Effects of IV and ICV Hypocretin-1 (Orexin A) in Hypocretin Receptor-2 Gene Mutated Narcoleptic Dogs and IV Hypocretin-1 Replacement Therapy in a Hypocretin-ligand-deficient Narcoleptic Dog

Nobuhiro Fujiki, MD, PhD; Yasushi Yoshida, MD, PhD; Beth Ripley, MA; Emmanuel Mignot, MD, PhD; Seiji Nishino, MD, PhD

Center for Narcolepsy, Stanford University School of Medicine

Study Objectives: Using two different canine models of narcolepsy, we evaluated the therapeutic effects of hypocretin-1 on cataplexy and sleep.

Measurements and Results: Intracerebroventricular administration of hypocretin-1 (10 and 30 nmol per dog) but not intravenous administration (up to 6µg/kg) induced significant wakefulness in control dogs. However, hypocretin-1 had no effect on cataplexy or wakefulness in hypocretin receptor-2 gene (Hcrtr2) mutated narcoleptic Dobermans. Only very high intravenously doses of hypocretin-1 (96 - 384 µg/kg) penetrated the brain, to produce a short-lasting anticyataplectic effect in a hypocretin-ligand-deficient animal.

Conclusions: Hypocretin-1 administration, by central and systemic routes, does not improve narcoleptic symptoms in Hcrtr2 mutated Dobermans. Systemic hypocretin-1 hardly crosses the blood-brain barrier to produce therapeutic effects. The development of more centrally penetrable and longer lasting hypocretin analogs will be needed to further explore this therapeutic pathway in humans.

Key Words: hypocretin, orexin, narcolepsy, cataplexy, ICV, canine narcolepsy

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INTRODUCTION

NARCOLEPSY IS A CHRONIC SLEEP DISORDER AFFECTING 0.03% TO 0.16% OF THE GENERAL POPULATION.1,3 Its major symptoms include excessive daytime sleepiness, cataplexy, rapid eye movement (REM) sleep-related symptoms (such as sleep paralysis,) and hypnagogic hallucinations.1 Cataplexy is pathognomonic for the disease and manifests itself as sudden loss of muscle tone in response to strong emotional stimuli.1 Current treatments include amphetamine-like stimulants (monamine release enhancers) or modafinil for excessive daytime sleepiness and antidepressants (monoamnergic uptake inhibitors) for cataplexy and REM sleep-related symptoms.1 These treatments are purely symptomatic and are often insufficient to fully control narcolepsy, emphasizing the need for developing more effective treatments.

The major pathophysiology of human narcolepsy is now believed to be hypocretin deficiency.1-3 This discovery originated from observations in a canine model of the disorder and in preprohypocretin (preproorexin) knockout mice. Null mutations in the hypocretin receptor-2 gene (Hcrtr2) cause narcolepsy in a genetically defined model of narcolepsy in dogs (Dobermans and Labradors).4 In addition, the phenotype of the hypocretin-ligand gene (preprohypocretin gene) knockout mice is similar to that in human and canine narcolepsy.5 In humans, hypocretin-related gene mutations are extremely rare,2 but a defect in hypocretin transmission is observed in most narcolepsy cases with cataplexy: hypocretin measurements in cerebrospinal fluid (CSF) as well as postmortem brain studies indicate decreased hypocretin peptide and mRNA.1-3,6 Hypocretin-ligand deficiency is also observed in sporadic cases of canine narcolepsy. Thus these animals share more similar pathophysiological mechanisms to the human disorder.7 Hypocretin-peptide replacement therapy or nonpeptide hypocretin agonists could thus represent promising new therapeutic avenues for ligand-deficient narcolepsy.

Using Hcrtr2-mutated narcoleptic Dobermans, John et al8 recently reported that intravenous (IV) administration of hypocretin-1, at doses up to 3 µg/kg, reduces cataplexy and consolidates sleep fragmentation. These results were unexpected, since hypocretin receptor-2 in these animals is nonfunctioning8,10 and hypocretin neurons and hypocretin production are not altered in these animals.7 To revisit this issue, we studied the effects of IV and intracerebroventricular (ICV) administration of hypocretin-1 on cataplexy and sleep in narcoleptic and control Dobermans. The CNS penetration of hypocretin-1 after the IV administration of hypocretin-1 was also investigated. Furthermore, the effect of the IV administration of hypocretin-1 on cataplexy was studied in our only available hypocretin-ligand-deficit narcoleptic dog, the result of which should be more predictive for the treatment of human narcolepsy.

MATERIALS AND METHODS

Animals

Twelve Dobermans born at the Stanford University Center for Narcolepsy Canine Colony were used in this study. A total of 4 narcoleptic and 2 control Dobermans were implanted with electroencephalogram (EEG), electrooculogram (EOG), and electromyogram (EMG) electrodes. An ICV cannula was implanted for 2 narcoleptic and 2 control Dobermans. The exact number of animals used for each experiment is described in each section. A 3-year-old male Schipperke (7 kg) with hypocretin deficiency confirmed by CSF analysis (< 40 pg/mL tested at 2.5 years of age) was also used for the IV hypocretin study. The onset of cataplexy in this dog was 10 months of age, and the animal was donated to Stanford Canine Narcolepsy Colony at 2.5 years of age. This animal was not used for ICV or sleep-recording experiments because of a prior agreement with the donor of the dog. Animals were housed in individual stainless-steel cages (1.0 x 1.8 m). All experiments were carried out in accordance with the guidelines described in The National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgical Preparation and Polygraph Recordings

The EEG, EOG, and EMG electrodes and ICV guide cannulas were implanted under general anesthesia with the assistance of a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, Calif) and a canine brain atlas.13 Electrodes for EEG recording were secured to the skull over the mid frontal, lateral parietal, and occipital cortices. Electrodes for EOG
recording were secured into the orbit of the frontal bone. Stranded stainless-steel wires were inserted into the dorsal neck muscle for EMG recordings. Wires from all electrodes were connected and soldered to a 20-pin electrical plug. Stainless-steel tubes (20-gauge, 20-mm) were used as guide cannulas for ICV injection needles, and the tips were placed on the surface of the cortical dura. The electrodes, guide cannulas, and electrical plug were cemented to the skull using acrylic cement. A detailed description of the surgical techniques employed has been described previously. The effects of hypocretin-1 administration on wakefulness were analyzed using 6-hour daytime polygraph (EEG, EOG, and EMG) recordings. Dogs were alone in the experimental room (3 m²) while being observed continuously through a 1-way mirror and video camera from the adjacent recording room where the polygraph recorder was installed. The electrical plug of the headstage of the animal was connected to a shielded cable that was wired to the recording room through the ceiling. Prior to actual experimental recording, animals were habituated to the experiment room. Recording was started at 9:00 AM and continued for 6 hours. Lights were on at all recording times. Vigilance stages of each animal were scored every 30 seconds based on EEG, EOG, and EMG recording as well as behavior observations as previously reported.

**Biologic Assay for Quantifying the Severity of Cataplexy**

The Food Elicited Cataplexy Test (FECT) was used to assess the effects of the IV and ICV administration of hypocretin-1 on cataplexy. The animal was brought to the experiment room where 12 pieces of wet dog food were placed in a circle on the floor. As wet canned food is a powerful emotional stimulus for dogs, multiple cataplectic attacks are easily elicited in narcoleptic animals. The experimenter recorded the number of attacks (NA) and the duration of each cataplectic attack, as well as the total elapsed time (ET) required for the dog to eat all 12 pieces of food. In order to contrast the results by John et al., results of the NA and ET are presented in the figures, but analyses for the total time spent in cataplexy (sum of duration of each attack) were also carried out. Averaged data, obtained from 2 FECT trials in succession, were used as 1 session data point.

**Hypocretin-1**

For all experiments, we used hypocretin-1, which acts at both hypocretin receptor-1 and hypocretin receptor-2; hypocretin-1 is more stable than hypocretin-2 in biologic fluids. We obtained hypocretin-1 from 2 different sources (Orexin A: Neurocrine, San Diego, Calif, and American Peptide, Sunnyvale, Calif), and each drug batch was used during approximately half of the sessions in each experiment. Hypocretin-1 was dissolved on the day of each experiment using 12-mm x 75-mm Borosilicate glass tubes (Fisher Scientific, Pittsburgh, Penn) in physiologic saline and artificial CSF (125 mM NaCl, 0.5 mM NaH₂PO₄, 2.5 M Na₂HPO₄, 1.3 mM CaCl₂, 2.5 mM KCl, 1 mM MgCl₂) for IV and ICV administration, respectively. Concentrations of hypocretin-1 used for each experiment are described in each corresponding section. All apparatus—

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**Figure 1**—Effects of a single (upper 2 panels) and repeated (lower panels) intravenous (IV) injections of hypocretin-1 on cataplexy in Hcrtr2-mutated narcoleptic Dobermans. The severity of cataplectic attacks in 6 genetically narcoleptic dogs was evaluated using the Food Elicited Cataplexy Test. Mean (± SEM) of elapsed time (ET) and of number of attacks (NA) as well as mean (± SEM) changes in ET and NA from the respective baselines are shown. (A) Single IV injection: Cataplexy data 2 hours before and 5, 60, 120, and 240 minutes after injection of test solutions (saline and 1, 3, 4, and 6 µg/kg of hypocretin-1 solutions). A single IV administration of hypocretin-1 had no significant effect on cataplexy in 6 narcoleptic Dobermans (Time Effect: F₄,₈₀ = 1.2, P = 0.31, Time x Treatment: F₁₂,₈₀ = 0.87, P = 0.57, NA; Time Effect: F₄,₈₀ = 1.3, P = 0.29, Time x Treatment: F₁₂,₈₀ = 0.97, P = 0.48). In 4 animals, 24 µg/kg of intravenously administered hypocretin-1 was also tested, but no effect on cataplexy was observed. (B) Repeated IV administration: 3 µg/kg hypocretin-1 was injected on 3 sequential days (arrows).
1-mL plastic Tuberculin SlipTip syringes with 27-gauge half-inch needles (Becton, Dickinson and Company, Franklin Lakes, NJ) and 25-µL Hamilton glass syringes (Hamilton, Reno, Nev) with PE20 polyethylene tubing (Becton, Dickinson and Company)—used for drug injections were presoaked with a silicon solution (SIGMACOTE, Sigma-Aldrich, Natick, Mass) to minimize absorption of hypocretin-1.

Experimental Design

Effect of Single and Repeated IV Administration of Hypocretin-1 on Cataplexy in Hcrtr2-mutated Dobermans

Six narcoleptic Dobermans (50% males, without head-stage implantation) were used to determine whether a single IV administration of hypocretin-1 has any effects on cataplexy. For these acute experiments, we used saline and 1, 3, 4, and 6 µg/kg of hypocretin-1 solutions. The IV injections were administered through the brachial vein. This dose range was chosen in accordance with the previously published study by John et al.8 In addition, 24 µg/kg of hypocretin-1 was tested in 4 narcoleptic Dobermans. Each dose was tested once a week. Dogs were brought to the experiment room at 9:00 AM, 2 hours prior to the injection. Two hours before and 5, 60, 120, and 240 minutes after the injection, FECTs were performed. The same 6 dogs were used to examine the effects of repeated IV administration of hypocretin-1 (3 µg/kg). In this protocol, we carried out FECT once each day for 8 sequential days at 10 AM. We injected the saline on the first day and 3µg/kg hypocretin-1 on 3 consecutive days to examine the effects of repeated administration of hypocretin-1. Injections were carried out after the FECT assessments in the morning. The FECT was administered for the following 4 days without any injections. This protocol was adapted from John et al8 with minor modifications.

Effect of IV Administration of Hypocretin-1 on Sleep and Wakefulness in Hcrtr2-mutated Dobermans

Four genetically narcoleptic and 2 control Dobermans were used. Prior to the actual experiment, dogs were habituated to the recording room and the cable connection. Recordings were started at 9:00 AM. The animal was brought to the experiment room, and saline or 3 µg/kg or 6 µg/kg of hypocretin-1 solution was injected intravenously through the brachial vein 5 minutes before the sleep recording. The order of the injections (saline, 3µg/kg, 6 µg/kg) was randomized for each session, and at least 2 days were allowed between each drug injection. Two sessions of 3 recordings (saline, 3µg/kg, 6 µg/kg) were repeated in 2 control animals, and a total of 4 polygraph sessions were analyzed for each diagnostic group.

Acute Effect of ICV Administration of Hypocretin-1 on Cataplexy in Hcrtr2-mutated Dobermans

Two narcoleptic Dobermans (females) were used for this study. One to 2 days prior to the experiment day, a 27-gauge needle with 15 cm of PE20 polyethylene tubing (the total inner volume of the tubing and the needle was 20 µL) was inserted into the lateral ventricle (AP: 24-26, L: 4-9, H: 20) through a guide cannula of the head stage after puncturing the dura with a fine 25-gauge needle. The tip of the tubing was attached to a 250-µL Hamilton glass syringe, and the location of the lateral ventricle for each injection site in each animal was verified by the observation of a constant CSF flow from the needle by slowly pulling the plunger. At the depth where we observed the constant CSF flow, the needle was anchored using a micro drive mechanism. The Hamilton syringe was disconnected from the tubing, and the tip of the tubing was sealed with a heated hemostat. After a baseline

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**Figure 2**—Effects of intravenous (IV) and intracerebroventricular (ICV) injections of hypocretin-1 on wake and sleep in control and genetically narcoleptic dogs. (A) Typical 6-hour hypnograms obtained in control and Hcrtr2-mutated narcoleptic Dobermans after IV (upper left panel) and ICV (lower left panel) administration of hypocretin-1. In each hypnogram, the levels of vigilance (ie, wake, rapid eye movement [REM] sleep, drowsy, light sleep, and deep sleep) are displayed from top to bottom. (B) Percentage changes in wake amounts after IV (upper right) and ICV (lower right) administration of hypocretin-1in 2 control and 4 genetically narcoleptic dogs (dose response in 2 sessions for each animal when only 2 animals are included). Data are expressed as mean ± SEM.
JECT session, 2 mm of the tip of the tubing was cut and connected to a 250-µL Hamilton syringe, and the constant CSF flow was confirmed again on each experiment day. The syringe was switched to a 25-µL Hamilton glass syringe, and 25 µL of test solution (artificial CSF or 30, 60, or 120 nmol per dog of hypocretin-1 solution) was injected into the lateral ventricle followed by an injection of 25 µL of artificial CSF. The test solution was injected over a 5-minute period. The Hamilton syringe was disconnected from the tubing, and the tip of the tubing was again sealed with a heated hemostat. The order of injections of each test solution was randomized, and the injection was performed once a week. The mean results of 2 dogs (1 session for each animal) are presented.

**Effect of ICV Administration of Hypocretin-1 on Vigilance States in Hcrtr2-mutated and Control Dobermans.**

Two genetically narcoleptic female and 2 control male Dobermans were used for this study. Experiment protocols for polygraph recordings were the same as those described above. The animal was brought to the experiment room, and artificial CSF or 10 nmol per dog or 30 nmol per dog of hypocretin-1 solution (25 µL) was injected 5 minutes before starting the sleep recording. Injections were separated by 2 days, and the order of the test solution was randomized. Two polygraphic recording sessions (artificial CSF, 10 nmol per dog, and 30 nmol per dog) were carried out for each animal, and a total of 4 polygraph sessions were analyzed for each diagnostic group.

**Assessment of Central Penetration of IV Administration of Hypocretin-1 in Narcoleptic Dobermans**

To assess penetration of hypocretin into the central nervous system, changes in hypocretin levels in the CSF of 2 narcoleptic Dobermans were measured after IV administrations of hypocretin-1. The dogs were injected with saline and 24 µg/kg, 96 µg/kg, and 386 µg/kg of hypocretin-1, and cisternal CSF taps were carried out 30 minutes after injection under general pentobarbital anesthesia (20 mg/kg IV, induction was 20 minutes after the drug administration). Three consecutive 2-mL CSF fractions were collected separately, and the mean hypocretin-1 level in the 3 fractions was determined.

Ten milliliters of blood collected with EDTA were also taken before and 30 minutes after injection, the plasma of which was used for the hypocretin-1 measurements in the blood.

Two consecutive CSF taps were carried out at least 1 month apart. Detailed methods for CSF taps are described elsewhere. The hypocretin-1 levels in the CSF and plasma were measured with a direct assay using a commercially available hypocretin-1 radioimmunoassay kit (Phoenix, Belmont, Calif).

**Acute Effect of IV Administration of Hypocretin-1 on Cataplexy in a Sporadic Narcoleptic Dog**

The acute effects of the IV administration of hypocretin-1 on cataplexy were also assessed in a sporadic hypocretin-deficient dog. After baseline FECTs, IV saline or hypocretin-1 at doses of 3, 6, 24, 48, 96, 192, and 384 µg/kg were administered, and effects on cataplexy were evaluated at 5, 15, 30, and 60 minutes after injection. Experiments were repeated 3 times for each dose, and the mean result of the 3 sessions are presented.

**Statistical Analysis**

Data are presented as mean ± SEM. Cataplexy and sleep data were transformed (rank or log) to obtain homogeneity of variance within groups (Bartlett test) before analysis of variance (ANOVA) was performed. For time-course experiments on cataplexy, significance (P < .05) of drug effects were compared with control values (saline injection) using repeated measures ANOVA (drug dose as a grouping factor). For dose-dependent effects of IV and ICV administration of hypocretin-1 on each sleep parameter and effects of repeated IV administration on cataplexy, significance of drug effects were assessed using a repeated measures 1-way ANOVA. Statistical sig-
nificance of the effect of the drug at individual time points was assessed by posthoc comparisons. All computations were performed using SYSTAT 5.2® (Systat Inc., Evanston, Ill) on an Apple G4 computer.

RESULTS

Effect of a Single and Repeated IV Administration of Hypocretin-1 on Cataplexy in Hcrtr2-mutated Dobermans

A single IV administration of hypocretin-1 at doses ranging from 1 µg/kg to 6 µg/kg had no significant effect on cataplexy in 6 narcoleptic Dobermans in time and time x treatment (see Figure 1A for ET and NA, data not shown for time spent in cataplexy). Similarly, repeated IV administration of 3 µg/kg of hypocretin-1 for 3 consecutive days had no effect on cataplexy (ET: F7, 35 = 1.9, P = .11; NA: F7, 35 = 0.75, P = .63) (Figure 1B). In addition, a higher-dose (24 µg/kg) was administered in 4 narcoleptic dogs, and no significant changes in cataplexy were observed (ET: F4,12 = 0.46, P = .77; NA: F4,12 = 1.07, P = .41). No changes in appetite, behavior, or heart rate were observed during and after drug injections.

Effect of IV Administration of Hypocretin-1 on Vigilance States in Hcrtr2-mutated Dobermans

Typical hypnograms obtained from 6-hour polygraph recordings as well as mean changes in wake amounts (4 sessions in Hcrtr2-mutated narcoleptic and control Dobermans) are shown in Figure 2A. No effects on amounts of any vigilance stage were observed after IV injection of hypocretin-1 at 3 µg/kg and 6 µg/kg in control or narcoleptic dogs either during the initial 4-hour or 6-hour period or during any of the 2-hour bin periods. Wake, sleep, and sleep fragmentation (mean duration of episodes for each vigilance stage) did not change statistically either during the initial 4-hour or 6-hour period or during any 2-hour bin periods.

Effect of ICV Administration of Hypocretin-1 on Vigilance States in Hcrtr2-mutated and Control Dobermans

Typical hypnograms obtained from 6-hour polygraph recordings after ICV administration of hypocretin-1 in control and narcoleptic dogs are shown in Figure 2B. The ICV administration of hypocretin-1 dose dependently increased wakefulness in control dogs (F2, 6 = 32.1, P < .001). Hypocretin-1 also significantly reduced REM sleep (by 72% at 30 nmol, F2, 6 = 5.3, P < .05) and slow wave sleep (by 67% at 30 nmol, F2, 6 = 32.1, P < .001). The wake-promoting effect of the ICV administration of hypocretin-1 in control animals (30 nmol per dog) was very potent and equivalent to the effect of the IV administration of 200 µg/kg of amphetamine (see Shelton et al1). Incidentally, it was also noted that control dogs tended to drink more water in the first 2 hours after ICV injection, but this effect was not formally assessed.

In contrast, the ICV administration of hypocretin-1 did not significantly change wake (F2, 6 = 2.72, P = .14) or slow wave sleep (F2, 6 = 3.44, P = .10) in Hcrtr2-mutated narcoleptic dogs. Of note, REM sleep was reduced by 40% at 30 nmol, a reduction that is statistically significant (F2, 6 = 6.23, P < .05).

Effect of ICV Administration of Hypocretin-1 on Cataplexy in Hcrtr2-mutated Dobermans

Time courses of mean values for FECT, ET, and NA after ICV administration of hypocretin-1 in Hcrtr2-mutated narcoleptic Dobermans are shown in Figure 3. Although we could only carry out 1 session for each animal, no effect on cataplexy was seen in either dog. The highest ICV dose injected (120 nmol per dog) was 4 times greater than the total amount of hypocretin-1 used for the IV dose at 3 µg/kg, but no change in cataplexy was observed even at this dose. Additionally, no changes in appetite, behavior, or heart rate were noticed in these animals.

Central Penetration of Hypocretin-1 in Dogs After IV Injection

The IV administration of hypocretin-1 rapidly increases plasma hypocretin-1 from undetectable (< 40 pg/mL) to 12 x 10^5 pg/mL (Figure 4A). In contrast, we observed no significant increase in hypocretin-1 levels in the CSF at the dose range we used for the behavior-protocol studies. We observed that higher doses (96 to 384 µg/kg) of the IV administration of hypocretin increased hypocretin-1 levels in the CSF to levels up to 2.4 fold, but this increase was small when compared to that observed in plasma (Figure 4B).

Acute Effect of IV Administration of Hypocretin-1 on Cataplexy in a Sporadic Narcoleptic Dog

Similar to results observed in Hcrtr2-mutated narcoleptic Dobermans, the IV administration of hypocretin-1 in doses up to 24 µg/kg did not induce any change in cataplexy in the ligand-deficient sporadic dog (Figure 5A). Interestingly, doses higher than 48 µg/kg of hypocretin-1 had a very short-lasting anticaataplectic effect (less than 30 minutes) (NA, Time Effect: F2, 6 = 7.24, P < .001; Time x Treatment: F2, 6 = 1.26, P = .21) (Figure 5B). The suppression of cataplexy was dose dependent, and in 2 out of 3 sessions that administered 384 µg/kg, cataplexy was completely suppressed 5 minutes after the injection (Figure 5C). No
changes in appetite, behavior, or heart rate were observed during after drug injections.

**DISCUSSION**

John et al. previously reported that a single IV administration of 3 µg/kg of hypocretin-1 significantly reduced, while a dose of 4µg/kg significantly aggravated, cataplexy in Hcrtr2-mutated narcoleptic Dobermans. They also reported that IV administration of hypocretin-1 at 3 µg/kg significantly reduced REM sleep and improved sleep fragmentation in narcoleptic Dobermans. Two possible mechanisms were proposed for these observations. First, John et al argued that despite mutations in the Hcrtr2 in dogs, there may be sufficient receptor activity for responding to a high dose of exogenous hypocretin-1. Second, they argued that some of their observed effects could be due to hypocretin-1 acting at the hypocretin receptor-1, which is abundant in the pontine locus coeruleus (LC), or acting at other unidentified hypocretin receptors. Their first possibility can now be excluded because our recent studies have shown that mutated receptors do not localize to the cell surface and are not functional in vitro by calcium-mobilizing assessment, as well as receptor- and hypocretin-stimulated [35S]γ-GTP bindings in Hcrtr2-mutated expressed cell line.9,10 With regards to the possibility of hypocretin receptor-1 or other hypocretin-receptor mediation for the effect, this hypothesis is also difficult to dispose, as central penetration of hypocretin-1 at the IV doses used was not assessed, and the effects of centrally administered hypocretin-1 in Hcrtr2-mutated animals were not examined in these animals.

Contrary to the results observed by John et al, we did not observe any significant effects of IV administration of hypocretin-1 on cataplexy and sleep in Hcrtr2-mutated Dobermans at doses up to 6 µg/kg. A higher IV dose (24 µg/kg) was also tested in 4 animals, but again no significant effect on cataplexy was observed. In addition, repeated IV administration of hypocretin-1 (3 µg/kg) for 3 consecutive days did not induce any change in cataplexy severity, which is also not consistent with the result reported by John et al.9 We studied the possibility that these discrepancies were due to the quality of the peptides used in these studies. We tested hypocretin-1 from 2 different commercial sources and found no differences. Along with these behavioral assays, central penetration of hypocretin-1 was estimated by measuring hypocretin-1 in the CSF after IV administration of hypocretin-1. Our results indicate that a small portion of the intravenously administered hypocretin-1 can penetrate the brain, as has been previously reported in rats.15,16 Of note, however, is that the large increase in the hypocretin-1 levels in the CSF was observed only after the highest IV dose of hypocretin-1 (384 µg/kg) was injected. Several groups have reported that IV administration of hypocretin-1 produces significant increases in arousal.15-16 We found that IV administration of hypocretin-1 (10 nmol per dog and 30 nmol per dog) had significant wake-promoting effects in control Dobermans. The wake-promoting effects of hypocretin-1 were very potent and comparable to those observed after IV administration (200 µg/kg) of d-amphetamine,17 a classic stimulant used for the treatment of human narcolepsy. In contrast, Hcrtr2-mutated narcoleptic Dobermans did not react to the IV administration of hypocretin-1. There was no change in any sleep-wake patterns, except a moderate reduction in REM sleep (when up to 30 nmol was given per dog). The IV dose for cataplexy testing was increased to 120 nmol per dog, but no effect in cataplexy was observed. The dose we used for IV administration was very high, with 30 nmol per brain roughly corresponding to the amounts used in the systemic IV administration of 3 µg/kg. Based on these results, we conclude that Hcrtr2-mutated narcoleptic Dobermans do not respond to hypocretin supplementation. This result is in agreement with our previous in vitro studies showing lack of biologic activity of the mutated receptors.9,10 Several authors have suggested that the wake-promoting and REM-suppressing effect of hypocretins are mediated through the activation of LC neurons, since hypocretins significantly excite adrenergic LC neurons in vitro and vivo.16,18-20 The LC neurons express hypocretin recep-

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**Hypocretin receptor-1, while histaminergic tuberomammillary neurons express hypocretin-receptor-2 exclusively.21 The hypocretin receptor-2 is also enriched in the basal forebrain cholinergic and dopaminergic midbrain, while the serotonergic raphe nuclei express both receptors.21 Considering the fact that the wake-promoting effect of the IV administration of hypocretin-1 was abolished in Hcrtr2-mutated Dobermans, it is questionable that the wake-promoting effects of the IV administration of hypocretin-1 are mediated by the activation of LC neurons. The amount of REM sleep was moderately reduced, but due to the low baseline REM amount under our experiment conditions (0.8% of recording time), it is difficult to conclude whether hypocretin receptor-1 plays a significant role in REM-sleep regulation.

We have previously demonstrated that hypocretin-containing neurons in Hcrtr2-mutated Dobermans are intact, with no change in morphology, number, or distribution.7 Hypocretin peptide levels in the brain and in the CSF of adult Hcrtr2-mutated narcoleptic Dobermans are comparable to those of control Dobermans.7 Hypocretin receptor-1-mediated function is thus probably intact in these animals yet is not sufficient to compensate for the Hcrtr2-mutated narcolepsy phenotype. The role of each hypocretin receptor has also been analyzed using hypocretin receptor-1 gene (Hcrtr1) knockout, Hcrtr2 knock-out, and double-receptor knockout mice.22 These manipulations indicate that hypocretin receptor-1 status could also influence the narcolepsy phenotype, since double-receptor knockout mice are more severely affected with narcolepsy than are Hcrtr2 knockout mice.23 Double-receptor knockout mice were also found to exhibit similar symptoms as prepseudohypocretin knockout mice. However, Hcrtr1 knockout mice exhibit only a mild sleep abnormality and do not exhibit cataplexy, suggesting a more critical role of hypocretin receptor-2-mediated function for the narcolepsy phenotype. This may explain why hypocretin supplementation, even when using central administration, did not improve the narcolepsy phenotype in Hcrtr2-mutated dogs and further confirms the critical role of hypocretin receptor-2 in the control of sleep and sleep-related motor function. It is possible, however, that individual receptors have different functions in different species.

The fact that the IV or ICV administration of hypocretin is inactive in Hcrtr2-mutated dogs does not mean that hypocretin supplementation is likely to be inactive in human narcolepsy. Studies in human narcolepsy have shown that despite the fact that hypocretin-gene mutations are rare in human cases, functional loss of hypocretin ligands is observed in most human cases. Similarly, although primarily Hcrtr2-mutated familial cases of canine narcolepsy have been studied in the laboratory, a sporadic form of the disease with hypocretin deficiency equivalent to the human disease has been observed in 17 breeds (Nishino et al,1 Baker et al,21 and unpublished observations). In this study, we used a male Schipperke with hypocretin deficiency to test the effect of intravenously administered hypocretin on narcolepsy symptoms (ICV experiments were not permitted, see methods). A short-lasting anticitaplectic effect was observed after high doses of hypocretin (48 - 384 µg/kg), suggesting the possibility of therapeutic effects. These effects may be centrally mediated, since increased CSF hypocretin-1 levels were observed after high IV doses of hypocretin-1 were administered (Figure 4). However, the effects were extremely short lasting and could be secondary to peripheral effects. Hypocretin receptors have been identified in the peripheral organs,24 and the functional roles of these receptors are not yet fully understood.

Further studies are needed to examine whether hypocretin-replacement therapy is feasible at higher doses in hypocretin-deficient patients. Additionally, the functionality of hypocretin receptors in patients who have had hypocretin deficiency for decades may also have to be tested before proceeding. Our results, however, clearly suggest that more stable, centrally active hypocretin analogs need to be developed for hypocretin-supplement therapy to be a viable alternative.
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