Hypocretin Receptor Expression in Canine and Murine Narcolepsy Models and in Hypocretin-Ligand Deficient Human Narcolepsy

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Study Objective: To determine whether hypocretin receptor gene (*hcrtR1* and *hcrtR2*) expression is affected after long-term hypocretin ligand loss in humans and animal models of narcolepsy.

Design: Animal and human study. We measured *hcrtR1* and *hcrtR2* expression in the frontal cortex and pons using the RT-PCR method in murine models (8-week-old and 27-week-old orexin/ataxin-3 transgenic (TG) hypocretin cell ablated mice and wild-type mice from the same litter, 10 mice for each group), in canine models (8 genetically narcoleptic Dobermans with null mutations in the *hcrtR2*, 9 control Dobermans, 3 sporadic ligand-deficient narcoleptics, and 4 small breed controls), and in humans (5 narcolepsy-cataplexy patients with hypocretin deficiency (average age 77.0 years) and 5 control subjects (72.6 years).

Measurement and Results: 27-week-old (but not 8-week-old) TG mice showed significant decreases in *hcrtR1* expression, suggesting the influence of the long-term ligand loss on the receptor expression. Both sporadic narcoleptic dogs and human narcolepsy-cataplexy subjects showed a significant decrease in *hcrtR1* expression, while declines in *hcrtR2* expression were not significant in these cases. *HcrtR2*-mutated narcoleptic Dobermans (with normal ligand production) showed no alteration in *hcrtR1* expression.

Conclusions: Moderate declines in *hcrtR* expressions, possibly due to long-term postnatal loss of ligand production, were observed in hypocretin-ligand deficient narcoleptic subjects. These declines are not likely to be progressive and complete. The relative preservation of *hcrtR2* expression also suggests that hypocretin based therapies are likely to be a viable therapeutic options in human narcolepsy-cataplexy.

Keywords: Narcolepsy, cataplexy, hypocretin/orexin, mice, dog, human, hypocretin/orexin receptors, RT-PCR

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NARCOLEPSY IS A DISABLING SLEEP DISORDER CHARACTERIZED BY EXCESSIVE DAYTIME SLEEPINESS (EDS), SLEEP PARALYSIS, HYPNAGOGIC HALLUCINATIONS, and cataplexy.[1,2] Narcolepsy is currently treated with amphetamine-like stimulants (or modafinil for EDS) and antidepressants for cataplexy and REM sleep related symptoms.[1] These treatments are purely symptom-oriented and are often problematic due to their limited effectiveness and undesirable side effects.[1]

Recent studies in animals and humans revealed that a deficit in hypocretin (hcrt, also known as orexin) transmission is the major pathophysiology. Doberman pinschers with autosomal recessive narcolepsy possess null mutations in the gene encoding hcrt receptor2 (*hcrtR2*),[3] one of two hypocretin receptors. Sporadic cases of canine narcolepsy show undetectable levels of the brain and cerebrospinal fluid (CSF) hcrt neuropeptide.[4] Preprohypocretin gene knockout mice as well as orexin/ataxin-3-transgenic mice (TG mice) with postnatal death of hcrt-producing neurons by hcrt neuron-specific expression of a truncated Machado-Joseph disease gene product (ataxin-3), also exhibit the narcolepsy phenotype.[5,6] In humans, genetic mutation in hcrt-related genes is rare, but a large majority of narcolepsy-cataplexy is found to be associated with the loss of hcrt production in the brain and CSF, possibly due to the postnatal cell death of hypocretin neurons.[7-11]

Since most human narcolepsy-cataplexy is caused by the loss of the hcrt peptide, hcrt replacement is a promising future therapeutic option. In this regard, the result that central administration of hctr-1 rescues the sleep abnormalities and cataplexy in orexin/ataxin-3-TG mice is very encouraging.[12]

The potential efficacy of hypocretin replacement therapies in human narcolepsy will however, require the integrity of hypocretin receptors. Functional hcrtRs may disappear gradually following the loss of hypocretin in narcolepsy. In previous studies, the effect of hcrt replacement was only examined in 14- to 15-week-old hypocretin cell deficient narcoleptic mice; whether longer-term changes in the responsiveness to hypocretin occur is unknown. Additionally, the etiological mechanisms of hypocretin cell death in human narcolepsy is currently unknown, and consequent hcrtR changes may well be different from those found in orexin/ataxin-3-TG mice. Thannickal et al. suggested that the loss of hypocretin function in human narcolepsy results from a cytotoxic or immunologically mediated attack focused on hcrtR2.[13] If this is the case, then a loss of function of hcrtR2 might also exist in human narcolepsy, and patients may not respond to hcrt replacement, as previously shown in *hcrtR2*-mutated narcoleptic dogs.[14]

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In the present study, we evaluated the influences of long-term hcrt deficiency on *hcrtR1* and *hcrtR2* expression in brain samples from canine and murine models of narcolepsy, as well as from a limited number of human narcolepsy-cataplexy patients. The evaluation in humans is critical, since the results may predict responsiveness to the hcrt replacement therapy and the potential usefulness of cell transplantation or gene-based therapies.

MATERIALS AND METHODS

Experimental Subjects

Narcoleptic Dogs

Eight genetically narcoleptic Dobermans with null mutations in the *hcrtR2* (male/female = 4/4, age 14.2 ± 1.9 months, range 5.2–56.9 months), 9 control Dobermans (m/f = 5/4, 22.4 ± 5.3 months, range 4.4–44.9 months), 3 sporadic narcoleptics (one male dachshund, 118 months; one male poodle, 46 months; and one male cocker/poodle mix, 200 months), and 4 small breed controls (one male beagle, 3 months; one male foxhound, 3 months; and two male mixed breed, 50 months and 100 months) were used in this study. All narcoleptic Dobermans exhibited cataplexy before 6 months, and the ages of onset of cataplexy of sporadic dogs were between 1 and 3 years of age. The small breed control dogs were purchased for neurochemical studies. The dogs were housed in individual cages (1.0 × 1.8 m) at the Stanford Department of Comparative Medicine Research Animal Facility, where temperature (22–24°C), humidity (40%), and 12 h light:dark cycle (ZT = 07:00) were maintained at constant levels.

The animals used were given a lethal dose of pentobarbital, and the brains were removed immediately postmortem, frozen by immersion in liquid 2-methylisobutane (−40°C), and stored at −80°C. Afterward, cortical cube tissues 1 cm on a side from the medial surface of the superior frontal gyrus were dissected and stored at −80°C until RNA extraction. All Dobermans were sacrificed between 07:00 and 09:00, while small breed dogs were sacrificed between 13:00 and 15:00.

Orexin/Ataxin-3-Transgenic Mice

The orexin/ataxin-3 TG mice (N3, 87.5% C57BL6 and 6.3% to 12.5% DBA1) were obtained from University of Tsukuba, Ibaraki, Japan, and breeding was initiated at the Stanford Center for Narcolepsy. Before being used in the experiments, the TG mice were backcrossed to C57BL/6J wild-type (Wt) mice (The Jackson Laboratory, Bar Harbor, Maine, USA), and the N7 offspring were genotyped. Wt mice from the same litter were used as controls. Finally, a total of 40 mice (10 mice for each of 4 groups) were used in this study: 8-week-old TG mice (m/f = 1/9, age 56.1 ± 2.1 days, range 53–58 days), 27-week-old TG mice (m/f = 4/6, age 193.1 ± 23.0 days, range 165–233 days), 8-week-old Wt mice (m/f = 1/9, age 54.9 ± 2.3 days, range 53–58 days), and 27-week-old Wt mice (m/f = 4/6, age 185.6 ± 15.3 days, range 165–206 days). All mice were provided with food and water *ad libitum* and housed under conditions where temperature, humidity, and 12 hr light:dark cycle (Zeitgaber time (ZT) 0 = 07:00) were maintained at constant levels.

All mice were sacrificed between 09:00 and 11:00 (light phase) by cervical dislocation followed immediately by decapitation. The brains were removed and dissected into 8 brain regions; cerebral cortex (the frontal and parietal cortex), thalamus, hypothalamus, basal forebrain, hippocampus, cerebellum, pons, and medulla, according to method described by Terao et al.,[15] and the cortex, pons, and hypothalamus were used for measuring the *hcrtR* and *pphcrt* gene expressions, respectively. Tissue samples were frozen on dry ice and stored at −80°C until RNA extraction. All animal experiments were carried out in strict accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. No animals in this study were used for any systematic pharmacological studies prior to collection of brain samples.

Human Narcolepsy-Cataplexy Patients

Postmortem brain samples from 5 narcolepsy-cataplexy patients and 5 control subjects were used in this study. Narcolepsy and control brain donors were primarily recruited through the Stanford narcolepsy brain donation program and the Stanford Brain Bank. All the narcoleptic subjects met the diagnostic criteria for narcolepsy with cataplexy in the 2nd edition of International Classification of Sleep Disorders.[16] Patients were all HLA *DQB1*0602* positive with cataplexy. Demographical data for the human subjects are shown in Table 1. There was no significant difference in either the mean age (77.0 vs. 72.6 y) or mean postmortem hours until brain autopsy (22.6 vs. 31.6 h) between the narcoleptic and control subjects. The hypocretin ligand deficiencies of all 5 narcolepsy-cataplexy subjects were confirmed by measurements of hypocretin-1 in selected regions in the cortex and pons using the method previously described.[8]

Frozen blocks from the middle and inferior frontal gyrus and the portion of pontine nuclei at the level of the origin of superior cerebellar peduncles without tegmentum were excised and processed. In one control subject (C8), the pontine tissue at the same anatomical level was not available, and only 4 narcoleptic subjects were included for the data analysis. Samples were stored at −80°C until RNA extraction.

Procedure

Isolation of Total RNA

For the real-time quantitative PCR procedure, we applied TaqMan assay (Applied Biosystems, Foster City, CA) for samples from mice and dogs, and LightCycler system (Roche, UK) for human brain samples, according to the standard protocol in the institutes that shared the measurements. The total RNA was isolated using Absolute RNA RT-PCR Miniprep kit (Stratagene, La Jolla, CA, USA) with RNase-free DNase treatment for the cortex, pons, and hypothalamus from mice, and cortex from dogs, and using Trizol reagent (Life Technologies, Inc., Rockville, MD) for the cortex and pons from human narcoleptic and control subjects from 20 mg of each sample according to the manufacturer's protocols. To secure the quality of extracted RNA from the human brain, we measured 28S/18S rRNA ratio using Agilent 2100 bioanalyzer (Agilent Technologies, Santa

Table 1—Demographical Data of the Study Subjects

	Age	Gender	PMI hr	Ethnicity	Comorbidity	Source
N1	72	M	18	caucasian	CA (bile duct)	Oslo Univ
N3	89	F	10.3	caucasian	CHF	SLC
N4	n/a	F	n/a	caucasian		Baffalo
N6	58	F	42	caucasian	dementia	Manitoba Univ
N7	89	F	20	caucasian	dementia	Oregon
C1	77	F	6	caucasian		Oslo Univ
C2	61	M	48	caucasian	CA (colon) Crohn AS	Oslo Univ
C4	73	F	48	caucasian	HF	Oslo Univ
C5	77	M	48	caucasian	CA (prostate)	Oslo Univ
C8	75	F	8	caucasian	CA (kidney) metabolic encephalopathy	VA(Ahmad)

PMI hr: postmortem interval hour

Clara, CA). The 28S/18S rRNA ratio for the human cortex and pons samples ranged from 1.21 to 2.04 (average, 1.81) and from 0.98 to 1.88 (average, 1.57), respectively, indicating the high integrity of RNA samples used for experiments. The A260/A280 ratio for the cortex and pons samples ranged from 1.86 to 2.05 (average, 1.97) and from 1.51 to 2.04 (average, 1.90). Also, the A260/A280 ratio for the canine cortex, murine cortex and pons samples ranged from 1.70 to 2.03 (average, 1.91), 1.88 to 2.01 (average, 1.95), and 1.86 to 2.00 (average, 1.93), showing the purity of RNA samples. The amount of extracted RNA was quantified by measuring the absorbance at 260 nm.

Reverse Transcription-PCR Conditions

First strand cDNA was synthesized using Superscript First-Strand Synthesis kit (Invitrogen, Carlsbad, CA, USA) for all samples. Starting total RNA amounts of murine and canine (human) samples were 1 μg (0.5 μg). Synthesis was started by priming template total RNA with 50 ng of random hexamers (0.5 μg of oligo(dT)12-18 primers), 1 μL of 10 mM dNTP mix in 10 μl reaction volume with DEPC-treated water by heating at 65°C for 5 min, snap-cooling on ice for 1 min. Reverse transcription was performed in the presence of 2 μl of 10X first strand buffer, 4 μL of 25 mM $MgCl_2$, 2 μL of 100 mM DTT, and 1 μL of RNase inhibitor (RNaseOUT) by incubating at 25°C for 2 min. Then, the template with 50 units of superscript II (final volume, 20 μL) was incubated at 25°C for 10 min (only for murine and canine samples), with additional incubation at 42°C for 50 min (for all samples). Reaction was quenched with heating at 70°C for 15 min, subsequent cooling on ice. Then, 2 units of RNase H was added and incubated at 37°C for 20 min. The final first strand cDNA samples were stored at −20°C until real-time PCR quantification.

PCR Primers and Fluorogenic Probes

For the murine and canine *hcrtR1*, *hcrtR2*, *pphcrt,* and for canine beta-actin (*actb*; internal standard), the TaqMan fluorogenic probes and corresponding primer sets were designed using Primer Express Software (Applied Biosystems, Foster City, CA) from the coding regions of the target genes as described in Table 2. The primers and the TaqMan fluorogenic probe for murine *actb* were supplied by Applied Biosystems. The TaqMan fluorogenic probe for the canine *hcrtR2* was designed on the exon1-exon2 boundary to detect the defective *hcrtR2* expression in the *hcrtR2*-mutated narcoleptic Dobermans in which the SINE insertion was observed in the 5' splice site of the exon 4.[3]

For the human *hcrtR1*, *hcrtR2*, and *actb*, the LightCycler oligohybriprobes and corresponding primer sets were designed and manufactured by Nihon Gene Research Laboratories Inc. (Sendai, Japan) as described in Table 2. All probes were labeled at the 3' end with fluorescein and at the 5' end with the LC-640 label.

Plasmid DNA Controls for Human Samples

Dilutions of the respective plasmid standards were used as template DNA for semi-quantitative RT-PCR for human *hcrtR1*, *hcrtR2* and *actb*. Plasmid DNA was supplied by Nihon Gene Research Laboratories Inc. (Sendai, Japan). Plasmid DNA was purified by standard methods using DNA ligation kit (Mighty Mix, Takara, Tokyo), pT7 Blue T-Vector (Novagen, Inc.), and transformed into Competent Cell/DH5-alpha (Takara, Tokyo) according to the manufacturer's protocols; sequences of primer sets were same as F- and R-primers for the LightCycler oligohybriprobes as shown in Table 2. The plasmid preparations were diluted in logarithmic steps to give a dilution range of 8.90 x 10^1~10^5 copies for *hcrtR1*, 3.56 × 10^2~10^6 copies for *hcrtR2*, and of 3.05 × 10^4~10^8 copies for *actb*. Each dilution was employed in duplicate per run.

Assay Condition

Real-time quantitative PCR was performed in triplicate for each gene and sample on the ABI PRIZM 7000 Sequence Detection System (Applied Biosystems) for murine and canine samples and on the LightCycler real time PCR system (Roche, UK) for human samples. For the TaqMan assay, the reaction mixture containing 2.5 μL of primers and probes, 25 μL of 20X TaqMan Universal PCR Master Mix, 17.5 μL of water was prepared and dispersed in a 96-well MicroAmp Optical Tube. For each optical tube, 5 μL of cDNA sample was added to give a final volume of 50 μL. Real-time PCR assay was done under the following universal conditions: 2 min at 50°C, 10 min at 95°C, 50 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min. A PCR mixture without cDNA template was used as a negative control.

Table 2—Primer and Hybriprobe sets for LightCycler and Taqman Systems

Species	Primer/Probe	Sequence	Assay location	Reference Seq.
Hypocretin receptor 1 (*hcrtr1*)				
Human	F primer	5'-CTgCCACCgAgATTTTAAg-3'		
	R primer	5'-CAAgTTAAgCAgACCTggTg-3'	3'-UTR	NM_001525
	Oligohybriprobe	5'-gTCCCTgTTCCTCTCAgCAgAgTAgCAC-3'-Flu		AY070269
		5'-LC-TCAggCTgCACTCCACgTCCTTCT-3'-p		
Mouse	F primer	5'-CCATCAgTgTCCTCAATgTCCTT-3'		
	R primer	5'-ggTCgCTggCTTggC-3'	Ex7/Ex8	NM_198959
	Taqman probe	FAM-5'-ACATCCCgAACACTCTCTT-3'-NFQ		
Canine	F primer	5'-CTACgTggCTgTgTTCCTAgTg-3'		AAEX01011943
	R primer	5'-gTCCTCATgTggTggTTCCT-3'	Ex1/Ex2	XM_544446
	Taqman probe	FAM-5'-CAACACgCTggTCTgC-3'-NFQ		
Hypocretin receptor 2 (*hcrtr2*)				
Human	F primer	5´-CCACTCAAATCAgCAACTT-3´		
	R primer	5´-gTTTTACTCAggTATCCTTgTC -3´	Ex7	NM_001526
	Oligohybriprobe	5'-CTCACTAgCATAAgCACACTCCCAgCA-3'-Flu		
		5'-LC-CCAATggAgCAggACCACTTCAAAA-3'-p		
Mouse	F primer	5'-gCTCACCAgCATAAgCACACT-3'		
	R primer	5'-TggACAggAgTgAAgATggTACT-3'	Ex7/Ex8	AY336085
	Taqman probe	FAM-5'-CCgCTTCAAAACTggTATCT-3'-NFQ		
Canine	F primer	5'-CTggCTACATCATCgTgTTCgT-3'		
	R primer	5'-CCTCATgTggTggTTCTTCCA-3'	Ex1/Ex2	AF164626
	Taqman probe	FAM-5'-ACAAACCAggACgTTgCC-3'-NFQ		
Preprohypocretin (*pphcrt*)				
Mouse	F primer	5'-CAggCACCATgAACTTTCCTTCTA-3'		
	R primer	5'-CAgCAgCAgCgTCACg-3'	Ex1/Ex2	NM_010410
	Taqman probe	FAM-5'-CCCAgggAACCTTTg-3'-NFQ		
Beta-actin (*actb*)				
Human	F primer	5'-AgTCCCTTgCCATCCTAA-3'		
	R primer	5'-ggAgACCAAAAgCCTTCA-3'	Ex7	NM_001101
	Oligohybriprobe	5'-gCAATgCTATCACCTCCCCTgTgTg-3'-Flu		
		5'-LC-ACTTgggAgAggACTgggCCATTCT-3'-p		
Mouse	F primer			
	R primer	(Mm00607939_s1)	Ex6	NM_007393
	Taqman probe			
Canine	F primer	5'-CgTgTTCCCgTCCATCgT-3'		
	R primer	5'-CCCACgTAggAgTCCTTCTg-3'	Ex2/Ex3	AF021873
	Taqman probe	FAM-5'-CATCACgCCCTggTgCC-3'-NFQ		

Flu:Fluorescein, LC:LCRed640, p:phosphorylation
FAM:6-carboxyfluorescein, NFQ:nonfluorescent quencher

For the LightCycler assay, Faststart DNA Master Hybridization Probe kit (Roche, UK) was used for amplification according to the manufacturer's instructions, with some modification. For the human *hcrtR1* (*hcrtR2*, or *actb*), a final concentration of 3 mM (3 mM, 4 mM) $MgCl_2$, 0.2 μM hybridization probes and 0.3 μM (0.5 μM, 0.3 μM) forward and reverse primers were used in addition to 2 μL of cDNA template. The thermocycling conditions included a denaturation step at 95°C for 10 min followed by amplification at 62°C (60°C, 62°C) for 10 min and extension at 72°C for 6 min (6 min, 8 min) for 45 cycles. The melt curve profile was as follows: 95°C for 0 s, 70°C for 15 s and 95°C for 0 s. A PCR mixture without cDNA template was used as a negative control.

Quantification of mRNA Expression

For the Taqman system, the relative quantification of the *hcrtR1*, *hcrtR2*, *pphcrt,* and *actb* expression among samples to be compared was performed with the comparative cycle threshold (C_T) method, in which the C_T parameter is defined as the cycle number at which the normalized reporter signal of a given reaction crossed the threshold value.

For the LightCycler system, we used external plasmid calibration curves to determine the *hcrtR1*, *hcrtR2,* and *actb* expression. Fitted lines were obtained using standard linear regression analysis of C_T values on the log of the plasmid copy number for each gene within the given dilution ranges with the slope (–3.326 ~ –3.602 for *hcrtR1*, –3.129 ~ –3.446 for *hcrtR2*, –3.378 ~ –3.489 for *actb*) and error rate (0.0295 ~ 0.0986 for *hcrtR1*, 0.0187 ~ 0.0273 for *hcrtR2*, 0.0117 ~ 0.0354 for *actb*).

To take into account the variation in RNA quantity and quality, each gene expression was normalized to the *actb* expression as an internal standard by dividing the target cDNA amount by *actb* reference.

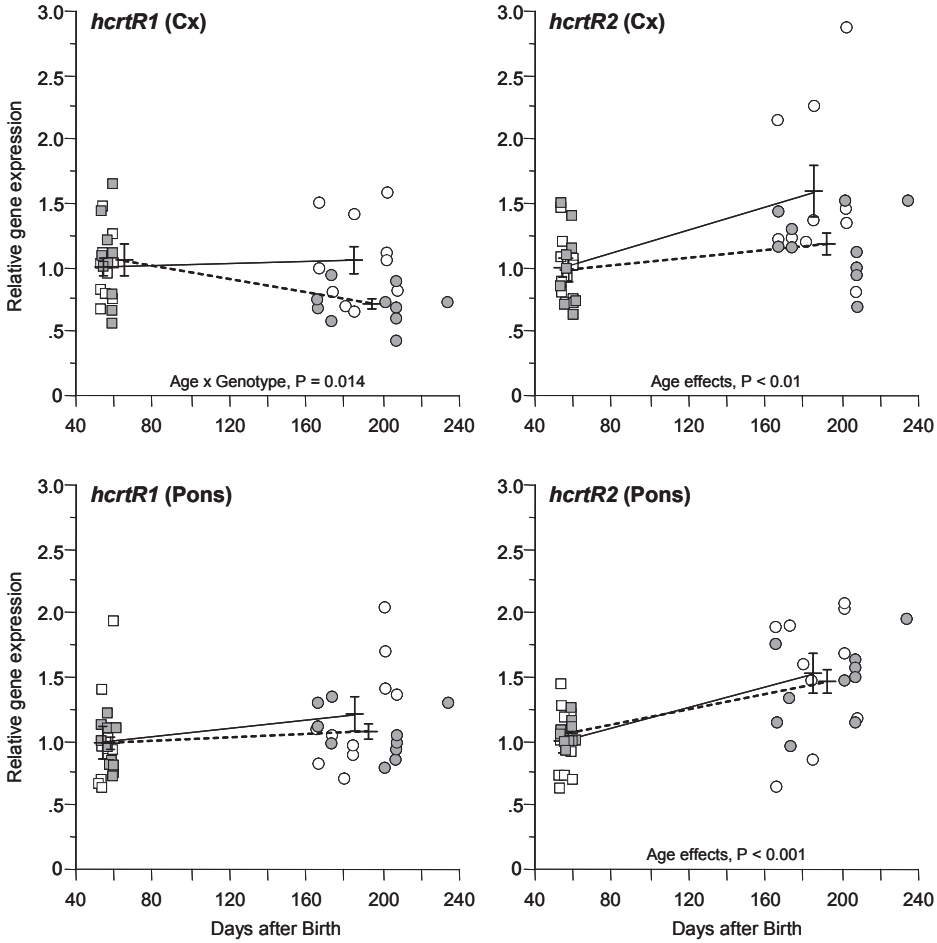

Figure 1—Postnatal changes in the *hcrtR1* and *hcrtR2* expression in the cortex and pons of wild (Wt) and orexin/ataxin-3-transgenic mice (TG) mice.

Data are expressed as relative values to average expression level (= 1) in the Wt mice at 8 weeks of age in each panel. Horizontal bars indicate days after birth. Open and closed squares (circles) represent data for Wt and TG mice at 8 weeks (27 weeks) of age. Solid and dotted lines represent mean ± SEM for the Wt and TG mice groups, respectively.

Two-way ANOVA showed a significant age × genotype interaction (8-week-old × 27-week-old × Wt × TG groups) in the *hcrtR1* expression ($F_{1,18}$=6.214, P = 0.023) in the cortex, resulting in significantly lower expression of the *hcrtR1* in the TG group compared to that in the Wt group at 27 weeks of age (P = 0.014). We also observed a significant age effect in the *hcrtR2* expression level in either the cortex ($F_{1,18}$ = 8.52, P < 0.01) or the pons ($F_{1,18}$ = 24.57, P < 0.001).

Statistical Analysis

Two-way ANOVA was used to identify significant age and genotype related effects in each gene expression level between 4 age- and genotype mice groups. Mann Whitney test was used for comparison of each gene expression between age groups or between narcoleptic and control groups. Results are shown as mean and SEM values. A P value < 0.05 was considered to indicate significance. In the gene expression analysis, data are expressed as relative values to average expression level of each gene (= 1) in the control animals or human subjects.

RESULTS

Murine *pphcrt, hcrtR1*, and *hcrtR2* Expression

There was no significant difference in *hcrtr1* and *hcrtr2* expression overall and in each age and genotype group, and thus the data analyses were performed on the combined data of both sexes. Hypothalamic *pphcrt* expression in the TG mice was almost completely suppressed compared to that in the Wt mice at 8 weeks (0.017 ± 0.004 vs. 1.000 ± 0.281, P < 0.005) and at 27 weeks (0.013 ± 0.002 vs. 0.973 ± 0.185, P < 0.001) of age, suggesting the sufficient postnatal ablation of pphcrt synthesis in the TG mice group by 8 weeks of age. The Wt mice showed no significant difference in the hypothalamic *pphcrt* expression level between the 2 age groups.

The *hcrtR1* and *hcrtR2* expression in the cortex and pons of the Wt and TG mice at 8 weeks and 27 weeks of age are summarized in Figure 1. In the cortex, there was no difference in either the *hcrtR1* (1.000 ± 0.078 vs. 1.077 ± 0.106) or *hcrtR2* (1.000 ± 0.071 vs. 0.987 ± 0.096) expression level between the Wt and TG groups at 8 weeks of age. We observed a significant age × genotype interaction in *hcrtR1* expression level in the cortex (P = 0.023), and the 27-week-old TG group showed a moderate decline in the *hcrtR1* expression compared to the 8-week-old mice. These changes resulted in significantly lower expression of the *hcrtR1* in the TG group compared to that in

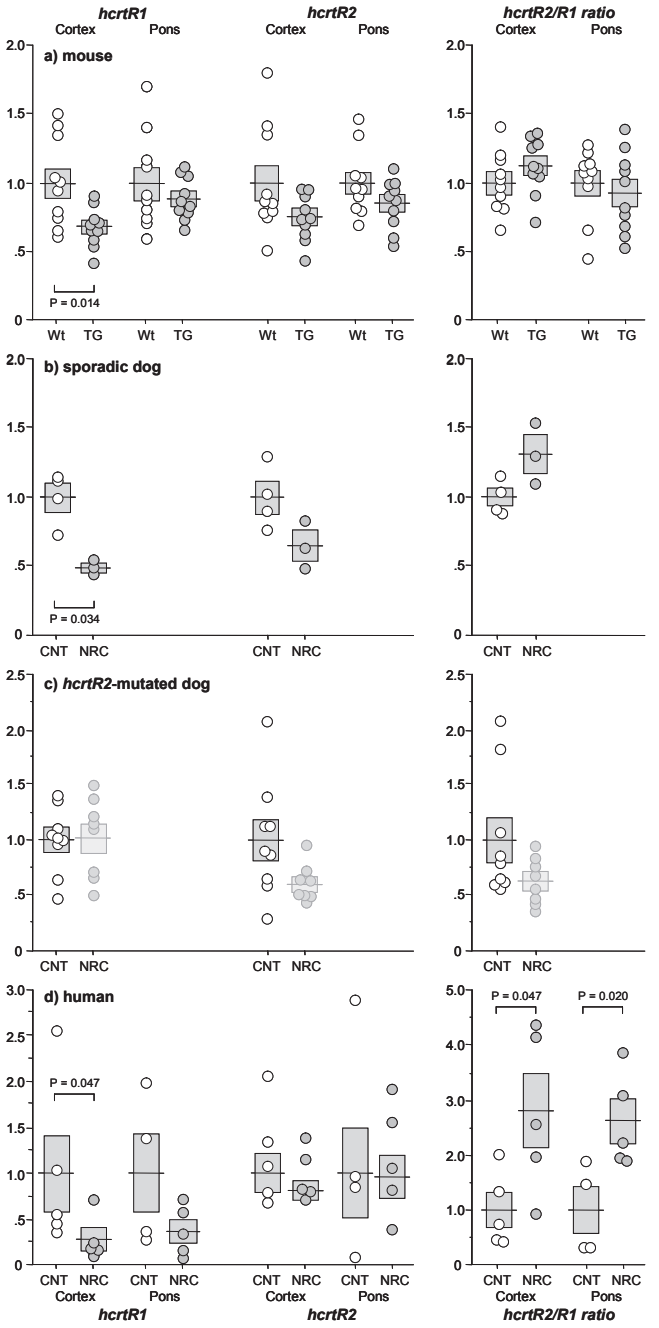

Figure 2—*hcrtR1* and *hcrtR2* expression and their ratio in the narcoleptic (TG/NRC) and control (Wt/CNT) groups.
Data are expressed as relative values to average expression level (= 1) in the corresponding Wt/CNT group. The mice data are from 27-week-old groups. Open and closed circles represent the Wt/controls and TG/narcoleptics, respectively. The number of the control subjects for the pontine analysis is 4, due to the lack of the availability of the tissue. Data for defective *hcrtR2* expression in the *hcrtR2*-mutated narcoleptic Dobermans (2c) are indicated in light gray. Columns with solid horizontal bars cover mean ± SEM.

the Wt group at 27 weeks of age (0.672 ± 0.044 vs. 1.000 ± 0.101, P = 0.014, Figure 2a). We observed a significant age effect in the *hcrtR2* expression level in the cortex (P < 0.01). Increases in the *hcrtR2* expression in 27-week-old mice were more prominent in Wt group. However, difference in *hcrtR2* expression between the TG group and the Wt group at 27 weeks of age was not statistically significant (0.744 ± 0.052 vs. 1.000 ± 0.124, P = 0.11, Figure 2a).

In the pons, there was also no difference in either the *hcrtR1* (1.000 ± 0.126 vs. 0.985 ± 0.051) or *hcrtR2* (1.000 ± 0.090 vs. 1.067 ± 0.030) expression level between the Wt and TG groups at 8 weeks of age (Figure 1).

As seen in the cortex, we observed significant age effects in the *hcrtR2* expression (P < 0.001), and the increases in the 27-week-old group were observed in both Wt and TG groups. The trend toward a lower expression of the *hcrtR1* and *hcrtR2* in the TG group was not observed in the pons (Figure 1 and 2a), and no significant genotype effects or genotype × age interactions were observed in the pons. We also calculated the *hcrtR2*/*hcrtR1* expression ratios in the cortex and the pons, and no statistical significant difference was observed.

Canine *hcrtR1* and *hcrtR2* Expression

In our previous study, we examined hcrt contents in CSF and/or brains and confirmed that these sporadic narcoleptic dogs are hcrt ligand-deficient.[4] In contrast, hcrt contents in the brain and CSF were not altered in *hcrtR2*-mutated narcoleptic Dobermans; the hcrt levels were comparable to those in control Dobermans and sporadic controls.[4]

HcrtR1 and *hcrtR2* expression levels in the cortex of the sporadic and genetic *hcrtR2*-mutated narcoleptic dogs are shown in Figure 2b and 2c. The sporadic ligand-deficient narcoleptic dogs showed a significant decrease in the *hcrtR1* expression (0.493 ± 0.028 vs. 1.000 ± 0.093, P = 0.034), while a decrease in the *hcrtR2* expression (0.656 ± 0.101 vs. 1.000 ± 0.116, P = 0.077) was not statistically significant. The *hcrtR2*/*hcrtR1* expression ratio in the sporadic narcoleptic dogs was higher than that in the non-affected controls, but the difference was not statistically significant (1.316 ± 0.130 vs. 1.000 ± 0.062, P = 0.077).

In contrast, *hcrtR2*-mutated narcoleptic Dobermans with normal hcrt ligand production showed the same level of the *hcrtR1* expression as that in the control Dobermans (1.020 ± 0.127 vs. 1.000 ± 0.100, P = 0.56). We observed a decrease in the defective *hcrtR2* expression in the narcoleptic Dobermans, but the difference was not significantly different (0.604 ± 0.061 vs. 1.000 ± 0.174, P = 0.068). Similarly, decrease in the *hcrtR2*/*hcrtR1* expression ratio in the affected group compared to that in the non-affected controls was (0.622 ± 0.076 vs. 1.000 ± 0.189, P = 0.068) was not statistically significant.

Human *hcrtR1* and *hcrtR2* Expression

The gene expression of the *hcrtR1* (1168 ± 234 copies in the cortex, 585 ± 181 copies in the pons), *hcrtR2* (2055 ± 438, 2536 ± 887) and *actb* (3531 ± 850 × 10^3, 3032 ± 772 × 10^3) were within the dilution range of each plasmid control. Relative expression of the *hcrtR1* and *hcrtR2* in human narcolepsy-cataplexy patients are illustrated in Figure 2d. We observed a significant decrease by 71% in the *hcrtR1* expression in the cortex of narcoleptic subjects compared to control subjects (0.285 ± 0.110 vs. 1.000 ± 0.404, P = 0.047). A similar degree of reduction (by 63%, 0.373 ± 0.119 vs. 1.000 ± 0.413, P = 0.221) in the *hcrtR1* expression in the pons of narcoleptic subjects was observed, but

this did not reach statistical significance. The *hcrtR2* expression in the cortex of narcoleptic subjects was also decreased by 19% (0.813 ± 0.103 vs. 1.000 ± 0.205, P = 0.917), but this decline was not statistically significant. No reduction in the *hcrtR2* expression in narcoleptic subjects was observed in the pons (by 3%, 0.971 ± 0.223 vs. 1.000 ± 0.496, P = 0.807).

The degree of the decrease in *hcrtR1* expression was larger than that of *hcrtR2* expression, resulting in significant increases in the *hcrtR2*/*hcrtR1* expression ratio in narcoleptic subjects compared to non-affected controls, both in the cortex (2.791 ± 0.655 vs. 1.000 ± 0.297, P = 0.047) and in the pons (2.719 ± 0.399 vs. 1.000 ± 0.446, P = 0.02).

DISCUSSION

We found a significant decrease in *hcrtR1* expression (by 33% vs. controls) in the cortex of 27-week-old TG mice, while there was no significant difference in *hcrtR1* expression at an early stage of hcrt neuronal loss (8 weeks of age). These age-dependent changes are likely due to the long-term loss of the hypocretin ligand. Similarly, a significant decrease in *hcrtR1* expression (by 50%) was observed in the cortex of 3 sporadic narcoleptic dogs. The sample population included a 200-month old dog (cataplexy onset at 24 months old) and *hcrtR1* expressions were comparable to those in other sporadic dogs, suggesting the declines may not be progressive.

The moderate declines in *hcrtR2* expressions were also observed in 27-week-old TG mice and sporadic narcoleptic dogs, but these declines were not statistically significant.

A significant decrease in *hcrtR1* (reduced by 71% in the cortex vs. controls) was also observed in human narcoleptic subjects (mean age = 77.0 y). Similar degree of decline in *hcrtR2* (reduced by 63% in the cortex vs. controls) was also observed in the pons of narcoleptic subjects, this change did not reach statistical significance, possibly because of the small sample size and the larger interindividual variations in expression (likely due to variations in postmortem interval, cause of death, length of agonal state, and RNA quality). However, significant increases in *hcrtR2*/*hcrtR1* expression ratio were observed in both the cortex and pons in these hypocretin deficient narcoleptic humans, suggesting relative preservation of hcrtR2. Therefore our human findings also support the assumption that the long-term postnatal hcrt cell loss induces moderate alterations in hcrtR production, with primary effects on the hcrtR1 system.

The conclusions of our study are strengthened by results obtained in multiple species at various ages, and by using different models of hypocretin deficiency. Several limitations should however be discussed. Foremost, we only studied *hcrtR* mRNA expression in the cortex and the pons (mice and humans). The reasoning for selecting these structures was based on anatomical importance (abundant hcrt projection and quantifiable signals for both hcrt 1 and hcrt 2)[17] and tissue sample availability. Although our pons samples included several brain structures involved in sleep regulation (such as the locus coeruleus, raphe nuclei, pedunculopontine tegmental nucleus, and laterodorsal tegmental nuclei in mice and the pontine reticular formation and adjacent pontine structures in humans), less significant changes in *hcrtR* expressions were observed in this region when compared to the cortex. Our dissections in the pons are likely to contain a largely heterogeneous group of nuclei. This suggests that hcrtR mRNA changes may be more evident in more finely dissected substructures, some of which could have functional relevance in sleep regulation (e.g., the histaminergic tuberomammillary nucleus and basal forebrain cholinergic neurons).[17,18] Another concern is the correlation between receptor function and gene expression, as we did not study the hcrtR functions. However, there are only a few methods to evaluate hcrtR functions, such as the gamma-GTP autoradiography,[19] and these evaluations generally require tissues without postmortem changes. The evaluation of the gene expression is still one of the most informative methods to assess receptor functions. However, the positron emission tomography (PET) study may be conducted in human subjects once a PET ligand for hypocretin receptors is developed.

The exact mechanisms underlying hcrt neuronal loss in humans are not yet known. Thannickal et al. reported that axon loss of hcrt neurons and associated gliosis occurred across various brain regions in narcoleptic patients. They also found that the degree of axonal loss correlated with the amount of *hcrtR2* expression expected from data reported in rat brain.[13] The authors went on to speculate that *hcrtR2* or a molecular target anatomically linked to *hcrtR2*, may be involved in the pathological process (such as inflammatory or autoimmune). In this model, hypocretin cell loss would only occur later as axonal cell loss progressed until the cell body is reached. Our limited human data do not support this hypothesis, as *hcrtR1* changes (over *hcrtR2* changes) were more prominent. Additionally, the decline in *hcrtR* expression was age dependent in the mice model and more likely to be due to the long-term postnatal loss of ligand production. This concept is also supported by findings in *hcrtR2*-mutated narcoleptic Dobermans, where normal expression of *hcrtR1* was observed (while a large decline in the defective *hcrcR2* exon-skipped mRNA expression was observed). Thus, gene expression of both receptors is partially regulated by ligand availability, and no evidence was found to support the hypothesis that *hcrtR2* itself is the potential target of the pathological process in hypocretin-deficient human narcolepsy.

Mieda et al.[12] reported that replacing hcrt (either genetically or pharmacologically) could rescue the narcolepsy-cataplexy phenotype in mice lacking hypocretin cells. The studies included 14- to 15-week-old mice. As more than 90% of cell death is completed before 8 weeks in this model,[5] these results suggest that the hcrt receptors and other neural pathways responsible for narcolepsy phenotype remain anatomically and functionally intact even after a considerable period of ligand deficiency. These authors also reported enhanced arousal response to hcrt1 supplementation in the TG mice when compared to Wt mice, suggesting increased sensitivity. Although we have not analyzed *hcrtR* expression in 14- to 15-week-old mice, we have confirmed that hcrt ligand gene expression was significantly reduced at 8 weeks of age without significant alteration in the *hcrtR* expression. Functional compensation in systems downstream of hcrt neurotransmission may thus exist in these mice, explaining the enhanced arousal response to hcrt in the model. It is therefore important to examine the sleep phenotype changes in these mice over the long term, as well as to evaluate the therapeutic response of the hypocretin replacement at different ages.

Although alternations in *hcrtR1 and hcrtR2* expressions were observed in human narcolepsy, we believe it is not likely to be problematic in the context of developing effective hypocretin replacement therapies. Several reasons can be advanced. First, the *hcrtR* expression declines are indirectly due to the loss of the ligand/neurons itself; we have no evidence to suggest receptors are altered. Second, the decline is not progressive and complete, and only a moderate change in receptor expression is observed, even in aging human and sporadic narcoleptic canines. Third, a partial loss of hcrtR2 function is unlikely to produce a phenotype by itself. Indeed, *hcrt*R2-mutated heterozygotes are asymptomatic in canines and mice.[1,20] Fourth, compensation through postsynaptic mechanisms downstream of hcrt neurotransmission may be involved, as suggested by the hypocretin replacement mice study. Finally, the relative preservation of hcrtR2 synthesis over that of hcrtR1 may also be encouraging, since hcrtR2 function is more critical for mediating the narcolepsy phenotype in both dogs and mice.[14,20] A preferential target of hcrt ligand development for narcolepsy should also be focused on hcrtR2.

In summary, this report suggests that hypocretin receptor transmission is only mildly altered in various models of hypocretin deficiency, including human narcolepsy. This finding should encourage the development of hcrt replacement therapy for human narcolepsy. If hypocretin replacement therapy is found to be successful, further studies using cell transplantation of embryonic hypothalamic cells or neural stem cells, or gene therapy protocols could be attempted to cure the disease in the future.

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