Pulsatile LH release is diminished, whereas FSH secretion is normal, in hypocretin-deficient narcoleptic men

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Kok, S. W., F. Roelfsema, S. Overeem, G. J. Lammers, M. Frölich, A. E. Meinders, and H. Pijl. Pulsatile LH release is diminished, whereas FSH secretion is normal, in hypocretin-deficient narcoleptic men. Am J Physiol Endocrinol Metab 287: E630–E636, 2004. First published June 1, 2004; 10.1152/ajpendo.00060.2004.—Hypocretin (orexin) peptides are involved in the regulation of energy balance and pituitary hormone release. Narcolepsy is a sleep disorder characterized by disruption of hypocretin neurotransmission. Pituitary LH secretion is diminished in hypocretin-deficient animal models, and intracerebroventricular administration of hypocretin-1 activates the hypothalamo-pituitary-gonadal axis in rats. We evaluated whether hypocretin deficiency affects gonadotropin release in humans. To this end, we deconvolved 24-h serum concentrations of LH and FSH in seven hypocretin-deficient narcoleptic males (N) and seven controls (C) matched for age, body mass index, and sex. Basal plasma concentrations of testosterone, estradiol, and sex hormone-binding globulin were similar in both groups. Mean 24-h LH concentration was significantly lower in narcolepsy patients [3.0 ± 0.4 (N) vs. 4.2 ± 0.3 (C) μU/ml, P = 0.01], which was primarily due to a reduction of pulsatile LH secretion [23.5 ± 1.6 (N) vs. 34.3 ± 4.9 (C) μU/l·h⁻¹, 24 h⁻¹, P = 0.02]. The orderliness of LH and FSH secretion, quantitated by the approximate entropy statistic, was greater in patients than in controls. In contrast, all other features of FSH release were similar in narcoleptic and control groups. Also, LH and FSH secretions in response to intravenous administration of 100 μg of GnRH were similar in patients and controls. These data indicate that endogenous hypocretins are involved in the regulation of the hypothalmo-pituitary-gonadal axis activity in humans. In particular, reduced LH release in the face of normal pituitary responsivity to GnRH stimulation in narcoleptic men suggests that hypocretins promote endogenous GnRH secretion.

orexin; luteinizing hormone; follicle-stimulating hormone; testosterone; estradiol; circadian rhythm; deconvolution analysis

NARCOLEPSY IS CHARACTERIZED by excessive daytime sleepiness, cataplexy, hypothalamic hallucinations, and sleep paralysis (30). It is caused by the progressive loss of hypocretin neurons in the brain (28, 31). Hypocretin neuronal cell bodies are located exclusively in the lateral hypothalamus/perifornical area (41), but axonal projections throughout the central nervous system predict diverse biological actions (7). Indeed, hypocretin peptides 1 and 2 (also called orexin A and B) appear to affect feeding behavior and energy expenditure, arousal, autonomic outflow, and a variety of neuroendocrine ensembles (9, 10, 43, 49). Therefore, apart from its well-recognized neuroendocrine manifestations, the clinical syndrome of hypocretin deficiency in humans may encompass a broad spectrum of behavioral, endocrine, and metabolic anomalies. In keeping with this postulate, we recently identified aberrations of the pituitary-adrenal and somatotropic ensembles and circulating leptin concentrations in hypocretin-deficient narcoleptic men (15, 17, 29).

The septal preoptic and arcuate nuclei are among the many brain sites receiving hypocretin inputs (7, 26, 32, 46). These nuclei are involved in the control of the hypothalamo-pituitary-gonadal (HPG) axis (50). Furthermore, intracerebroventricular administration of hypocretins acutely induces LH release in ovarian-steroid-primed ovarioctomized rats (37). Also, hypocretin-1 promotes gonadotropin-releasing hormone (GnRH) release in hypothalamic explants from male rats (40), and intracerebroventricular administration of anti-hypocretin antibody completely abolishes the preovulatory LH surge in intact female rats (14). Thus the available data suggest that endogenous hypocretin peptides stimulate LH release in rodents. It is currently unknown whether these peptides exert analogous effects in humans. As far as we are aware, the impact of hypocretins on FSH secretion has not been investigated to date.

The present study was conducted to evaluate the role of endogenous hypocretins in the regulation of gonadotropin release in the human. We specifically hypothesized that hypocretin deficiency in narcoleptic men would blunt spontaneous pituitary LH release.

METHODS

Subjects

We included seven male patients from the outpatient clinic of the Department of Neurology, Leiden University. The diagnosis of narcolepsy with cataplexy was made on clinical grounds by a physician experienced with narcolepsy (G. J. Lammers). Also, the result of a multiple sleep latency test was typical for narcolepsy in all patients (23). In addition, all patients were HLA-DR2/DQβ1*0602 positive (22) and lacked hypocretin-1 in their cerebrospinal fluid [measurements as previously described (28)]. All subjects were free of medication (or 3 patients) had discontinued medication for ≥2 wk before the study. Two of these three narcoleptic subjects had used psycho-stimulants (methylphenidate and modafinil) and one a tricyclic antidepressant (clomipramine).

Body weight and height of the subjects were measured, as well as waist and hip circumference. The waist-to-hip ratio (WHR) was used as a relative measure of abdominal fat mass. Total body fat mass was determined by dual-energy X-ray absorptiometry (Hologic QDR4500, Waltham, MA). Seven male control subjects were recruited through advertisements in local newspapers. Controls were matched for age, body mass index, total fat mass, and WHR, since narcoleptics are (moderately) obese (16).

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Subjects were eligible for the study after exclusion of hypertension (defined as a repeated blood pressure measurement of systolic $>160\text{ mmHg}$ or diastolic $>90\text{ mmHg}$), any known (history of) pituitary disease, recent body weight change ($>3\text{ kg weight gain or loss within the previous 3 mo}$), and fasting blood glucose $>7.0\text{ mmol/l}$.

Written informed consent was obtained from all subjects. The study was approved by the ethics committee of the Leiden University Medical Center.

Clinical Protocol

Subjects were admitted to the Clinical Research Center in the morning. Drawing of blood was performed under standardized dark-light and alimentary conditions. During the 24-h study occasion, three standardized meals were served, at 0900, 1300, and 1800 (Nutridrink 1.5 kcal/ml; 1,500 standard meals were served, at 0900, 1300, and 1800.

Upon arrival, an intravenous cannula was inserted in an antecubital vein 1 h before the start of blood sampling, which was performed at 10-min intervals for 24 h through a long line to prevent sleep disturbance. Samples were allowed to clot and were centrifuged at $4\text{ °C}$ for 20 min, and serum was frozen at $-20\text{ °C}$ until assay. Sleep registration, using a portable electroencephalogram system (Porti; Twente Medical Systems, Enschede, Netherlands), confirmed abnormally distributed 24-h total sleep as well as REM sleep in narcoleptic subjects and normal distribution of sleep stages in control subjects. On a separate occasion, blood was drawn at 0800 after an overnight fast for measurement of LH, FSH, testosterone, estradiol, and sex hormone-binding globulin (SHBG) concentrations. Subsequently, a GnRH test was performed (42), whereby 100 µg of gonadorelin (Aventis Pharma, Hoewelaken, Netherlands) were given as an intravenous bolus, and serum LH concentrations were measured every 10 min for 90 min thereafter.

Assays

Serum LH and FSH concentrations were measured with time-resolved immunofluorometric assays (Wallac, Turku, Finland), with coefficients of variation (CV) of $3.9–6.8\%$ in the concentration range of $5.5–147\text{ U/l}$ for LH and $3.7–5.3\%$ in the concentration range of $5.1–41.3\text{ U/l}$ for FSH. Testosterone was measured with a coated-tube radioimmunoassay (RIA; Diagnostic Products, Los Angeles, CA) with a CV of $10.6–18.6\%$ in the concentration range of $2.7–44.3\text{ nmol/l}$. SHBG was measured with an immunoradiometric assay (Spectria, Esppo, Finland) and serum estradiol with an RIA (Diagnostic Systems Laboratory, Webster, TX). All samples of each gonadotropin 24-h profile were processed in the same assay procedure. In addition, the leptin concentration was measured in every 20-min sample by RIA (Linco Research, St. Charles, MO). The detection limit of the assay was $0.5\text{ µg/l}$ and the CV was $3.4–8.3\%$ in the concentration range of $4.9–25.6\text{ µg/l}$.

Details on leptin secretion in narcolepsy were previously published, but here we report only the 24-h mean leptin concentration in relation to LH parameters and testosterones (15).

Calculations and Statistics

Deconvolution analysis. Deconvolution analysis (47) was used to estimate the four secretory and clearance measures of interest: 1) the number and locations of secretory events; 2) the amplitudes of secretory bursts; 3) the durations of randomly dispersed LH and FSH secretory bursts; and 4) the endogenous single-component, subject-specific plasma half-lives of LH and FSH. It was assumed that the gonadotropin distribution volumes and LH and FSH half-lives were time and concentration invariant. The following parameters were calculated: half-duration of secretory bursts (duration of the secretory burst at half-maximal amplitude), hormone half-life, burst frequency, amplitude of the secretory burst (maximal secretory rate attained within a burst), mass secreted per burst, basal secretion rate (only for LH), pulsatile secretion rate (product of burst frequency and mean burst mass) and total secretion (sum of basal and pulsatile). Based on recent validation studies in men, deconvolution analysis was carried out at 95% joint statistical confidence intervals for all calculated LH and FSH amplitudes (13, 25).

Approximate entropy. The univariate approximate entropy (ApEn) statistic was developed to quantify the degree of irregularity, or disorderliness, of a time series (33). Technically, ApEn quantifies the

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<th>Table 1. Deconvolution-derived features of LH secretion</th>
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<tr>
<td>Burst number per 24 h</td>
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<td>Burst interval, min</td>
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<td>Burst duration, min</td>
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<td>Burst amplitude, U/l</td>
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<td>Burst mass, U/l</td>
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<td>Half-life, min</td>
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<td>Basal secretion, U/l</td>
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<td>Pulsatile secretion, U/l</td>
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<td>Total secretion, U/l</td>
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Data are expressed as means ± SE. Group statistical differences were calculated by the 1- or 2-tailed Student’s t-test for unpaired data (see METHODS) after log transformation where required.
The summed logarithmic likelihood that templates (of length \( m \)) of patterns in the data that are similar (within \( r \)) remain similar (within the same tolerance \( r \)) on next incremental comparison and has been formally defined elsewhere (34). The ApEn calculation provides a single nonnegative number, which is an ensemble estimate of relative process randomness, wherein larger ApEn values denote greater irregularity. Cross-ApEn (X-ApEn) quantifies joint pattern synchrony between two separate but parallel time series after standardization (\( z \)-score transformation) (36, 38). In the present analysis, we calculated X-ApEn assuming \( r/|H|1005 \leq 20\% \) of the SD of the individual time-series and \( m/|H|11005 \leq 1; \) hence, use of the designation X-ApEn \((1, 20\%)\). This parameter set affords sensitive, valid, and statistically well-replicated ApEn and X-ApEn metrics for assessing hormone time series of this length (35, 36). Both ApEn and X-ApEn results are reported as the ratio of the absolute value to that of the mean of 1,000 randomly shuffled data series, values approaching 1.0 denoting complete randomness.

**Statistical analysis.** Results are expressed as means ± SE unless stated otherwise. Student’s one-tailed \( t \)-test was used to evaluate the a priori hypothesis that LH secretion is reduced in narcoleptic men. All other comparisons were statistically evaluated by two-tailed Student’s \( t \)-test or the Kolmogorov-Smirnov test for two samples, where appropriate. Statistical analysis was performed using Systat (release 10.0; Systat, Richmond, CA).

**RESULTS**

**Subjects**

Narcoleptic patients (N) and controls (C) had a comparable age \([46.1 ± 15.9 \text{ (N)} \text{ vs. } 46.9 ± 16.1 \text{ (C)} \text{ yr}]\) and body mass index \([28.3 ± 2.6 \text{ (N)} \text{ vs. } 28.4 ± 2.2 \text{ (C)} \text{ kg/m}^2]\).

**Serum Hormone Concentrations**

Gonadal steroid hormone concentrations were similar in narcoleptics and controls \([\text{testosterone, } 23.3 ± 1.8 \text{ (N) vs. } 19.9 ± 0.8 \text{ (C)} \text{ nmol/l, } P = 0.11; \text{ estradiol, } 73.9 ± 5.8 \text{ (N) vs. } 78.3 ± 8.2 \text{ (C)} \text{ pmol/l, } P = 0.18; \text{ SHBG, } 28.5 ± 2.4 \text{ (N) vs. } 27.6 ± 2.3 \text{ (C)} \text{ nmol/l, } P = 0.79]\). The mean plasma LH concentration was significantly lower in narcoleptic patients \([3.0 ± 0.4 \text{ (N) vs. } 4.2 ± 0.3 \text{ (C)} \text{ U/l, } P = 0.01]\), whereas the

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**Table 2. Deconvolution-derived features of FSH secretion**

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<thead>
<tr>
<th></th>
<th>Narcolepsy</th>
<th>Controls</th>
<th>( P ) Value</th>
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<tbody>
<tr>
<td>Burst number per 24 h</td>
<td>19.7±2.7</td>
<td>19.7±0.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Burst duration, min⁻¹</td>
<td>3.23±0.64</td>
<td>4.80±1.70</td>
<td>0.40</td>
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<tr>
<td>Burst height, U/l⁻¹min⁻¹</td>
<td>0.073±0.019</td>
<td>0.062±0.008</td>
<td>0.60</td>
</tr>
<tr>
<td>Burst mass, U/l</td>
<td>0.481±0.092</td>
<td>0.567±0.075</td>
<td>0.49</td>
</tr>
<tr>
<td>Half-life, min</td>
<td>363±33</td>
<td>350±17</td>
<td>0.74</td>
</tr>
<tr>
<td>Total secretion, U/l⁻¹24h⁻¹</td>
<td>10.6±3.2</td>
<td>11.3±1.8</td>
<td>0.85</td>
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</table>

Data are means ± SE. Group statistical differences were calculated by 2-tailed Student’s \( t \)-test for unpaired data after log transformation where required.

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Fig. 2. Representative 24-h LH-secretory profiles (C and D) with their corresponding plasma concentration series and intrasample dose-dependent standard deviations and fitted curves calculated by deconvolution (A and B) in a narcoleptic patient (A and C) and his matched control (B and D). Note the decreased pulsatile secretion in the narcoleptic patient. Time is depicted in minutes elapsed after the 1st sample was taken.
FSH concentration was similar in both groups [3.7 ± 0.9 (N) vs. 4.0 ± 0.4 (C), P = 0.74]. Fig. 1 shows the 24-h serum LH and FSH concentration vs. time in narcoleptic subjects and controls.

**LH and FSH Secretion**

The deconvolution-derived parameter estimates of LH and FSH secretion and elimination are given in Tables 1 and 2, respectively. Pulsatile secretion of LH was diminished in narcoleptic subjects [23.5 ± 1.6 (N) vs. 34.3 ± 4.9 (C) U·h⁻¹·24 h⁻¹, P = 0.02] as a result of the concerted effects of reduced burst frequency and burst mass. Total LH secretion was also less in narcoleptic patients (P = 0.04). In contrast, all features of FSH release were similar in narcoleptics and controls. Also, plasma LH and FSH half-lives were not different in both groups. Representative 24-h LH- and FSH-secretory profiles calculated by deconvolution analysis with the corresponding curves of serum concentration data are shown in Figs. 2 and 3, respectively.

**GnRH Test**

Both basal serum gonadotropin levels [LH, 3.2 ± 0.4 (N) vs. 4.4 ± 0.4 (C) U/l, P = 0.18; FSH, 4.8 ± 1.1 (N) vs. 3.8 ± 5.9 (C) U/l, P = 0.43] and their maximal concentrations in response to GnRH stimulation [LH, 16.6 ± 2.8 (N) vs. 22.8 ± 4.9 (C) U/l, P = 0.27; FSH, 9.3 ± 3.0 (N) vs. 6.8 ± 0.9 (C) U/l, P = 0.43] were similar in both groups. The individual LH and FSH responses are displayed in Fig. 4.

**Leptin and LH Relationship**

The mean 24-h leptin concentration in patients was 5.9 ± 1.1 μg/l, and in controls 11.2 ± 1.3 μg/l (P = 0.01). No significant correlations were present between 24-h LH secretion and mean 24-h leptin concentration either in the combined groups (R = 0.07) or separately (patients: R = 0.20; controls: R = 0.39).

**Leptin and Testosterone**

Between the 24-h leptin concentration and serum testosterone a significant inverse linear correlation was found, as displayed in Fig. 5.

**ApEn**

ApEn ratio values for LH were lower in patients compared with controls, denoting a more regular secretion (N 0.653 ± 0.035; C 0.718 ± 0.014, P = 0.046). The individual data are displayed in Fig. 6. Similarly, the ApEn ratio of FSH was also smaller in patients (0.837 ± 0.032 vs. 0.927 ± 0.020, P = 0.03). The X-ApEn ratio between the LH and FSH concentration series was lower in patients than in controls, indicating increased synchrony between both hormones in patients compared with controls (N, 0.725 ± 0.017; C, 0.786 ± 0.015, P = 0.02). X-ApEn ratio between the leptin concentration series (20-min data) and the LH series (with only the 20-min data) did not differ between the groups (N, 0.889 ± 0.021; C, 0.911 ± 0.026, P = 0.53).
DISCUSSION

This study shows that the average 24-h plasma LH concentration is reduced in hypocretin-deficient narcoleptic men, whereas gonadal steroid hormone levels are normal. The reduction of the plasma LH concentration in narcoleptics is accounted for by a diminution of pulsatile LH release (by 30% compared with healthy controls), which cannot be attributed to insensitivity of the pituitary gonadotrophs to GnRH stimulation. LH burst frequency and burst mass were reduced in narcoleptic men. In contrast, plasma FSH concentrations and various features of FSH secretion were similar in narcoleptics and controls.

These data suggest that endogenous hypocretin peptides stimulate pulsatile LH release in men. In particular, the fact that spontaneous LH secretion is blunted, whereas pituitary responsiveness to GnRH stimulation is intact in narcoleptic subjects, suggests that hypocretin deficiency primarily inhibits hypothalamic GnRH release. This inference agrees with studies in male rats showing that hypocretin-1 promotes GnRH release from hypothalamic explants (40). Moreover, hypocretin inputs are abundant in the preoptic area that contains many GnRH neurons (50), which coexpress hypocretin-1 receptors and are in direct contact with hypocretin fibers (3). These physiological and anatomical observations obviously support the notion that hypocretin peptides are involved in the regulation of GnRH release. The fact that FSH secretion is not diminished (whereas LH release is) in narcoleptic subjects may imply that hypocretins specifically modulate GnRH burst frequency, as LH secretion is particularly enhanced by high-frequency bursts, whereas low-frequency bursts predominantly promote FSH release (20).

ApEn quantitates the relative orderliness or reproducibility of subordinate (nonpulsatile) secretory patterns in neurohormone time series and reflects feedforward and feedback adjustments driven by (patho)physiological changes in interglan- dular communication. In view of the unchanged testosterone feedback signal in the patients, the increased LH and FSH regularity and increased synchrony of both hormones might reflect a diminished GnRH input signal with unchanged pituitary responsiveness toward this neurohormone (48).

Alternatively, hypoleptinemia may blunt LH release in narcoleptic patients. We have previously shown (15) that the plasma leptin concentration is considerably reduced in narcoleptic men. Leptin has emerged as an important modulator of the HPG axis (5). Indeed, leptin-deficient ob/ob mice are hypogonadotropic and sterile, and recombinant leptin administration rescues these defects in ob/ob males (24) and females (6). Moreover, the circadian rhythm of plasma leptin levels is synchronous with that of LH concentrations (18), and the reduction of LH secretion induced by fasting in the rat and in humans can be reversed by leptin administration (1, 4, 8).

These data indicate that leptin stimulates LH release and that genetic and/or physiological alterations of circulating leptin...
levels can impact pituitary gonadotropin release. In this context, it is conceivable that the reduction of the plasma leptin concentration in narcoleptics (15) is involved in the diminution of pituitary LH release in these patients, but it should be mentioned that we could not demonstrate a (linear) correlation between mean circulating leptin concentration and LH production.

Notwithstanding the diminished LH production, testosterone concentration was unchanged in patients. Recently, it has become clear that leptin is involved in the (local) regulation of testosterone synthesis and secretion in the male rat gonad (44). Direct evidence for such action in humans is not available, but an inverse relation between serum testosterone and leptin has been demonstrated (19). Therefore, the inhibitory effect of leptin on testosterone (and adrenal hormone) secretion might be relevant in narcoleptic patients, because narcoleptics exhibit a 50% reduction in circulating leptin concentration, which may unleash gonadal testosterone secretion to compensate for diminished LH drive (15). The finding of an inverse relationship between testosterone and leptin in our subjects reinforces this view.

Prepro-orexin mRNA and the orexin receptors are expressed in various endocrine and nonendocrine tissues (11), and, for instance, orexins can modulate directly human and porcine adrenal steroidogenic function (21, 27). Relevant for the present investigation was the recent finding of the mRNA expression of the OX1 but not of the OX2 receptor in the male rat gonad (2). Activation of the OX1 receptor stimulates testosterone secretion, both in vivo and in vitro, suggesting a physiological (modulating) role of the orexin system in concert with leptin and ghrelin at the testicular level (45). The significance of these findings for human physiology needs to be explored. However, OX1 and OX2 receptor expression was demonstrated in the male human reproductive system, including the testis, suggesting that orexins can directly impact gonadal hormone release in humans as well as in rodents (12).

The impact of the alterations in the HPG ensemble described here on the fertility of narcoleptic men remains to be determined. Some early reports have suggested that narcolepsy is associated with impaired fertility (39), but since then these suggestions have not been confirmed, as far as we are aware. Moreover, it is not our clinical impression that narcoleptic (male) patients are less fertile, and gonadal hormone concentrations in plasma were normal in narcoleptic (male) patients are less fertile, and gonadal hormone concentrations in plasma were normal in narcoleptic males.

In conclusion, hypocretin-deficient narcoleptic men have reduced circulating LH levels, which are brought about by a diminution of pulsatile LH release. In contrast, FSH secretion is normal in these patients. The pituitary sensitivity to GnRH stimulation is normal in narcoleptics, which suggests that a reduction of hypothalamic GnRH release underlies their blunted LH secretion, and in accordance with this view is the increased orderliness of secretion of gonadotropins. Both hypocretin deficiency per se and hypothalaminemia may be involved in changes in the HPG axis in narcoleptic males.

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