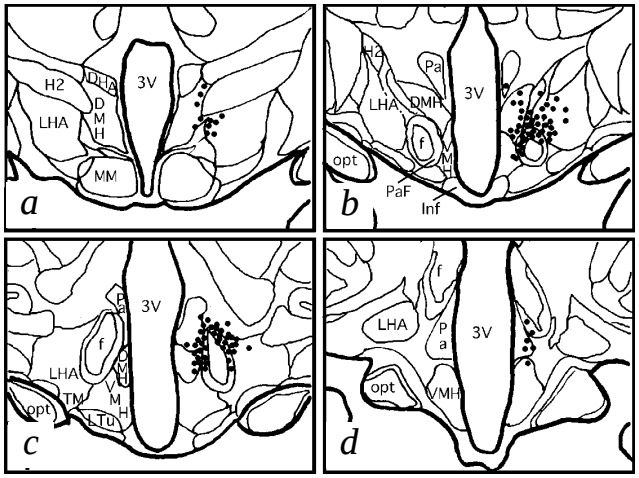


**Fig. 2** Distribution of hypocretin-containing cells in the human hypothalamus. The *preprohypocretin* mRNA expressing neurons are localized discretely in the perifornical area. Their distribution is illustrated on schematic diagrams of representative coronal planes through the human hypothalamus. Each black circle represents 3–5 cells detected in emulsion-coated sections. DHA, dorsal hypothalamic area; DMH, dorsal hypothalamic nucleus; f, fornix; H2, lenticular fasciculus; Inf, infundibular nucleus; LHA, lateral hypothalamic area; MM, mammillary nucleus; opt, optic tract; Pa, paraventricular hypothalamic nucleus; PaF, parafornical nucleus; TM, tuberomammillary nucleus; VMH, ventromedial hypothalamic nucleus.

formed over a 16-year follow up period consistently showed extremely short sleep latencies and multiple SOREMPs. Examples of cataplectic attacks in this patient triggered by laughter may be viewed at http://www.med.stanford.edu/school/psychiatry/narcolepsy/. His symptoms are partially controlled with methylphenidate and either imipramine, chlomipramine or fluoxetine. Additional clinical features include severe periodic leg movements and episodic nocturnal bulimia focused on sweets from the age of 5. HLA typing indicates DRB1*0402, DRB1*0701; DQB1*0202, DQB1*0302. Hcrt-1 concentrations are undetectable in lumbar CSF (concentrations <40 pg/ml versus 263 ± 15.3 pg/ml in three unrelated controls from the same geographical area). Magnetic resonance imaging focused on the hypothalamic region was unremarkable.

### Impaired processing and trafficking of mutant Hcrt

We examined the cellular phenotypes of the wild type and mutant *Hcrt* alleles fused to either green fluorescent protein (GFP) or the V5 epitope tag transiently transfected into Neuro-2A neuroblastoma cells (Fig. 1*a* and *b*). The wild-type protein passed through the secretory pathway from the Golgi apparatus through the *trans*-Golgi network (TGN) and finally into mature secretory vesicles, as seen by immunostaining with antibodies to these structures (Fig. 1*c*). The mutant phenotype was markedly different, with diminished localization to the Golgi, TGN and scant amounts in mature secretory granules (Fig. 1*d*). The mutant peptide accumulated in a membrane system with a characteristic branching tubular appearance (Fig. 1*b*). Following transfection, the bolus of expressed protein followed a visible time course with respect to the cellular localization. At 12 h post-transfection, 77% of cells expressing the wild-type construct showed Golgi localization versus 54% for the mutant ($\chi^2 = 11$, $P < 0.001$). At this time, 5% of cells expressing wild type had localization in branching tubules, versus 54% of cells with the mutant construct ($\chi^2 = 54.1$, $P < 10^{-9}$). At 72 hours post-transfection, 35% of wild type versus 10% for mutant showed Golgi localization ($\chi^2 = 7.1$, $P < 0.01$). Although 8% of wild-type expressing cells contained branching tubules, these were seen in 85% of cells expressing the mutant construct ($\chi^2 = 57.8$, $P < 10^{-9}$).

Immunostaining with anti-calnexin antibodies indicated that the tubular network was not rough endoplasmic reticulum (ER) (data not shown). Instead, the appearance of the network was reminiscent to that of syntaxin 17, a protein thought to localize to the smooth ER (SER) (ref. 14 and R. Scheller, personal communication). When the mutant allele was co-transfected with a syntaxin 17 construct, the two proteins showed partial co-localization, indicating that the structures containing the mutant peptide probably consist of SER (Fig. 1*f*). When wild type and mutant *Hcrt* constructs were co-expressed, the presence of tubular networks did not apparently affect the trafficking of the wild type (Fig. 1*e*).

The V5 tagged wild type and mutant constructs were also used in a cell free transcription/translation assay to investigate processing by signal peptidase (data not shown). In the presence of canine pancreatic microsomal membranes, signal peptide cleavage was observed (≥10%) for the wild type whereas the mutant form was processed at markedly decreased concentrations (<1%). These results indicate abnormal trafficking and processing of the mutant peptide but do not prove nor refute dominance for the mutant allele.

### Localization of Hcrt neurons in the hypothalamus

We mapped Hcrt-containing neurons in 13 control subjects using *in situ* hybridization. As illustrated in Fig. 2*a–d*, most Hcrt-hybridizing cells were detected in the tuberal region of the hypothalamus. The radiolabeled cells were localized throughout the dorsal part of the parafornical nucleus, the adjacent areas of the dorsomedial (DMH) and the ventromedial (VMH) hypothalamic nuclei, and the tuberal lateral hypothalamic area (Fig. 2*b* and *c*). A few radiolabeled cells were also present in the mammillary (lateral part of the DMH) and anterior (lateral part of the paraventricular nucleus) hypothalamic areas (Fig. 2*a* and *d*). Cell distribution and signal intensity were independent of age (32–94 years), sex, HLA-DQB1*0602 status and post mortem delay (6–26 hours). The same distribution was obtained with two independent oligoprobes complementary to the *prepro-hypocretin* sequence. Sense oligoprobes or RNase pretreatment of adjacent sections produced no signal (data not shown). Emulsion-dipped sections disclosed that silver grains are mainly distributed over medium-sized cells of oval shape with maximum apparent diameter of 15–20 μm (Fig 3*b*, insert). The population of Hcrt-expressing cells was estimated at 15,000–20,000 from a series of representative sections across the entire hypothalamus of two control subjects.

### Absence of *Hcrt* mRNA in the hypothalami of patients

*In situ* hybridization studies were conducted for melanin concentrating hormone (MCH), a peptide also expressed in the perifornical area of the human hypothalamus[15]. *MCH* mRNA expressing cells were more widely distributed than Hcrt positive cells, as previously reported[8,15,16]. Although partial overlap between MCH- and Hcrt-expressing cells was suggested, especially dorsal and dorsolateral to the fornix, the respective patterns of radiolabeling were generally distinct. We next performed Hcrt and MCH *in situ* hybridizations on adjacent

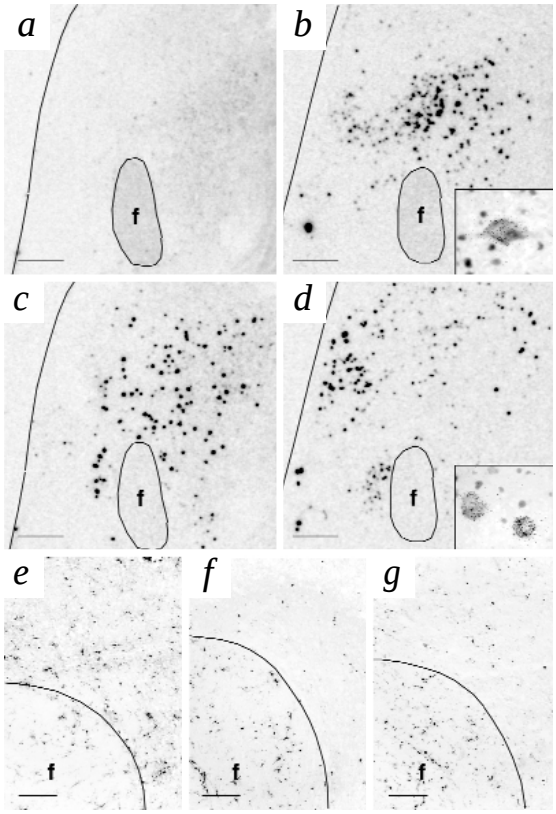


**Fig. 3** Hypocretin, MCH and HLA expression studies in the hypothalamus of control and narcoleptic subjects. *Preprohypocretin* transcripts are detected in the hypothalamus of control (***b***) but not narcoleptic (***a***) subjects. *MCH* transcripts are detected in the same region in both control (***d***) and narcoleptic (***c***) sections. Immunohistochemical staining of HLA-DR discloses normally distributed resting microglia in both white and gray matters of control and narcoleptic subjects (***e,f,*** 2 narcoleptic subjects; ***g,*** 1 control subject). *f*, fornix. Scale bar represents 10 mm (*a–d*) and 200 μm (*e–g*).

sections in control and narcoleptic tissues. Sections from four controls and two narcoleptic subjects were processed in parallel. We found no signal for Hcrt in the hypothalamus of human narcoleptic subjects (Fig. 3*a*). In contrast, MCH neurons were observed on adjacent sections (Fig. 3*c*). In control tissues, both peptides were highly expressed (Fig. 3*b* and *d*). MCH expression was similar in control and narcoleptic brains. Of note, both narcoleptic patients and 3 of 13 controls were HLA-DQB1*0602 positive and one narcoleptic subject had a family history for narcolepsy–cataplexy.

### Absence of Hcrt peptides in the CNS of patients

We measured the concentrations of Hcrt-1 and Hcrt-2 peptides in brain tissue from eight control and six narcoleptic subjects using radioimmunoassays (Fig. 4). Two of the narcoleptic subjects and four of the controls were also used in the *in situ* hybridization study. Peptide concentrations were measured in cortex (14 subjects) and available pons samples (4 subjects); these structures are known to receive hypocretin projections. Hcrt-1 and Hcrt-2 were detectable in all control samples, independent of their DQB1*0602 status. As previously described in rat brain[17,18], hypocretin concentrations in controls were 10–20-fold higher in the pons (Hcrt-1: 21,516 ± 2001 pg/g, and Hcrt-2: 13,340 ± 1231 pg/g; $n = 2$) than in the cortex (Hcrt-1: 939 ± 239 pg/g; Hcrt-2: 1561 ± 323 pg/g; $n = 8$). However, in the pons of two narcoleptic subjects, one of which was also tested using *in situ* hybridization, Hcrt-1 and Hcrt-2 were in the undetectable range (<332 pg/g). Both peptides were also undetectable in cortex samples, with the exception of one subject with low cortical concentrations (Hcrt-1: 347 pg/g and Hcrt-2: 485 pg/g) and undetectable concentrations in the pons. These results show that Hcrt-1 and Hcrt-2 are absent in narcoleptic patients.

### Lack of overt immunopathology in the perifornical area

The absence of hypocretin signal, together with the established HLA association in narcolepsy, suggests the possibility of an autoimmune destruction of Hcrt-containing cells in the hypothalamus. Thionin, crystal violet and glial fibrillary acidic protein (GFAP) staining of brain sections from two narcoleptic subjects disclosed no obvious lesions or gliosis in the perifornical area (data not shown). Increased HLA Class II (HLA-DR) expression and microglial activation is a sensitive indicator of pathological events in the CNS (ref. 19). HLA-DR immunocytochemistry was performed in tissue from two narcoleptics and four controls. The sections taken were adjacent to those used for *Hcrt* and *MCH in situ* hybridization experiments. Resting HLA-DR positive microglia were detected in the white and gray matter of control (Fig. 3*g*) and narcoleptic (Fig. 3*e*,*f*) subjects. Staining in the perifornical area was moderate and none of the cases were associated with activated, amoeboid microglia. Microglial HLA labeling was higher in the white matter (fornix) than the gray matter (perifornical area), but did not differ between control and disease status (Fig. 3*e–g*). *In situ* hybridization with tumor necrosis factor (TNF)-alpha, a cytokine strongly expressed in many inflammatory CNS disorders, including multiple sclerosis and experimental autoimmune encephalomyelitis[20], also produced no significant signal in control and narcoleptic tissue (data not shown). These results exclude the possibility of persistent inflammation or extensive neuronal loss in the region.

### Discussion

Our results point to a central role for hypocretin neurotransmission in the etiology of human narcolepsy based on two parallel approaches: mutation screening of three hypocretin loci and neuropathological analysis. Only one severe, early onset case of narcolepsy was associated with a predicted dominant mutation in the *Hcrt* locus. This case displays all the classical features of the narcolepsy syndrome as well as associated periodic leg movements during sleep, a commonly reported co-morbidity in narcolepsy[21,22]. The early onset and severe clinical picture of this patient is reminiscent of genetically induced narcolepsy in animals. Most cases of human narcolepsy appear during adolescence (age 15–25 years)[23,24]; this is the only known case starting before 2 years of age[24,25]. Narcolepsy in *Hcrtr2* mutated canines and *Hcrt* knockout mice typically begins early and before sexual maturity[10,26].

Dominant effects of signal peptide mutations have been frequently reported[27,28]. An analogous Leu->Arg substitution in the signal peptide of the parathyroid hormone gene causes dominant hypoparathyroidism[27]. The mutant polypeptide has impaired translocation into the ER and is poorly cleaved by signal peptidase[27], as observed in our hypocretin mutant. In autosomal dominant neurohypophyseal diabetes insipidus, mutations impairing signal peptide cleavage result in the accumulation of mutant polypeptides in the rough ER (ref. 29). These accumulations cause loss of viability in stably transfected, differentiated

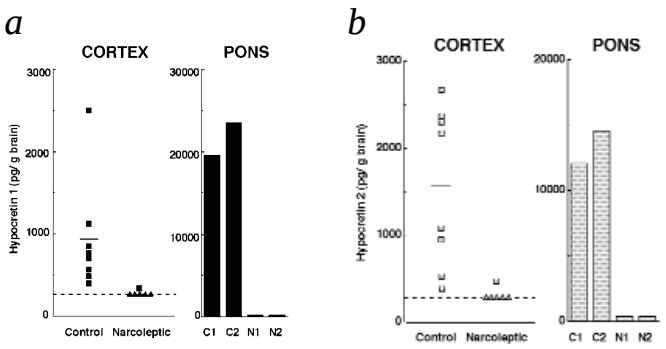


**Fig. 4** Hypocretin concentrations in the cortex and pons in narcoleptic and control subjects. Cortical Hcrt-1 (***a***) and Hcrt-2 (***b***) concentrations were detectable in all controls (—, mean) and undetectable (----, detection limit, 332 pg/g) in 5 of 6 narcoleptic subjects ($P < 0.002$ and $P < 0.003$ for Hcrt-1 and Hcrt-2, narcolepsy versus control). The difference in hypocretin concentrations (2 narcoleptic versus 2 controls) was more pronounced in the pons where high concentrations are observed in control subjects. Note that the narcoleptic subject with low but detectable cortical Hcrt-1 and Hcrt-2 concentrations was also tested in the pons (N2) and found to have undetectable concentrations.

neuro-2A cells, but do not affect the viability of transiently transfected cells[28]. Neurodegeneration has also been documented *in vivo* by magnetic resonance imaging studies of the posterior pituitary[30]. Our mutant allele has a similar, but not identical, phenotype with preferential localization to the smooth ER (SER). The mutant peptide is poorly cleaved and accumulates in the SER. The SER is not extensive in neurons; rather, it is enriched in cells that synthesize or modify large amounts of steroids. We propose that the accumulation of the mutant peptide within the SER exerts a dominant effect through degeneration of Hcrt neurons *in vivo*, although it remains possible that the cells are intact, but do not secrete hypocretin. This would be consistent with the undetectable CSF concentrations of Hcrt-1 in this patient. The small number of *Hcrt*-expressing neurons may confer particular vulnerability.

The low frequency of mutations among our 74 narcoleptics was unexpected considering the emphasis on HLA-DQB1*0602 negative and familial cases. For example, there was no mutation detected in a three generation HLA-DQB1*0602 negative family, with normal Hcrt-1 CSF concentrations in two affected members tested. The normal Hcrt-1 concentrations suggested a receptor defect, as in *Hcrtr2* mutant narcoleptic canines, which also have normal Hcrt-1 concentrations. By contrast, other subjects with familial HLA-DQB1*0602 positive narcolepsy had undetectable Hcrt-1 CSF concentrations but no *Hcrt* mutations. All these familial cases had delayed, peripubertal onset, in contrast to our patient with the *Hcrt* signal peptide mutation. These findings indicate etiologic and genetic heterogeneity in familial narcolepsy–cataplexy. As most human narcolepsy cases are sporadic and HLA associated[2], frequent hypocretin mutations were not expected. Indeed, the sporadic cases, with or without HLA-DQB1*0602, were not associated with common informative *Hcrtr1* and *Hcrtr2* polymorphisms. Further studies will be needed to explore the effects of these polymorphisms on disease expressivity, onset and drug response. We conclude that these loci are not typically involved in predisposition to human narcolepsy.

We next investigated hypocretin system function in HLA-DQB1*0602 positive patients through neuropathological experiments. *In situ* hybridization studies demonstrated an absence of *Hcrt* transcripts in the hypothalamus of all patients tested. These results indicate either a lack of transcription in intact cells or a previous destruction of Hcrt-containing neurons. Radio-immunoassays of Hcrt-1 and Hcrt-2 indicate an absence of both peptides in two projection areas, extending our finding that Hcrt-1 concentrations are undetectable in the CSF of most narcoleptic patients[11]. This result was especially striking in the pons where hypocretin projections are dense[8,17,18], indicating a total loss of neurotransmission. The lack of excitatory Hcrt projections to monoaminergic cell groups containing Hcrtr2 receptors might be involved in generating the narcolepsy phenotype[31]. The dopaminergic ventral tegmental area and histaminergic tuberomammillary nucleus, two wake-promoting systems, might be critical in this model[9,10,31,32].

An autoimmune mediation for human narcolepsy has been suspected since 1984, when the disorder was first shown to be associated with HLA-DR2 (ref. 33). Further studies extended the association across ethnic groups, using HLA-DQ (ref. 34), but no evidence for immunopathology has been found[4,5]. The absence of Hcrt signal in human narcolepsy is consistent with the hypothesis that Hcrt neurons may be the target of an autoimmune process leading to cell destruction. *In situ* hybridization for TNF-alpha and immunocytochemistry for HLA-DR disclosed no sign of recent inflammation in the two brains examined. This might be explained by the fact that the two subjects were examined long after the Hcrt cells were putatively destroyed (more than 50 years after disease onset). More surprisingly, however, we also did not detect significant residual gliosis and/or cellular loss in the region. The disease process did not affect MCH positive neurons. This result is remarkable, considering that MCH and Hcrt-positive cells are intermingled in the region of interest. Hcrt-containing neurons are few in number (15,000–20,000 neurons), and dispersed within a limited area of the tuberal hypothalamus. This might be an explanation for the absence of an overt lesion in our histopathological studies.

Narcolepsy is now treated symptomatically using monoaminergic amphetamine-like and antidepressant compounds[35]. These compounds have many untoward side effects and only partially control the condition. The finding that hypocretins are absent in most cases, together with the report of a rare *Hcrt* mutation in a young child with severe symptoms, strongly supports a central role for hypocretin deficiency in the human disorder. A more suitable and effective treatment strategy may therefore be to supplement hypocretin transmission, for example, using hypocretin receptor agonists. Our findings suggest a loss of neurons that may be irreversible once the disease is fully established. Understanding the mechanism leading to this process will be needed before preventative or truly curative strategies can be developed.

## Methods

**Subjects**. All narcoleptic subjects had unambiguous cataplexy, and eighty percent had undergone polysomnography as well as Multiple Sleep Latency testing, showing abnormalities diagnostic of narcolepsy. For the mutation screening, we studied 19 DQB1*0602 negative and 28 DQB1*0602 positive sporadic patients, and 10 DQB1*0602 negative and 17 DQB1*0602 positive familial probands. These included two early onset DQB1*0602 negative patients (isolated cataplexy, onset 3 months; narcolepsy/cataplexy, onset 6 months) and two previously described patients from Leiden, with de-

tectable and high CSF Hcrt-1 levels[11]. Complete sequences for all exons of the three genes were obtained for 14 DQB1*0602 negative and 22 DQB1*0602 positive sporadic cases and 8 DQB1*0602 negative and 16 DQB1*0602 positive familial probands. Fifteen Caucasian controls were sequenced completely for each gene. Allelic frequencies were studied among 118 controls. For the neuropathological studies, eighteen controls (13 males, 5 females) and 6 narcoleptic subjects (4 males, 2 females) were included. All but one African-American control subject were Caucasians. No signs of cerebral infarction or neurodegenerative disorders were observed. One subject had a family history of narcolepsy-cataplexy in first-degree relatives. Three controls (out of 9 typed) and all narcoleptic subjects were HLA-DQB1*0602 positive. Age was 71.94±4.41 (23 to 94 years) in controls and 63.8±8.4 years (23 to 77 years) in narcoleptics.

**Exon/Intron Boundaries.** To determine exon-intron boundaries of the *Hcrtr1* gene, lambda clones were isolated and subcloned from a human genomic phage library (Clontech, Palo Alto, California) using a human *Hcrtr1* cDNA probe. *Hcrtr2*-containing BAC clones 106-C-7, 575-E-23 and 575-M-3 were identified through PCR screening of BAC superpools (Research Genetics, Huntsville, Alabama). Exon-intron boundaries and flanking sequence of the *Hcrtr2* locus were determined by directly sequencing BAC DNA as previously described[9]. Exon sequences have been submitted to Genbank (accession numbers AF202078-AF202084 (*Hcrtr1*), and AF202085-AF202091 (*Hcrtr2*)).

**PCR primers, sequencing and analysis.** For Hcrtr1/2 primers and amplification conditions, refer to the Genbank entries. Hcrt primers were as follows: PPex-1-F5:5′-ATCTTAGACTTGCCTTTGTCTGGC-3′, PPex-1-R5: 5′-CAAACACAGGCTCTTAGCAAAACA-3′, PPex-1-F1: 5′-ATCTCCAGGGAGCAGATAGACAGA-3′, PPex-1-R1 5′-GCATTCTGGAGTCTTGCTAGTTCCTG-3′, PPex-2-F4: 5′-AGTGACAGCCAGAAAGGAGTGCAG-3′, PPex-2-R4: 5′-ACCAGTCACACGAATGGAGACTCG-3′, PPex-2-F2: 5′-AGCAAGGAGAACTAAGTGACAGCC-3′, PPex-2-R2: 5′-GGTTGAATCGGAAACACTCTTGGTT-3′. Sequence reactions were performed using standard procedures, and run on an ABI 377 (PE Biosystems). Sequence alignments and trace comparisons were made using Sequencher 3.1 (Gene Codes, Ann Arbor, Michigan).

**Constructs.** An artificial cDNA construct was made by amplifying *Hcrt* exons 1 and 2 and fusing them with a chimeric primer before cloning into pCR2.1topo (Invitrogen, Carlsbad, California). Resulting wild type (p25) and mutant (p4) artificial cDNA constructs contained no additional mutations. These two constructs were used to create the GFP and V5 fusions by cloning into pCDNA3.1topo-CTGFP (Invitrogen), or by addition of the V5 epitope tag using patch-PCR. Syn17-Myc DNA was a kind gift of R. Scheller (Stanford University).

**Cell culture, transfections and immunofluorescence.** Neuro-2A cells (ATCC-CCL131) were maintained using standard techniques. The day before transfection, cells were seeded onto sterile coverslips. Cells were transfected using Effectine (Qiagen), and mixture was removed after 1 h. Cells were harvested at 12, 24, 48, 72, or 96 hour timepoints, and fixed in 4% paraformaldehyde. Standard techniques were used for immunostaining. Primary antibodies included Calnexin (Rough ER), P115(Golgi): VAMP4 (TGN), Syn17(SER), S V2a (Synaptic vessicles, secretory vessicles) (all gifts of R. Scheller), and V5 (Invitrogen). Secondary antibodies included anti rabbit Texas red, anti mouse Texas red (Vector Laboratories, Burlingame, California) anti goat Cy3 (Jackson Immunoresearch, Westgrove, Pennsylvania). Coverslips were mounted in Vectashield (Vector Laboratories). Immunofluorescence images were captured on film. The investigator was blinded to transfected constructs during quantification of mutant versus wild type phenotypes and 48-96 cells were analyzed for each timepoint.

***In vitro* translation and peptide processing studies.** Preprohypocretin plasmid constructs were transcribed and translated in rabbit reticulocyte lysates including [$^3$H]-Leucine and with or without the addition of canine pancreatic microsomal membranes (Promega, Madison, Wisconsin). Products were analyzed on SDS-PAGE gels with Glycine or tricine buffer. Signals were enhanced using Amplify (Amersham Pharmacia Biotech, Piscataway, New Jersey).

**Preparation of brain tissue.** Post mortem delays were 13.46 ± 1.88 h (5 to 26 h) in controls and 24.6 ± 15.2 h (4.5 to 98 h) in patients. Coronal slices of brains (1 cm thick) including the entire hypothalamus region, the pons (locus coeruleus area) and the frontal cortex were immediately frozen on dry ice and stored at –80 ºC. Neuroanatomical experiments were conducted in 13 control subjects. Only 2 narcoleptic samples contained the hypothalamus and used for *in situ* hybridization and immunocytochemical studies. These 2 subjects were a 77 year old female and a 67 year old male (postmortem delays 6.75 and 17 h, respectively). Cryostat sections (15 µm thick) were made throughout the hypothalamus (from the mammillary bodies to the optic chiasm region). Radioimmunoassays were performed in 9 control and 6 narcoleptic brains. Samples (1 g) were taken from the frontal cortex (8 control and 6 narcoleptic subjects) and the pons (2 control and 2 narcoleptic subjects). Diced tissue samples were boiled for 10 min in 10 ml of distilled water, cooled to room temperature, acidified using glacial acetic acid and HCl (final concentration: 1 M and 20 mM respectively), homogenized and cleared in three rounds of centrifugation. The supernatant of the third spin was acidified again with an equal volume of 0.1% trifluoroacetic acid (TFA) and purified on a C-18 Sep-Column (Phoenix Pharmaceuticals, Belmont, California). Samples were eluted with 3 ml of 0.1% TFA containing 60% acetonitrile, and dried.

***In situ* hybridization of Hypocretin, MCH and TNF-alpha.** The following antisense oligonucleotide probes were used: S1HCRTHUM (bases 198-238) and S2HCRTHUM (bases 365-407) (GeneBank, NM001524) of human *prepro-hypocretin*, and S1MCHHUM (bases 501-541) of human *pro-MCH* (GeneBank, NM002674). Oligonucleotides for human *TNF-alpha* were obtained from Oncogene Research Products (Boston, Massachusetts). Oligoprobes were 3'end labeled with [$^{35}$S]-dATP to a specific activity of at least $1 \times 10^8$ cpm/µg and column purified. Corresponding sense oligoprobes were used as controls. Coronal sections were fixed in 4% paraformaldehyde for 10 min. Slides were rinsed 5 minutes in 2xSSC, immersed 10 min in 0.1M triethanolamine (pH 8) containing 0.25% of acetic anhydride, rinsed again in 2xSSC, dehydrated in ascendant concentrations of ethanol, delipidated 10 min in chloroform, dipped in ethanol 100% and 95%, and airdried. *In situ* hybridization was conducted as previously described[36]. Signal was detected using hyperfilm for 8-10 days at 4 °C. Sense oligoprobe and RNase pretreatment controls were conducted using adjacent sections. Cell mapping was performed using a digital camera and Adobe Photoshop software. The hypothalamic subdivisions were identified and named according to Mai *et al*. (ref 37). The total number of *Hcrt* mRNA expressing cells was estimated using a series of emulsion-coated sections taken every 100 µm along the entire hypothalamus of 2 subjects. Cell counts of radiolabeled cells were made using a computerized image analysis system (SAMBA, Alcatel, France).

**Immunohistochemistry of HLA and GFAP.** HLA and GFAP immunostaining was performed on adjacent sections in the perifornical area. Frozen sections were dried and fixed with 4% paraformaldehyde, rinsed twice in 0.1 × M PBS and blocked with BSA. Sections were incubated sequentially with mouse anti-human DR-alpha (clone TAL.1B5, Dako Corp., Carpinteria, California) or monoclonal mouse anti-GFAP (Chemicon, Temeluca, California), biotinylated horse anti-mouse IgG (Vector Laboratories), and exposed to avidin–biotin–HRP complexes (Vector Elite Kit, Vectastain). Sections were rinsed twice in PBS after each incubation. Immunoperoxidase was localized by immersion in 0.025% DAB and 0.003% $H_2O_2$ in 0.05 M Tris-HCl buffer (pH 7.6). By including 6% ammonium nickel (II) sulfate hexahydrate (Nacalai Tesque, Kyoto, Japan) microglia (HLA) and astrocytes (GFAP) were stained in black. Sections were blindly scored by 3 investigators as previously described[38].

**Radioimmunoassays of hypocretins in human CSF, cortex and pons.** CSF levels of Hcrt-1 were determined as previously described[11]. Evaporated brain tissue samples were re-suspended in 500 µl of RIA buffer. Hcrt-1 and Hcrt-2 were measured using commercially available RIA kits (Phoenix Pharmaceuticals). Recovery efficiency during extraction was determined

using an internal standard ($^3$H Hcrt-2, American Peptide, approx. 50,000 dpm [68 pmol]) and was found to be 58.3 ± 2.5%. All reported values (pg/g of wet brain tissue) were adjusted to reflect the estimated original values before extraction. All measurements were conducted in a single RIA and in duplicate using 10-100 µl of sample. The intra-assay variability was 3.8% and the detection limit was 332 pg/g.

**Statistical analysis.** All values are reported as means ± SEM when applicable. Comparisons were made using the Mann-Whitney U-test or $\chi^2$ when appropriate.

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