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Plasma total ghrelin and leptin levels in human narcolepsy and matched healthy controls: Basal concentrations and response to sodium oxybate

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ABSTRACT

Study objectives: Narcolepsy is caused by a selective loss of hypocretin neurons and is associated with obesity. Ghrelin and leptin interact with hypocretin neurons to influence energy homeostasis. Here, we evaluated whether human hypocretin deficiency, or the narcolepsy therapeutic agent sodium oxybate (SXB), alter the levels of these hormones.

Methods: Eight male, medication free, hypocretin deficient, narcolepsy with cataplexy patients, and 8 healthy controls matched for age, sex, body mass index (BMI), waist-to-hip ratio, and body fat percentage were assessed. Blood samples of total ghrelin and leptin were collected over 24 hours at 60 and 20-min intervals, respectively, during 2 study occasions: baseline, and during the last night of 5 consecutive nights of SXB administration (2 × 3.0 g/night).

Results: At baseline, mean 24-h total ghrelin (936 ± 142 vs. 949 ± 175 pg/mL, $P = 0.873$) and leptin (115 ± 5.0 vs. 79.0 ± 32 mg/L, $P = 0.18$) levels were not different between hypocretin deficient narcolepsy patients and controls. Furthermore, SXB did not significantly affect the plasma concentration of either one of these hormones.

Conclusions: The increased BMI of narcolepsy patients is unlikely to be mediated by hypocretin deficiency-mediated alterations in total ghrelin or leptin levels. Thus, the effects of these hormones on hypocretin neurons may be mainly unidirectional. Although SXB may influence body weight, the underlying mechanism is unlikely to involve changes in total ghrelin or leptin secretion.

INTRODUCTION

The hypocretin system, also known as the orexin system, is of major importance in the regulation of sleep and sustained wakefulness. Moreover, hypocretin neurons are responsive to metabolites and hormones helping to translate signals of metabolic state into adaptive levels of activity and consciousness.[156;157] Hypocretin deficiency leads to narcolepsy, a sleep-wake disorder characterized by excessive daytime sleepiness, cataplexy, and disrupted nocturnal sleep. Obesity is associated with the disorder,[18] yet the cause of the increased body weight has been challenging to discern due to inconsistent findings on the hormonal and metabolic characteristics of this population.[22;30;31;33;81;151] However, altered ingestive behavior has been observed in these patients,[81;158-160] suggesting hypocretin deficiency may dysregulate feeding behavior, and possibly energy homeostasis.

Ghrelin is a peptide hormone mainly produced by endocrine cells in the stomach and gastrointestinal tract, and is an important endogenous regulator of energy balance and growth hormone (GH) secretion.[161] Its expression is complex89 and influenced by sympathetic nervous system activity.[90] Across the wake period, plasma concentrations wax and wane episodically, providing an appetite-stimulating signal to the brain.[91] During sleep, ghrelin levels rise sharply in the early part of the night and decrease gradually toward morning. During sleep deprivation, however, levels gradually rise toward a plateau in the morning.[162] Hypocretin neurons directly sense and are excited by ghrelin, and an interaction between these two systems has been shown to be involved in ingestive behavior.[87] A study by Toshinai et al. first identified this connection.[92] In that study, ghrelin-induced feeding was attenuated in rats pretreated with anti-hypocretin-1 IgG and anti-hypocretin-2 IgG, and suppressed in hypocretin knockout mice. Later, it was demonstrated that ghrelin plays a key role in the rewarding aspects of eating, but it requires the presence of intact hypocretin signaling to impart this effect.[163]

Leptin is another peptide hormone involved in energy homeostasis, the dominant role of which is to signal energy deficiency to the brain.[164] It is an adipokine produced primarily by subcutaneous white adipose tissue, and its expression is stimulated by various hormones, sympathetic outflow, energy intake, and output.[164;165] Under normal conditions, blood levels display circadian variation as levels rise across the day and peak in the middle of the night.[166] During sleep deprivation, blood leptin levels show a reduced and flattened profile.[167] Receptors for leptin are found on hypocretin cells,[86] and leptin can directly inhibit the expression of isolated hypocretin neurons.[87] Indirectly, leptin can affect the activity of hypocretin cells via energy regulating neurons in the arcuate nucleus of the

hypothalamus.[88] Conversely, because the hypocretin system greatly influences autonomic control,[156] it is plausible that hypocretin deficiency may alter leptin expression via inhibited sympathetic activity. Indeed, obese hypocretin deficient mice have lowered sympathetic vasoconstrictor outflow, while greater heart rate variability has been observed in hypocretin deficient narcolepsy patients.[168;169] Thus, leptin and hypocretin may interact to affect levels of physical activity and wakefulness in response to energy needs, and the loss of hypocretin neurons may dysregulate leptin expression and signaling. While ghrelin levels have not been previously reported in hypocretin-deficient narcoleptic patients, abnormal leptin levels have been observed.[30;31] It is unknown if the associations between hypocretin and total ghrelin or leptin are uni-or bi- directional. Because hypocretin influences sympathetic outflow and sympathetic nervous system activity affects the expression of both leptin and ghrelin, hypocretin deficiency may lead to altered levels of these hormones. This study of hypocretin deficient narcoleptic patients provides a unique opportunity to further explore the nature of these relationships. We hypothesized that both total ghrelin and leptin levels would be abnormal in hypocretin-deficient narcolepsy patients, which might help explain the increased BMI and abnormal ingestive behavior seen in this population.[22;30;81;149;170;171]

Additionally, we explored if the narcolepsy therapeutic agent, sodium oxybate (SXB), has an effect on these hormones. In a narcolepsy population, SXB improves disrupted nocturnal sleep, impaired wakefulness, and cataplexy, and promotes weight loss.[74;172] Like ghrelin, SXB administration also stimulates GH release.[75] We hypothesized that its administration would alter total ghrelin levels, the effect of which might be involved in its GH-promoting effects. Here, we investigate whether total blood ghrelin or leptin levels are altered in hypocretin-deficient narcoleptic patients compared to controls, and whether total ghrelin or leptin levels are influenced by SXB.

MATERIALS AND METHODS

Subjects

We included 8 medication-free, male hypocretin-deficient narcolepsy with cataplexy patients and 8 healthy male controls, matched for age, BMI, and body fat percentage. Hypocretin measurement was performed according to international standards.[46] Body fat percentage was measured with bioelectrical impedance analysis (Bodystat, Douglas, Isle of Man, UK). Two patients were drug naive, one patient was tapered from antidepressants ≥ 2 weeks prior

to the study, and 2 patients had prior history with SXB; however, no subject took sodium oxybate within 20 days of study initiation. The other patients did not take any medication for at least several months prior to beginning the study.

Subjects were eligible for participation after exclusion of chronic conditions, with particular attention to the absence of sleep disorders in control subjects, hypertension, pituitary disease, and weight change (> 3 kg weight gain or loss within the last 3 months) as assessed by structured clinical interview. None of the participants had previously undergone gastrectomy. Written informed consent was obtained from all subjects. The study was approved by the ethics committee of the Leiden University Medical Center.

Clinical protocol

All subjects were admitted to the Clinical Research Center for 24-h blood sampling before and after 5 days of SXB administration. A cannula was inserted into an antecubital vein ≥ 45 min before the start of blood sampling at 12:00. Blood samples were collected with S-Monovette (Sarstedt, Etten-Leur, The Netherlands) from a 3-way stopcock that was attached to a 0.9% NaCl and heparin (1 U/mL) infusion (750 mL/24 h) to keep the cannula from clotting. For total ghrelin measurements, blood was collected in EDTA tubes at 60-min intervals, and these tubes were immediately put on ice. Ghrelin samples were acidified with 50 µL of 1 N HCL. Within 5 min of sampling, tubes were centrifuged at 1,250 *g* at 4°C for 20 minutes. For leptin measurements, blood was collected at 20-min intervals. After clotting, the blood was centrifuged within 30 min of sampling (20 min, 1,250 *g*, 4°C). Serum was then stored at -80°C until hormonal assays. Three standardized meals were served at 08:30, 13:00, and 18:00 (Nutridrink, 1.5 kcal/mL, 2,100 kcal/d; macronutrient composition per 100 mL: protein, 6 g; fat, 5.8 g; carbohydrate, 18.4 g; Nutricia, Zoetermeer, The Netherlands). Subjects were asked to complete each meal provided. Food-induced suppression of total ghrelin release was defined as the ratio between total ghrelin levels one hour post- prandially to the levels immediately before the meal (lunch and dinner) or 30 min postprandially to 30 min before the meal (breakfast). Subjects remained sedentary except for bathroom visits. In both study occasions, lights were switched off (dark period) at 23:00 and then switched on at 07:30.

Sodium oxybate

In the drug-intervention study occasion, SXB was administered in a total nightly dose of 6 grams/night for 5 consecutive nights in both the narcoleptic patients and the controls. Each

night, 3 grams of SXB were administered orally at 23:00 and 03:00. Lights were turned off after ingestion of the first dose.

Assays

Plasma total ghrelin and leptin levels were measured by radioimmunoassay (LINCO Research, St. Charles, MO, USA) with a detection limit of 93 pg/mL, and an interassay variation ranging from 14.7% to 17.8% for total ghrelin and a detection limit of 0.5 μg/L and an interassay variation ranging from 3.0% to 5.1% for leptin. Samples from each patient and matched control were handled in the same run.

Deconvolution analysis

Leptin concentration time series were analyzed via a recently developed automated deconvolution method, empirically validated using hypothalamo-pituitary sampling and simulated pulsatile time series.[121] The Matlabbased algorithm first detrends the data and normalizes concentrations to the unit interval [0, 1]. Second, the program creates multiple successive potential pulse-time sets, each containing one fewer burst via a smoothing process (a nonlinear adaptation of the heat-diffusion equation). Third, a maximum-likelihood expectation estimation method computes all secretion and elimination parameters simultaneously conditional on each of the multiple candidate pulse-time sets. The fast half-life was represented as 3.4 min constituting 19% of the decay amplitude. The slow half-life was estimated as an unknown variable between 6 and 70 min. Here we present only results for pulse frequency (pulses per 24 h), basal secretion, pulsatile secretion, and total secretion per 24 h, all expressed as μg per liter distribution volume.

Data analysis and statistics

Results are expressed as mean ± SD unless otherwise specified. Unpaired t-tests were used to assess differences in means between the 2 groups, while paired t-tests were applied to assess changes in means within each group. All tests were 2-tailed, and significance level was set at $P < 0.05$. Statistical analyses were performed using SPSS for Windows (release 17.0, SPSS, Inc., Chicago, IL).

RESULTS

Subjects

Patients and controls did not differ with respect to age, BMI, waist-to-hip ratio, and body fat percentage (Table 4.1). SXB was well tolerated by all participants. Apart from mild drowsiness, no other side effects were reported during the study.

Sleep and wakefulness differences

When compared to controls, during baseline conditions and after SXB administration, narcolepsy patients spent significantly less time awake across a 24 h period, and during the day (defined as the lights-on period between 07:30-23:00) they spent less time awake and more time in slow wave sleep ([SWS] $P = 0.004$ and $P = 0.005$, respectively; Table 4.2).

Effect of sodium oxybate administration on sleep and wakefulness

In both groups, administration of SXB resulted in a significant decrease in stages I/II NREM and REM sleep over 24 h ($P = 0.011$ and $P = 0.009$, respectively), while at night, awakenings were significantly reduced ($P = 0.002$) and the percentage of SWS more than doubled (narcolepsy: 6.5% ± 5.5% vs.16.5% ± 8.4%, controls: 7.1% ± 5.5% vs. 18.5% ± 6.4%; $P = 0.001$ for administration effect). During the day, time spent in stages I/II NREM and REM sleep ($P = 0.038$ and $P = 0.041$, respectively) was reduced, while there was a trend towards longer periods of wakefulness ($P = 0.098$).

Table 4.1 Demographics, body composition, baseline parameters

	Patients	Controls	*P*-value
Age (yrs)	38.0 ± 4.7	37.9 ± 4.1	0.98
BMI (kg/m^2)	28.1 ± 1.6	27.4 ± 1.4	0.74
Waist/hip ratio	0.92 ± 0.03	0.90 ± 0.02	0.58
Body fat (%)	23.6 ± 2.1	23.4 ± 1.7	0.95

Data are shown as mean ± SEM.

Table 4.2 Sleep patterns before and after SXB administration

	Narcolepsy		Controls		Narcolepsy vs. controls (Baseline)	Narcolepsy vs. controls (SXB)	Treatment effect	Interaction (Group × Treatment)
	Baseline	SXB	Baseline	SXB				
Wake total (%)	60.8 ± 2.9	60.8 ± 2.2	68.7 ± 2.0	70.1 ± 2.4	0.044*	0.013*	0.58	0.57
Wake day (%)	79.4 ± 4.2	82.9 ± 3.2	95.6 ± 2.1	97.3 ± 1.0	0.004**	0.001**	0.098	0.60
Wake night (%)	25.8 ± 5.7	19.2 ± 4.3	18.4 ± 4.0	19.2 ± 5.8	0.31	1.00	0.40	0.087
Stage I/II total (%)	29.1 ± 1.4	26.3 ± 1.4	25.0 ± 2.4	21.1 ± 2.2	0.16	0.063	0.011*	0.62
Stage I/II day (%)	14.6 ± 3.0	11.1 ± 2.5	2.5 ± 1.6	1.6 ± 1.0	0.003**	0.005**	0.038*	0.23
Stage I/II night (%)	55.1 ± 2.5	53.5 ± 3.7	65.6 ± 5.7	56.4 ± 5.3	0.11	0.65	0.056	0.13
SWS total (%)	3.7 ± 0.7	7.6 ± 1.2	2.5 ± 0.7	6.6 ± 0.9	0.24	0.53	0.001**	0.90
SWS day (%)	2.1 ± 0.6	2.7 ± 1.1	0.03 ± 0.03	0.05 ± 0.05	0.005**	0.041*	0.49	0.56
SWS night (%)	6.5 ± 1.9	16.5 ± 3.0	7.1 ± 1.9	18.5 ± 2.4	0.84	0.61	0.001**	0.76
REM total (%)	6.3 ± 1.8	4.7 ± 1.0	3.7 ± 0.8	2.1 ± 0.8	0.19	0.070	0.009**	0.93
REM day (%)	2.9 ± 1.4	1.2 ± 0.5	0.8 ± 0.5	0.0 ± 0.0	0.20	0.032*	0.041*	0.51
REM night (%)	12.6 ± 3.0	10.8 ± 2.1	8.8 ± 1.8	5.8 ± 2.3	0.31	0.13	0.063	0.53
No. of awakenings	50.5 ± 10.5	35.0 ± 4.8	35.5 ± 7.1	15.3 ± 1.7	0.26	0.005**	0.002**	0.85
Sleep efficiency (%)	66.9 ± 7.0	81.5 ± 4.9	81.2 ± 4.0	81.9 ± 6.0	0.10	0.96	0.082	0.06

Data are shown as mean ± SEM. Percentages of sleep stages during the 24 h of study, before and after SXB administration. Unpaired t- tests were used to assess differences between the 2 groups. Mixed-effects models were applied to assess the effect of treatment and potential interaction effects between group (i.e. narcolepsy or control) and treatment. * $P < 0.05$ and ** $P < 0.01$.

Baseline total ghrelin levels

Mean 24-h total ghrelin levels at baseline were virtually identical between narcolepsy patients and controls (P = 0.873; Figure 4.1A). Mean total ghrelin levels were also not different between the 2 groups when the analyses were restricted to the dark period (P = 0.973). In fact, at no single time-point an intergroup difference could be detected (all $P \geq 0.232$). Food induced suppression of total ghrelin concentration (expressed as the ratio between postprandial to preprandial total ghrelin concentration) was similar in the 2 groups (lunch: P = 0.413, dinner: P = 0.301, breakfast: P = 0.437, and mean postprandial total ghrelin levels averaged over the 3 occasions [P = 0.540]) (Table 4.3).

Effect of sodium oxybate on total ghrelin levels

Twenty-four hour mean total ghrelin levels during SXB administration were not different between narcolepsy patients and controls (P = 0.642; Figure 4.1B). Similar to baseline, mean

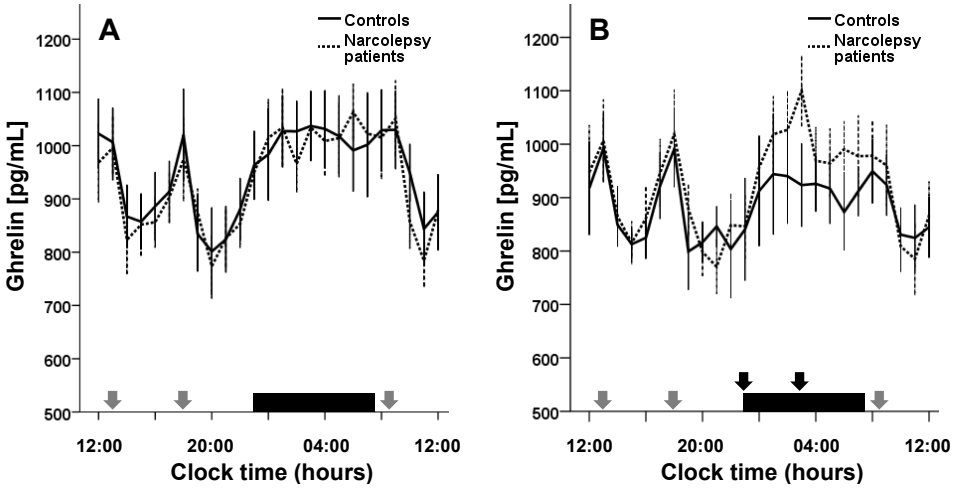

Figure 4.1 Mean 24-h ghrelin levels in narcolepsy patients and matched controls. The diurnal plasma ghrelin levels, as well as food induced suppression of ghrelin release were not significantly different between narcolepsy patients and matched controls, either during basal conditions **(A)** or after five days of sodium oxybate administration **(B)**. Hourly blood sampling started at noon and continued for 24 hours. The black bar on the abscissa indicates the dark period (23:00-7:30 h). The grey arrows indicate the timings of the lunch, dinner and breakfast at 13:00 h, 18:00 h and 08:30 h, respectively. The black arrows indicate the timings of sodium oxybate administrations during the second occasion at 23:00 h and 03:00 h. Error bars show the means ± SD.

Table 4.3 Plasma ghrelin concentrations and deconvolution of leptin levels before and after administration of sodium oxybate in both narcoleptic patients and controls

	Baseline			Sodium oxybate		
	Patients	Controls	*P*	Patients	Controls	*P*
Ghrelin						
24-h total integrated concentration (pg/mL)	936 ± 142	949 ± 175	0.873	920 ± 142	886 ± 150	0.642
Dark period[a] (pg/mL)	1012 ± 156	1009 ± 196	0.973	983 ± 163	910 ± 211	0.449
Food induced suppression of ghrelin concentration[b] (pg/mL)						
Lunch	0.83 ± 0.10	0.86 ± 0.09	0.413	0.87 ± 0.08	0.88 ± 0.16	0.920
Dinner	0.93 ± 0.16	0.83 ± 0.17	0.301	0.89 ± 0.20	0.80 ± 0.10	0.261
Breakfast	1.05 ± 0.10	1.01 ± 0.09	0.437	0.98 ± 0.12	0.98 ± 0.06	0.880
Postprandial total ghrelin[c] (pg/mL)	0.93 ± 0.08	0.90 ± 0.11	0.540	0.91 ± 0.08	0.88 ± 0.06	0.428
Leptin						
Total 24-h secretion (µg/Lx24h)	115 ± 98	79.0 ± 88	0.18	100 ± 113	64.0 ± 35	0.58
Basal 24-h secretion (µg/Lx24h)	64.7 ± 63	37.9 ± 30	0.96	56.0 ± 70	47.6 ± 63	0.94
Pulsatile 24-h secretion (µg/Lx24h)	50.3 ± 36	25.6 ± 11	0.11	43.8 ± 46	31.0 ± 27	0.29
Pulse frequency (no/24h)	18.5 ± 2.7	15.3 ± 4.8	0.04	19.8 ± 2.4	19.0 ± 3.0	0.04

[a] In both study occasions, lights were switched off (dark period) at 2300 h and then switched on at 0730 h.
[b] Expressed as the ratio between post- to preprandial ghrelin concentration.
[c] Averaged over three occasions.

total ghrelin levels during the dark period did not differ between the 2 groups ($P = 0.449$), and at no single time-point a difference could be detected between groups (all $P \geq 0.05$). Postprandial total ghrelin suppression, as defined above, was also similar between the 2 groups after SXB administration: lunch ($P = 0.920$), dinner ($P = 0.261$), and breakfast ($P = 0.880$); mean postprandial total ghrelin levels averaged over the 3 occasions ($P = 0.428$) (Table 4.3). The average change in 24-h total ghrelin levels between the second and first occasion amounted to -15 ± 72 pg/mL in narcolepsy patients and -63 ± 87 pg/mL in controls, but was not significantly different from zero in either group (paired t-tests: $P = 0.56$ and $P = 0.078$, respectively).

Baseline leptin levels

Mean 24-h total leptin levels at baseline were not significantly different between narcolepsy patients and controls ($P = 0.18$; Figure 4.2A). Mean pulse frequency was different between the 2 groups ($P = 0.04$), but mean 24-h basal and pulsatile secretion levels were not different ($P = 0.96$; $P = 0.11$, respectively; Table 4.3).

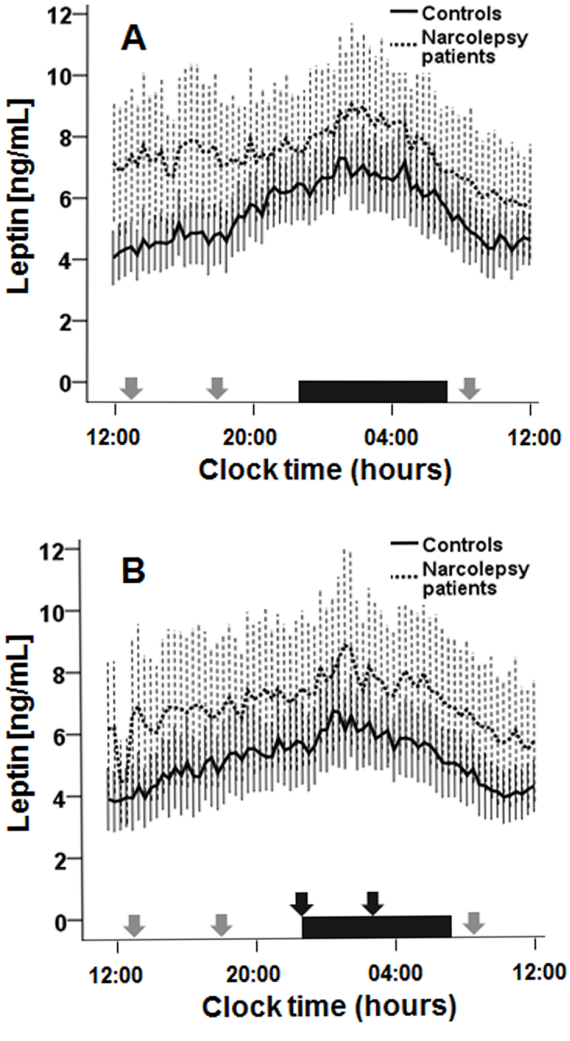

Figure 4.2 Mean 24-h plasma leptin concentration ± SD, before **(A)** and during sodium oxybate administration **(B)** in narcolepsy patients and matched controls. The black horizontal bar on the abscissa indicates the lights off period. The grey arrows indicate the timing of meals and the black arrows indicate timing of sodium oxybate administration **(B)**.

Effect of sodium oxybate on leptin levels

Mean 24-h total leptin levels during SXB treatment were not significantly different between narcolepsy patients and controls ($P = 0.58$; Figure 4.2B); neither were mean 24-h basal and pulsatile secretion rates ($P = 0.94$; $P = 0.29$, respectively). Mean pulse frequency was different between the 2 groups ($P = 0.04$; Table 4.3).

DISCUSSION

We found no differences in mean 24-h total plasma ghrelin levels or food-induced suppression of ghrelin concentrations between narcolepsy patients and controls, nor any influence of 5 days of SXB administration in both groups. In view of the capacity of ghrelin to stimulate growth hormone secretion, it is worth noting that a report from this same research protocol showed no differences in mean hourly GH levels between patients and controls, supporting our conclusion that total ghrelin levels are not altered with hypocretin deficiency.[173]

Despite the excitatory influence of ghrelin on hypocretin neurons and the interaction of the ghrelin-hypocretin systems to influence food reinforcement, our finding did not show the total ghrelin level to be influenced by hypocretin deficiency, suggesting a unidirectional relationship. These findings also suggest that disturbed ingestive behavior is unlikely mediated by an altered total ghrelin level in narcolepsy patients. Notably, we measured total ghrelin levels and not the biologically active, octanoylated-ghrelin fraction. While there is a high correlation between the total and octanoylated fraction ghrelin level,[174] it remains possible that the active fraction may be altered in this population.

In contrast to earlier reports,[30;31] more recent, larger, controlled studies have not demonstrated an abnormal leptin level in humans with hypocretin deficiency.[32;33] Similar to the recent research on this subject, we found that the mean 24-h total leptin level, and basal and pulsatile secretion levels were not significantly different between narcolepsy patients and controls. The mean leptin pulse frequency was slightly but significantly higher in narcolepsy patients in both conditions, but the clinical relevance of this finding is unclear. Because sleep disruption and insulin resistance have been shown to affect leptin levels,[175] it is plausible that previous investigations showing decreased leptin in narcolepsy may have resulted from a study sample of narcoleptic patients with relatively poor sleep or a difference in insulin sensitivity compared to the control group.

There were several limitations to the study. The small number of patients and controls raise the possibility of a type II statistical error. However, the intergroup differences were very small; therefore, a large sample size would be needed to detect a difference if present. As with many chronic diseases, compensatory mechanisms are likely involved in narcolepsy as the condition progresses from onset into the chronic stage. Studying narcoleptic patients only during the chronic stage challenges the interpretation that loss of hypocretin cells in the hypothalamus does not alter leptin and ghrelin levels since compensatory adaptation may have already taken place. However, alterations in appetite and weight regulation remain present and clinically relevant in the chronic stage of the disease, and therefore our findings remain relevant despite putative compensations. Additionally, since sleep-wake state instability is intrinsic to hypocretin deficiency, standardizing research parameters such as study environment, meal timing and composition, and predefined bedtimes may have created a setting not representative of real-life conditions for these patients. Therefore, although we did not find alterations in total ghrelin and leptin concentrations in this controlled and standardized environment, it remains possible that the release of these hormones is affected by the altered sleep, wake, and eating patterns described in this population.

As expected, in both groups nighttime administration of SXB increased SWS and reduced awakenings, and the narcoleptic patient group showed a trend towards increased wakefulness the following day. As demonstrated in other studies, acute SXB administration corresponds with a significant increase in GH release.[75;173] However, we found no evidence that the GH-elevating effect is mediated through an influence on total ghrelin secretion. Various treatment effects of SXB exhibit discrete temporal dynamics with some effects occurring acutely and other effects taking place only after chronic exposure. Although the difference in total ghrelin levels between patients and controls after SXB administration was not significant, it is possible that significant differences would be seen with higher doses, prolonged periods of nightly administration, or in a larger group of subjects. Lastly, we did not see an effect of SXB on the leptin level, and to our knowledge, an interaction between this drug and hormone has not been reported elsewhere. Therefore, mechanisms underlying increased BMI and altered ingestive behavior in narcolepsy and the effects of SXB administration on GH release and weight loss are unlikely to involve changes in total plasma ghrelin or leptin concentrations. Future investigations should further evaluate if the sleep-wake instability intrinsic to hypocretin deficient narcolepsy promotes ingestive and activity patterns that promote positive energy balance.