Narcolepsy beyond sleepiness : endocrine, metabolic and other aspects

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Altered circadian rhythm of melatonin concentrations in hypocretin-deficient men

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ABSTRACT

Hypocretin deficiency causes narcolepsy. It is unknown whether melatonin secretion is affected in this sleep disorder. Therefore, in both narcolepsy patients and matched controls we measured plasma melatonin levels hourly for 24 h before and after five days of sodium oxybate (SXB) administration. Although mean melatonin concentrations were similar between patients and controls, in narcoleptics the percentage of 24-h melatonin secreted during the daytime was significantly higher, and melatonin secretion exhibited a weaker coupling to sleep. SXB did not affect melatonin secretion. These findings suggest that hypocretin deficiency might disturb both the circadian control of melatonin release and its temporal association with sleep.

INTRODUCTION

Narcolepsy in humans is caused by hypocretin deficiency due to loss of hypocretin-producing neurons in the hypothalamus by an as yet unknown mechanism.[47;48] An autoimmune cause is assumed but has not been proven yet.[62] Hypocretins play an important role in the regulation of various homeostatic functions, most notably the consolidation of the sleep/wake cycle.[176] Therefore, it is not surprising that the inability to stay awake during the day and asleep during the night are among the major symptoms of narcolepsy.[102] Another potential modulator of sleep is melatonin, high levels of which have been associated with sleepiness.[93] The synthesis and secretion of melatonin in the pineal gland is under the influence of the circadian clock genes in the suprachiasmatic nucleus (SCN) of the hypothalamus.[93] Apart from its circadian control, melatonin production is also regulated by the light/dark cycle and primarily occurs at night.[93] Specialized melanopsin-containing cells in the retina detect a narrow band of blue wavelengths and subsequently signal the SCN, resulting in melatonin suppression during exposure to (day)light.[177] Furthermore, accumulating evidence derived from experiments on various animal models indicates that the hypocretinergic innervation of the pineal gland could also be important for the regulation of diurnal melatonin synthesis and secretion.[94;178-181]

Sodium oxybate (SXB), or γ-hydroxybutyrate, has been shown to be highly effective in the treatment of narcolepsy-associated sleep disturbances.[73] It reduces cataplexy, improves nocturnal sleep fragmentation, and, at higher doses, it may also reduce excessive daytime sleepiness (EDS). The mechanisms of action of SXB are poorly understood. It is known to act on the GABAB receptor, whereas the existence of a special GHB receptor is still a matter of debate. SXB might act via modulation of melatonin release, which is known to affect sleep. Indeed, previously we have shown that GABAergic mechanisms affect melatonin release.[182-184] Several papers have delineated melatonin secretion in narcolepsy patients, primarily focusing on whether altered melatonin secretion could contribute to EDS and nocturnal sleep disturbances.[185-187] Although most of these studies found melatonin levels within the normal range in narcolepsy patients, in a more recent study Blazejova et al. discovered elevated saliva melatonin levels during the day in almost half of the patients.[188] These patients had a shorter sleep onset on the Multiple Sleep Latency Test (MSLT) compared to those without elevated daytime melatonin levels. However, the MSLT and melatonin sampling were performed on separate occasions. Therefore, as comparison of findings from these previous studies is hampered by significant methodological differences, it remains to be elucidated whether, how, and to which extent melatonin secretion is altered in narcolepsy patients.

Given the recent indications for a hypocretinergic innervation of the pineal gland, we hypothesized that melatonin secretion and/or its circadian rhythmicity might be altered in hypocretin-deficient narcolepsy patients, and that SXB might reverse these alterations.[94;178-181] To evaluate this hypothesis we performed 24-h blood sampling studies in combination with polysomnography on a group of hypocretin-deficient narcolepsy patients and rigorously matched healthy controls, both before and after SXB administration.

SUBJECTS AND METHODS

Subjects

Seven male narcolepsy patients with definite cataplexy, who fulfilled the diagnostic criteria of the second edition of the International Classification for Sleep Disorders were included.[118] All narcolepsy patients had hypocretin levels under the detection limit using a standardized cerebrospinal fluid assay[132] and were free of medication for at least 2 weeks before study. Seven healthy controls, matched for sex, age, body mass index (BMI), waist-to-hip ratio (WHR) and fat percentage were included for comparison (see Table 5.1 for subjects' characteristics). Subjects were eligible for study after exclusion of hypertension, any known (history of) pituitary, psychiatric or neurological disease (other than narcolepsy), alcohol or drug abuse, recent weight change (> 3 kg weight change within the last 3 months), a sleep disorder history (controls), and endurance sports. Routine laboratory tests were performed to rule out diabetes, anaemia, hepatic, and/or renal disease. The study was approved by the ethics committee of the Leiden University Medical Centre and adhered to the ethical standards outlined in the Helsinki Declaration.[189] Written informed consent was obtained from all subjects.

Table 5.1 Group characteristics

	Narcolepsy	Controls	*P*-value
Age (yrs)	34.3 ± 3.4	34.7 ± 3.0	0.93
BMI	28.2 ± 1.8	27.8 ± 1.6	0.89
Fat %	22.9 ± 2.3	23.9 ± 1.9	0.76
WHR	0.90 ± 0.04	0.90 ± 0.01	0.99

Data are shown as mean ± SEM. Comparisons were made using independent t-test.

Protocol

Subjects were admitted to the Clinical Research Centre for 24-h blood sampling. A cannula was inserted into an antecubital vein 45 min before the start of blood sampling at 12:00 h. Blood samples were collected with S-Monovette (Sarstedt, Etten-Leur, The Netherlands) from a three-way stopcock attached to a 0.9% NaCl and heparin (1 U/ml) infusion (750 ml/24 h) to keep the cannula from clotting. Sampling was performed through a long line to prevent sleep disruption by investigative manipulations. For melatonin measurements, blood was collected in EDTA tubes at 60-min intervals, except the sample at 23:30 h which was taken 30 min after the previous sample. Blood was put on ice immediately after sampling and centrifuged within 5 min (1250 *g* at 4°C for 20 min). Subsequently, plasma was divided into separate aliquots in Sarstedt tubes and stored at -80°C until assay. Three standardized meals were served at 08:30, 13:00, and 18:00 h (Nutridrink, Nutricia, Zoetermeer, The Netherlands; 1.5 kcal/ml, 2100 kcal/d; macronutrient composition/100 ml: protein, 6 g; fat, 5.8 g; carbohydrate, 18.4 g). A 24-h urine sample was collected for the determination of catecholamine concentrations. Patients and controls were always studied in the same room, mostly in parallel to keep circumstances equal and rule out seasonal differences. Subjects remained (semi)supine except for bathroom visits. Daytime naps were allowed. Light intensity was between 300 and 400 lux during L and < 10 lux during D. Lights were switched off at 23:00 h and turned on at 07:30 h the next day. Twenty-four hour sampling was performed at baseline and on the fifth day of SXB administration.

Sodium oxybate

SXB was administered in two nighttime doses of three grams each, at 23:00 h and 03:00 h. To monitor for possible side effects, the first night of administration was done on the neurology ward.

Sleep analysis

Sleep was polygraphically recorded throughout both sampling occasions, using an Embletta X100 recorder (Embla, Broomfield, CO, USA). The recordings were scored visually by an experienced sleep technician at 30-s intervals according to AASM criteria.[190] To allow assessment of the association between changes in plasma melatonin levels (measured every 60 min) and sleep stages (scored every 30 s), sleep profiles were divided into 30-min segments as described previously.[120] These segments were condensed from the 30-s scoring intervals by calculating the percentage of time spent in stages I and II non-REM sleep, slow

wave sleep (SWS), and REM sleep in the 30 min immediately preceding each melatonin measurement. The temporal relation between melatonin levels and the conjoining sleep segments was subsequently assessed by cross-correlation analysis.

Melatonin assay

Melatonin levels were measured by radioimmunoassay after extraction from plasma with dichloromethane.[191] Duplicate aliquots each containing 100 µl of extracted plasma were assayed by adding 100 µl of a specific rabbit antiserum (R19540, INRA, Nouzilly, France) and 300 µl of labelled [125I]-2-iodomelatonin. The mixture solution was incubated overnight at 4°C. Then 800 µl of an anti-rabbit gamma globulin was added, and the tubes were put on ice for 1 h. Tubes were then centrifuged and the supernatant discarded. The pellet was counted using a γ-ray counter. The detection limit of the assay was 1 pg/tube (10 pg/ml plasma).

Statistical analysis

Results are expressed as mean ± standard error (SEM), unless otherwise specified. Statistical comparisons were made with either the Student's t-test or repeated-measures ANOVA, as appropriate. The area under the curve (AUC) of the melatonin versus time plots was calculated as a measure of the total 24-h melatonin secretion. As there was considerable interindividual heterogeneity in absolute melatonin concentrations, diurnal variations in melatonin secretion were assessed by calculating the percentage of the 24-h AUC during the lights-on period (i.e., 07:30 - 23:00 h). Cross-correlation analysis was applied to assess the association between melatonin levels and the percentage of time spent in various sleep stages in the preceding 30 min.[127] Cross-correlation analysis is a standard method of assessing the degree of correlation between two time series, and was defined here as:

$$r = \frac{\sum_{t=0}(m_t - \bar{m})(s_t - \bar{s})}{\sqrt{\sum_{t=0}(m_t - \bar{m})^2}\sqrt{\sum_{t=0}(s_t - \bar{s})^2}}$$

Where *t = 0,1,2,...,24*, and m_t = the melatonin concentration at each time point, $\bar{m}$ = the mean 24-h melatonin concentration, s_t = the percentage of time spent in various sleep stages in the preceding 30 min at each time point, and $\bar{s}$ = the 24-h mean of each s_t. For cross-correlations during nighttime only, *t* ranged from 0 to 9. Statistical calculations were performed using SPSS (release 17.0, SPSS, Inc., Chicago, IL). All tests were two-tailed, and the significance level was set at 0.05.

RESULTS

Subjects

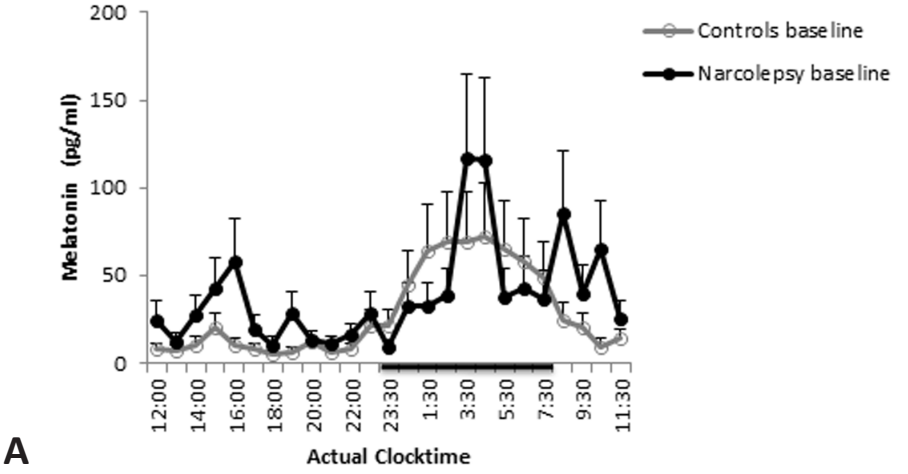
Narcolepsy patients and controls did not differ with respect to age, BMI, fat percentage, and WHR (all $P \geq 0.76$, Table 5.1). There were also no differences in urinary epinephrine, norepinephrine, and dopamine levels either at baseline (all $P \geq 0.70$) or after SXB administration (all $P \geq 0.16$). Ingestion of SXB was well-tolerated; apart from mild drowsiness, no other side-effects were reported.

Melatonin profiles

Mean 24-h melatonin concentrations did not differ between narcolepsy patients and controls, either before (39.2 ± 16.4 vs. 28.6 ± 4.6 pg/mL, $P = 0.56$) or after SXB administration (39.8 ± 9.2 vs. 31.0 ± 5.1 pg/mL, $P = 0.43$) (Figure 5.1). Similarly, differences in mean melatonin levels were not significant between the two groups when analyzed for day- and nighttime separately, either during the basal condition or after SXB treatment (all $P \geq 0.11$). As expected, with repeated-measures ANOVA, we found a significant effect of *Circadian Time* on melatonin levels in both groups ($P < 0.005$). However, the effect of *Circadian Time* on melatonin levels differed between narcolepsy patients and controls ($P = 0.042$ for *Time × Group* interaction effect). The percentage of total 24-h melatonin secreted during the day was significantly higher in narcolepsy patients compared to controls, both during the basal condition (46.6 ± 4.1 vs. 32.5 ± 5.8%) and after SXB intake (51.5 ± 3.5 vs. 34.4 ± 4.0%), ($P = 0.007$ for *Group* effect; Figure 5.2). Administration of SXB did not significantly affect the percentage of melatonin secreted during the daytime ($P = 0.415$ for the *Treatment* effect and $P = 0.718$ for *Group × Treatment* interaction effect).

Sleep recordings

Compared to controls, narcolepsy patients spent significantly less time awake both during basal conditions and SXB treatment (Table 5.2). During the day (defined as the lights-on period between 07:30 - 23:00 h), narcolepsy patients spent significantly less time awake, while significantly more time was spent in non-REM sleep, regardless of treatment (Table 5.2). SXB administration resulted in a significant decrease in stages I/II non-REM and REM sleep over the 24 h in both groups ($P = 0.011$ and $P = 0.009$, respectively), while it significantly



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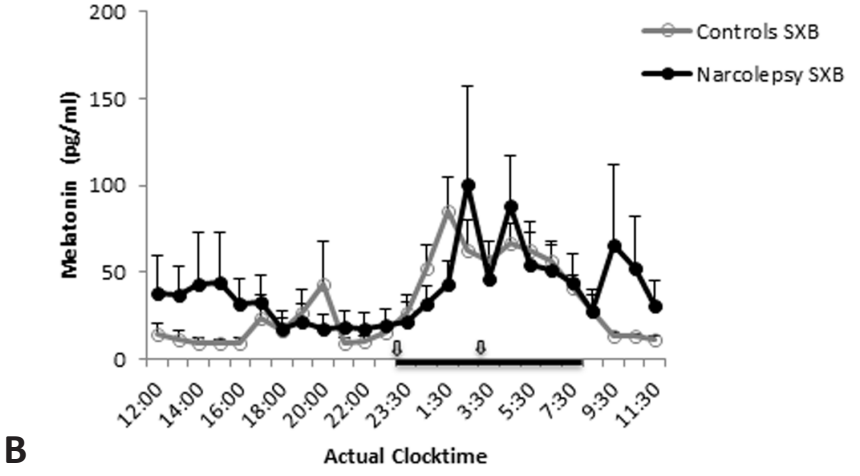

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Figure 5.1 Mean melatonin concentrations plus standard error of the mean in narcolepsy patients and controls at baseline **(A)** and during SXB administration **(B)**. No difference in mean concentrations was found between groups in both conditions. Black bar on the abscissa indicates the dark period (23:00–07:30 h). Downward arrows **(B)**) indicate ingestion of SXB (23:00 h and 03:00 h). Time is displayed in actual clock time.

increased the time spent in SWS ($P = 0.001$). During the day, SXB treatment reduced the time spent in stages I/II non-REM and REM sleep ($P = 0.038$ and $P = 0.041$, respectively), while it tended to increase wakefulness ($P = 0.098$). The percentage of SWS during the night more than doubled in both groups in response to SXB treatment (narcolepsy: 6.5 ± 1.9 vs.16.5 ± 3.0%, controls: 7.1 ± 1.9 vs. 18.5 ± 2.4%; $P = 0.001$ for treatment effect), whereas there were trends for decreases in the percentages of stages I/II non-REM and REM sleep.

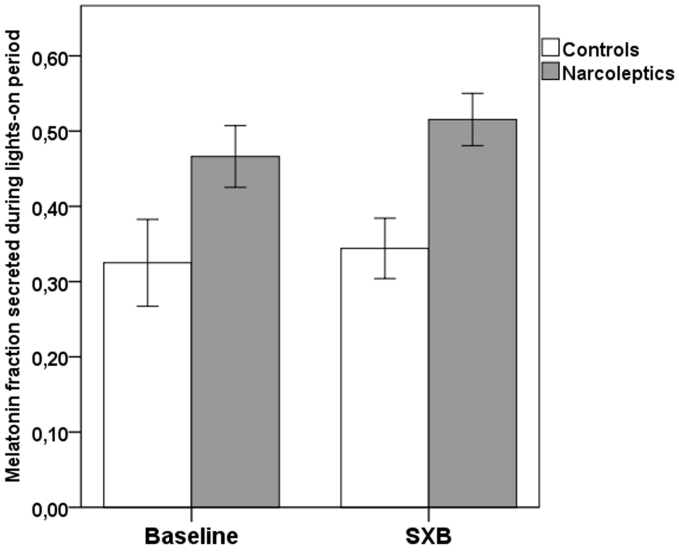

Figure 5.2 Fraction of melatonin secreted during the day in narcolepsy patients and controls, both at baseline and during sodium oxybate administration (lights-on period 07:30-23:00 h). Fraction of melatonin secreted during daytime is higher in narcolepsy patients compared to matched controls (P = 0.007 for group effect, P = 0.415 for treatment effect, and P = 0.718 for group × treatment interaction effect). Bars represent standard errors of the mean.

Table 5.2 Sleep patterns before and after sodium oxybate administration

	Baseline			Sodium oxybate		
	Patients	Controls	*P*	Patients	Controls	*P*
Wake total (%)	60.8 ± 2.9	68.7 ± 2.0	0.044*	60.8 ± 2.2	70.1 ± 2.4	0.013*
Wake day (%)	79.4 ± 4.2	95.6 ± 2.1	0.004*	82.9 ± 3.2	97.3 ± 1.0	0.001*
Wake night (%)	25.8 ± 5.7	18.4 ± 4.0	0.310	19.2 ± 4.3	19.2 ± 5.8	0.999
Stage I/II total (%)	29.1 ± 1.4	25.0 ± 2.4	0.155	26.3 ± 1.4	21.1 ± 2.2	0.063
Stage I/II day (%)	14.6 ± 3.0	2.5 ± 1.6	0.003*	11.1 ± 2.5	1.6 ± 1.0	0.005*
Stage I/II night (%)	55.1 ± 2.5	65.6 ± 5.7	0.114	53.5 ± 3.7	56.4 ± 5.3	0.647
SWS total (%)	3.7 ± 0.7	2.5 ± 0.7	0.239	7.6 ± 1.2	6.6 ± 0.9	0.534
SWS day (%)	2.1 ± 0.6	0.03 ± 0.03	0.005*	2.7 ± 1.1	0.05 ± 0.05	0.041*
SWS night (%)	6.5 ± 1.9	7.1 ± 1.9	0.843	16.5 ± 3.0	18.5 ± 2.4	0.611
REM total (%)	6.3 ± 1.8	3.7 ± 0.8	0.191	4.7 ± 1.0	2.1 ± 0.8	0.070
REM day (%)	2.9 ± 1.4	0.8 ± 0.5	0.203	1.2 ± 0.5	0.0 ± 0.0	0.032*
REM night (%)	12.6 ± 3.0	8.8 ± 1.8	0.305	10.8 ± 2.1	5.8 ± 2.3	0.127

* $P < 0.05$. Percentages of sleep stages during the 24 h of study, before and after SXB administration. Data are shown as mean ± SEM. Unpaired t-tests were used to assess differences between the two groups.

Table 5.3 Cross-correlations between melatonin concentrations and percentages of time spent in each sleeping stage in the preceding 30 min during the total 24-h period or during the nighttime (23:00-07:30) only

	Patient baseline	Patient SXB	Control baseline	Control SXB	P for group effect	P for treatment effect	P for group x treatment interaction
SWS (24 h)	0.04 ± 0.09	0.00 ± 0.07	0.33 ± 0.10	0.25 ± 0.07	0.023*	0.264	0.561
I/II NREM sleep (24 h)	0.24 ± 0.07	0.22 ± 0.09	0.53 ± 0.13	0.52 ± 0.04	0.007**	0.857	0.959
REM sleep (24 h)	0.19 ± 0.11	0.07 ± 0.07	0.34 ± 0.09	0.18 ± 0.07	0.349	0.106	0.979
Awake (24 h)	-0.31 ± 0.05	-0.36 ± 0.09	-0.73 ± 0.13	-0.63 ± 0.08	0.007*	0.709	0.376
SWS (night)	0.12 ± 0.16	-0.15 ± 0.10	-0.01 ± 0.11	-0.04 ± 0.10	0.517	0.266	0.634
I/II NREM sleep (night)	0.17 ± 0.10	0.23 ± 0.12	0.46 ± 0.15	0.35 ± 0.12	0.105	0.877	0.524
REM sleep	0.10 ± 0.15	-0.29 ± 0.09	0.21 ± 0.09	-0.02 ± 0.11	0.221	0.030*	0.538
Awake (night)	-0.32 ± 0.07	0.08 ± 0.08	-0.60 ± 0.08	-0.35 ± 0.11	0.003**	0.006**	0.457

Data are shown as mean ± SEM. Comparisons were made using repeated measurements ANOVA. * $P < 0.05$. ** $P < 0.01$.

Melatonin and sleep

The cross-correlations between melatonin levels and the percentages of time spent in either SWS, phase I/II non-REM sleep, or time awake were significantly weaker in narcolepsy patients compared to controls during the 24-h period (all $P < 0.023$; Table 5.3). Although SXB treatment did not influence the association between melatonin levels and sleep during the total 24-h period, restriction of the analyses to the lights-off period showed that, in both groups, SXB treatment inverted the association between melatonin concentrations and REM-sleep, while it attenuated the negative association between melatonin concentrations and time spent awake (Table 5.3).

DISCUSSION

Recent publications have suggested an association between altered temporal patterning of melatonin secretion in persons complaining of poor sleep and medical conditions linked with disordered sleep.[192-194] However, to our knowledge, this is the first study which combines measurements of diurnal plasma melatonin concentration profiles and polysomnography in hypocretin-deficient narcolepsy patients and matched controls. Although mean 24-h levels of plasma melatonin were similar between narcolepsy patients and controls, the circadian distribution of melatonin release was altered in narcolepsy, since the proportion of melatonin secreted during daytime was substantially higher in narcolepsy patients. Moreover, our findings indicate that the temporal coupling between sleep and plasma melatonin levels is diminished in narcolepsy patients, suggesting that hypocretin deficiency differently affects the circadian distribution of sleep and melatonin release because otherwise, despite an altered diurnal pattern of sleep and melatonin secretion, their cross-correlation would have been expected to remain unchanged.

In agreement with earlier reports, we did not find differences in mean melatonin levels between narcolepsy patients and controls.[185;188;195] Moreover, we were able to detect daytime melatonin secretion in all our patient and control subjects. This is in line with an earlier study in which daytime melatonin secretion was established in less than half of the narcolepsy patients.[188] Two reasons are likely to account for the fact that we did find an increase in all patients and Blazejova in less than half of them: first, we measured (daytime) melatonin levels much more frequently, and, second, we assessed melatonin levels in plasma instead of saliva in which melatonin concentration is estimated to be only 24% of that in plasma.[196]

The proportion of total 24-h melatonin secreted during the daytime was substantially higher in narcolepsy patients. This finding may reflect disruption of the circadian control of melatonin secretion as a consequence of hypocretin deficiency. Several studies – on sheep,[181] pig,[179] rat,[180] and zebrafish[94] – have found evidence for hypocretinergic innervation of the pineal gland as indicated by the presence of hypocretin fibers and/or receptors in this region. Functional evidence for the connection between the hypocretin and melatonin system was recently provided in a study on zebrafish by Appelbaum et al.[94] Zebrafish have only one hypocretin receptor gene (humans have two), and serve as a simplified model for the study of the hypocretin system.[197] Hcrtr-/- null-mutant fish display the disturbed nocturnal sleep phenotype, but not the other symptoms of narcolepsy.[94] The mutant fish exhibit reduced mRNA expression of arylalkylamine-N-acetyltransferase, a key enzyme in the melatonin production pathway, during the night,[94] suggesting that melatonin secretion may be stimulated by hypocretin signalling during the night. As our data indicate that hypocretin deficiency results in a smaller amount of the total diurnal melatonin being released during the nighttime, our findings are in line with those of Appelbaum et al. and suggest that hypocretin is mainly involved in the consolidation of the diurnal rhythm of melatonin release, confining it mainly to the dark phase of the circadian cycle. Indeed, another study on zebrafish demonstrated synaptic plasticity of the hypocretinergic projections to the pineal gland that exhibited marked circadian rhythmicity, again suggesting that hypocretin signalling may modulate circadian variations in melatonin secretion. A recent study byMcGregor et al. provides further clues as how hypocretin might be involved in the modulation of circadian rhythms.[198] Although these authors did not assess melatonin release, they found decreased responsivity to light in hypocretin deficient mice. Since light is the major suppressor of melatonin synthesis, it is conceivable that defects in hypocretin signalling might disrupt light-induced suppression of melatonin secretion, resulting in relatively higher daytime concentrations as described in the present study. However, the effect of hypocretin on melatonin secretion in humans is likely to be modest as one might expect larger differences in the absence of hypocretin.

A potential limitation of this study is the relatively small number of participants. However, this limitation is partly offset by the large number of melatonin samples and the standardized conditions under which the sampling was performed. All subjects were assessed at the same location, during the same time of year, and under the same lighting conditions. This is essential because these factors have been shown to be major modulators of melatonin secretion.[93] Furthermore, our subjects were closely matched and all narcolepsy patients were proven to be hypocretin deficient.

In summary, in narcolepsy patients, melatonin secretion was relatively higher during the day and exhibited a weaker coupling to sleep, suggesting that hypocretin deficiency might disturb both the circadian control of melatonin release and its temporal association with various stages of sleep. Furthermore our findings indicate that SXB does not affect melatonin secretion per se but might act to modulate its temporal coupling with sleep.

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