Hypocretin deficiency : neuronal loss and functional consequences

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Neuronal Loss and Functional Consequences

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Hypocretin Deficiency

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Overig Lid
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πέμπε δέ μιν πομποῖσιν ἅμα κραιπνοῖσι φέρεσθαι,
῞Υπνῳ καὶ Θανάτῳ διδυμάοσιν, οἵ ῥα μιν ὦκα.

Then Sleep and Death, two twins of winged race,
Of matchless swiftness, but of silent pace.
- Homer, The Iliad (XVI:831), *(Pope's translation)*

For My Parents

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General Introduction and Scope of the Thesis

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The dual discovery of hypocretin

The hypocretins were discovered in 1998 nearly simultaneously by two different groups. One group named these newly found peptides *hypocretins* because of their *hypo*thalamic origin and a weak sequence homology to the in*cretin* hormone family.[1] Only six weeks later, another group named the same peptides *orexins*, because intracerebroventricular injection of these neurotransmitters stimulated food intake in rats (ορεξη = appetite).[21]

From the precursor molecule preprohypocretin two peptides are produced: hypocretin-1 and -2.[1] Hypocretin 1 is 33 amino acids in length, with an N-terminal pyroglutamyl residue and an amidated C-terminal. Four cystein residues in the peptide form two sets of intrachain disulfide bonds. Hypocretin-2 is a 28 amino acid peptide with an amidated C-terminal (Figure 0.1).[2] There are two types of hypocretin receptors. Both are 7-transmembrane G-protein coupled receptors encoded by 7 exons. The hypocretin receptor 1 has a preferential affinity for hypocretin-1, whereas hypocretin receptor 2 binds both hypocretins with equal affinity.[2]

Anatomy of the hypocretin system

Hypocretin is produced by neurons in a subregion of the hypothalamus (see Box 1), the dorsolateral hypothalamus (see Box 2), centered around the fornix and adjacent areas. In rats, estimates of the number of hypocretin containing neurons range from 1,000 to 4,000, depending on the antiserum and/or estimation method.[3] In the human brain, this number was estimated at 15,000-20,000 using in situ hybridization[3] and 50,000-80,000 using immunocytochemistry.[4] The cell bodies of hypocretin producing neurons all lie together in a rather small area, but this does not hold at all for their projections, which are found throughout the brain.[5,6] In accordance with this finding, hypocretin receptors are also found throughout the brain.

1 Currently, 'orexin' is used more by basic researchers studying animal models and metabolism, while 'hypocretin' is used more by clinical sleep specialists. In this thesis 'hypocretin' will be used, since this is the name given by the group that was the first to describe these peptides. Furthermore, in the OMIM and MGD genetic databases, the term hypocretin is used.

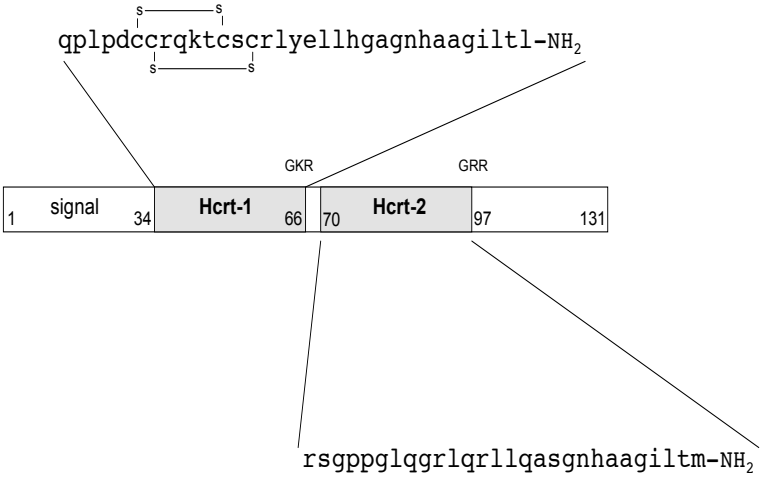


Figure 0.1 **Diagram of preprohypocretin**

GKR and GRR depict dibasic residues, that are potential cleavage sites for prohormone convertases. The derived aminoacid sequence for hypocretin-1 and hypocretin-2 are shown as well. The C-terminal end of both peptides are amidated. Note the two intrachain disulfide bridges in hypocretin-1.

Function of the hypocretin system

When hypocretins were first discovered they were thought to be mainly involved in the regulation of food intake. Local injection of hypocretin-1 in several hypothalamic areas, such as the dorsomedial nucleus, induced feeding behaviour,[7] while administration of hypocretin-1 antibodies suppressed feeding in rats.[8]

Hypocretin administration does not however alter total 24 hour food consumption and neither does prolonged administration affect body weight in rats.[9] Furthermore, the appetite-inducing activity of hypocretin is much less compared with for example that of the most well-known appetite inducing peptide Neuropeptide Y (NPY) and sometimes even absent.[10] These findings suggest that the major function of hypocretin must be another than the regulation of food intake.

The prevailing view that the main function of the hypocretin system regulates food intake underwent a change following the discovery that dogs (Dobermans and Labradors) suffering from an autosomal recessive inheritable form of the sleep disorder narcolepsy (see Box 3) have a mutation in the type 2 receptor for hypocretin. This prompted the view that hypocretins are crucial for the regulation of sleep. As said, hypocretin neurons project widely throughout the brain, but closer scrutiny revealed a notable concentration in wake stimulating areas.[5] Soon further evidence for the role of hypocretin in regulating sleep and activity/arousal was found. In a number of animal studies central administration of hypocretin-1 resulted in general hyperactivity together with stereotypical motor activities, such as burrowing and grooming.[10-12] Both hypocretins

Box 1: The Human Hypothalamus

The human hypothalamus represents only a very small portion of the adult human brain: with 4 cm^3 it amounts to only 0.3% of the adult brain. It is nevertheless extraordinarily complex, containing as it does many different cell groups with different structural and molecular organizations that are critically involved in a great many physiological, endocrine and behavioral processes.[29] Among these are the regulation of food intake, autonomic tone, the sleep-wake cycle and temperature.

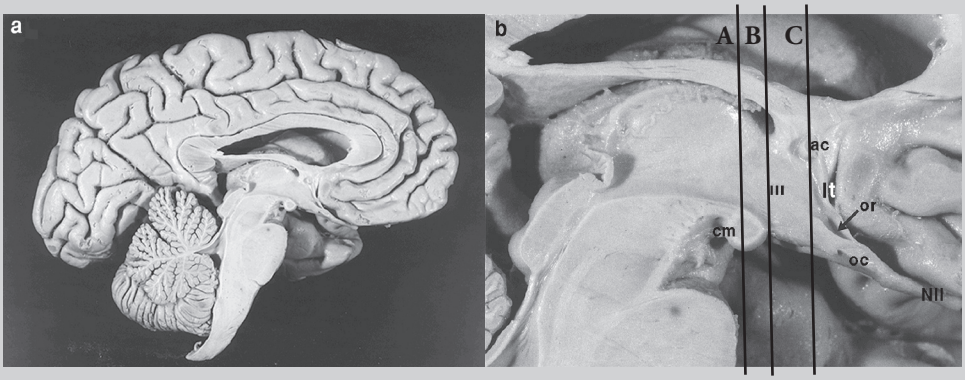


Figure 0.2 | **Medial surface of the human brain (a: overview), (b: detail with the hypothalamus)**
ac = anterior commissure, NII = optic nerve, lt = lamina terminalis, oc = optic chiasm, or = optic recess, III = third ventricle, cm = corpus mamillare.

The exact location of the boundaries of the hypothalamus itself is quite arbitrary.[30,31] Moreover, the various cell types within the hypothalamus do not respect the anatomical boundaries of the different nuclei. However, the borders of the hypothalamus are generally considered to lie as follows. *Rostrally*, the border is the lamina terminalis, and caudally it is the plane through the posterior fissure and the posterior edge of the mamillary body (Figure 0.2). It should be noted, however, that the nucleus basalis of Meynert (that does not belong to the hypothalamus *sensu stricto*) extends even more caudally than the mammillary bodies (Figure 0.2). The *ventral* border of the hypothalamus includes the floor of the third ventricle that blends into the infundibulum of the neurohypophysis. The exact location of the *lateral* boundaries is less clear, i.e. the striatum/nucleus accumbens, amygdala, the

increase blood pressure and heart rate in rats when injected intracerebroventricularly.[13,14] Moreover, hypocretin-1 and -2 increased the firing rate of the histaminergic neurons, which play a prominent role in arousal.[15]

Lack of hypocretin: Narcolepsy

Shortly after these animal discoveries **narcolepsy** in man (see Box 3) was also shown to be due to a malfunction of the hypocretin system. In healthy persons hypocretin

(Box 1 continued)

posterior limb of the internal capsule and basis pedunculi and, more caudodorsally, the lateral border of the subthalamic nucleus.[31,32]

Most authors distinguish three hypothalamic regions (Figure 0.3),[31] (A) the chiasmatic or preoptic region, (B) the cone-shaped tuberal region (which surrounds the infundibular recess and extends to the neurohypophysis) and (C) the posterior or mammillary region, which is dominated by the mammillary bodies that abut the midbrain tegmentum.[32]

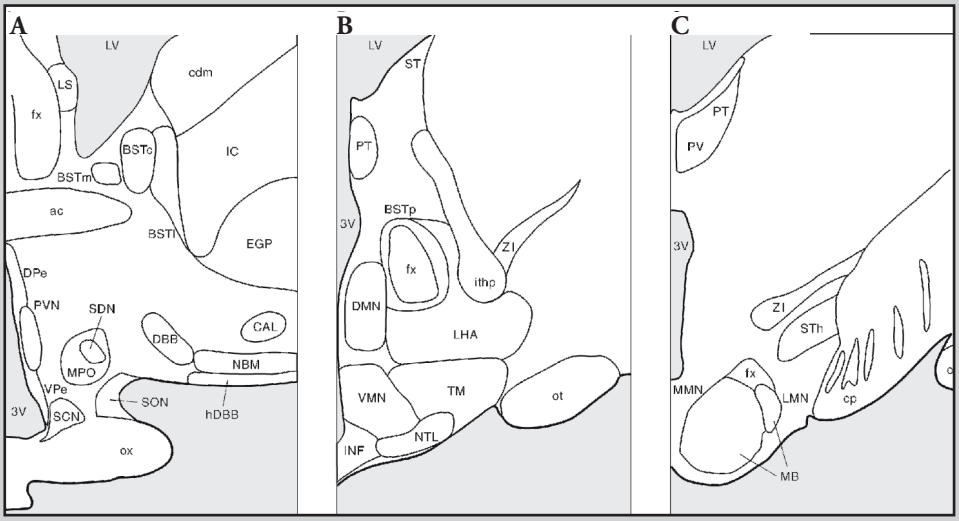
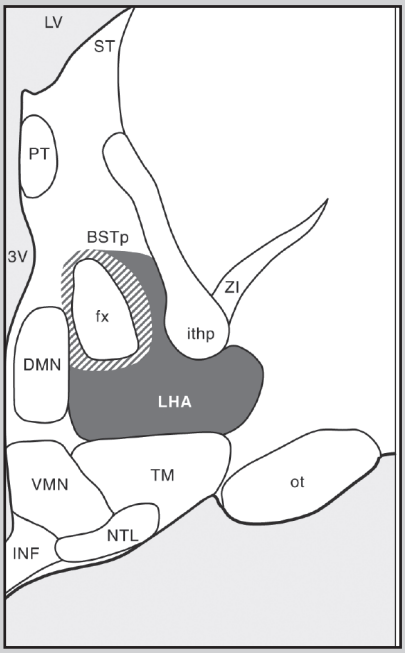


Figure 0.3 | **Nuclei of the human hypothalamus in three representative coronal cuts**

Abbreviations: Ox: optic chiasma, NBM: nucleus basalis of Meynert, hDBB: horizontal limb of the diagonal band of Broca, SDN: sexually dimorphic nucleus of the preoptic area, SCN: suprachiasmatic nucleus, BST: bed nucleus of the stria terminalis, (c = centralis; m = medialis; l = lateralis; p = posterior); PVN: paraventricular nucleus, SON: supraoptic nucleus, DPe: periventricular nucleus dorsal zone, VPe: periventricular nucleus ventral zone, fx: fornix, 3V: third ventricle, ac: anterior commissure, VMN: ventromedial hypothalamic nucleus, INF: infundibular nucleus, OT: optic tract, MB: mamillary body i.e. MMN: medial mamillary nucleus + LMN: lateromamillary nucleus, cp: cerebral peduncle. (Adapted from Fernández-Guasti et al., 2000; Fig. 2.)

could be detected in the cerebrospinal fluid, but in narcoleptic patients the amount was so low that its presence could not be detected.[16] Further research showed that the lack of hypocretin was caused by a specific loss of hypocretin containing neurons.[3] At present it is not clear how the amount of cell loss translates to disease severity. How these cells are lost is also at yet unknown. There is only one report about a genetic mutation causing the narcolepsy phenotype showing an autosomal dominant mode of inheritance.[17] The most popular hypothesis concerns an autoimmune process that selectively targets hypocretin neurons, but no direct proof for such a process has yet been found.[18] The strongest argument for this hypothesis is the fact that almost all

Box 2: The Lateral Hypothalamus



The area lateral of the preoptic nucleus and the paraventricular nucleus (PVN) does not belong to a well circumscribed nucleus and is called the lateral hypothalamic area (LHA) or lateral hypothalamic zone (figure 0.4). The relatively sparse neurons in this zone, which include the hypocretin producing neurons, are interspersed around the fibres of the fornix.[31] Large, darkly staining neurons are found scattered through the lateral hypothalamic area. These cells merge with the tuberomamillary nucleus neurons. The cells of the lateral hypothalamic area project on other hypothalamic areas, on the cerebral cortex, brainstem and spinal cord.[31] The LHA is involved in the regulation of food intake and body weight, together with the infundibular nucleus, paraventricular nucleus (PVN), dorsomedial nucleus (DMN), and the ventromedial hypothalamic nucleus (VMN).[32]

Figure 0.4 | Schematic representation of the nuclei of the human hypothalamus. The lateral hypothalamus is indicated in grey.

narcolepsy with cataplexy patients share the same major histocompatibility complex (MHC) subtype of immune system (HLA, Human Leukocyte Antigen, DQB1*0602).[19]

The hypocretin system in other disorders

Abnormalities of sleep resembling those seen in narcolepsy, inspired an interest in hypocretin function in neurodegenerative disorders, such as **Alzheimer's Disease**, **Parkinson's Disease** and **Huntington's Disease**. Furthermore, intriguing reports about sleep disturbances and even a state resembling cataplexy in the **Prader-Willi Syndrome** have led to an interest in hypocretin functioning in this genetic disorder that affects the hypothalamus.[20] There are not many tools to assess hypocretin functioning. Electrophysiological tests, imaging techniques, blood- and even CSF measurements provided inconclusive results.[21-23] Therefore, we decided to study post-mortem brain material from these disorders.

Involvement of hypocretin in narcoleptic symptoms other than sleep

Although the link between hypocretin deficiency and the sleep-related symptoms of narcolepsy has been well established, there are other consequences of a lack of hypocretin that have to be studied in more detail. Most importantly, it is still unknown how a

lack of hypocretin results in the emotion-triggered cataplectic attacks that characterize narcolepsy (see box 3). In fact, the relationship between hypocretin deficiency and cataplexy is stronger than that with excessive daytime sleepiness. Virtually all patients suffering from cataplexy are hypocretin-deficient, but narcoleptic patients that do not have cataplexy often still have detectable amounts of hypocretin in their CSF.[24]

Metabolism

Hypocretin-deficient narcoleptic patients are more obese than healthy controls and subjects suffering from idiopathic hypersomnia, a disorder resembling narcolepsy in that subjects also suffer from EDS, but in whom there is no hypocretin deficiency. [25] Obesity in narcolepsy thus seems to be related in some way to hypocretin deficiency. Involvement of the hypocretin system in metabolism is also indicated by effects of hypocretin administration and hypocretin antagonists administration on food intake, as well as by anatomical connections between the hypocretin system and the hypothalamic circuitry responsible for the regulation of metabolism.

Thermoregulation

Relationships between skin temperature and sleep have been discovered in the 1930's but were largely neglected afterwards until recently.[26] Core body temperature is higher during the day than during the night. In contrast, skin temperature follows the opposite pattern, i.e., it is higher during the night and lower during the day.[27] The core body temperature rhythm is intrinsically linked to that of sleep and wakefulness. Warmer hands and feet promote the onset of sleep, while active manipulation of the skin temperature affects sleepiness.[27] Thermoregulation and sleep/wake regulation are both major functions of the hypothalamus and as such hypocretin deficiency may be involved in the regulation of temperature.

Box 3: Narcolepsy

Narcolepsy is a sleep/wake disorder that affects between 25 and 50 per 100,000 people.[33] It is a severely disabling disorder characterized by an instability of wakefulness and the various sleep stages, meaning that these cannot be maintained for long periods, so frequent unwanted transitions between these states ensue.[34] The classical symptoms of narcolepsy are:[35,36] excessive daytime sleepiness, cataplexy (a sudden, bilateral loss of muscle tone luxated by strong emotional stimuli -such as laughter- with preserved consciousness), hypnagogic hallucinations (very vivid, often frightening dream-like experiences that occur during the transition between wakefulness and sleep) and sleep paralysis (an inability to move during the onset of sleep or upon awakening, while patients are subjectively awake). Other important symptoms are fragmented nocturnal sleep, disturbed vigilance[37] and obesity[25]. Determination of the hypocretin levels in the cerebrospinal fluid (CSF) has become a diagnostic test for narcolepsy with cataplexy.[24,38] Treatment of narcolepsy is currently based on antidepressants working against cataplexy, sleep paralysis and hypnagogic hallucinations. Stimulants, such as modafinil and methylphenidate, are used to treat excessive daytime sleepiness. Gammahydroxybutyrate is a relatively new hypnotic that may improve all symptoms.[39]

Autonomic Nervous System

Fat tissue is densely innervated by both sympathetic and parasympathetic fibers. Metabolism is increased when sympathetic tone is higher.[28] A higher temperature of the distal skin is linked to an increased loss of heat. This can in turn be due to peripheral

Scope of the Present Thesis

In this thesis many of the above-mentioned links of the hypocretin system are investigated.

Part I - The Hypothalamus and its Hypocretin Neurons

The first three chapters deal with the hypothalamic hypocretin system in disorders that are accompanied by narcolepsy-like sleep disturbances, i.e. **Prader-Willi Syndrome** (chapter 1), **Parkinson's Disease** (chapter 2) and **Huntington's Disease** (chapter 3). To determine whether the hypocretin system is affected in these disorders, the total number of hypocretin neurons was determined using quantitative techniques in post-mortem human hypothalami. Furthermore, hypocretin levels in both post-mortem CSF and brain tissue were measured in patients with Parkinson's and Huntington's Disease.

The reason why hypocretin neurons disappear in **narcolepsy** is still a mystery. A putative autoimmune aetiology has been hypothesized, but a screening for **auto-antibodies** and a n=1 trial with **intravenous immunoglobulins** yielded no unequivocal results in favor of this hypothesis (chapters 4 and 5).

Part II - When Hypocretin Neurons are Absent: Narcolepsy

The consequences of hypocretin deficiency in narcoleptic patients are explored, focussing on **vigilance** (chapter 7), **metabolism** and the **autonomic nervous system** (chapter 8) and **skin temperature regulation** (chapters 9-11).

The ability of a specific neuropsychological test to measure **vigilance** as a severity indicator for narcolepsy is explored in chapter 7.

Two possible causes for the obesity commonly seen in narcolepsy are a decreased **basal metabolic rate** and a changed **autonomic tone**, reflected in an abnormal heart rate and blood pressure variability. In chapter 8 both elements are examined in hypocretin-deficient narcoleptic subjects.

To assess the influence of hypocretin deficiency on **skin temperature regulation**, thermoregulatory profiles of the proximal and distal skin of narcoleptic subjects were compared to profiles of healthy controls during a daytime sleep registration in chapter 9. To further study whether changes in skin temperature regulation can causally affect sleep, both core body and skin temperatures were **manipulated** while sleep and vigilance were measured in chapters 10 and 11.

vasodilatation caused by a decrease in sympathetic tone.[26] The regulation of body weight, metabolism and body and skin temperature is influenced by the autonomic nervous system. Since integration of autonomic function with many other bodily functions is situated in the hypothalamus, it is possible that the autonomic nervous system has a role in narcoleptic symptoms.

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The Number of Hypothalamic Hypocretin (Orexin) Neurons is Not Affected in Prader-Willi Syndrome

Based On: *Fronczek R, Lammers GJ, Balesar R, Unmehopa UA, Swaab DF. J Clin Endocrinol Metab. 2005;90:5466-70.*

CHAPTER 1

The Number of Hypothalamic Hypocretin (Orexin) Neurons Is Not Affected in Prader-Willi Syndrome

Context **Narcoleptic patients with cataplexy have a general loss of hypocretin (orexin) in the lateral hypothalamus, possibly due to an autoimmune-mediated degeneration of hypocretin neurons. In addition to excessive daytime sleepiness, Prader-Willi syndrome (PWS) patients may show narcolepsy-like symptoms, such as sleep onset rapid eye movement sleep and cataplexy, independent of obesity-related sleep disturbances, which suggests a disorder of the hypocretin neurons.**

Objective **We hypothesized that the narcolepsy-like symptoms in PWS are caused by a decline in the number of hypocretin neurons.**

Design **We estimated the number of hypocretin neurons in postmortem hypothalami using immunocytochemistry and an image analysis system.**

Setting **This study was conducted at the Netherlands Institute for Brain Research.**

Patients **Eight PWS adults, three PWS infants, and 11 controls were studied.**

Results **There was no significant difference in the total number of hypocretin-containing neurons among the seven PWS patients (in whom sufficient hypothalamic material was available to quantify total cell number) and seven age-matched controls, either in adults or in infants. A significant decline with age was found in adult PWS patients ($r = -0.9$; $P = 0.037$).**

Conclusion **We conclude that a decrease in the number of hypocretin neurons does not play a major role in the occurrence of narcolepsy-like symptoms in PWS.**

Narcolepsy is a sleep disorder characterized by excessive daytime sleepiness (EDS), cataplexy, premature transitions to rapid eye movement (REM) sleep, known as sleep-onset REM periods, sleep paralysis, and hypnagogic hallucinations.[1] In addition, obesity is a common feature in narcoleptic patients.[2] Patients with cataplexy have lowered cerebrospinal fluid (CSF) levels of the neuropeptide hypocretin (orexin) as an indirect reflection of a loss of hypocretin neurons in the perifornical area of the hypothalamus, possibly due to an autoimmune process.[3,4] Prader-Willi syndrome (PWS), the most common syndromal cause of human obesity, is characterized by an insatiable hunger from childhood onward, mental retardation, hypogonadism and growth deficiency, whereas hypotonia, feeding problems, and failure to thrive are the predominant features in the neonatal period.[5] The molecular genetic cause is nonexpression of the paternal genes in the PWS region on chromosome 15q11-13.[6] EDS in PWS is a symptom that has only recently attracted attention because it was first thought to be due to sleep apnea

related to obesity.[7] There have been several reports, however, that PWS patients show EDS, sleep onset with REM, and in some cases even cataplexy, independent of obesity-related sleep disturbances.[8,9] Interestingly, there are preliminary studies reporting lower CSF levels of hypocretin in several patients, which suggests hypocretin neurons are affected in PWS.[10-12] We determined the number of hypocretin-containing cells in the postmortem lateral hypothalamus of PWS adults, infants, and matched controls using immunocytochemistry.

Patients and Methods

Hypothalamic material

Hypothalami from eight PWS adults and three PWS infants from different clinical centers were used. Eight adult controls and three control infants, matched for age, sex, postmortem delay (PMD), fixation time, and premortal illness duration, were obtained through The Netherlands Brain Bank. Clinicopathological details are given in Table 1. Permission was obtained for a brain autopsy and for the use of human material and clinical information for research purposes. Exclusion criteria for control subjects were: primary neurological or psychiatric disease, glucocorticoid therapy during premortal illness, and weight problems, such as excessive weight loss before death or tube feeding. An exception was control 91-009 (Table 1), who suffered from tetraplegia secondary to cervical birth trauma. The clinical histories of the PWS adults and infants have been described previously, except for 03-021, 00-028, and 02-074.[13-16] No direct mentioning of the occurrence of EDS, sleep onset REMs, or cataplexy could be found in the records of either the previously published or unpublished PWS medical histories. All PWS patients met Holmes clinical criteria, and six had genetically confirmed diagnoses (Table 1). Tissues were fixed in 10% PBS (pH 7.4) formalin at room temperature. Hypothalami were paraffin-embedded and serially sectioned at 6µm from rostral to caudal. Every 100th section was stained with thionin for orientation.

Hypocretin-1 immunocytochemistry

Every 100th section in the expected hypocretin-1 cell area, from the level where the fornix touches the paraventricular nucleus to the level where the fornix reaches the corpora mammillaria, was stained using a hypocretin-1 (orexin A) antibody (Phoenix Pharmaceuticals, Inc., Belmont, CA; catalog no. H-003-30, batch no. R2626) and visualized according to the avidin-biotin complex method using diaminobenzidinenickel solution to finish the staining as described previously by Goldstone et al.[17] If these slices did not cover the whole hypocretin-1 area, extra sections were added at equal distances, both rostral and caudal, until no more hypocretin cells were present. Mean (± sd) number of sections added per subject was 1.75 ± 2.79.

Antibody specificity

To test the specificity of the antibody, a dot blot was performed, adding a dilution of 1:1250 antihypocretin onto 2% gelatin-coated nitrocellulose paper (0.1-µm pore size) containing different spots with 30 µl hypocretin-1, somatostatin (1–14), somatostatin (1–28), galanin, melanin-concentrating hormone-1 receptor, β-lipotropin, substance-P,

Table 1.1

NBB no.	Sex	Age (years)	PMD (hours)	Fixation time (days)	Brain weight (g)	Cause of death	Other clinical problems	Premorbid illness duration (days)	Hcrt-1 cell number (x 1000)
Prader-Willi syndrome (sufficient material available for quantification)									
98-168	F	6 Mo	9.75	60	772	Asphyxia	PW71B maternal methylation pattern, severe hypotonia	1.0	78.7
03-021	M	3	41.0	63	1360	Unknown (possible asphyxia)	Gastro-enteritis	1.0	52.7
96-034	F	25	35.1	26	1300	DIC post operation. Repair perforated gastric ulcer	BMI 24.6, ch 15q11-13 del	1.5	90.2
00-028	M	32	<48.0	59	1550	Sudden death following 2 days of fever, diarrhoea and vomiting	Weight 76 kg, ch 15q11-13 del	3	87.9
91-058	F	33	5.0	33	1223	Pneumonia	Congestive cardiac failure, BMI 27.1	4	82.5
02-074	M	49	-	50	-	-	Diabetes	-	42.7
90-111	F	64	20.0	14	1150	Respiratory failure	BMI 30.9	4	74.2
Mean		29.5	22.7	43.6	1226			2.4	72.7
Median		32.0	20.0	50.0	1262			2.3	78.7
SD		22.9	18.9	19.2	261			1.4	18.1
Controls (used in means between groups analysis)									
86-041	M	6 Mo	6.5	14	800	SIDS	-	1	88.8
88-050	M	9 Mo	41.0	164	940	SIDS	-	1	82.2
02-076	M	27	-	31	1520	Drowning	-	1	79.9
85-041	F	28	5.4	44	1365	Cardiogenic shock post myocardial infarction	Crohn's disease	1	77.3
91-009	F	36	71.5	61	1348	Faecal peritonitis from perforated peptic ulcers	Tetraplegia secondary to cervical birth trauma	1	111.9
94-035	M	49	7.7	40	1404	Cardiac arrhythmia	Hypertension	1	78.5
01-069	F	68	5.75	32	1153	Respiratory insufficiency	-	-	55.2
Mean		29.9	23.0	55.1	1219			1.0	82.0
Median		28.0	7.1	40.0	1348			1.0	79.9
SD		24.4	27.5	50.0	265			0.0	16.8

Table 1.1 (Continued)

NBB no.	Sex	Age (years)	PMD (hours)	Fixation time (days)	Brain weight (g)	Cause of death	Other clinical problems	Premorbid illness duration (days)	Hcrt-1 cell number (x 1000)
Prader-Willi Sydrome (insufficient material available for quantification)									
99-079	F	9 Mo	10.0	76	-	Cardiovascular failure after bronchopneumonia	Hypoglycemia, hypothermia, ch 15q11-13 del	2.0	ND*
83-011	F	30	4.5	365	1310	Sepsis post operation. Repair enterocutaneous fistula	Jejuno-ileal bypass and small bowel resection 6-10 y ago, BMI 42.2	35	ND*
93-056	M	38	45.0	385	1540	Diabetic ketoacidosis	BMI 38.5, ch 15q11-13 del	1	ND*
95-104	M	51	16.0	32	1570	Pneumonia	Hypertension, testicular seminoma 28y, BMI 33.8, ch15 UPD	7	ND*
Mean		29.9	18.8	214.5	1473			11.3	
Median		34.0	13.0	220.5	1540			4.5	
SD		21.3	18.0	186.4	2142			16.0	
Controls (used in regression analysis)									
97-153	F	7 Mo	20.4	39	760	SIDS	-	1	56.0*
92-037	F	32	30	45	1280	Bronchopneumonia/bronchitis	Hyperventilation	-	80.5*
99-071	M	39	16.50	130	1400	Myocardial infarction	Hypercholesterolemia	1	127.3*
94-118	M	49	22.3	33	1254	Faecal peritonitis post revision ileocolonic anastomosis	Adenocarcinoma	32	54.9*
Mean		30.2	22.3	61.8	1174			11.3	79.7
Median		35.5	21.4	42.0	1267			1.0	68.3
SD		20.9	5.7	45.8	283			17.9	33.9

BMI, Body Mass Index (in kg/m2); ch, chromosome; del, deletion; DIC, Disseminated Intravascular Coagulation; F, female; M, male; Mo, months, NBB no., Netherlands Brain Bank number; ND, not determined; PMD, post mortem delay; SIDS, Sudden Infant Death Syndrome; SD, standard deviation; UPD, uniparental disomy; -, Unknown. * Incomplete patient, or control matched with an incomplete patient

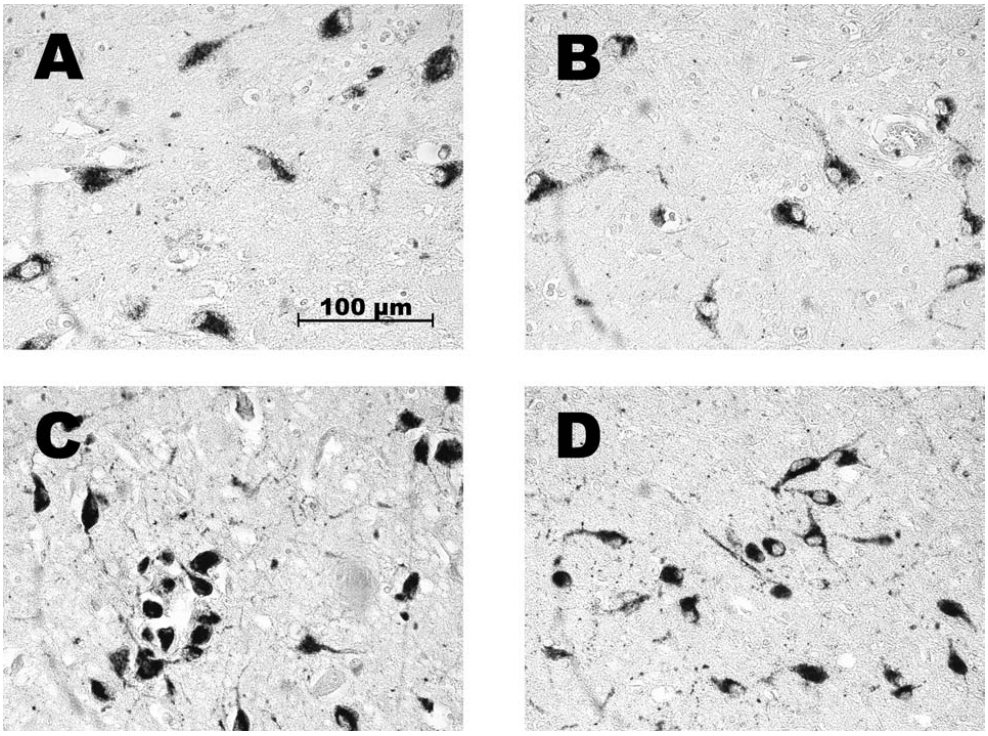

Figure 1.1 **Examples of staining of hypocretin-IR cell bodies**

Examples of staining of hypocretin-IR cell bodies in the lateral hypothalamus of an adult control subject #02-076 (A), an adult Prader-Willi patient #91-058 (B), a control infant #97-153 (C) and a Prader-Willi infant #99-079 (D). There was no significant difference in the intensity of staining and the distribution pattern. Note that the density of cell bodies is higher in the infant subjects, which is accompanied by a smaller volume of the hypothalamic area containing these neurons.

γ-melanocyte-stimulating hormone, LHRH, adrenocorticotropic hormone (1–39), neurotensin, oxytocin, CRH , agouti-related protein (83– 132), neuropeptide-Y, GHRH (1– 40), arginine-vasopressin, desacetylmelanocyte-stimulating hormone, neuropeptide EI, β-melanocyte-stimulating hormone, glycoprotein hormone receptor, cocaine- and amphetamine-regulated transcript, or melanin-concentrating hormone. The next day, the nitrocellulose sheet was incubated with secondary antibody, avidin-biotin peroxidase complex, and diaminobenzidinenickel solution to finish the staining. The only spot that showed staining was the one containing hypocretin-1. Specificity was further confirmed by the absence of staining in hypothalamic sections using antiserum preadsorbed with human hypocretin-1 peptide fixed overnight with 4% formaldehyde onto gelatin-coated nitrocellulose filter paper, 0.1 µm, and the presence of staining when preadsorbed with α-melanocyte-stimulating hormone peptide, which did not differ from unadsorbed serum.

Immunocytochemistry quantification

An estimate of the total number of hypocretin-1 immunoreactive (IR) cells was made using an image analysis system (ImagePro version 4.5, Media Cybernetics, Silver Spring) connected to a camera (JVC KY-F55 3CCD) and plain objective microscope (Zeiss Axioskop with Plan-NEOFLUAR Zeiss objectives, Carl Zeiss GmbH, Jena, Germany). Randomly selected fields were counted in every section, covering in total 15% of a manually outlined area containing hypocretin-1 IR cells. This was done by one person while blinded for the diagnosis. Each positively stained profile containing a nucleolus was counted. Calculation of the total number of hypocretin-1 IR neurons was performed by a conversion program based upon multiplication of the neuronal counts by sample frequency of the sections, as was described previously by Goldstone et al.[17] Mean (±sd) number of sections quantified per subject was 13.9±3.5. The coefficient of variation (sd/mean x 100%) of this method was 7.6% (calculated by counting one complete patient five times). Reliability was further confirmed by graphically presenting the actual numbers of neurons counted in every section from rostral to caudal to review the distribution pattern (figures not shown due to space restrictions).

Statistics

Spearman's ρ correlation was performed to assess the effect of age, PMD, fixation time, and duration of premortal illness on hypocretin-1 IR cell number. Means between groups were tested by Mann-Whitney U test, considering $P < 0.05$ to be significant.

Results

Distribution of hypocretin-1-containing neurons

The location and intensity of the hypocretin-1 IR cell bodies was similar in controls, PWS adults, and infants. Hypocretin-1 IR neuronal cell bodies were restricted to the peri-fornical region in the lateral hypothalamus. On the level where the fornix crosses the paraventricular nucleus, some hypocretin-1 IR cell bodies started to appear in the supraoptic area. In subsequent levels, the fornix migrated to the corpora mammillaria while passing through an area with a high number of hypocretin-1 IR cell bodies. When the fornix reached the corpora mammillaria, there were still many hypocretin-1 IR cell bodies visible.

Hypocretin-1 cell number in PWS and controls

In four PWS patients (three adults, one infant), the area showing hypocretin-1 IR cell bodies was not completely present in the available hypothalamic material. Therefore, the total cell counts of these patients and their matched controls were not included in this final analysis. Extrapolation of the data obtained from the material that was available by comparing the distribution patterns of the incomplete patients with those of complete cases did not point to a different number of cells and would thus not have changed the final outcome. Controls 94-035 and 94-118 were an equal match to incomplete patient 95-104. Exclusion of either one of these controls did not influence the outcome. In the analysis presented here, control 94-118 was excluded. There were no significant differences among sex, PMD, fixation time, or premortal illness duration

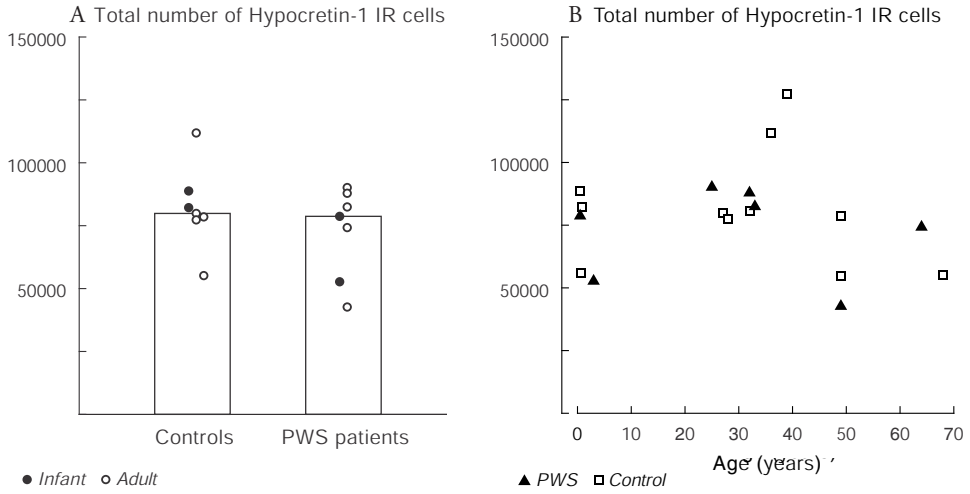

Figure 1.2 Results

(A, Left) Median number of hypocretin IR cells in Prader-Willi Syndrome patients (right bar) and controls (left bar). Open circles represent adults, filled circles represent infants. There is no significant difference between the two groups (Mann-Whitney U: n=14, p=0.56). (B, Right) Correlation between age and total number of hypocretin IR cells. Squares represent controls; triangles represent Prader-Willi Syndrome patients. The total number of hypocretin-1 neurons declines with age. In PWS adults, a correlation between age and total IR cell number was found (n=5, r=0.900, p=0.037).

between groups. Furthermore, there was no significant correlation of these variables with hypocretin-1 cell number in PWS, controls, or the combined group. The mean (±sd) number of cells found in controls was approximately 82,000±16,800. There was no significant difference in hypocretin-1 IR cell number in PWS adults or infants compared with controls (n = 14; P = 0.56; Figs. 1 and 2A).

Effects of age on cell number

The total number of hypocretin-1 IR neurons declines with age (Figure 1.2B). In PWS adults, a negative correlation between age and total hypocretin-1 IR cell number was found (n = 5, r = -0.900, P = 0.037). In controls (all eight adults included), this was not the case (n = 8, r = -0.395, P = 0.333), whereas after pooling of all adult subjects, a trend remained present (n = 13, r = -0.537, P = 0.059).

Discussion

In this study, the number of hypocretin-1 IR neurons in postmortem material in PWS patients was not different from that in controls. A significant decrease in hypocretin-1 IR neurons with age was found in PWS adults but not in controls. This lack of significance is caused by two control cases with a remarkably high number of hypocretin-1 IR neurons (91-009 and 99-071). Excluding these two controls leads to a significant correlation with age in the combined adult group (n = 11, r = -0.699, P = 0.017). The decrease in hypocretin-1 IR neurons with age and its functional implications in relation to sleep

homeostasis, endocrine changes, and the autonomic nervous system need further study. An effect of age on hypocretin gene expression and brain content has been found in rats,[18] but in a study by Kanbayashi et al.,[19] human lumbar CSF hypocretin-1 levels did not seem to be related to age. The estimated total number of some 80,000 neurons is similar to the number reported by Thannickal et al.[20] using immunocytochemistry on paraffin-embedded material. A lower total number of hypocretin-expressing cells (15,000–20,000) was found by Peyron et al.[3] using in situ hybridization on frozen material.

Although we found relatively low numbers of hypocretin-1 IR neurons (45,000 –55,000) in one PWS adult (02-074) and one PWS infant (03-021), similar numbers were also found in two control adults (94-118 and 01-069) and one control infant (97-153). This lower number of hypocretin-1 IR cells is not likely to cause any narcolepsy-like symptoms because narcoleptic patients have a 90–95% reduction of hypocretin-1 IR cells, and we found the same low numbers in controls. In agreement with the main findings of this paper, we recently measured a normal level of hypocretin-1 in the CSF of one PWS patient (Lammers, G. J., unpublished data). Because it is still unclear to what extend CSF levels reflect the total number of hypocretin-1 neurons in the brain, the lowered levels of hypocretin-1 in the CSF of PWS patients measured by Mignot et al., Nevsimalova et al., and Arii et al.[10-12] could be caused by other, unknown factors. No CSF samples were available for the PWS patients and control subjects in this study. Furthermore, it is not known whether the PWS subjects had narcoleptic features. The clinical records available to us were either incomplete in this respect, and the appropriate investigations (e.g. electrophysiology, sleep studies) were not performed. It is still possible that individual PWS subjects with clear narcoleptic features may turn out to have a disturbed hypothalamic hypocretin system, reflected in a lower number of hypocretin IR neurons.

In conclusion, although the determination of hypocretin-1 mRNA and receptors may give additional information in the future, neither the hypocretin cell number nor the intensity of staining was different in PWS patients tested. It is not conclusive whether a decrease in hypocretin neurotransmission explains the occurrence of the narcolepsy-like symptoms associated with some patients afflicted with PWS.

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Visualizing the Hypocretin Receptor in Prader-Willi Syndrome

Fronczek R, Wong K, Unmehopa UA, Lammers GJ, Swaab DF. Unpublished

CHAPTER 1A

Visualizing the Hypocretin Receptor in Prader-Willi Syndrome

Introduction

In the study described in chapter 2 we found no lowered number of hypocretin neurons in PWS patients. There have been several reports, however, that the hypocretin receptors may be involved in narcolepsy-like symptoms.[1,2] We hypothesized, therefore, that the number of hypocretin-receptor-1 and/or -2 expressing cells may be decreased in the hypothalamus of PWS patients. The aim of this study was to quantify hypocretin-receptor-1 and -2 expressing cells in PWS patients and controls. Therefore, we had to determine the optimal antibody for the hypocretin-receptors in human hypothalami. We tested several antibodies on hypothalami and pituitaries of healthy controls obtained from the Netherlands Brain Bank.

Methods

Post-mortem material and Antibodies

Hypothalami from four control subjects (Table 1b.1) and pituitaries from three control subjects (Table 1b.2) were used to test 7 different antibodies against both hypocretin receptor 1 and 2 (Table 1b.3).

Staining procedure

Sections were stained using the different antibodies at the manufacturer's recommended dilution and 1:500, and visualized according to the ABC-method using DAB-nickel solution to finish the staining as described previously.[5]

Antigen retrieval techniques (used before incubation with the first antibody)

Proteinase K treatment at 10µg/ml at 37°C
Citrate Buffer pH 6.0, 10 min boiling
pH 4.0 (citrate buffer), pH 7.0 (TBS) and pH 9.0 (0.05 M Tris-HCl buffer)
Microwave treatment at 900W for 2x5 min.

Blocking steps (added to pre-incubation rinsing and incubation solution)

1%, 3%, 5% milk (ELK, Melkunie, The Netherlands)
0.1% Bovine Serum Albumin (BSA) + 1.5% Normal Goat Serum (NGS)
TRIS-pH 9.0 high salt 0.05%

Table 1b.1 Overview of the hypothalami used in the experiments

NBB Number	Age (yrs)	Sex	Brain-weight (g)	pH	Post-Mortem Delay (hours)	Diagnosis
93-025	68	M	1157	-	<41:00	Respiratory Insufficiency
97-088	78	F	1351	6.20	4:15	Braak 1
91-207	75	F	920	-	5:35	-
96-081	61	F	1311	-	5:15	Space Occupying Process

Table 1b.2 Overview of the pituitaries used in the experiments

NBB Number	Age (yrs)	Sex	Brain-weight (g)	pH	Post-Mortem Delay (hours)	Diagnosis
93-025	68	M	1157	-	<41:00	Respiratory Insufficiency
96-013	68	F	1122	6.80	10:30	-
96-075	76	M	1449	7.31	5:45	Dementia with Senile Involutive Cortical Canges

Table 1b.3 Overview of the antibodies used in the experiments

Company	Code	Target	Publication
Lifespan Biosciences	LS-A6638	hypocretin receptor 1	-
	LS-A6641	hypocretin receptor 1	
	LS-A6677	hypocretin receptor 1	
Chemicon	AB3092	hypocretin receptor 1	Suzuki et al. 2002[3]
	AB3094	hypocretin receptor 1	
Alpha Diagnotics	OX1R11-A	hypocretin receptor 1	Blanco et al. 2001[4]
	OX2R22-A	hypocretin receptor 2	

Results

Both the Chemicon and Alpha Diagnostic antibodies did not produce any staining in the hypothalamus. The hypocretin receptor 2 antibody of both companies did produce a very weak staining in a few pituitaries (Figure 1b.1). Antigen retrieval or blocking steps did not lead to any improvement.

The Lifespan antibodies did not produce any stained cell bodies in the hypothalamus or the pituitary. However, what could be seen in some cases was a dense network of 'boutons' in the striatum, especially using Lifespan 1 (Figure 1b.2). However, this staining was not seen in all hypothalami. Antigen retrieval or blocking steps did not lead to any improvement.

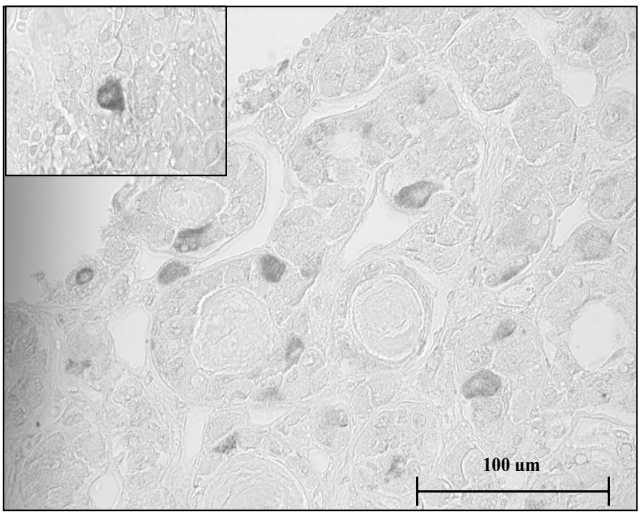

Figure 1b.1
Example of staining
Alpha Diagnostics OX2R22-A (receptor 2) 1:500 40x: Pituitary 95-207 (large) and Pituitary 96-013 (inset).

Discussion

In this study, none of the antibodies produced a reliable staining. Two antibodies directed against hypocretin receptor 2 (Chemicon and Alpha Diagnostics) did produce a weak signal in some pituitaries, but this signal could not be improved. Furthermore, the three different Lifespan antibodies produced a dense network of stained boutons in the striatum of some hypothalami.

In contrast, previous studies have reported results using some of the antibodies we studied: Suzuki et al. identified hypocretin receptor 1 immunoreactivity in chemically identified target neurons in the rat (not human) hypothalamus using the Chemicon antibody.[6] These hypocretin-containing neurons induced a phospoholipase C-mediated release of Ca^{2+} from intracellular stores. Blanco et al. studied the cellular localization of hypocretin receptors in the human pituitary, using the Alpha Diagnostic antibodies.[7] The results demonstrated that hypocretin receptors 1 and 2 were expressed by somatotrope and corticotrope cells, respectively in the human pituitary. The fact that we only found a weak and vague hypocretin receptor 2 signal in the pituitaries we studied, could be due to the type of material we used (older subjects, longer fixation times), but non-specific staining in the Blanco study cannot be excluded, since only preabsorbtion of the primary antibodies with hypocretin-1 and 2 before incubation was used as a test of specificity in that study.[8] Suzuki et al not only used preabsorbtion with the antigen to determine specificity, but performed a western blot analysis as well, which showed that the Chemicon antibodies recognize a hypothalamic rat polypeptide of 50 kDa, the expected molecular mass of orexin receptors given the amino acid sequence of orexin receptors in the rat brain.[9] Note that all these results were obtained from rat material.

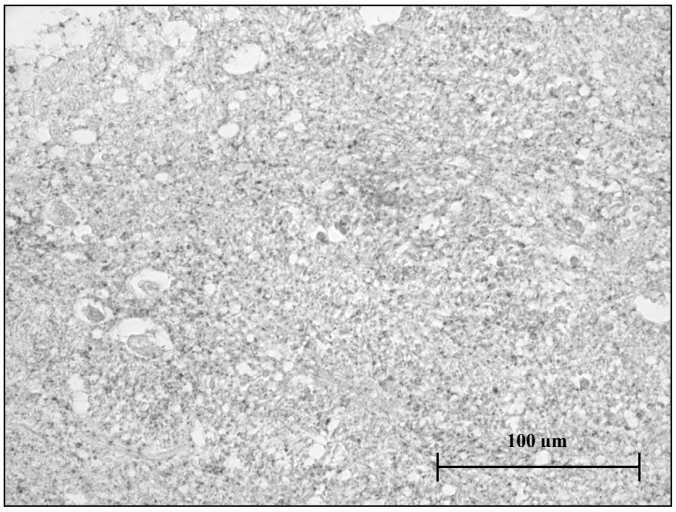

Figure 1b.2
Example of staining
Lifespan Biosciences Ls-A6638: 9713/14AP3-1 (receptor 1) 1:500 40x: NBB 93-025 (M68).

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Hypocretin (Orexin) Loss in Parkinson's Disease

CHAPTER 2

Based On: *Fronczek R, Overeem S, Lee SY, Hegeman IM, van Pelt J, van Duinen SG, Lammers GJ, Swaab DF. Brain. 2007;130:1577-85.*

Hypocretin (Orexin) Loss in Parkinson's Disease

Objective **The hypothalamic hypocretin (orexin) system plays a central role in the regulation of various functions, including sleep/wake regulation and metabolism. There is a growing interest in hypocretin function in Parkinson's disease (PD), given the high prevalence of non-motor symptoms such as sleep disturbances in this disorder. However, studies measuring cerebrospinal fluid hypocretin levels yielded contradictory results so far.**

Methods **In PD patients and controls we (1) estimated the number of hypocretin neurons in post-mortem hypothalami using immunocytochemistry and an image analysis system and (2) quantified hypocretin levels in post-mortem ventricular cerebrospinal fluid (CSF) and (3) prefrontal cortex using a radioimmunoassay. Furthermore, presence of Lewy bodies was verified in the hypothalamic hypocretin cell area.**

Results **Data are presented as median (25^{th}-75^{th} percentile). We showed a significant decrease between PD patients and controls in (1) the number of hypocretin neurons (PD: 20,276 (13,821 – 31,229); controls: 36,842 (32,546 – 50,938); p = 0.016), (2) the hypocretin-1 concentration in post-mortem ventricular CSF (PD: 365.5 pg/ml (328.0 – 448.3); controls: 483.5 (433.5 – 512.3); p = 0.012) and (3) the hypocretin-1 concentrations in prefrontal cortex (PD: 389.6 pg/g (249.2 – 652.2); controls: 676.6 (467.5 – 883.9); p = 0.043).**

Conclusion **Hypocretin neurotransmission is affected in PD. The hypocretin-1 concentration in the prefrontal cortex was almost 40% lower in PD patients, while ventricular CSF levels were almost 25% reduced. The total number of hypocretin neurons was almost half compared to controls.**

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder in which motor symptoms such as hypokinesia, tremor and rigidity are the most well-known. However, there is a growing interest in the non-motor symptoms, such as autonomic dysfunction and cognitive disturbances.[1] Of these, sleep disorders are one of the most striking.[2-4] Sleep disturbances occur often in patients with PD and can even precede the motor symptoms. Excessive daytime sleepiness with frequent naps and so-called 'sleep-attacks' have been reported in 15-50% of patients.[5,6] Furthermore, there are clear nighttime sleep disturbances, such as fragmented nocturnal sleep, REM-sleep behavior disorder and periodic leg movements, as well as daytime sleep-onset REM periods.[3,4,7] The

combination of these symptoms suggest a common etiology with narcolepsy.[8]

Narcolepsy is a primary sleep-wake disorder characterized by excessive daytime sleepiness and REM-sleep dissociation phenomena such as cataplexy. Moreover, there are core symptoms of narcolepsy that resemble the nighttime sleep disturbances commonly seen in PD, most notably fragmented nocturnal sleep and REM-sleep behavior disorder.[9] Furthermore, sleep-onset REM periods form one of the neurophysiological characteristics of narcolepsy. Narcolepsy is caused by a loss of hypocretin (orexin) producing neurons, reflected in undetectable cerebrospinal fluid (CSF) levels.[10] Hypocretin neurons are exclusively located in the lateral hypothalamus and project widely throughout the central nervous system,[11] where they have an excitatory effect on several autonomic, metabolic, neuro-endocrine and arousal systems.[12]

In PD, there is a progressive and irreversible degeneration of dopaminergic neurons projecting from the substantia nigra to the striatum. In addition, there are degenerative changes in many other parts of the brain, including the hypothalamus.[13] Lewy bodies, the pathophysiological hallmark of PD, have been found in various brain regions, again including the hypothalamus.[14] These observations suggest that there are hypothalamic changes in PD, thus possibly involving the hypocretin system.
Several studies have been conducted to detect damage to the hypocretin system in PD. However, these only assessed CSF hypocretin levels. Moreover, results have been conflicting. One study reported decreased levels in ventricular CSF in late stage PD patients,[15] but three other groups found normal concentrations in spinal CSF.[16-18]

Here we used a combination of approaches in three brain compartments to detect whether the hypocretin system is affected in PD. First, we measured hypocretin levels in post-mortem ventricular CSF. Second, hypocretin content was determined in peptide extracts from cerebral cortex, as this has been shown to be a more sensitive technique compared to CSF measurements.[10] Third, we directly counted the total number of hypocretin neurons in the lateral hypothalamus of PD patients versus matched controls.

Materials and Methods

Post Mortem Material

Hypothalami and ventricular CSF were provided by The Netherlands Brain Bank. Frozen prefrontal cortex tissue was obtained from the Leiden PD Brain Bank. Permission was obtained for a brain autopsy and for the use of human material and clinical information for research purposes. The controls were matched for age, sex and Alzheimer Braak stage (for both groups ≤2).[19] Clinicopathological details are given in tables 1 and 2. Exclusion criteria for control subjects were use of corticosteroids and primary neurological or psychiatric disease, unless stated otherwise. This was verified by a systematic neuropathological analysis.[20,21] In all PD patients the clinical diagnosis was confirmed by a systematic neuropathological examination;[20] all patients were late-stage PD. No direct mentioning of the occurrence of EDS, sleep onset REMs or cataplexy could be found in the medical records of PD patients.

Table 2.1 Clinicopathological details of subjects used for hypocretin cell count and ventricular CSF measurement

	NBB no.	Age	Sex	PMD (hours)	Fixation time (days)	Cause of death	Hcrt-1 cell number x10^3	CSF (pg/ml)
PD	00-102	56	M	5.08	37	Malignant neuroleptic syndrome	N.D.	404
	02-057	62	M	9.25	43	Combined kidney and liver failure	22.1	327
	93-064	73	F	41.00	34	Massive pneumonia of the left lung	40.6	N.A.
	94-092	77	F	9.67	24	Cachexia	14.2	327
	02-013	80	F	5.50	30	Cachexia	18.4	331
	98-043	81	F	4.17	43	Pneumonia	28.9	377
	00-034	86	M	8.50	45	Unknown	12.5	463
	01-122	86	M	5.58	32	Aspiration pneumonia	13.7	354
	02-064	87	M	7.33	36	Respiratory insufficiency	32.0	486
Median		80.0		7.33	36.0		20.3	366
Percentiles (25th-75th)		67.5-86.0		5.12-9.45	31.0-43.0		13.8-31.3	328-448
Controls	98-127	56	M	5.42	35	Myocardial infarction	49.1*	554
	99-101	69	M	19.25	41	Pneumonia, unexpected death in sleep	39.2	468
	97-156	77	F	2.67	47	Unknown	32.4	495
	93-139	78	F	6.42	32	Respiratory insufficiency	17.4	472
	00-142	82	F	5.50	36	Myocardial infarction	35.5	368
	00-022	83	F	7.75	34	Myocardial infarction	53.2	N.A.
	95-106	74	M	8.00	60	Myocardial infarction	33.5	422
	94-076	78	M	8.42	24	Cardiac arhythmia	65.9	495
	00-072	78	M	18.00	45	Kidney failure, dehydration	44.2	518
Median		78.0		7.75	36.0		38.2	484
Percentiles (25th-75th)		71.5-80.0		5.45-13.20	33.0-46.0		32.9-51.2	434-512

NBB no, Netherlands Brain Bank number; PMD, postmortem delay; M, male; F, female; N.D., not determined; N.A., not available; Hcrt-1, Hypocretin-1.
* This control was matched with the incomplete PD patient and thus excluded from the group analysis.

Table 2.2 Subjects used for hypocretin brain tissue measurement

	LBB no	Age	Sex	Cause of death	Hcrt-1 tissue content (pg/g)
Controls	91-124	37	M	Myocardial infarction	996
	04-117	46	M	Cardiac arhythmia	364
	92-062	48	M	Myocardial infarction	665
	93-194	52	M	Stroke	688
	04-072	60	F	Pneumonia	433
	04-063	63	F	Leptomeningeal metastasis	737
	04-118	64	M	Myocardial infarction	1368
	00-064	67	F	Aortic Dissection	858
	00-041	68	M	Pneumonia	697
	93-306	70	M	Metastasized carcinoma	1282
	04-071	71	M	Cardiomyopathy	573
	00-067	74	M	Metastasized carcinoma	243
	00-083	76	M	Aortic Dissection	638
	93-303	79	F	Stroke	892
	04-054	83	M	Leptomeningeal metastasis	358
	00-061	86	M	Myocardial infarction	570
Median		68			677
Percentiles (25th-75th)		54-76			468-884
PD	95-008	62	M	Unknown	198
	96-145	70	M	Gastrointestinal Bleeding	534
	87-329	74	F	Unknown	296
	89-215	75	F	Pneumonia	202
	98-057	77	F	Lung Carnicoma	390
	04-055	80	F	Cardiac Asthma	906
	98-136	80	M	Pneumonia	771
	89-199	83	M	Myocardial infarction	304
	89-032	84	M	Cachexia	432
Median		77			390
Percentiles (25th-75th)		72-82			249-653

LBB no, Leiden Brain Bank number; M, male; F, female; N.D., not determined; N.A., not available; Hcrt-1, Hypocretin-1.

Immunocytochemistry

Hypothalami from nine PD patients and nine matched controls were used. Tissues were fixed in 10% PBS (pH 7.4) formalin at room temperature and were paraffin-embedded and serially sectioned at 6 µm from rostral to caudal. Every 100th section in the expected hypocretin cell area, from the level where the fornix touches the paraventricular nucleus to the level where the fornix reaches the corpora mammillaria, was stained using a hypocretin-1 antibody (Phoenix Pharmaceuticals, Inc., Belmont, CA; catalog no. H-003-30). The specificity of this antibody was confirmed in a previous study.[22] Antibody binding was visualized according to the avidin-biotin complex method using diaminobenzidinenickel solution to finish the staining as described previously by Goldstone et al.[23] If these sections did not cover the whole hypocretin area, extra sections were added at equal distances, both rostral

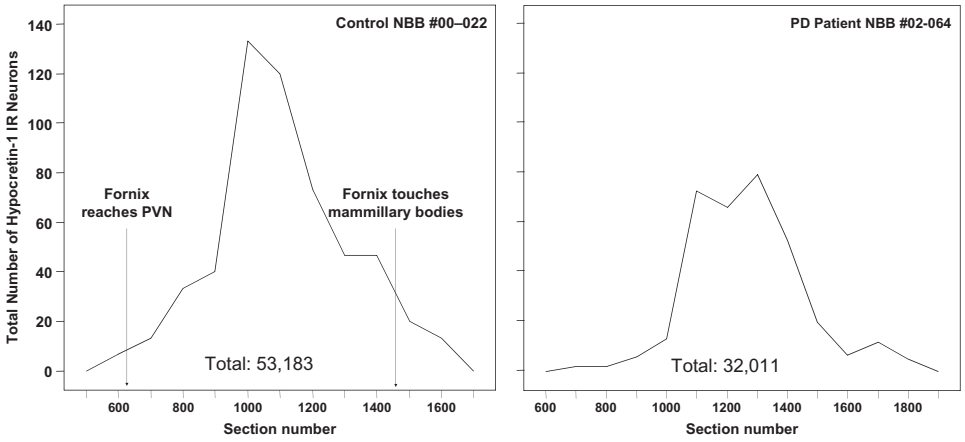

Figure 2.1 **Sample distribution patterns**
Sample distribution patterns of a PD patient (NBB #02-064, left) and a control subject (NBB #00-022, right). For each slide, the total number of hypocretin neurons is shown. The total number of cells is determined by calculating the total area under the curve. Only when the complete hypocretin cell area is contained within the measured slides, a subject can be included.hypothalamic area containing these neurons.

and caudal, until no more hypocretin cells were present.

For each subject, three sections were taken from the middle of the verified hypocretin cell area and double-stained for hypocretin-1 and Lewy Bodies, using a cocktail of the aforementioned hypocretin-1 antibody and an alpha-synuclein antibody (Zymed, Carlsbad, CA; catalog no. 32-8100). Hypocretin-1 antibody binding was visualized using the avidin-biotin complex method described above, while alpha-synuclein antibody binding was visualized using the alkaline-phosphatase blue method.[24,25]

Immunocytochemistry quantification

An estimate of the total number of hypocretin-1 immunoreactive (IR) cells was made using an image analysis system (ImagePro version 5.1, Media Cybernetics, Silver Spring) connected to a camera (JVC KY-F55 3CCD) and plane objective microscope (Zeiss Axioskop with Plan-NEOFLUAR Zeiss objectives, Carl Zeiss GmbH, Jena, Germany). Randomly selected fields were counted in every section, covering in total 15% of a manually outlined area containing the hypocretin-1 IR cells. This was done by one person while blinded for the diagnosis. To prevent influence of cell size, only positively stained cell profiles containing a nucleolus (~2 µm) were counted. This counting procedure, which was judged to be the best for the thin (6 µm) sections used, is based on the principle that nucleoli can be considered as hard particles that will not be sectioned by a microtome knife but, instead, are pushed either in or out of the paraffin when hit by the knife.[26-28] Calculation of the total number of hypocretin-1 IR neurons was performed by a conversion program based upon multiplication of the neuronal counts by sample frequency of the sections, as was described previously by Goldstone *et al.*[23] Mean (±SD) number of sections needed to cover the complete hypocretin area was 9.7±3.1 per subject. The coefficient of variation (SD/mean x 100%) of this method

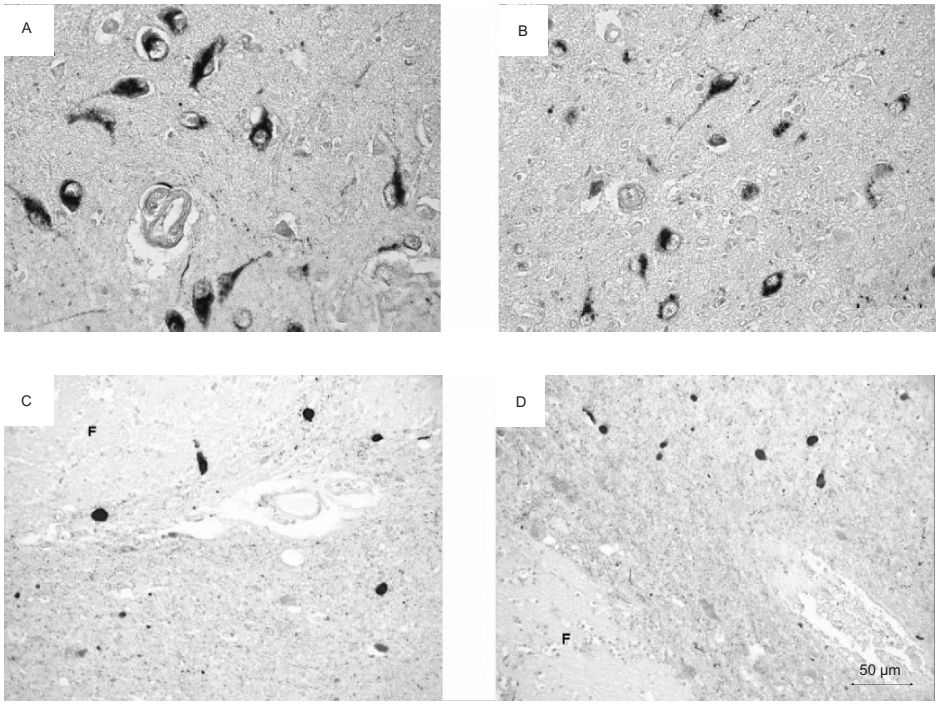

Figure 2.2 Example of staining

Examples of hypocretin-1 cell bodies in the lateral hypothalamus of a control subject NBB #94-191 (A) and a PD patient NBB #91-272 (B). There was no significant difference in the intensity of staining and the distribution pattern of hypocretin neurons. (C, D) Lewy bodies (alpha-synuclein) in the perifornical region of the lateral hypothalamus of two PD patients NBB #01-122 and NBB #94-092. 'F' indicates the fornix.

was 7.3% (calculated by counting one complete subject five times). Reliability and completeness of the cell counting was further confirmed by graphically presenting the actual numbers of neurons counted in every section from rostral to caudal to review the distribution pattern (see sample control and patient in Figure 2.1).

Hypocretin-1 measurements in cortex and CSF

One gram of frozen pre-frontal cortex was used from nine (5 male) PD patients and sixteen (4 male) healthy controls. We used the most rostral part of the pre-frontal cortex, as this cortical region is densely innervated by hypocretin neurons resulting in high hypocretin concentrations.[10] Diced tissue samples were boiled for 10 minutes in 10.0 ml of MilliQ water, cooled to room temperature, acidified using glacial acetic acid and HCl (final concentration: 1.0 M and 20.0 mM respectively), homogenized and centrifuged. The supernatant was acidified again with an equal volume of 0.1% trifluoracetic acid (TFA) and vacuum dried. Samples were re-suspended in 500 µl of RIA buffer before measurements.

Ventricular CSF was available from eight Parkinson's patients and eight matched controls (all these subjects were also included in the immunocytochemistry study).

After collection, ventricular CSF was centrifuged at 2500rpm for 10 minutes and the supernatant immediately stored at -80°C until measurements.

Hypocretin-1 levels were measured using a commercially available radioimmunoassay (RIA) (Phoenix Pharmaceuticals, Belmont, USA). All measurements were conducted in duplicate 100 µl aliquots in a single assay run. The detection limit was 50 pg/ml and intra-assay variability was less than 5%. We used a validated reference sample to adjust levels to previously reported values.[10]

Statistics

All data are given as median (25^{th} – 75^{th} percentile). Group differences were analyzed using the Mann-Whitney U and the chi-square test. Correlations between hypocretin-1 tissue concentration, CSF concentration, cell number, post-mortem delay, fixation time and age were evaluated using Spearman correlation. All reported p-values are two-sided, with 0.05 as the significance threshold.

Results

Hypocretin-1 histochemistry

The location and staining intensity of the hypocretin-1 IR cell bodies was similar in controls and PD patients (Figure 2.2A, B). Hypocretin-1 IR neurons showed the same distribution pattern as described before:[22] cell bodies were restricted to the perifornical region of the lateral hypothalamus. On the level where the fornix crosses the paraventricular nucleus, some hypocretin neurons started to appear in the supraoptic area. In the subsequent levels, the fornix migrated to the corpora mammillaria while passing through an area with a high number of hypocretin cells. When the fornix reached the corpora mammillaria, there were still many hypocretin-1 IR cell bodies visible. In all PD patients Lewy bodies were abundantly present in the perifornical region of the lateral hypothalamus (Figure 2.2C, D), while only a few Lewy bodies could be discerned in one control patient (NBB #00-320). However, hypocretin neurons

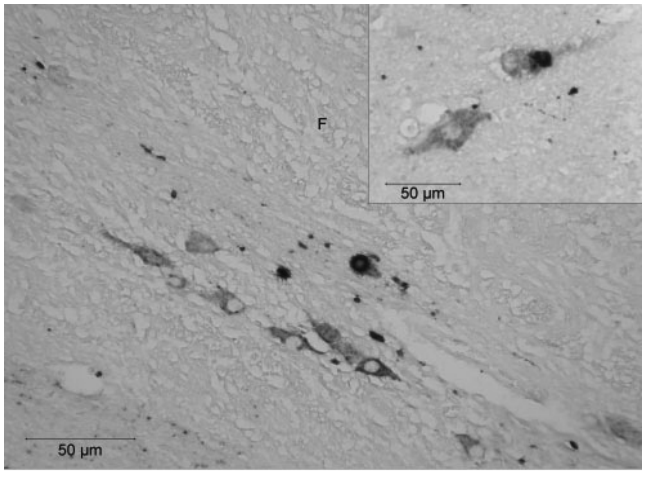

Figure 2.3
Example of Staining
Example of hypocretin neurons (DAB staining in grey) that contain a Lewy body (alpha-synuclein, AP-blue staining, in black) in PD patients NBB #01-280 and #94-245 (insert). Several hypocretin neurons that do not show this colocalization can be seen as well. 'F' indicates the fornix.

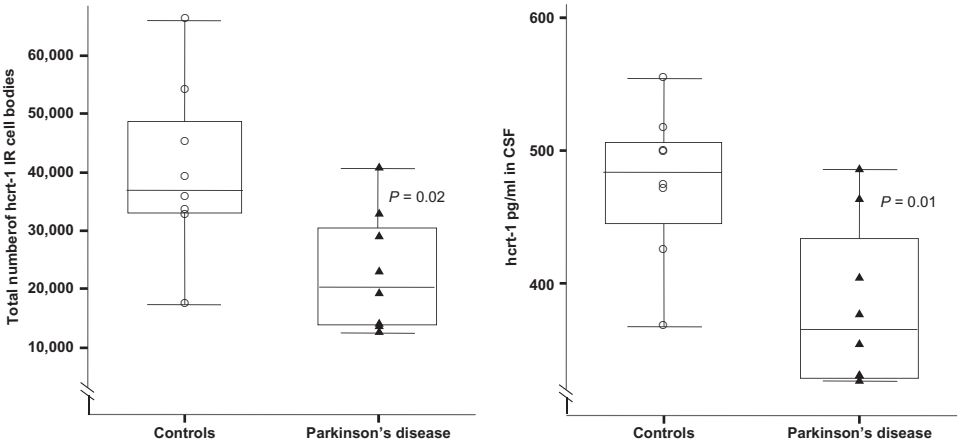

Figure 2.4 Boxplots showing total number of hypocretin neurons and CSF concentration

Boxplots showing the median, 25th-75th percentiles and the range of the number of Hypocretin neurons (A) and the hypocretin-1 concentration in post-mortem ventricular cerebrospinal fluid (B) in PD patients (right) and controls (left). Open circles represent controls, closed triangles represent PD patients.

that contained a Lewy body (Figure 2.3) were rare and only 1-2 double-stained neurons could be discerned in sections that contained numerous hypocretin neurons.

In one PD patient (NBB #00-102, table 1) the area showing hypocretin-1 IR cell bodies was not completely present in the available hypothalamic material. Therefore, the total counts of this patient and the matched control (NBB #98-127) were not included in the final analysis.

Hypocretin-1 cell number

There were no significant differences in age, sex, post-mortem delay (PMD) and fixation time between groups (all $p > 0.43$). Furthermore, there was no significant correlation of these variables with hypocretin-1 cell number in PD patients (all $p > 0.55$), controls (all $p > 0.14$), or the combined group (all $p > 0.33$).

In PD, the total number of hypocretin neurons was almost half compared to controls (PD: 20,276 (13,821 – 31,229); controls: 36,842 (32,546 – 50,938); $p = 0.016$, Figure 2.4).

Ventricular CSF Hypocretin-1 Content

Post-mortem ventricular CSF was not available for one PD patient (NBB #93-064) and one control (NBB #00-022). There were no significant differences in age, sex, PMD and fixation time between groups (all $p > 0.51$). Furthermore, there was no significant correlation of these variables with hypocretin-1 CSF content in PD patients (all $p > 0.13$), controls (all $p > 0.44$), or the combined group (all $p > 0.44$).

There was a significant reduction in hypocretin-1 ventricular CSF content in PD patients compared to controls (PD: 365.5 pg/ml (328.0 – 448.3); controls: 483.5 (433.5 – 512.3); $p = 0.012$, Figure 2.4).

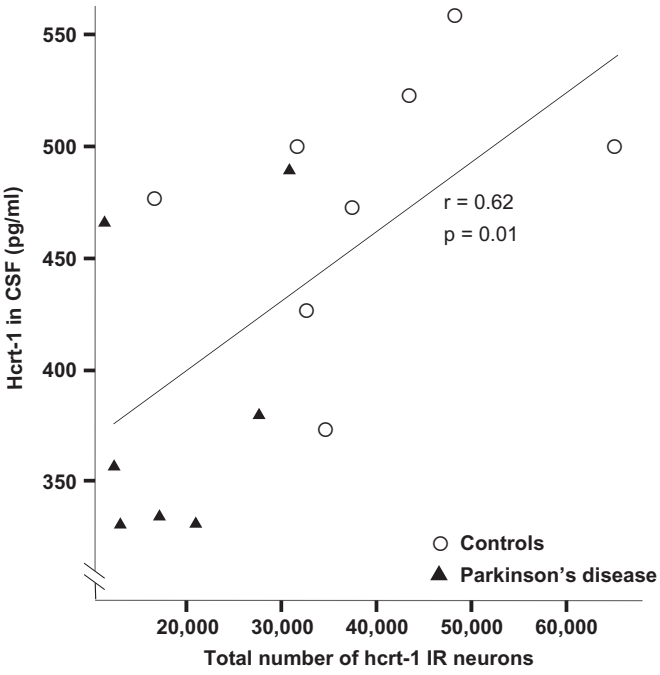

Figure 2.5
Correlation
Correlation between hypocretin-1 IR cell number and post-mortem ventricular CSF hypocretin-1 content. Open circles represent controls, closed triangles represent PD patients.

Relation between hypocretin cell number and CSF levels
As hypocretin cell counts and CSF levels were available in the same subjects, we were able to correlate these two variables directly. There was a significant correlation between cell number and ventricular CSF content in the combined group (n=15, r=0.62, P=0.010, Figure 2.5), but not within the separate groups (controls: p = 0.23; PD: p = 0.70).

Hypocretin-1 concentration in prefrontal cortex
There was no significant correlation between age or sex and hypocretin-1 concentrations in PD patients (all p > 0.19), controls (all p > 0.37), or the combined group (all p > 0.34). The effect of Braak grade could not be evaluated, since all PD subjects were late-stage. Hypocretin-1 concentration in controls was 676.6 (467.5 – 883.9) pg/gram of wet brain tissue, comparable to previously reported values.[10] Hypocretin levels were almost 40% lower in PD patients (389.6 pg/g (249.2 – 652.2); p = 0.042; Figure 2.6).

Discussion

In this study we show that the hypocretin system is affected in PD by examining three brain compartments. Hypocretin-1 tissue concentrations in the prefrontal cortex were almost 40% lower in PD patients, while ventricular CSF levels were almost 25% reduced. The total number of hypocretin neurons was almost half compared to controls. Lewy bodies were abundantly present in the perifornical hypothalamus as a sign of an active disease process in that region. Hypocretin neurons that contained a Lewy body were discernable in every PD patient, but the majority of hypocretin neurons did not

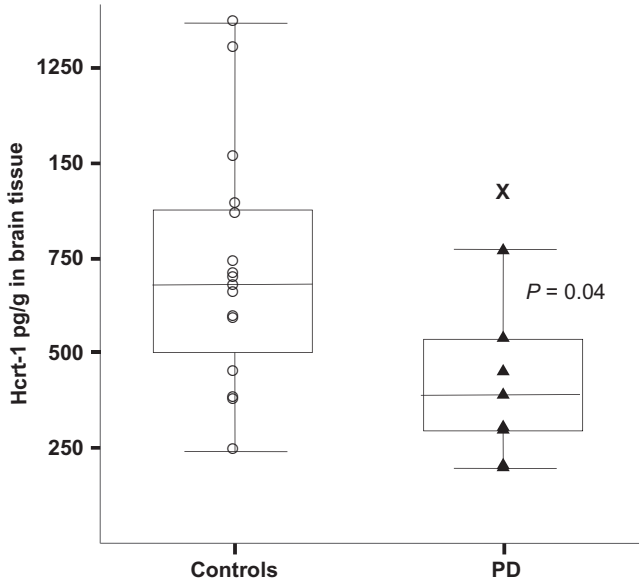

Figure 2.6
Boxplot
Boxplot showing the median, 25th-75th percentiles and the range of the hypocretin-1 concentration in the prefrontal cortex of PD patients (right) and controls (left). The 'X' indicates an out-of-range value. Open circles represent controls, closed triangles represent PD patients.

show this colocalization.

These results convincingly show that the hypocretin system is damaged in PD and are thus in line with and extend upon one of the previous CSF studies, in which low hypocretin-1 levels were found in ventricular CSF in patients with late-stage PD.[15] In that study an inverse correlation between hypocretin-1 levels and disease severity was reported. We could not correlate hypocretin concentrations with disease severity, since all our subjects were late-stage PD.

Studies using spinal CSF have all found normal hypocretin-1 levels. Even PD patients who were selected because of clear sleep abnormalities did not show lowered hypocretin-1 concentrations in spinal CSF.[16,18] The discrepancies between the measurements in spinal CSF and ventricular CSF could be due to the fact that the results of Drouot et al. were obtained in much more advanced PD patients than the studies using spinal CSF. Another explanation could be that CSF hypocretin-1 concentrations are more representative in the area around the hypothalamus, where hypocretin-1 is produced and released by fibers protruding into the lumen of the ventricles, as was shown in the rat.[29] However, in one human study, hypocretin-1 was measured in six subsequent fractions of spinal CSF, using up to 12 ml and no clear gradient between ventricular and spinal CSF levels was found.[17]

It has been shown that hypocretin levels in spinal CSF can be decreased in subjects with acute brain pathology, such as head trauma or a vascular event.[30] Two of the control subjects included in the brain tissue measurement in our study died of stroke (#93-303 and #93-194). However, the hypocretin concentrations in their prefrontal cortex were well within the control range .

There was one control subject with a relatively low number of hypocretin neurons (#93-193). As a specific HLA subtype (DQB1*0602) is an almost invariably necessary factor to develop the sporadic form of narcolepsy with cataplexy. Therefore, an intriguing explanation for this finding could be HLA DQB1*0602 positivity of this subject. Regrettably, we could not obtain frozen brain tissue of the subjects that were included in the cell counts. It was thus not possible for us to determine HLA subtypes. However, HLA DQB1*0602 positivity could be a factor involved in a lower hypocretin cell number, even in the normal population, and this should be explored in future studies.

We used the same hypocretin-1 RIA that has been used by many authors.[16-18,31] It is a well-known fact that the inter-assay variability of this particular RIA is not optimal. However, the intra-assay variability is very low. This stresses the importance of running all samples in a single assay in these types of study, which we did. To compare values with previous reported results, we included a standard reference sample to correct for inter-assay variability.[31] In all tested samples, hypocretin-1 levels were well above the detection limit, and therefore measured reliably.

Hypocretin measurements in CSF have been widely used as a reflection of hypocretin function. However, the relation between actual hypocretin cell number and CSF concentrations was not known as of yet. In a recent rodent study, lesioning about 15% of hypocretin cells did not alter CSF hypocretin-1 levels, but a loss of more than 70% of neurons resulted in a 50% decline in CSF levels.[32] Apparently, in young adult rats it is possible to loose a substantial number of hypocretin cells without changes in CSF levels. This is the first human study that shows a possible correlation between hypocretin cell number and ventricular CSF levels.

Whether our findings fully explain the sleep symptoms in PD remains an intriguing question. Due to the retrospective character of this brain bank study, we had no clinical data on sleep disturbances. However, between one third and half of all PD patients have been reported to experience excessive daytime sleepiness and during sleep registrations even a narcolepsy-like phenotype, including sleep-onset REM periods and fragmented nocturnal sleep, is found on a regular basis.[2-4] This implicates that a significant proportion of the cases we studied would have suffered from sleep disturbances. In a recent rodent study, microinjection of prepro-hypocretin short interfering RNA's (siRNA) in the perifornical hypothalamus resulted in a 60% reduction of prepro-orexin mRNA and a persistent increase in the amount of REM-sleep.[33] In the aforementioned rodent study by Gershchenko, where 70% of hypocretin neurons were lesioned, an increase in REM-sleep was seen as well.[32] Although these results were obtained in rodents, it is not improbable that the reduction in hypocretin neurotransmission found in our human study contributes to the sleep problems commonly seen in PD.

It is likely that the loss of hypocretin neurons is not limited to this cell group in the hypothalamus. Many cell types are affected in PD throughout the brain, but vulnerability seems to be different. To gain more insight into the specificity of the reduction in hypocretin neurons, it would be of interest to count melanin concentrating hormone (MCH) neurons in the peri-fornical region in future studies. Deficiencies

in other neurotransmitters besides hypocretin have been proposed as an explanation for the sleepiness in PD. For example, Rye et al. mention of the possible involvement of midbrain dopaminergic and noradrenergic neurons that influence sleep/wake state through thalamocortical pathways.[3] Both the loss of dopamine and hypocretin neurons can thus contribute to sleep disturbances in PD.

Although sleep-onset REM and REM-sleep behavior disorder are described frequently in PD,[8] there are no reports about cataplexy. Cataplexy is the essential feature of narcolepsy with cataplexy, which is characterized by REM sleep abnormalities and undetectable levels of hypocretin in the spinal CSF.[9] In contrast, hypocretin is usually detectable in narcolepsy without cataplexy, where REM sleep disturbances occur without cataplexy, comparable to the findings in PD. It has been proposed that narcolepsy without cataplexy may be caused by a milder form of hypocretin deficiency compared to the almost complete loss of hypocretin in narcolepsy with cataplexy.[34] Indeed, Thannickal et al. described the highest number of surviving hypocretin neurons in the brain of a narcoleptic patient that did not suffer from cataplexy.[35] Our findings may support this hypothesis, since we found a reduction in number of hypocretin neurons but not a complete loss in PD, possibly leading to REM sleep disturbances and sleep/wake abnormalities, but not to cataplexy.

To conclude, our data shows that the disease process in PD also affects the hypothalamic hypocretin system. It is now important to establish the correlation between hypocretin impairment and the occurrence of the various sleep disturbances. Furthermore, our findings implicate that in the future, hypocretin agonists may have a place in the treatment of PD.

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Hypocretin and Melanin-Concentrating Hormone in Patients with Huntington Disease

CHAPTER 3

Based On: *Aziz NA, Fronczek R, Maat-Schieman MLC, Unmehopa UA, Roelandse FWC, Overeem S, Van Duinen SG, Lammers GJ, Swaab DF, Roos RAC. Brain Pathology, in press.*

Hypocretin and Melanin-Concentrating Hormone in Patients with Huntington disease

Context To evaluate whether hypocretin-1 (orexin-A) and melanin-concentrating hormone (MCH) neurotransmission are affected in patients with

Methods Huntington disease (HD), we immunohistochemically stained hypocretin and MCH neurons and estimated their total numbers in the lateral hypothalamus of both HD patients and matched controls. In addition, hypocretin-1 levels were determined in prefrontal cortical tissue and post-mortem ventricular cerebrospinal fluid (CSF) using a radioimmunoassay.

Results The total number of hypocretin-1 neurons was significantly reduced by 30% in HD brains ($p=0.015$), while the total number of MCH neurons was not significantly altered ($p=0.100$). Levels of hypocretin-1 were 33% lower in the prefrontal cortex of HD patients ($p=0.025$), but ventricular CSF levels were similar to control values ($p=0.306$). Neuronal intranuclear and cytoplasmic inclusions of mutant huntingtin were present in all HD hypothalami, although with a variable distribution across different hypothalamic structures.

Conclusion We found a specific reduction in hypocretin signalling in patients with HD as MCH cell number was not significantly affected. It remains to be shown whether the moderate decrease in hypocretin neurotransmission could contribute to clinical symptoms. As the number of MCH expressing neurons was not affected, alterations in MCH signalling are unlikely to have clinical effects in HD patients.

Introduction

Huntington disease (HD) is an autosomal dominant progressive neurodegenerative disorder caused by an expanded CAG trinucleotide repeat in the IT15 gene on chromosome 4. Choreiform movements, psychiatric and behavioural problems and cognitive impairment characterize HD.[1] Other debilitating but less well-known features of the disease are weight loss, sleep disturbances and autonomic nervous system dysfunction,[2] the causes of which are poorly understood. HD is neuropathologically characterized by generalized atrophy and cell death in the striatum and cerebral cortex and the presence of neuronal intranuclear and cytoplasmic inclusions of mutant huntingtin, particularly in the neocortex and neostriatum.[3,4] Substantial hypothalamic atrophy and cell death have also been reported,[3-7] however, the presence of HD inclusions has not been investigated so far in different hypothalamic structures.

Hypocretin/orexin and melanin-concentrating hormone (MCH) are neuropeptides that are synthesized in the lateral hypothalamus by two distinct neuronal populations.[8-10] These neuropeptides both play a key role in the regulation of body energy metabolism, sleep-wake cycles and autonomic functions.[8,11-13] Moreover, recent animal studies implicate the MCH system in the modulation of several behavioural modalities, most notably stress, depression and sexual behaviour.[8,14] A relation has also been demonstrated between hypocretin release and some psychiatric symptoms.[15,16] Potential alterations in hypocretin and/or MCH signalling might thus contribute to some symptoms in HD patients, particularly weight loss, sleep disturbances and autonomic dysfunction as well as some behavioural disorders like increased rates of depression and anxiety in these subjects.[1]

Recently, it was demonstrated that the R6/2 mouse, the most widely used model of HD that expresses the first exon of the HD gene with ~150 CAG repeats, exhibits a progressive and massive loss of hypocretin-1 immunopositive neurons in the lateral hypothalamic area.[17] This loss amounted to 71% at the end stage (12 weeks) and was accompanied by loss of neuronal nuclear antigen (NeuN)-immunopositive neurons.[17] On the other hand, the YAC128 mouse model of HD with the full-length mutant HD gene with ~120 CAG repeats, shows a 10% loss of hypocretin-1 immunopositive neurons at 12 months.[18] Furthermore, MCH cell number was reported to be decreased by 38% in the hypothalamus of twelve week old R6/2 mice, while MCH peptide levels were reduced by 57%.[19] Atrophy and a decreased density of hypocretin-1 expressing neurons in single coronal sections from the lateral hypothalamus have also been observed in HD patients.[17] Nevertheless, four recent papers reported normal hypocretin-1 concentrations in the cerebrospinal fluid (CSF) of HD patients.[18,20-22]

In order to validate and extend the above findings in patients with HD we applied a four-way approach. First, we estimated the total numbers of both hypocretin-1 and MCH expressing neurons in the lateral hypothalamus of HD patients and matched controls. This allowed testing for the specificity of potential changes in the neuronal numbers. Second, we measured hypocretin-1 levels in post-mortem ventricular CSF because this could better reflect hypocretin-1 production than spinal measurements.[23,24] Third, hypocretin-1 contents in peptide extracts from cerebral cortex were assessed, since this has been shown to be a more sensitive technique compared to CSF measurements.[24,25] And finally, we also investigated various hypothalamic regions, including the lateral hypothalamus, for the presence of neuronal intranuclear and cytoplasmic inclusions.

Materials and methods

Post-mortem material

Autopsy hypothalami from eight HD patients and eight controls (matched for age, sex, post-mortem delay and fixation time) were obtained through the Netherlands Brain Bank (NBB). Ventricular CSF was available in 7 of these HD patients and one of the controls; therefore CSF from six additional controls (matched for age, sex and post-mortem delay) was used for comparison (Table 3.1). Frozen prefrontal cortical

Table 3.1 Clinicopathological details of HD patients and control subjects used for neuronal cell counts and hypocretin-1 measurements in ventricular CSF.

		Sex	*Age (yrs)*	*Age of onset (yrs)*	*PMD (h)*	*Fix (d)*	*Brain weight (g)*	*Grade*	*CAG repeat length*	*Cause of death*	*MCH cell no.*	*Hcrt-1 cell no.*	*Hcrt-1 levels in CSF (pg/ml)*
Patients	HD-1	M	57	42	07:30	53	1162	3-4	46	Cachexia	76987	42088	536
	HD-2	F	50	35	05:40	55	1292	2-3	47	Pneumonia	N.A.	24022	595
	HD-3	M	79	54	06:15	34	1001	4	44	Pneumonia and sepsis	75652	29890	422
	HD-4	F	67	56	06:05	41	1289	1	45	Unknown	102436	50863	531
	HD-5	M	49	40	05:45	49	1122	3	54	Cachexia secondary to pneumonia.	N.A.	20448	468
	HD-6	F	80	58	07:15	49	906	2	41	Pneumonia	76620	36023	404
	HD-7	M	61	39	10:25	48	1380	3	43	Pneumonia	77846	27880	481
	HD-8	M	54	41	03:50	80	1212	2	-	Sudden death	N.A.	42569	N.A.
Median *Percentile*			59.0	41.5	6:10	49.0	1187.0	-	45.0		76987	32957	
25th:			51.0	39.3	5:41	42.8	1031.3		43.0		76136	24987	
75th:			76.0	55.5	7:26	54.5	1291.3		47.0		90141	42449	
Controls	C-1[a]	M	58	-	<17:00	96	1408	-	-	Aorta dissection	77237	49105	N.A.
	C-2[a]	F	49	-	<13:30	165	1437	-	-	Metastasized cervix carcinoma.	89347	56250	N.A.
	C-3[a]	M	79	-	<3:00	53	1435	-	-	Haemorrhage from leaking aorta prosthesis	84652	36231	N.A.
	C-4[a]	F	68	-	05:45	32	1153	-	-	Unknown	N.A.	52900	N.A.
	C-5[a]	M	49	-	<12:40	40	1404	-	-	Sudden death	118604	54638	N.A.
	C-6[a,b]	F	82	-	05:30	36	1280	-	-	Myocardial infarction	N.A.	60972	409
	C-7[a]	M	61	-	13:50	52	2220	-	-	Carcinoma of the oesophagus	94776	27912	N.A.
	C-8[a]	M	54	-	<08:00	59	1350	-	-	Hepatocellular carcinoma	86792	53216	N.A.
	C-9[b]	M	56	-	5:25	-	1522	-	-	Cardiac infarction	N.A.	N.A.	536
	C-10[b]	F	51	-	7:40	-	1156	-	-	Sepsis	N.A.	N.A.	640
	C-11[b]	M	79	-	6:00	-	1392	-	-	Mestastasized adenocarcinoma	N.A.	N.A.	631
	C-12[b]	F	69	-	4:20	-	1186	-	-	Heart failure	N.A.	N.A.	549
	C-13[b]	M	53	-	14:25	-	1341	-	-	Heart failure	N.A.	N.A.	581
	C-14[b]	M	61	-	12:05	-	1460	-	-	Heart failure	N.A.	N.A.	336
Group a: Median *Percentiles*			59.5		10:20	52.5	1406.0				88070	53058	-
25th:			50.3		5:33	37.0	1297.5				82798	39450	
75th:			76.3		13:45	86.8	1436.5				100733	55847	
Group b: **Median** ***Percentiles***			61.0		6:00	-	1341.0				-	-	549.0
25th:			53.0		5:25		1186.0						409.0
75th:			79.0		12:05		1460.0						631.0

[a]) Hypothalamic material from these controls was available for Hcrt-1 and/or MCH immunohistochemistry.

[b]) Ventricular CSF from these controls was available for Hcrt-1 radioimmunoassay.

Legend: Fix = fixation time, PMD = post-mortem delay, Grade = Vonsattel et al's grade, N.A. = not available. Hcrt-1, hypocretin-1.

tissue from a second group of 19 HD patients and 16 controls was obtained through the Leiden University Medical Centre HD pathology archives (Table 3.2). HD brains were graded according to the scheme of Vonsattel et al. for neuropathological disease severity.[4] All HD patients had clinical features and a positive family history of the disease; one patient (#1; Table 3.2) had infantile HD and was studied separately as there are indications that infantile and classical HD may differ neuropathologically.[26] The diagnosis of HD was genetically confirmed (i.e. CAG repeat lengths ≥ 40) in all but one of these patients (#HD-8, Table 3.1). However, the latter patient's brain showed HD pathology grade II and the presence of neuronal intranuclear and cytoplasmic inclusions combined with a positive family history of the disease further consolidated the diagnosis. Exclusion criteria for control subjects were primary neurological and/or psychiatric disorders and glucocorticoid therapy during the final premortal illness

Table 3.2 Clinicopathological data of subjects used for hypocretin brain tissue measurement.

	subject no.	Age	Sex	Grade	CAG	Cause of death	Hcrt-1 Tissue content (pg/g)
HD	1[a]	19	F	3	86	Cachexia	1097
	2	34	M	3	52	Unknown	389
	3	40	F	3	41	Suicide	530
	4	44	M	N.A.	50	Pneumonia	343
	5	45	M	4	53	Recurrent aspiration pneumonia	198
	6	49	M	3	47	Respiratory insufficiency	743
	7	51	M	3	46	Bronchopneumonia	360
	8	51	M	3	45	Unknown	584
	9	53	F	2	47	Pulmonary embolism	511
	10	54	M	3	43	Pneumonia	198
	11	55	F	3	47	Metast. Grawitz tumor	617
	12	57	F	3	43	Pulmonary embolism	278
	13	57	M	4	49	Bronchopneumonia	275
	14	57	M	3	47	Unknown	665
	15	63	F	3	43	Basilar artery thrombosis	1066
	16	66	F	3	41	Squamous cell lung carcinoma	443
	17	69	F	2	42	Aspiration pneumonia	725
	18	75	M	3	43	Unknown	302
	19	77	M	3	39	Bronchopneumonia	376
Median		54.5		3.0	45.5		416.4
Percentiles (25^{th} – 75^{th})		48.0 – 63.8		2.5 – 3.0	42.8 – 47.5		295.8 – 629.4
Controls	1	37	M	-	-	Myocardial infarction	996
	2	46	M	-	-	Cardiac arhythmia	364
	3	48	M	-	-	Myocardial infarction	665
	4	52	M	-	-	Stroke	688
	5	60	F	-	-	Pneumonia	433
	6	63	F	-	-	Leptomeningeal metastasis	737
	7	64	M	-	-	Myocardial infarction	1368
	8	67	F	-	-	Aortic Dissection	858
	9	68	M	-	-	Pneumonia	697
	10	70	M	-	-	Metastasized carcinoma	1282
	11	71	M	-	-	Cardiomyopathy	573
	12	74	M	-	-	Metastasized carcinoma	243
	13	76	M	-	-	Aortic Dissection	638
	14	79	F	-	-	Stroke	892
	15	83	M	-	-	Leptomeningeal metastasis	358
	16	86	M	-	-	Myocardial infarction	570
Median		67.5					676.6
Percentiles (25^{th} – 75^{th})		54.0 -75.5					467.5-883.9

[a]) This patient had juvenile HD and was therefore excluded from subsequent analyses.
Legend: Grade = Vonsattel et al's grade, CAG = CAG repeat length, Hcrt-1, hypocretin-1, N.A. = not available.

period, except two controls who had suffered strokes and were used for prefrontal cortex hypocretin-1 measurements (#C-4 and #C-14; Table 3.2).

Table 3.3 Correlations

	Age at death	Age of onset	Disease duration	Grade	CAG repeat no.
Hcrt-1 cell no.	0.452 (0.260)	0.643 (0.086)	-0.096 (0.820)	-0.482 (0.227)	-0.429 (0.337)
MCH cell no.	-0.500 (0.391)	-0.200 (0.747)	-0.821 (0.089)	-0.700 (0.188)	0.300 (0.624)
Ventricular CSF Hcrt-1 levels	-0.607 (0.148)	-0.607 (0.148)	-0.436 (0.328)	-0.090 (0.848)	0.571 (0.180)
Prefrontal cortex Hcrt-1 levels	0.078 (0.760)	-0.417 (0.265)	0.192 (0.620)	-0.666 (0.004)*	-0.179 (0.476)

- Correlation is significant at the 0.01 level.

Spearman's ρ correlations (p-values) between post-mortem findings and other (clinical) disease parameters in HD patients. The only significant correlation was between the prefrontal cortex Hcrt-1 levels and Vonsattel et al.'s grade of neuropathological disease severity. Trends were visible for the relations between Hcrt-1 cell number and age of onset and between MCH cell number and disease duration.

Hypocretin-1 and MCH immunohistochemistry

The hypothalami were fixed in 10% PBS (pH 7.4) formalin at room temperature and were paraffin embedded and serially sectioned at 6 µm in rostro-caudal direction. Every 100th section was stained with thionin for orientation. The lateral hypothalamus, from the level where the fornix abuts the paraventricular nucleus up to the posterior border of the corpora mamillaria, was stained at 600 µm intervals in every two consecutive sections. One of the sections was stained with a hypocretin-1 and the other with a MCH monoclonal antibody (Phoenix Pharmaceuticals, Inc., Belmont, CA; catalog no. H-003-30 and H-070-47, respectively). The sections were visualized according to the avidin-biotin complex method using diaminobenzidine-nickel solution to finish the staining as described previously.[27]

Table 3.4 Neuronal inclusions in nuclei of HD hypothalami and adjacent regions.

	SCN		SON		PVN		DBB/ NBM		INF		VMN		DMN		LH/ PFA		NTL		TMN		SMA	
Type	**C**	**I**	**C**	**I**	**C**	**I**	**C**	**I**	**C**	**I**	**C**	**I**	**C**	**I**	**C**	**I**	**C**	**I**	**C**	**I**	**C**	**I**
HD-1	-	-	+	-	+	-	+	-	+	-	+	-	+	-	+	+	+	-	+	+	+	-
HD-2	-	-	-	-	-	-	+	-	+	+	+	-	+	-	+	+	+	-	+	-	+	-
HD-3	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	+	+	-
HD-4	-	-	-	-	+	-	+	-	+	-	+	-	+	-	+	-	-	-	+	+	+	-
HD-5	+	-	-	-	-	-	+	-	+	-	+	-	+	-	+	-	+	-	+	+	+	-
HD-6	-	-	-	-	+	-	+	-	+	-	+	-	+	-	+	-	-	-	+	-	+	-
HD-7	+	-	+	-	-	-	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	-
HD-8	+	-	+	-	+	-	+	+	+	-	+	-	+	-	+	-	+	-	+	+	+	-
% patients	**50**	**0**	**50**	**0**	**63**	**0**	**100**	**25**	**100**	**25**	**100**	**13**	**100**	**0**	**100**	**25**	**75**	**13**	**100**	**75**	**100**	**0**

Legend: Type = type of neuronal inclusions; C = cytoplasmic inclusions (i.e. inclusions in dystrophic neurites); I = intranuclear inclusions; - = inclusions absent; + = inclusions present. The last row indicates, per structure, the percentage of HD patients who had neuronal inclusions of the specified type. **SCN**, suprachiasmatic nucleus; **SON**, supraoptic nucleus; **PVN**, paraventricular nucleus; **INF**, infundibular nucleus; **DBB**, diagonal band of Broca; **NBM**, nucleus basalis of Meynert; **VMN**, ventromedial nucleus; **DMN**, dorsomedial nucleus; **LH**, lateral hypothalamus; **PFA**, perifornical area; **NTL**, lateral tuberal nucleus; **TMN**, tuberomamillary nucleus; **SMA**, supramamillary area.

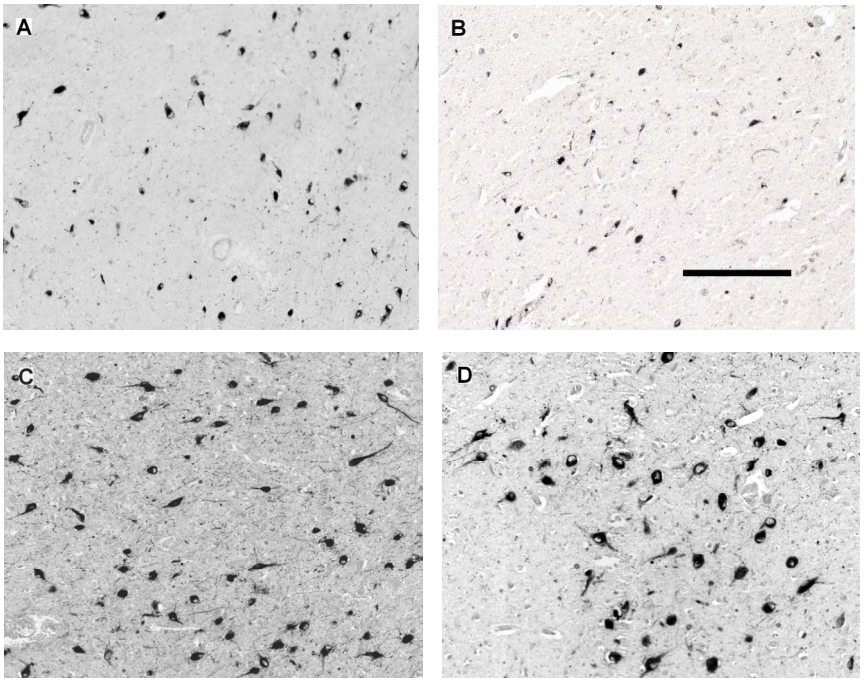

Figure 3.1 Representative photographs of hyocretin-1 and MCH IR neurons

Representative photographs of hyocretin-1 and MCH IR neurons in the lateral hypothalamus of two control subjects (A and C, #C-4 and #C-2 respectively) and two patients with HD (B and D, #HD-6 and #HD-1 respectively) with neuronal counts at or around the median (Table 1). The pictures were taken from slides with the highest numbers of immunoreactive neurons. The illustrations show a modest reduction in the number of hypocretin-1 IR neurons in the HD brain (A and B) while the number of MCH IR neurons is not significantly affected (C and D). Scale bar 250 μm.

N-terminal huntingtin immunohistochemistry

Using thionin staining for orientation, nine to ten sections were chosen so that large parts of the following hypothalamic (and adjacent) structures would be contained in at least two coronal cuts: the suprachiasmatic nucleus, the supraoptic nucleus, the paraventricular nucleus, the infundibular nucleus, the diagonal band of Broca, the nucleus basalis of Meynert, the ventromedial nucleus, the dorsomedial nucleus, the lateral hypothalamus/perifornical area, the tuberomamillary nucleus, the lateral tuberal nucleus and the supramamillary area (the supramamillary area was contained in only one coronal cut). These sections were stained with a monoclonal antibody against the N-terminus of human huntingtin (Chemicon, Temecula, CA; batch no. 5374) after pretreatment by boiling in citrate buffer (pH 6.0) for 20 minutes. The sections were processed according to the avidin-biotin complex method using diaminobenzidine-nickel solution and counterstained with Harris's Hematoxylin for nuclear staining.

Antibody specificity

The specificity of the hypocretin-1 antibody has been confirmed previously.[28] To test the specificity of the MCH antibody, a dot blot was performed,[29] adding a dilution of 1:1000 anti-MCH onto 2% gelatin-coated nitrocellulose paper (0.1-µm pore size) containing different spots with 20 pmol MCH, hypocretin-1, somatostatin (1–14), somatostatin (1–28), galanin, MCH-1 receptor, β-lipotropin, substance-P, α-melanocyte-stimulating hormone, luteinizing hormone-releasing hormone, adrenocorticotropic hormone (1–39), neurotensin, oxytocin, corticotropin-releasing hormone , agouti-related protein (83–132), neuropeptide-Y, growth hormone-releasing hormone (1– 40), arginine-vasopressin, desacetylmelanocyte-stimulating hormone, neuropeptide EI, glycoprotein hormone receptor and cocaine- and amphetamine-regulated transcript. The next day, the nitrocellulose sheet was incubated with secondary antibody, avidin-biotin peroxidase complex, and diaminobenzidinenickel solution to finish the staining. The only spot that showed staining was the one containing MCH. Specificity was further confirmed by the absence of staining in hypothalamic sections using antiserum preadsorbed with the human MCH peptide fixed overnight with 4% formaldehyde onto gelatin-coated nitrocellulose filter paper, 0.1 µm, and the presence of staining when preadsorbed with α-melanocyte-stimulating hormone peptide, which did not differ from unadsorbed serum.

Quantification of hypocretin-1 and MCH neuronal numbers

An estimate of the total number of hypocretin-1 and MCH immunoreactive (IR) cells was made using an image analysis system (ImagePro version 4.5, Media Cybernetics, Silver Spring) connected to a camera (JVC KY-F553CCD) and plain objective microscope (Zeiss Axioskop with Plan-NEOFLUAR Zeiss objectives, Carl Zeiss GmbH, Jena, Germany). In every section randomly selected fields, covering in total 15% of a manually outlined area containing all the hypocretin-1 or MCH IR cells, were counted by the same person (NAA) who was blinded to the diagnosis. Each positively stained profile containing a nucleolus was counted.[24] Calculation of the total number of hypocretin-1 IR neurons was performed by a conversion program based upon multiplication of the neuronal counts by sample frequency of the sections as described previously.[30] Mean (± SD) number of sections quantified per subject was 10.2 ± 1.5 for hypocretin-1 and 11.5 ± 2.3 for MCH. The coefficient of variation (i.e. SD / mean x 100%) of this method was 3.4% for hypocretin-1 and 4.5 % for MCH (calculated by counting one complete control twice).

Hypocretin-1 measurements in cerebral cortex and CSF

One gram of frozen prefrontal cortex was used for hypocretin-1 measurements (Table 3.2). The most rostral part of the prefrontal cortex was chosen, as this cortical region is densely innervated by hypocretin neurons resulting in high hypocretin-1 concentrations.[31] The tissue samples were boiled for 10 minutes in 10.0 ml of MilliQ water, cooled to room temperature, acidified using glacial acetic acid and HCl (final concentration: 1.0 M and 20.0 mM respectively), homogenized and centrifuged. The supernatant was acidified again with an equal volume of 0.1% trifluoracetic acid and vacuum dried. Samples were resuspended in 500 µl of radioimmunoassay buffer and centrifuged at 3000 rpm for 10 minutes before measurements. Immediately after collection, ventricular CSF was

centrifuged at 2500 rpm for 10 minutes and the supernatant was stored at -80°C until hypocretin-1 levels were measured using a commercially available radioimmunoassay (Phoenix Pharmaceuticals, Belmont, USA). All measurements were conducted in duplicate 100 µl aliquots in a single assay run. The detection limit was 50 pg/ml and intra-assay variability was less than 5%. We used a validated reference sample to adjust levels to previously reported values.[24,25]

Assessment of neuronal intranuclear and cytoplasmic inclusions

The presence or absence of neuronal intranuclear inclusions and cytoplasmic inclusions (i.e. inclusions in dystrophic neurites) in the hypothalamic regions of interest were assessed by one of us (NAA) at ×100 magnification.[3] For each region two coronal sections were investigated. The percentage of HD patients who had either neuronal intranuclear or cytoplasmic inclusions of mutant huntingtin was calculated per structure (Table 3.4).

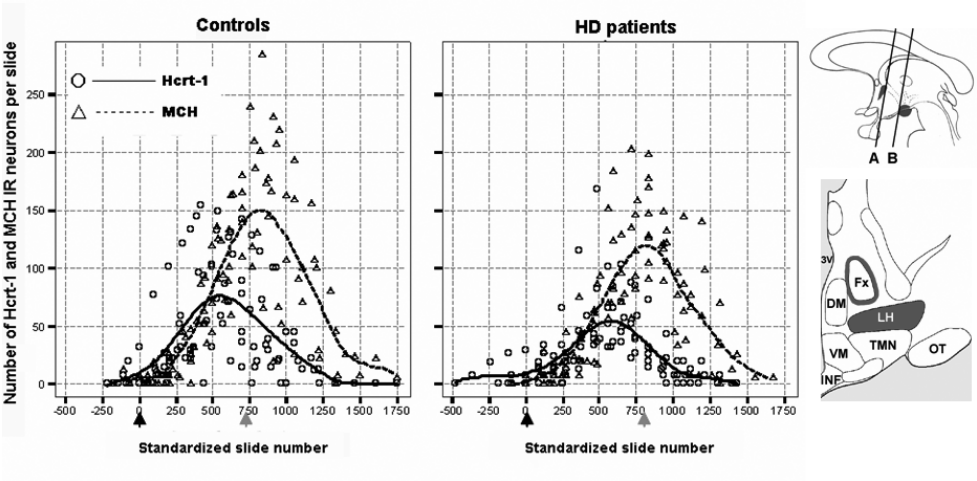

Figure 3.2 **Distribution patterns**

Distribution patterns of hypocretin-1 and MCH IR neurons in the lateral hypothalamus of controls and HD patients. In order to present data from all subjects in the same diagrams, the individual distribution patterns were standardized in rostro-caudal direction for the anatomical distance between the point where the fornix abuts the paraventricular nucleus (black arrows; at the level of line 'A' in the upper cartoon) and the fornicomamillary junction (grey arrows; at the level of line 'B' in the upper cartoon). This procedure was performed separately for the control and the HD group. In addition, local linear regression was used to fit curves to the standardized pooled data in order to clarify the underlying distribution patterns (kernel = Gaussian, bandwidth value = 1.0). Note that the overall rostro-caudal dispersion of hypocretin-1 and MCH IR neurons does not appear to be noticeably different between control and HD subjects. **Upper cartoon**: A schematic sagittal view of the hypothalamus; the paraventricular nucleus and the mamillary bodies are indicated in dark. **Lower cartoon**: A schematic coronal view of the hypothalamus midway between lines 'A' and 'B' in the upper cartoon; the perifornical area and the lateral hypothalamus are indicated in dark. Hcrt-1, hypocretin-1; 3V, third ventricle; Fx, fornix; LH, lateral hypothalamus; DM, dorsomedial nucleus; VM, ventromedial nucleus; TMN, tuberomamillary nucleus; INF, infundibular nucleus; OT, optic tract.

Statistics

All data are presented as medians (25th – 75th percentile). Differences between the groups were statistically evaluated by the Mann-Whitney U (MWU) test, the Wilcoxon signed rank (WSR) test or the χ2-test. Spearman's ρ correlation test was performed to assess all correlations. Differences in clock time of death (circular parameter) between controls and patients with HD were tested with the Watson's two-sample test of homogeneity.[32] Tests were two-tailed and values of $p < 0.05$ were considered to be significant. Local linear regression was used to fit curves to the standardized pooled data (Figure 3.2).[33]

Results

Hypocretin-1 and MCH neurons in HD patients and controls, subject characteristics

There were no significant differences in age, sex, post-mortem delay, fixation time and clock time of death between the HD and the control group (all $p \geq 0.27$ (Table 3.1)).

Distribution of hypocretin-1- and MCH neurons

The intensity of hypocretin-1 and MCH immunostaining showed no obvious differences between the HD and the control group (Figure 3.1). Hypocretin-1 IR neurons were restricted to the perifornical region in the lateral hypothalamus as previously described.[28] MCH IR neurons were mainly confined to the same areas although they were more widely disseminated than hypocretin-1 IR neurons. The first hypocretin-1 IR neurons emerged in the lateral hypothalamus at the junction of the fornix and the paraventricular nucleus and were followed by the first MCH IR neurons. However, the rostro-caudal distance between the location of the first hypocretin-1 neurons and that of the first MCH neurons did not significantly differ from zero (either in patients (n = 8) or controls (n = 8) or both groups combined (respective p-values by WSR test: 0.168, 0.336 and 0.085)) (Figure 3.2). At subsequent levels the fornix was surrounded by both hypocretin-1 and MCH IR cell bodies throughout its entire course up to the mamillary bodies. The number of hypocretin-1 IR neurons peaked just before the fornicomamillary

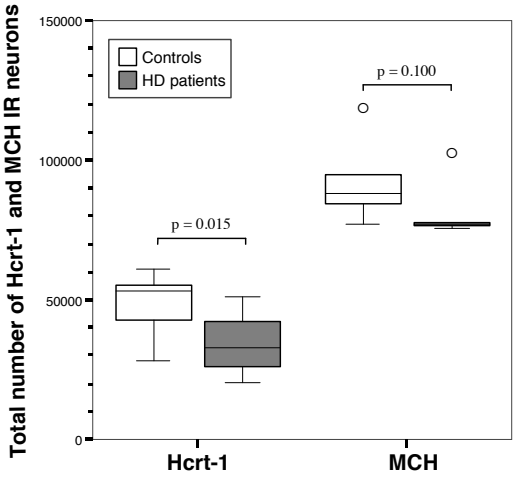

Figure 2.3

Total number of hypocretin neurons

The total numbers of hypocretin-1 and MCH IR neurons in the lateral hypothalamus of control subjects and HD patients. The total number of hypocretin-1 IR neurons is significantly decreased in HD, while there is only a trend towards a reduction in the total number of MCH IR neurons (MWU-test: n = 16, p = 0.015 for Hcrt-1 and n = 11, p = 0.100 for MCH). Outliers (defined as data points which lie 1.5 times the interquartile range below the first or above the third quartile) are symbolized by 'o '. Hcrt-1, hypocretin-1.

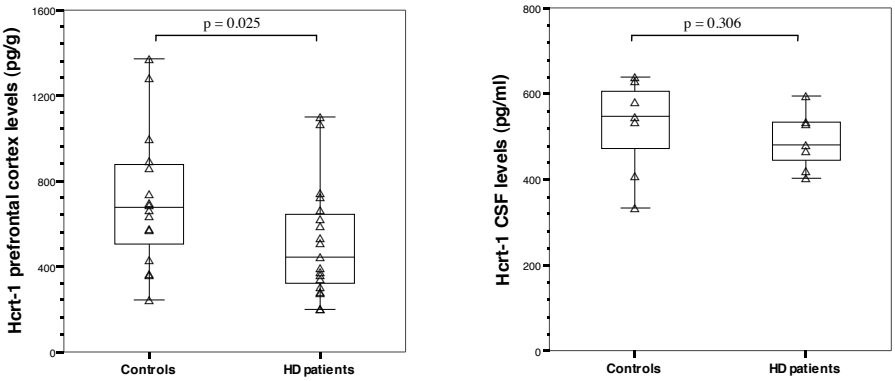

Figure 3.4 **Hypocretin-1 levels in the prefrontal cortex**

Compared to controls, hypocretin-1 levels were significantly lower in the prefrontal cortex of HD patients (MWU-test: p = 0.025), but not in their CSF (MWU-test: p = 0.306). Hcrt-1, hypocretin-1.

junction, whereas the peak number of MCH IR neurons was seen after this junction in the supramamillary area (Figure 3.2); the rostro-caudal distance between the two peaks was calculated in each individual and was significantly larger than zero (all $p \leq 0.011$ by WSR test, either in patients (1200 µm [1200-1800]) or controls (1500 µm [1200-2250]), or both groups combined (1200 µm [1200-1800]).

Hypocretin-1 and MCH cell numbers

The total number of hypocretin-1 IR neurons in the lateral hypothalamus of HD patients was significantly reduced by 30 % compared to values in matched controls (32,957 (24,987 – 42,449) vs. 53,058 (39,450 – 55,847); p = 0.015; Figure 3.3). As the available hypothalamic material of two controls and three HD patient (#C-4, #C-6 and #HD-2, #HD-5 and #HD-8, respectively (Table 3.1)) did not contain the caudal part of the MCH area, these cases were excluded from subsequent calculations of the total number of MCH IR neurons. Exclusion of these subjects did not alter group comparability. There was a trend towards a decrease in the total number of MCH IR neurons in HD patients (HD: 76,987 (76,136 – 90,141); controls: 88,070 (82,798 – 100,733); p = 0.100; Figure 3.3).

Effects of age of onset, CAG repeat length, disease duration and Vonsattel et al's grade on hypocretin-1 and MCH cell numbers in HD

There was a positive trend for the relation between age of onset of HD (defined as the age at which the clinical diagnosis was first made) and hypocretin-1 IR neuronal numbers (r = 0.64, p = 0.086), but not MCH neuronal numbers (r = 0.21, p = 0.645). In HD patients, the total hypocretin-1 and MCH cell numbers were not significantly associated with either CAG repeat length, duration of illness or Vonsattel et al's grades of neuropathological disease severity (Table 3.3).[4]

Ventricular CSF hypocretin-1 content

The two groups were well matched for age, sex and post-mortem delay (all p > 0.80) (Table 3.2). Furthermore, there were no significant correlations between these variables and hypocretin-1 CSF contents in HD patients, controls or the combined group (all p

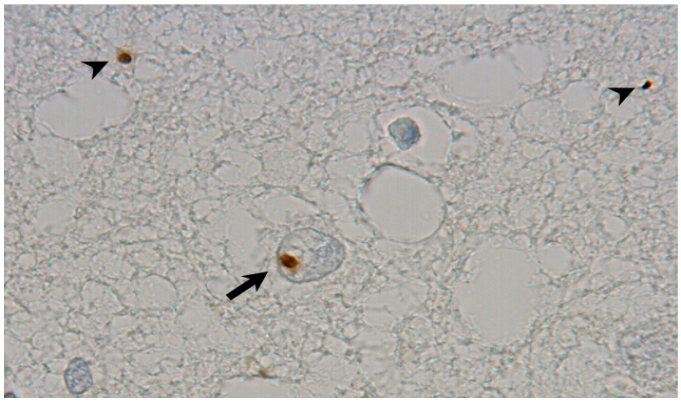

Figure 3.5
Huntingtin inclusions
Examples of neuronal intranuclear (arrow) and cytoplasmic (arrowheads) inclusions of mutant huntingtin in the tuberomamillary nucleus of one HD patient (#HD-7; obj. ×60 oil).

> 0.11). The CSF contents of hypocretin-1 was not different between HD patients and controls (HD: 481 pg/ml (422 – 536); controls: 549 pg/ml (409 – 631); p = 0.306, Figure 3.4), nor was there a significant correlation between the hypocretin cell counts and CSF levels in the 8 subjects (7 HD patients and 1 control) in whom both measurements were available (r = -0.238; p = 0.570). There were no significant correlations between CSF hypocretin-1 levels and other (clinical) disease parameters (Table 3.3).

Hypocretin-1 concentration in prefrontal cortex

The two groups did not differ with respect to gender, but the HD group was significantly younger (p = 0.034) (Table 3.2). However, hypocretin-1 concentrations were not significantly correlated with age (either in HD patients, controls, or the combined groups (all p ≥ 0.37)). Hypocretin-1 concentration in controls was 676.6 (467.5 – 883.9) pg/gram of wet brain tissue, which is comparable to previously reported values.[25] Compared to controls mean hypocretin-1 cortical levels were 33% lower in adult HD patients (416.4 pg/g (295.8 – 629.4); p = 0.025). Furthermore, the prefrontal hypocretin-1 levels in these patients were significantly associated with the Vonsattel et al's grades (r = -0.666, p = 0.004), but not with other (clinical) disease parameters (Table 3.3). The patient with infantile HD had the highest levels of cortical hypocretin among all the HD patients studied (Table 3.2). Since the prefrontal tissues belonged to subjects whose hypocretin cell counts were unavailable, we could not relate the prefrontal cortex levels of hypocretin-1 to the hypocretin cell counts.

Neuronal intranuclear and cytoplasmic inclusions in HD hypothalami

N-terminal huntingtin-positive neuronal intranuclear and cytoplasmic inclusions were observed in all HD hypothalami (Figure 3.5). Cytoplasmic inclusions were far more abundant than intranuclear inclusions which were seen only sporadically. Intranuclear inclusions were most consistently observed in the neurons of the tuberomamillary nucleus. The HD inclusions were not uniformly distributed among the various hypothalamic and adjacent structures (Table 3.4). Only in relatively few patients cytoplasmic inclusions were present in the most rostral hypothalamic structures (i.e. the suprachiasmatic, the supraoptic, and the paraventricular nuclei), while intranuclear inclusions could not be detected at all in these areas.

Discussion

In this study, we demonstrate a significant reduction by about 30% in the total number of hypocretin-1 neurons in the lateral hypothalamus of HD patients. This decrease appears to be relatively specific as the total number of MCH neurons was not significantly altered. Hypocretin-1 levels in the prefrontal cortex were reduced to the same extent, but ventricular CSF levels were unchanged. Furthermore, we describe the distribution of intranuclear and cytoplasmic inclusions of mutant huntingtin in the lateral hypothalamus and various other hypothalamic and adjacent structures in patients with HD.

Recently the density of hypocretin-1 neurons was assessed in single coronal sections from the lateral hypothalamus of HD patients and found to be decreased.[17] However, cell density is subject to substantial variation depending on both the rostro-caudal level of the sections (Figure 3.2) and the cutting direction. Therefore, in this study we systematically counted the total number of hypocretin-1 expressing neurons and were able to unequivocally confirm a significant but moderate decrease in the number of these neurons in HD patients. A positive trend was also visible for the relation between hypocretin-1 cell number and age of onset in HD patients paralleling findings by Kremer et al.[7] who found an association between neuronal numbers in the lateral tuberal nucleus and age of onset in HD.

We corroborated the specificity of the reduction in hypocretin-1 neuronal numbers by assessing the total number of MCH neurons as well. Since the reduction in hypocretin-1 neuronal numbers was more pronounced than that in MCH neuronal numbers, hypocretin neurons appear to be more vulnerable to the pathogenic mechanisms underlying HD. Interestingly, a recent study in rat hypothalamic slice cultures showed that 24-hours of incubation with N-methyl-D-aspartate (NMDA) resulted in a marked decrease in the number of hypocretin-1 neurons, whereas MCH neurons in the same cultures were relatively spared.[34] Moreover, examinations of the effects of several endogenous glutamate receptor agonists highlighted quinolinic acid as an endogenous excitotoxin that could cause selective loss of hypocretin-1 neurons as compared to MCH neurons by activating NMDA receptors.[34] Therefore, NMDA receptor-mediated excitotoxicity could be involved in the greater susceptibility of hypocretin-1 neurons in HD patients, a pathomechanism that has also been proposed for the massive neuronal loss in the lateral tuberal nucleus of these patients.[7]

To further assess hypocretin neurotransmission in HD, we also examined hypocretin-1 contents in the prefrontal cortex and ventricular CSF. Whereas the mean levels of hypocretin-1 were about 30% lower in the prefrontal cortex of HD subjects and correlated with Vonsattel et al's grades, ventricular CSF hypocretin-1 contents did not differ between patients and controls. The latter finding is in accordance with four recent papers that reported normal hypocretin-1 concentrations in the CSF of HD patients.[28,20-22] The apparent discrepancy between the findings in the CSF and those in the hypothalamus and the prefrontal cortex of HD patients could be accounted for by the fact that a mean reduction of approximately 30% in the number of hypocretin-1

neurons is probably not large enough to be reflected in the CSF.[20-22] This assumption is supported by the fact that a reduction by half in the number of hypocretin neurons in PD patients only causes a 25% decrease in hypocretin-1 levels in the ventricular CSF.[24] Accordingly, rat studies indicate that a 73% decline in hypocretin neuronal numbers is needed to decrease CSF hypocretin-1 levels by half.[35] Yet another possibility is impaired clearance of hypocretin-1 from the CSF in HD.

So far, hypocretin signalling has been studied in two animal models of HD. The R6/2 mouse model is reported to have a loss of more than 70% in both hypocretin-1 expressing neurons and hypocretin-1 CSF levels, whereas the YAC128 mouse model exhibits a reduction of 10% in the number of hypocretin-1 neurons. Moreover, R6/2 mice are reported to have a loss of almost 40% in the number of MCH expressing neurons while their hypothalamic MCH levels are reduced by nearly 60%.[19] The discrepancy between our findings and those from these transgenic mice could be accounted for by the existence of several confounding variables. First, these transgenic mouse models have very large CAG repeat expansions (> 120 repeats) and resemble juvenile HD more than the adult form of the disease.[36] An intriguing possibility is thus that juvenile HD patients might indeed exhibit more extensive pathology of the hypocretin system. In this study, we could measure hypocretin-1 levels in the prefrontal cortex of only one juvenile HD patient (Table 3.2). Surprisingly, this case appeared to have the highest levels of cortical hypocretin among all the HD patients studied. This finding may, however, be due to the stronger cortical atrophy that accompanies the juvenile variant of HD compared to the adult form of the disease.[26] Second, the mild reduction in hypocretin in the YAC128 mice may be due to the fact that these mice, unlike the R6/2 mice and several human patients, were not (close to) end stage at the time of assessment (i.e. 12 months). Thus, several variables may confound the comparisons between various animal models and the human condition and should, therefore, be taken into consideration when comparing animal and human data.

Hypocretin deficiency is the primary pathophysiological cause of narcolepsy, a sleep-wake disorder characterized by excessive daytime sleepiness and REM-sleep dissociation phenomena such as cataplexy, i.e. a sudden weakening of posture muscle tone usually triggered by emotion.[37] Although R6/2 mice exhibit episodes of behavioural arrest closely resembling those seen in hypocretin knock-out mice and transgenic mice with specific ablation of hypocretin-containing neurons,[38,39] it remains to be shown whether the modest decrease of hypocretin-1 signalling in adult HD patients could contribute to clinical symptoms, particularly sleep disturbances.[40] Unfortunately, due to the retrospective nature of our study we could not relate our post-mortem findings to clinical signs and symptoms. This stresses the need for systematic post-mortem brain tissue collection of clinically well-documented patients for future neuropathological studies.

In this report we also present an estimation of the total number of MCH neurons in the human brain and their relative distribution with respect to hypocretin-1 neurons. Our results confirm those from a recent study[41] and suggest that MCH neurons are indeed more abundant in the human hypothalamus and have a wider rostro-caudal distribution than hypocretin-1 neurons, which is also in accordance with findings in

rodents.[42,43] Even though MCH and hypocretin-1 neurons start to appear at about the same level rostrally, MCH neurons are relatively more abundant in the posterior hypothalamus. Whether this finding could be accounted for by the existence of several distinct MCH subpopulations along the rostro-caudal axis as opposed to a more homogenous hypocretin population[42,43] remains to be elucidated. As we could not find a clear reduction in the number of MCH neurons in HD patients, alterations in MCH levels are unlikely to have clinical implications in HD.

In this study we have assessed neuronal numbers by counting immunopositive neurons by means of a technique that has been validated and applied previously.[24,28,41] It should be stressed that it is in principle impossible to distinguish the loss of an immunocytochemical neuronal marker from the loss of the neurons in a heterogeneous and anatomically loosely defined brain structure such as the lateral hypothalamus.

Interestingly, neuronal intranuclear and cytoplasmic inclusions were not uniformly present in various hypothalamic and adjacent structures in HD patients. This finding may indicate that various hypothalamic nuclei are differentially affected by inclusion formation despite their close anatomical juxtaposition in the hypothalamus. Elucidation of the underlying mechanisms of this heterogeneity may lead to better understanding of why certain neuronal populations are more susceptible to HD pathology than others.

In conclusion, we found a specific reduction by about 30% in hypocretin signalling in patients with HD. It remains to be shown whether this moderate decrease in hypocretin signalling could contribute to clinical symptoms. As MCH cell number was not clearly affected in HD patients, alterations in MCH neurotransmission are unlikely to have clinical effects in HD.

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Immunohistochemical Screening for Autoantibodies against Lateral Hypothalamic Neurons in Human Narcolepsy

CHAPTER 4

Based On: *Overeem S, Verschuuren JJ, Fronczek R, Schreurs L, den Hertog H, Hegeman-Kleinn IM, van Duinen SG, Unmehopa UA, Swaab DF, Lammers GJ. J Neuroimmunol. 2006;174:187-91.*

Immunohistochemical Screening for Autoantibodies against Lateral Hypothalamic Neurons in Human Narcolepsy

Most human patients with narcolepsy have no detectable hypocretin-1 in their cerebrospinal fluid. The cause of this hypocretin deficiency is unknown, but the prevailing hypothesis states that an autoimmune-mediated mechanism is responsible. We screened for the presence of autoantibodies against neurons in the lateral hypothalamus in 76 patients and 63 controls, using immunohistochemistry. Antibodies were present in 2 patients, but also in 2 controls. However, one of the patients had a clearly different staining pattern, and nerve endings of recognized cells were found to project onto hypocretin-producing neurons, suggesting a possible pathophysiological role. Humoral immune mechanisms do not play an important role in the pathogenesis of narcolepsy, at least not in the clinical overt stage of the disease.

INTRODUCTION

Narcolepsy is a sleep disorder affecting approximately 5 per 10,000 people. Excessive daytime sleepiness, cataplexy and fragmented nocturnal slaap are the main symptoms, and pose a severe burden on the life of patients [1,2]. In humans, the disease occurs mostly in a sporadic form, with genetic factors influencing susceptibility [3]. Narcolepsy is associated with certain human leucocyte antigen (HLA-) subtypes, with over 90% of patients being positive for HLA-DQB1*0602 [4]. This strong HLA association has led to the hypothesis that narcolepsy is an autoimmune disorder, but direct evidence for this theory is lacking as of yet.

Recent studies pinpointed alterations in hypothalamic hypocretin (orexin) mediated neurotransmission as the primary cause of narcolepsy: more than 90% of human patients lack the neuropeptide hypocretin-1 in their cerebrospinal fluid (CSF) [5]. Post mortem studies showed that hypothalamic neurons staining for hypocretin were virtually absent in narcoleptics, probably due to a degenerative process [6-8].

It is possible that an autoimmune process destroying hypocretin producing neurons ultimately causes the hypocretin deficiency in human narcolepsy. In this study, we sought evidence for this hypothesis and screened serum and CSF of a large cohort of narcoleptic patients for circulating antibodies against lateral hypothalamic neurons using immunohistochemistry.

METHODS

Subjects and sample collection

We included, after informed consent, 76 narcoleptic patients (45 males) with an average age of 45.6±15.6 years. The average duration of illness was 23.6±10.6 years. The diagnosis of narcolepsy was made on clinical grounds combined with Multiple Sleep Latency Testing.[1] In 54 patients, CSF was available so hypocretin-1 levels could be measured as previously described;[9] 46 had undetectable levels. From 59 patients, HLA typing was known; 55 were DQB1*0602 positive. In addition, sera of 63 control subjects without any medical condition were used (33 males, age 32.8±16.3 years). After collection, both CSF and serum was aliquoted and immediately stored at –70°C.

Brain tissue

Immunohistochemistry was performed on sections of encoded human hypothalamus, obtained from the Netherlands Brain Bank and from the department of pathology of the Leiden University Medical Center (3 male subjects who died of non-neurological disease, age 63 (post-mortem delay [PMD] 1.7 hours), age 37 (PMD 5 hours) and age 48 (PMD 19 hours) respectively). Hypothalami were freshly dissected, fixed in buffered formaline for 60-70 days, paraffin-embedded and serially-sectioned at 6 µm. In the study we used the sections from the expected hypocretin area, from the level where the fornix touches the paraventricular nucleus to the level where the fornix reaches the corpora mammillaria.

Screening immunohistochemistry

After deparaffinization and rehydration, endogenous peroxidase activity was blocked in methanol-0.3% H_2O_2 for 20 minutes. Sections were pre-incubated in Tris-buffered-saline (TBS)-10% normal goat serum for half an hour and then incubated with serum at a dilution of 1:400 in supermix (0.05M Tris, 0.15M NaCl, 0.25% gelatin, 0.5% Triton X-100, pH 7.6) overnight at room temperature (RT). Subsequently, sections were incubated with biotinylated goat-anti-human-IgG (GaH, Vector Laboratories, USA) 1:2000 in supermix for one hour at RT, and labelled with ABC-Elite kit (Vector) in supermix for 30 minutes at RT, stained with 3,3'-diaminobenzidine as chromogen, and counterstained with Harris hematoxylin. In ambiguous cases, sera were tested again in different concentrations (1:200, 1:400, 1:800). CSF samples were tested according to the same protocol, except that they were used undiluted.

Of every 25 subsequent hypothalamic sections, one was stained with rabbit-anti-hypocretin-1 (Phoenix Pharmaceuticals, Belmont, CA) 1:5000 to identify the area of interest. Slides incubated only with supermix served as negative control.

Double staining with serum and anti-hypocretin-1

Serum staining was done as described above, with the following additions. Staining was intensified by proteinase K treatment [10], and a 48-hour incubation in patient serum. The reaction was visualized using AEC (3-amino-9-ethylcarbazole) solution (5 mg/ml, Vector) for 20 minutes. Subsequently, sections were stained with rabbit-anti-hypocretin-1 (1:1250 in supermix), alkaline phosphatase conjugated donkey-anti-rabbit

IgG (Vector) 1:50 in supermix for 1 hour and fast blue solution (Sigma Chemicals, Zwijndrecht, Holland) 2 mg/ml in Tris-HCl for 5 minutes.

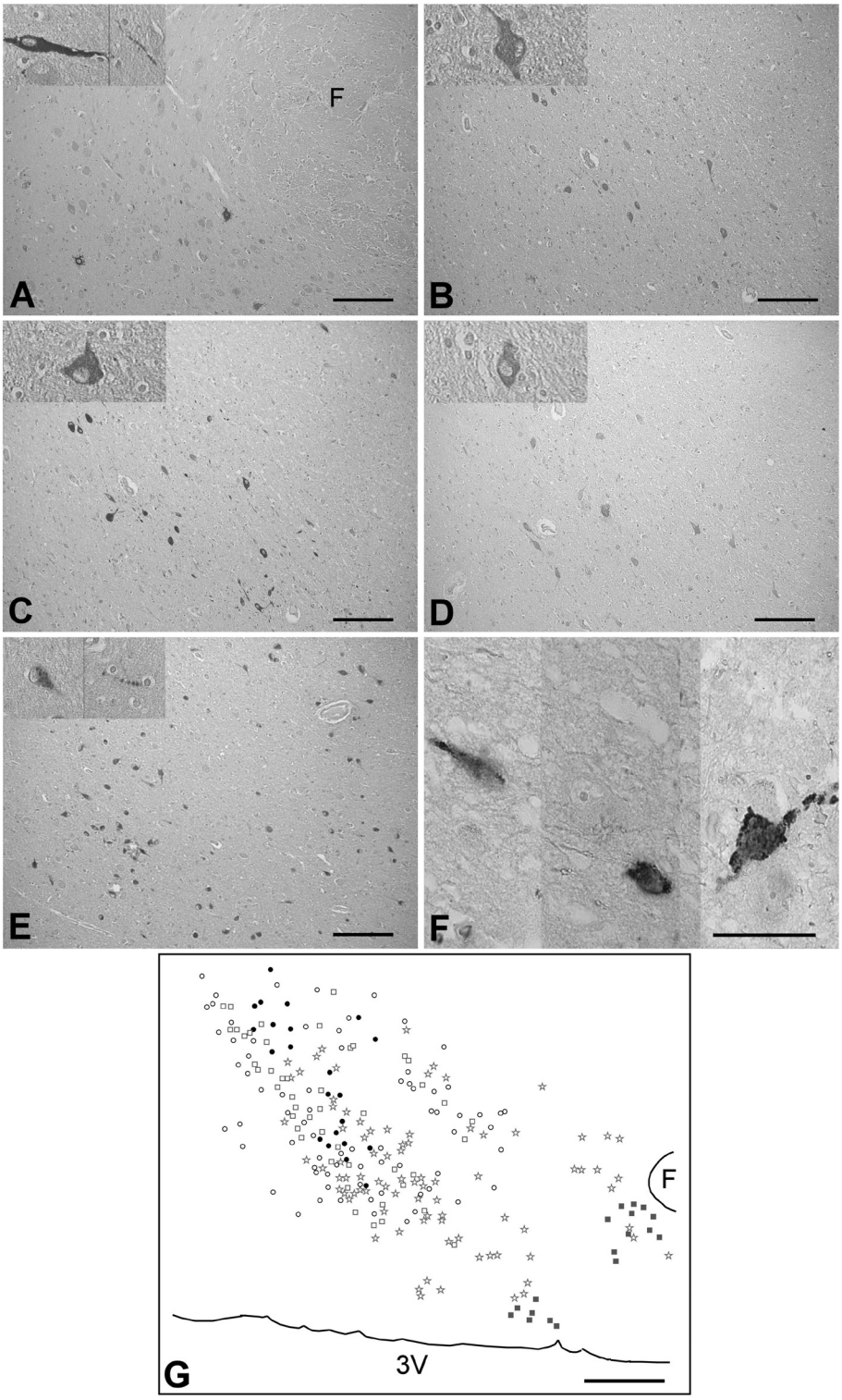
Enzyme-linked immunoadsorbent assay (ELISA)
ELISA was setup according to standard protocols [11]. In short, microtiter plates were coated with 1 µg hypocretin-1 or –2 per well and incubated with 100 µl of patient or control serum (diluted 1:500) or undiluted CSF, for 60 minutes at 37ºC. Peroxidase-conjungated rabbit-anti-human-IgG (Dako, Glostrup, Denmark) 1:4000 was used as the second step, and binding visualized with 3,3',5,5'-tetramethylbenzidine-0.1 mg/ml DMSO in 0.1 M Na-acetate-0.1% H_2O_2. Wells coated with 25 ng/ml of human immunoglobulin served as positive control. As a positive control for the hypocretin coating, 2 wells were primairly stained using rabbit-anti-hypocretin-1 or –2 at 1:4000, and secondly with peroxidase-conjungated swine-anti-rabbit-IgG 1:500. Staining intensity was quantified using spectrophotometry at 450 nm, with a positive cut-off value set at an absorbance of 1,000A.

RESULTS

Hypocretin staining
Hypocretin-1 positive neurons were found in all hypothalamic sections used (see Figure 4.1E for a representative section). Hypocretin-1 positive cell bodies were mainly located in the perifornical area of the lateral hypothalamus, as expected (Figure 4.1E and G). Fibers from hypocretin neurons, characterized by multiple bead-like varicosities, were found throughout the preoptic, anterior and tuberal hypothalamus (Figure 4.1E, insert).

Screening immunohistochemistry
From the 76 patient sera, we found 2 that consistently stained neurons in the lateral and tuberal hypothalamus. However, we also found 2 control subjects with similar immunoreactivity. Both patients had clear-cut cataplexy, and had no detectable hypocretin-1 levels in the CSF. See Figure 4.1A-D for adjacent sections of the hypocretin area stained with these sera. Patient 1 was HLA-DQB1*0602 positive and had a duration of illness of over 7 years. Serum of this patient stained a small number of neurons, closely surrounding the fornix (Figure 4.1A and F). Furthermore, multiple neuronal fibers were visible, including ones with boutons closely resembling those of hypocretin

Figure 4.1 Representative examples of staining and schematic overview (right page)
Adjacent sections from the lateral hypothalamus, stained with positive sera from 2 patients (A, B), 2 controls (C, D) and anti-hypocretin-1 (E). Magnifications of representative neurons are shown in the top left corners. (G) Sketch, overdrawn from the adjacent sections, to compare the distribution of stained cells and to indicate the relative number of neurons stained in the lateral hypothalamus (hypocretin neurons [stars], patient 1 [filled squares], patient 2 [open squares], control 1 [open circles], control 2 [black filled circles]). (F) 3 parts from sections double stained with anti-hypocretin-1 (light grey) and serum of patient 1 (dark grey). Note that no cell bodies are double stained. There are multiple bouton-like structures staining grey, in close proximity of hypocretin cell bodies, suggesting nerve endings.[21] Scale bars: (A-E): 200 µm, (F): 50 µm, (G): 1000 µm. Abbreviations: F = fornix, 3V = third ventricle.


F
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neurons (Figure 4.1A and E). CSF tested also positive, although the staining pattern with CSF was much weaker. In contrast, patient 2 did not have the HLA-DQB1*0602 genotype. Moreover, the staining pattern of this patient closely resembled those of the two control subjects with a high number of cells in a relatively large area of the lateral and tuberal hypothalamus (Figure 4.1B-D, and Figure 4.1F). None of these sera stained neuronal fibers resembling those of hypocretin neurons, although the distribution of positive neurons virtually overlapped with the hypocretin field (Figure 4.1G).

Double staining experiment

We further characterized the staining patterns by doubly-labelling hypothalamic sections with serum and anti-hypocretin. We found no neurons double-staining both with serum and anti-hypocretin-1 or –2. However, using serum from patient 1, we found several hypocretin-positive cell bodies surrounded by axons and bouton-like structures stained with patient serum (Figure 4.1F).

Enzyme-linked immunoadsorbent assay

None of the sera and CSF samples tested positive in the ELISA assay, using hypocretin-1 or -2.

DISCUSSION

In 2000, it was shown for the first time that human narcolepsy is caused by defects in hypothalamic hypocretin neurotransmission, most likely through a specific degeneration of hypocretin producing neurons [6,7,12,13]. Particularly the strong HLA association gave rise to the current hypothesis that narcolepsy is an autoimmune disorder. However, general markers of immune activation in the nervous system have not been found [4], and several studies screening for previously described neuronal antibodies were negative [14-16]. Smith et al reported that narcoleptics may harbour IgG interfering with peripheral cholinergic transmission when injected in rats, but the link to the pathophysiology of narcolepsy remains unclear and the results have not been confirmed [17].

Recently, the first study specifically looking for antibodies against the hypocretin peptides and some of their cleavage products was performed, with negative results [18,19]. However, it certainly is possible that circulating autoantibodies recognize other components of hypocretin producing neurons. In a first screening study using a pooled ELISA approach, Black et al. found that CSF from narcoleptic subjects showed immunoreactivity to rat hypothalamic protein extract on a group level [20]. Our current study is the first to use immunohistochemistry on human hypothalamic material as a screening method. Furthermore, it is the largest antibody screening to date. Our results confirm earlier studies showing that there are no specific antibody responses in narcolepsy to hypocretin-1 or -2, or other components of hypocretin neurons, at least not in the clinical stage of narcolepsy. We found 2 patients with serum containing antibodies recognizing parts of hypothalamic neurons, but this was a non-specific finding, as these were present in the same number of control subjects. The HLA positive patient turning up in our screen may still be of interest however. The pattern of immunoreactivity was clearly different

from the other positive subjects. Furthermore, although double labeling experiments showed that the neurons did not contain hypocretin themselves, some showed nerve endings projecting onto hypocretin-producing cells. It is tantalizing to hypothesize that pathogenic autoantibodies staining bouton-like structures recognize a possible synaps between nerve endings and hypocretin positive cells, implying that these antibodies might bind extracellular parts of synaptic proteins. Attractive candidates target cells are the recently described glutaminergic interneurons in the lateral hypothalamus that project directly to hypocretin cells to regulate their activity [21]. Future experiments may shed more light on this.

In conclusion, we found no disease-specific increase in the presence of antibodies against lateral hypothalamic neurons, although it remains possible that antibodies were present in earlier (pre-clinical) stages of the disease. However, other screening studies using different methodologies yielded similar results. It therefore is very important to keep an open mind to mechanisms other than autoimmunity explaining the hypocretin deficiency in human narcolepsy.

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Response to Intravenous Immunoglobulins and Placebo in a Patient with Narcolepsy with Cataplexy

Based On: *Fronczek R, Verschuuren JJ, Lammers GJ . J Neurology, In press*

CHAPTER 5

Response to Intravenous Immunoglobulins and Placebo in a Patient with Narcolepsy with Cataplexy

Introduction

Narcolepsy with cataplexy is caused by a loss of hypocretin producing neurons in the lateral hypothalamus.[1,2] The strong Human Leukocyte Antigen (HLA DQB1*0602) association supports an autoimmune aetiology.[3] Still, there is no direct evidence for anti-neuronal antibodies or T-cell mediated autoimmunity to support this hypothesis.[4,5] Treatment with high-dose prednisone after acute onset of hypocretin-deficiency in an 8-year-old boy without cataplexy was not effective.[6] However, two studies suggested that treating narcoleptics with intravenous immunoglobulins (IVIg) shortly after disease onset may dramatically reduce the frequency and severity of cataplexy.[7,8]

Methods

We present a n=1 study in a 55 year old female patient suffering from typical narcolepsy with cataplexy for 7 years, who was almost unresponsive to any regular treatment, but had a dramatic response on open label treatment with IVIg. Polysomnographic findings were typical of narcolepsy with cataplexy. She was HLADQB1*0602 positive, hypocretin deficient and used venlafaxine (75 mg/day) with limited effects. Cataplexy was frequent and disabling (according to her diary: mean ± standard deviation; 3.30±0.15 complete attacks per day; range 3-4). Together with her severe excessive daytime sleepiness, the patient was invalided with profound impact on quality of life. She was almost homebound and evaded social activities to avoid a provocation of her complaints. After informed consent, we treated her with open label IVIg (1gm/kg/day over 2 days). After treatment she reported a clear reduction of cataplectic attacks and several days without any attacks. This effect lasted three weeks and disappeared gradually. Repeated treatment six months later showed a similar response. We started a double-blind placebo-controlled n=1 trial to analyse this remarkable response.[9]

This consisted of four successive treatment periods in which IVIg (1gm/kg/day over 2 days) or placebo was randomly administered.[9] The patient could request the 'rescue' medication for that period, if she did not experience significant clinical improvement within 10 days after treatment. This rescue medication was IVIg when the treatment

period was started with placebo and placebo when the treatment period was started with IVIg. The next treatment period was started after the patient indicated that the treatment effect had disappeared, and at least 4 weeks after the previous treatment. During the entire study period the patient kept a diary in which she noted the number of complete cataplectic attacks. Venlafaxine was continued in an unchanged dose throughout the entire study. Differences between the placebo and the IVIg periods were analysed using t-tests, corrected for the number of days within each period.

Results

The study lasted for 188 days. The patient correctly identified placebo and/or IVIg treatment in half (50%) of the treatment periods: the second (IVIg, 63 days) and third (placebo, 65 days). She mistook placebo for IVIg in the first treatment period (26 days) and IVIg for placebo in the fourth treatment period (34 days). During the preceding two month long baseline situation 1.45±2.72 complete cataplectic attacks per day were scored. During the study period both treatments resulted in a decrease of cataplectic attacks. IVIg treatment decreased the attack rate to 0.27±0.73 per day, and placebo to 0.48±1.28 attacks per day. The reduction of attacks of both treatments was significantly lower compared to the pre-study period ($p<0.001$ for both IVIg and placebo.). There was, however, no significant difference between the two treatment modalities ($p=0.17$).

Discussion

In conclusion, open treatment with IVIg led to a striking improvement in the frequency of the cataplectic attacks in this patient. However, during a subsequent double-blind

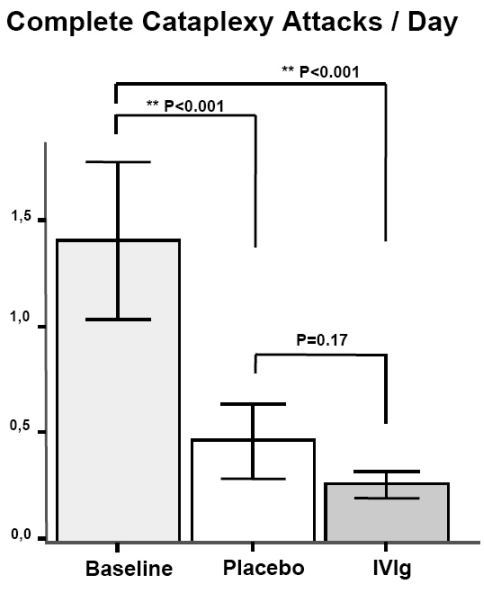

Figure 5.1
Cataplexy attacks / Day
Total number of cataplectic attacks for both treatment modalities and the baseline period. Error bars represent standard error of the mean. Differences were analysed using t-tests.

placebo-controlled n=1 trial there was no difference between placebo and IVIg treatment. Nevertheless, the placebo effect was impressive. The patient reported less cataplectic attacks after the first drug administration of the study, which was placebo. Carry-over effects of earlier received IVIg during the trial are thus unlikely. Earlier open studies found a decrease of cataplectic attacks around disease onset during IVIg treatment.[7,8] Although our patient did not receive IVIg near disease onset, there is no clear reason why there would be difference in placebo response between our case and the cases that were published earlier. Our findings stress the need for a large, double-blind placebo controlled study.

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Narcolepsie: Behandeling en Diagnostiek in Nieuw Perspectief

CHAPTER 6

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Narcolepsie: Diagnostiek en Behandeling in Nieuw Perspectief

De 5 klassieke symptomen van narcolepsie zijn overmatige slaperigheid overdag, kataplexie, slaapparalyse, hypnagoge hallucinaties en een verstoorde nachtslaap.
De aanwezigheid van kataplexie blijkt sterk samen te hangen met een deficiëntie van de neurotransmitter hypocretine.
Dit inzicht heeft recentelijk geleid tot een nieuwe diagnostische classificatie met een onderverdeling in narcolepsie zonder kataplexie, aangetoond met een multipele inslaaplatentietest, en narcolepsie met kataplexie, die bevestigd kan worden met een multipele inslaaplatentietest of met een hypocretine 1-deficiëntie in de liquor.
Er zijn diverse behandelingsmogelijkheden, bijvoorbeeld met psychostimulantia of gammahydroxyboterzuur.

Overmatige slaperigheid overdag komt vaak voor: bij navraag betreft dit 1 op de 7 mensen.[1] Bij veel personen ligt de oorzaak waarschijnlijk in de levensstijl, in de vorm van een zelf opgelegd tekort aan slaap,[1] bij anderen is wel degelijk sprake van een primaire slaapstoornis ofwel hypersomnie. De invloed op de kwaliteit van leven, thuis en op het werk, en de bijdrage aan gevaarlijke situaties in het verkeer maken tijdige opsporing van hypersomnie wenselijk ten behoeve van adequate behandeling.

Het prototype van een hypersomnie is narcolepsie. Deze aandoening is vaak al anamnestisch vast te stellen. Over het algemeen manifesteert deze ziekte zich op jeugdige leeftijd en beperkt deze daardoor niet alleen de kwaliteit van leven, maar ook de ontplooiingskansen van een patiënt. De mogelijkheden voor medicamenteuze behandeling zijn recent uitgebreid, zodat de vooruitzichten van behandeling beter zijn dan voorheen. Bovendien is er in de afgelopen jaren meer inzicht verkregen in de pathofysiologie, met name in de hypocretineneurotransmissie in de hypothalamus. Dit heeft zijn weerslag gehad in de nieuwe internationale classificatie van slaapstoornissen.[2] In dit artikel worden de diagnostiek, de therapie en de pathofysiologie in deze nieuwe context geplaatst.

Klinisch beeld

Op basis van epidemiologisch onderzoek in andere Europese landen zouden er in Nederland ruim 7000 mensen aan narcolepsie lijden;[1] echter, het aantal personen bekend wegens deze aandoening schat men op slechts 1000.

Overmatige slaperigheid overdag, het kernsymptoom van narcolepsie, is dagelijks aanwezig en uit zich zowel in een continue slaperigheid als in onweerstaanbare slaapaanvallen. Na in slaap gevallen te zijn worden patiënten meestal snel wakker, waarna ze zich tijdelijk verkwikt voelen. Slaapaanvallen treden vooral op tijdens monotone bezigheden, maar bij ernstige vormen kunnen ze ook voorkomen tijdens activiteiten zoals eten of fietsen. Verminderde vigilantie door de verhoogde slaapneiging kan leiden tot geheugenklachten en automatisch gedrag.[3] Dit laatste houdt in dat men weliswaar wakker genoeg is om handelingen te verrichten, maar onvoldoende alert om dit adequaat te doen: de patiënt doet bijvoorbeeld waspoeder in de koelkast of maakt aantekeningen met een onzinnige inhoud.

De slaapproblemen beperken zich niet tot overdag. Patiënten vallen 's avonds vlot in slaap, maar worden 's nachts herhaaldelijk wakker. De totale hoeveelheid slaap per etmaal is doorgaans dan ook niet toegenomen.[4] Bij narcolepsie is overmatige slaperigheid overdag niet het gevolg van een verstoorde nachtelijke slaap.

Kataplexie is een plotselinge spierverslapping die optreedt bij emoties, waarbij het bewustzijn behouden blijft, en die specifiek is voor narcolepsie. Dit fenomeen treedt uiteindelijk bij ongeveer 85% van alle patiënten met narcolepsie op, vrijwel altijd tegelijk met of binnen enkele jaren na de manifestatie van de verhoogde slaapneiging. Kataplexie is een plotseling optredend, kortdurend en bilateraal verlies van spiertonus met een behouden bewustzijn, uitgelokt door emotionele uitingen, zoals lachen. Ook andere situaties, zoals boosheid of een onverwachte ontmoeting met een bekende, kunnen aanvallen uitlokken.[3] Deze aanvallen duren seconden tot minuten, waarbij het herstel abrupt en volledig is. De frequentie varieert van minder dan 1 maal per maand tot tientallen malen per dag. De aanvallen van de meeste patiënten zijn uitsluitend partieel, waarbij bijvoorbeeld alleen de kaak of het hoofd gaat hangen of de knieën slap worden. Een ernstiger type is de complete aanval: patiënten vallen op de grond en kunnen in het geheel niet bewegen. Het duurt meestal enige seconden totdat de spierverslapping maximaal is, zodat patiënten vaak nog steun kunnen zoeken en zich meestal niet verwonden (Figuur 6.1). Voorts kunnen er trekkingen voorkomen, met name in het gelaat. Belangrijk voor het onderscheid met epilepsie of syncope is dat

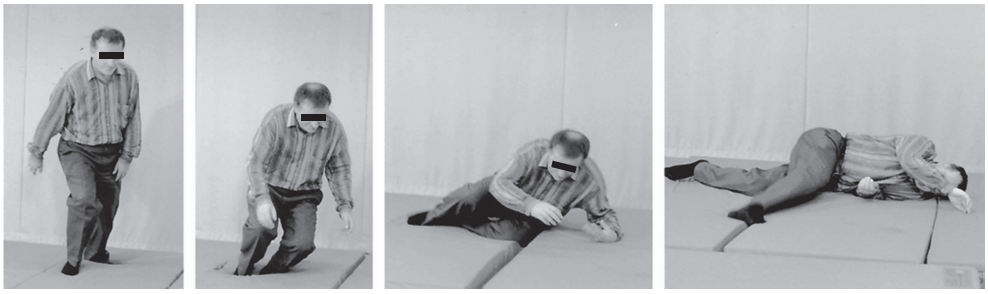

Figure 6.1 Fotoreeks van een complete kataplexieaanval

Fotoreeks van een complete kataplexieaanval, die ongeveer 2 s duurt. De spierverslapping is in eerste instantie niet volledig, zodat de patiënt steun kan vinden en zichzelf niet verwondt. Afgedrukt met schriftelijke toestemming van belanghebbende.

het bewustzijn tijdens een aanval volledig intact blijft, zodat patiënten achteraf precies kunnen vertellen wat er is gebeurd.[3]

Hypnagoge hallucinaties en slaapparalyse worden meestal met narcolepsie in verband gebracht en treden op bij de overgang tussen slaap en waak. Omdat ze ook geïsoleerd en bij andere slaapstoornissen voorkomen, zijn ze niet langer opgenomen in de diagnostische criteria voor narcolepsie (tabel). Hypnagoge hallucinaties zijn levensechte, vaak onaangename en beangstigende droomervaringen. Ze kunnen zowel visueel, auditief als tactiel van aard zijn. Pas na afloop beredeneren patiënten dat het niet om een werkelijke gebeurtenis ging. Ze kunnen ten onrechte als psychotisch verschijnsel worden geïnterpreteerd.[5] Bij een slaapparalyse zijn patiënten wakker, maar kunnen zij zich niet bewegen.

Zelfs een minder ernstige vorm van narcolepsie is invaliderend. Zo is de kwaliteit van leven van narcolepsiepatiënten sterker aangetast dan bijvoorbeeld van epilepsiepatiënten.[6] Doordat narcolepsie meestal in de adolescentie ontstaat, staan de verhoogde slaapneiging en de emotieafhankelijke kataplexie de normale ontwikkeling van relaties, het volgen van een opleiding en het vinden van werk in de weg. Ook hypnagoge hallucinaties maken patiënten onzeker. Hierbij komt dat met de huidige regelgeving narcolepsiepatiënten feitelijk de rijbevoegdheid ontzegd wordt,[7] hoewel een groot aantal patiënten succesvol behandeld wordt.

Pathofysiologie

In de afgelopen jaren is het begrip van de pathofysiologie van narcolepsie toegenomen. Dit begon eind jaren zeventig van de vorige eeuw met de ontdekking van narcolepsie bij honden, waarbij de ziekte sprekend lijkt op die bij mensen. Zo hebben de dieren complete kataplexieaanvallen, uitgelokt door spelen of het zien van een stuk vlees. Bij aangedane dobermanns en labradors bleek de ziekte autosomaal recessief over te erven. In 1999 werd ontdekt dat deze dieren een mutatie hebben in de type 2-receptor voor de neurotransmitter hypocretine.[8] Deze neurotransmitter, ook wel 'orexine' genoemd, wordt uitsluitend geproduceerd in de laterale hypothalamus.[9] Hypocretineproducerende neuronen hebben verbindingen door het hele brein, met name met waakstimulerende kernen.[10]

Al snel bleek dat ook bij mensen de oorzaak van narcolepsie in het hypocretinesysteem ligt. Bij gezonde individuen is de hypocretineconcentratie goed meetbaar in de liquor cerebrospinalis, maar bij vrijwel alle narcolepsiepatiënten met kataplexie is hypocretine niet aantoonbaar.[11] Dit tekort wordt veroorzaakt door een degeneratie van hypocretineproducerende neuronen.[12] Er zijn aanwijzingen dat een autoimmuunproces ten grondslag ligt aan het verdwijnen van de hypocretineneuronen, maar direct bewijs voor deze theorie is er nog niet.[13]

Er zijn modellen ontwikkeld, deels gebaseerd op anatomisch aangetoonde verbindingen, die verklaren hoe een hypocretinetekort tot symptomen van narcolepsie leidt. Een voorbeeld van zo'n model is de 'flip-flopschakeling' (Figuur 6.2).[14]

Criteria voor narcolepsie volgens de 'International classification of sleep disorders (ICSD-2)[2]

	narcolepsie met kataplexie	narcolepsie zonder kataplexie
dagelijks overmatige slaperigheid gedurende tenminste 3 maanden	aanwezig	aanwezig
typische kataplexie*	aanwezig	afwezig
andere verklaring voor hypersomnie, zoals een andere slaapstoornis, een andere organische aandoening, een psychiatrische stoornis of het (overmatig) gebruik van medicatie	afwezig	afwezig
diagnose bevestigd door:		
- multipele inslaaplatentietest (inslaaplatentie < 8 min en ≥ 2 maal remslaap	additioneel	noodzakelijk
- hypocretine-1 bepaling in liquor	additioneel: hypocretine concentratie verlaagd (< 110 pg/ml)	hypocretine concentratie soms verlaagd

*Typische kataplexie is gedefinieerd als een bilaterale spierverslapping, geluxeerd door sterke emotionele uitingen (de meest kenmerkende situaties zijn lachen of grappen maken), van korte duur (minder dan 2 min), met behoud van bewustzijn.

Diagnostiek

Recentelijk zijn er nieuwe diagnostische criteria voor slaapstoornissen opgesteld ('International classification of sleep disorders'; ICSD-2).[2] De genoemde samenhang tussen kataplexie en een deficiëntie van de neurotransmitter hypocretine heeft geleid tot een onderscheid tussen narcolepsie met en zonder kataplexie (zie de bovenstaande tabel en Figuur 6.3).

Narcolepsie met kataplexie

In principe kan narcolepsie met kataplexie op basis van de anamnese worden vastgesteld: er is overmatige slaperigheid overdag en typische kataplexie. Het is belangrijk te onderkennen dat met name partiële aanvallen regelmatig door patiënten niet worden herkend, zodat men daar gericht naar moet vragen. Wegens de sociale en therapeutische consequenties verdient het de voorkeur deze diagnose objectief te bevestigen.

De eerste mogelijkheid hiervoor is een zogenaamde multipele inslaaplatentietest (zie Figuur 6.3).[15] Deze test houdt in dat de patiënt 4 of 5 maal gedurende één dag in bed ligt in een stille, donkere kamer en 20 min de tijd krijgt om in slaap te vallen, waarbij de tijd tot inslapen wordt gemeten. Een gemiddelde inslaaplatentie over de gehele dag berekend van minder dan 8 min wijst op overmatige slaperigheid overdag, maar geeft nog weinig duidelijkheid over de oorzaak. Indien er minimaal 2 maal remslaap optreedt, wordt voldaan aan de criteria voor narcolepsie.

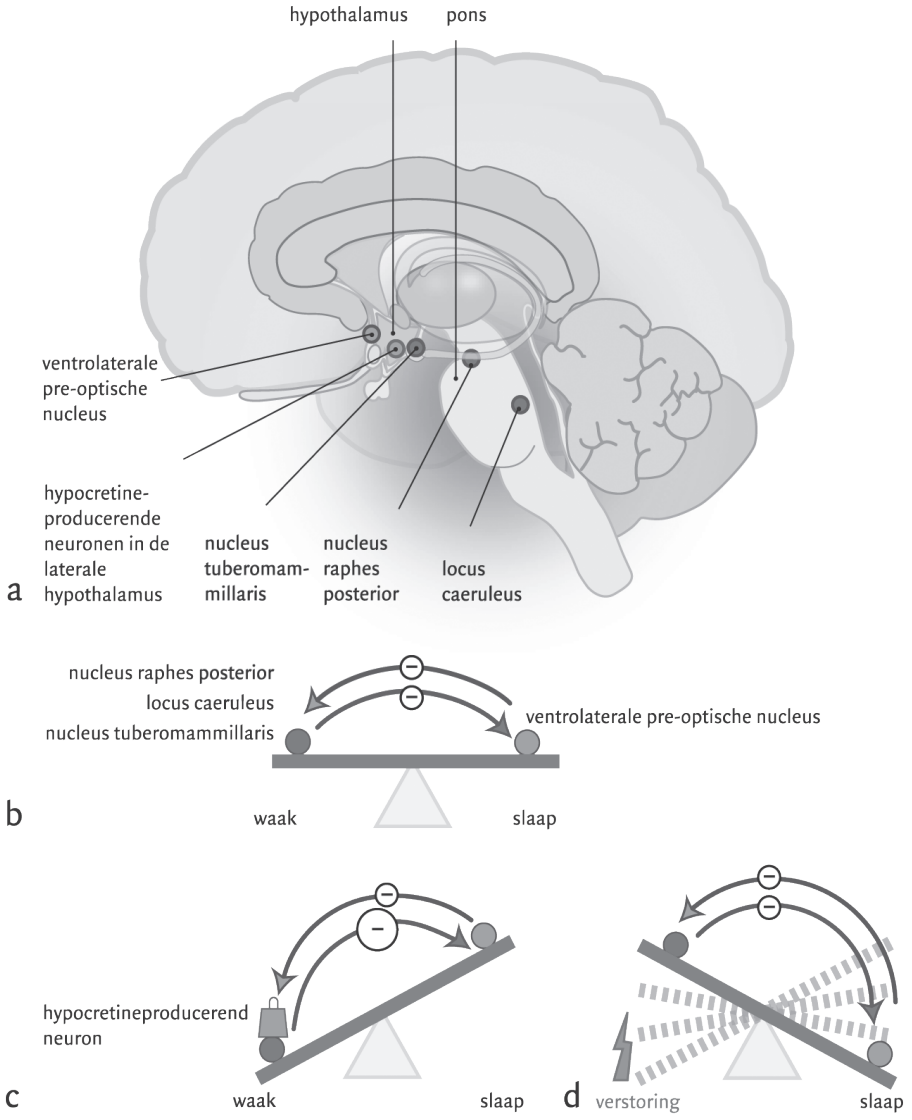

Figure 6.2 De 'flip-flopschakeling'-hypothese

De 'flip-flopschakeling'-hypothese beschrijft het slaap-waaksysteem als een schakelaar waarbij slaap- en waakcentra in het brein elkaar wederzijds remmen: (a) slaap- en waakcentra in het brein; (b) deze centra vormen een netwerk met de eigenschappen van een 'flip-flop', een term afkomstig uit de elektronica. Door het bistabiele karakter van deze slaapschakelaar worden overgangstoestanden vermeden; zodra een van beide toestanden sterker wordt, 'flipt' de schakelaar naar die toestand. De slaap-waakovergangen zijn relatief abrupt bij zowel mensen als dieren, wat een evolutionair voordeel lijkt te hebben; (c) in de waakstand wordt de slaap volledig geremd. Een kleine verstoring kan echter tot een zichzelf versterkende omschakeling naar de tegenovergestelde toestand leiden. Dit wordt voorkomen door het hypocretinesysteem, dat uitgebreide projecties naar waakstimulerende centra heeft; het is daarmee de externe stabilisator voor waak; (d) bij narcolepsie valt de stabiliserende invloed van hypocretine weg en wordt de schakelaar instabiel; een kleine verstoring overdag leidt tot een ongewilde overgang naar de slaaptoestand.

De tweede mogelijkheid is het bepalen van hypocretine 1-spiegels in de liquor cerebrospinalis.[11] Afwezigheid van deze neurotransmitter is in grote mate specifiek voor narcolepsie.

Narcolepsie zonder kataplexie

Men kan narcolepsie zonder kataplexie overwegen bij een verhoogde slaapneiging zonder kataplexie, maar dan moeten vaker voorkomende oorzaken van overmatige slaperigheid overdag eerst uitgesloten worden. Indien de oorzaak ligt in een tekort aan nachtslaap of externe verstoringen van de slaap, kan men nagaan of verlenging van de slaapduur tot vermindering van de slaperigheid leidt. Andere oorzaken, zoals het slaapapneusyndroom, vergen een gerichte anamnese en een nachtelijke slaapregistratie, met onder andere meting van de ademhaling.

Indien men geen andere oorzaak voor de overmatige slaperigheid vindt, wordt een multipele inslaaplatentietest verricht. Narcolepsie zonder kataplexie wordt dan alleen vastgesteld bij een inslaaplatentie korter dan 8 min en tenminste 2 perioden met remslaap. Als de inslaaplatentie verkort is en er geen remslaap optreedt, is er formeel een idiopathische hypersomnie. Wanneer daarnaast hypocretine 1 afwezig is in de liquor cerebrospinalis, heeft de patiënt toch narcolepsie zonder kataplexie. Dit is slechts bij minder dan 10% van deze patiënten het geval.

HLA-typering is niet zinvol: het subtype DQB1*0602 komt voor bij 90% van de narcolepsiepatiënten met kataplexie en bij 40% van deze patiënten zonder kataplexie, maar komt ook voor bij meer dan 25% van de algemene bevolking, zodat de specificiteit gering is.

Therapie

Zoals bij iedere slaapstoornis zijn leefregels van belang bij de behandeling van narcolepsie. Patiënten wordt geadviseerd een regelmatig leven te leiden met vaste tijden van opstaan en naar bed gaan, ook tijdens weekenden en vakanties. Voorts kunnen geplande dutjes vaak kortdurend tot betere prestaties leiden.[16]

Medicamenteuze therapie is vrijwel altijd geïndiceerd en uitsluitend gericht op symptoombestrijding. De klachten van hypersomnie kunnen erdoor verminderen en bij veel patiënten wordt kataplexie er volledig door onderdrukt.[17] De meest gebruikte middelen vallen onder de Opiumwet, maar vanwege de positieve invloed van deze middelen op de kwaliteit van leven dient deze bepaling niet tot terughoudendheid met voorschrijven te leiden. Bovendien zijn er eigenlijk nooit problemen met misbruik of verslaving van deze middelen bij narcolepsiepatiënten. Hypocretine speelt mogelijk een rol bij het ontstaan van verslaving,[18] hetgeen zou kunnen verklaren waarom hypocretinedeficiënte narcolepsiepatiënten minder gevoelig zijn voor het ontwikkelen van afhankelijkheid.

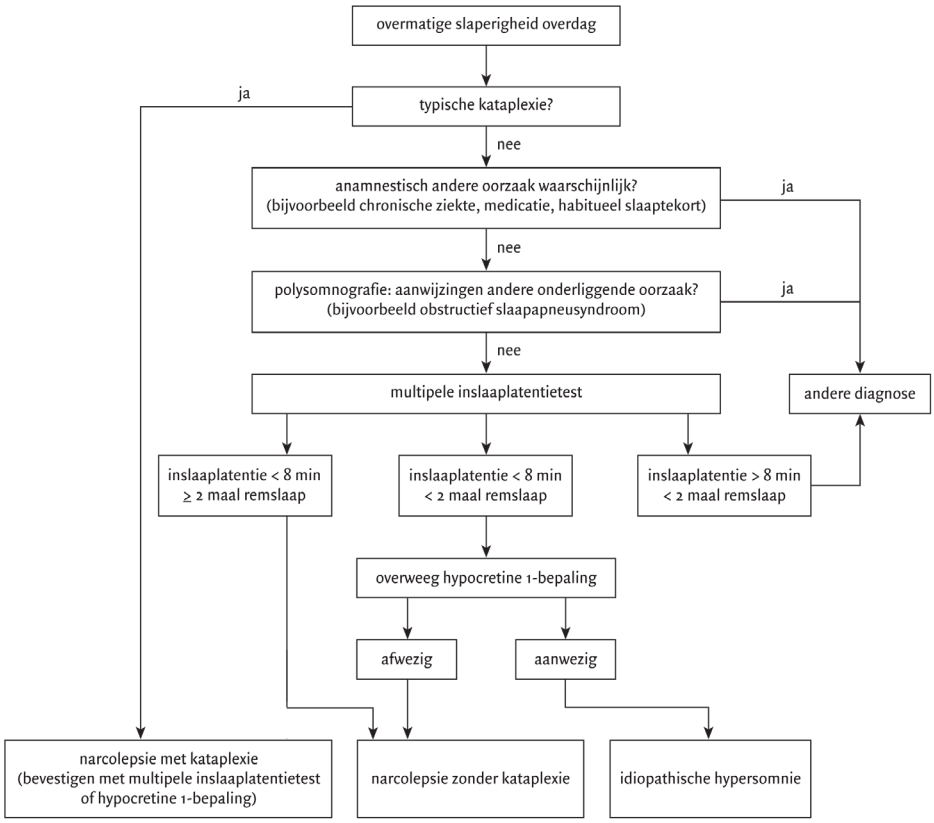

Figure 6.3 Stroomdiagram met diagnostische beslissingen om narcolepsie vast te stellen

De multipele inslaaplatentietest dient altijd voorafgegaan te worden door een nachtelijke polysomnografie om de kwaliteit van de voorafgaande nachtslaap te controleren. Vanwege beperkingen van de sensitiviteit en specificiteit van de multipele inslaaplatentietest dient men niet uitsluitend hierop af te gaan, maar dient het klinisch oordeel altijd mee gewogen te worden. Eventueel kan een hypocretine 1-bepaling zekerheid geven.[2]

Hypersomnie

Voor de behandeling van hypersomnie zijn stimulantia als modafinil en methylfenidaat beschikbaar. Modafinil wordt 1-2 maal per dag gedoseerd met een maximale dagdosis van 400 mg en methylfenidaat 3-4 maal per dag met een maximale dagdosis van 60 mg om gedurende de gehele dag werkzaam te zijn. Methylfenidaat kan bij minder ernstige vormen van narcolepsie gericht worden ingenomen wanneer de situatie dit vereist: bijvoorbeeld voor een vergadering of concertbezoek. De belangrijkste bijwerkingen zijn agitatie en hoofdpijn; terughoudendheid is geboden bij hypertensie. Over het algemeen wordt modafinil beter verdragen. Voor beide middelen geldt dat bij een derde van de patiënten op den duur tolerantie optreedt.[17]

Kataplexie

Van oudsher wordt kataplexie behandeld met middelen die voornamelijk een noradrenerge werking hebben, zoals tricyclische antidepressiva als clomipramine of

imipramine. Deze middelen zijn hier overigens niet voor geregistreerd. Doseringen van 10 mg/dag zijn soms al effectief. Selectieve serotonineheropnameremmers worden ook gebruikt, maar vaak zijn hiervan hogere doseringen noodzakelijk, waarschijnlijk omdat de noradrenerg aangrijpende metabolieten verantwoordelijk zijn voor het therapeutisch effect. Deze middelen kunnen tevens gunstige effecten hebben op slaapparalyse en hypnagoge hallucinaties. Als het gebruik hiervan acuut wordt gestaakt, bestaat er echter een risico op een zogenoemde status cataplecticus, waarbij kataplexie zeer frequent optreedt.[3]

Gammahydroxyboterzuur

Sinds juli 2006 is gammahydroxyboterzuur in Nederland geregistreerd en wordt dit middel vergoed. Het is een kortwerkend slaapmiddel waarvan het werkingsmechanisme niet duidelijk is. In ons land bestaat er redelijk veel ervaring mee vanwege onderzoek dat eind jaren tachtig bij narcolepsiepatiënten werd verricht.[19] Gammahydroxyboterzuur is werkzaam tegen kataplexie, de verhoogde slaapneiging en de gestoorde nachtslaap.[20] Er zijn tevens aanwijzingen voor een gunstig effect op hypnagoge hallucinaties. Vanwege de grote hoeveelheid die per keer moet worden genomen, 4,5-9,0 g per nacht verdeeld over 2 giften, wordt het als drank toegediend. Het werkt snel; de eerste dosis moet daarom direct vóór de nachtslaap in bed worden ingenomen nadat de tweede dosis voor diezelfde nacht is klaargezet. Deze tweede dosis kan na 3 tot 4 uur worden ingenomen, waarbij er een interval is van tenminste 3 uur tussen de tweede inname en de geplande tijd van opstaan. Dit schema wordt al snel routine voor de patiënt. In tegenstelling tot bij benzodiazepinegebruik, neemt de hoeveelheid diepe slaap toe en treedt nauwelijks gewenning op.

De bijwerkingen zijn merendeels dosisgerelateerd. Misselijkheid komt het vaakst voor, maar incontinentie voor urine en slaapwandelen worden door patiënten als de vervelendste bijwerkingen ervaren.[21] Deze laatste 2 treden zelden op; dosisverlaging is vaak de oplossing.

Men dient patiënten te waarschuwen het middel niet te gebruiken in combinatie met alcohol 's avonds. Vanwege het sederend effect is het niet verstandig het aan ouders met jonge kinderen voor te schrijven. Het eerste uur na inname is het middel nog zo werkzaam dat het bij ontwaken patiënten 'slaapdronken' maakt. Een ernstig probleem is dat gammahydroxyboterzuur illegaal onder andere als 'partydrug' wordt gebruikt.[22] Om die reden valt het onder de Opiumwet en mag het alleen door specialisten met voldoende deskundigheid worden voorgeschreven.

Welke plaats gammahydroxyboterzuur in Nederland zal krijgen, moet nog blijken; in de Verenigde Staten is het in korte tijd uitgegroeid tot het middel van eerste keuze.

Conclusie

In de afgelopen jaren is narcolepsie met kataplexie een duidelijk omschreven ziekte-entiteit geworden, met een opgehelderde pathofysiologie en een organisch substraat. Als

gevolg hiervan is het algehele begrip van slaap en met name de regulatie van slaap en waak toegenomen. Dit heeft geleid tot een nieuwe classificatie van slaapstoornissen.[2]

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Focusing on Vigilance instead of Sleepiness in the Assessment of Narcolepsy: High Sensitivity of the Sustained Attention to Response Task (SART)

Based On: *Fronczek R, Middelkoop HA, van Dijk JG, Lammers GJ. Sleep. 2006;29:187-91*

CHAPTER 7

Focusing on Vigilance Instead of Sleepiness in the Assessment of Narcolepsy: High Sensitivity of the Sustained Attention to Response Task (SART)

Context The severity of narcolepsy is commonly measured with the Multiple Sleep Latency Test (MSLT), focusing on the tendency to fall asleep. A neglected but perhaps equally important complaint is impaired performance in the waking state. We evaluated the Sustained Attention to Response Task (SART) for the quantification of vigilance in narcolepsy.

Methods The SART involves withholding key presses to 1 in 9 target stimuli. In the present study, it was administered prior to each of 5 MSLT sessions in a 1-day study. The Epworth Sleepiness Scale was administered to measure subjective sleepiness. SART and MSLT results (number of errors and sleep latency) were compared using Receiver Operator Curves, sensitivity, and specificity.

Patients Fifteen untreated narcoleptics and 15 matched controls.

Results The area under the receiver operating curve was 0.97 for the MSLT and 0.95 for the SART. Sensitivity and specificity for the MLST were 80% and 100% using a cutoff point of 5 minutes. For the SART, these values were 87% and 100%, using a 5-error cutoff. The SART and MSLT showed no correlation with each other or with the Epworth Sleepiness Scale.

Conclusion The SART, measuring attention, was abnormal as often as the MSLT, measuring sleepiness. The inability to remain vigilant during the day may be the most serious complaint in narcolepsy, since it impairs performance. The SART is valid in this respect, is easy to administer, and takes little time.

Introduction

Excessive daytime sleepiness (EDS), usually characterized as the tendency to fall asleep, is considered to be the main complaint in narcolepsy. However, this focus on inadvertently falling asleep may have led to undervaluation of a perhaps equally important complaint: impaired performance in the waking state. Broughton et al reported in the early 1980s that impaired performance in narcolepsy was linked not only to sleep but

also to lapses in vigilance.[1-3] Fluctuations in vigilance in narcolepsy may be considered as the counterpart of fragmented nocturnal sleep. In fact, the notion that both sleep and vigilance are disturbed has led to the concept of a loss of "state boundary control" to explain the pathophysiology of narcolepsy.[4,5] Moreover, a combined deficit of sleep and vigilance control has emerged as the core problem in narcolepsy in hypocretin-deficient mice.[6] In spite of the importance of the dual disturbance of vigilance and sleep, the most commonly used tests to measure the severity of narcolepsy focus solely on the tendency to fall asleep. The multiple sleep latency test (MSLT) is the most commonly used objective test to assess sleepiness and to diagnose narcolepsy.[7] Sleep latency is measured in 4 or 5 twenty-minute periods over 1 day, during which subjects lie in a dark and quiet room and try to fall asleep. Narcolepsy is likely when the mean sleep latency is 5 minutes or less and 2 or more sleep-onset rapid eye movement (REM) periods occur. Some authors have recently advocated an abnormality threshold of 8 minutes.[8] Various studies have questioned both the diagnostic yield and the validity of the MSLT in the diagnosis of narcolepsy.[8-14] For example, patients without sleep complaints may fulfil MSLT criteria for narcolepsy, whereas only 70% of patients with clear cataplexy do.[13,15] Although the mean sleep latency may well quantify sleepiness in healthy sleepdeprived subjects, it is debated whether it does so in narcolepsy.[9,14] On top of these limitations, the MLST is time and labor intensive. The maintenance of wakefulness test (MWT) is an alternative to the MSLT. Subjects are asked to remain awake instead of trying to fall asleep, which may better reflect daily life.[16] However, the validity of the MWT is also questionable, and it is equally time consuming to perform.[9,17] Therefore, the time seems ripe for tests aimed at impaired vigilance to measure this aspect of the severity of narcolepsy. Such a test may also have better properties to predict impaired performance.

We considered the Sustained Attention to Response Task (SART) to be a good candidate, since it reflects vigilance and sustained attention.[18,19] Furthermore, it only takes a short time to perform, has a high frequency of stimuli, and is easy to administer, which make it useful in a clinical setting. To explore the role of the SART in diagnosing and quantifying vigilance as an essential aspect of the severity of narcolepsy, we compared the SART with 2 current tools to measure sleepiness: the MSLT and the Epworth Sleepiness Scale (ESS).[20]

Materials and methods

Subjects

Fifteen unmedicated patients with narcolepsy were studied; all had daytime sleepiness and unequivocal cataplexy and thereby fulfilled the criteria of narcolepsy with cataplexy (International Classification of Sleep Disorders).[21] Thirteen patients were tested shortly after diagnosis and had never had treatment. Two patients had stopped medication to participate. These 2 patients were the only ones who had previously undergone an MSLT, but their diagnosis did not rely on MSLT results, as both had clear cataplexy. Unmedicated controls were recruited using an advertisement in a local newspaper and were matched in number, sex, age, and level of education with the patients. No

control had any complaints of excessive sleepiness or lowered vigilance. All subjects were instructed to follow their normal sleep routine the night before the testing day.

Design

The ESS was administered at 8:30 AM. This is a simple self-administered questionnaire, which is shown to provide a measurement of the subject's general level of daytime sleepiness.[20] The first sleep latency test began at 9:00 AM. The MSLT was performed according to the standards laid out by Carskadon et al,[22] with sleep latency tests at 9:00 AM, 10:30 AM, 12:00 noon, 1:30 PM, and 3:00 PM. The SART (see below) was administered 15 minutes prior to each sleep latency test, while subjects were seated on a chair in front of a computer screen. Before the sleep latency test at 9:00 PM, all subjects had to do a short version of the SART to become familiar with the test. Between sleep latency tests, participants were allowed to go for short walks in the hospital and eat or drink but not sleep.

Table 1—Clinical Characteristics and Test Results

	Controls	**Narcolepsy**
Men/women, no.[a]	9/6	8/7
Age, y	34 (28-39)	33 (30-36)
ESS score	4.3 (2.6-7.0)*	17.4 (16.1-19.8) *
SART error score	2.0 (1.3-4.0)*	10.6 (6.1-18.7)*
MSLT sleep latency, min	12.2 (8.6-14.2)*	2.5 (0.8-4.7*

Data are presented as medians with 25th and 75th percentile in parentheses unless otherwise indicated. ESS refers to Epworth Sleepiness Scale; SART, Sustained Attention to Response Task; MSLT, Multiple Sleep Latency Test.
Mann-Whitney U was used to assess group differences; *$p < .01$
[a]χ^2 test was used to assess group differences; *$p < .01$

Sustained Attention to Response Task

A number from 1 to 9 was shown 225 times in white on a black computer screen over a 4.3-minute period in a quiet room with dimmed lights. Each of the 9 numbers was shown 25 times in random order. The font size was chosen at random from 26, 28, 36, or 72 points. The numbers were presented in a predetermined and quasirandom way so that identical numbers were not clustered. Each number was presented for 250 milliseconds, followed by a blank screen for 900 milliseconds. Subjects had to respond to the appearance of each number by pressing a small button, except when the number was a 3. Subjects had to press the button before the next number appeared and were instructed that accuracy was more important than speed. A complete SART takes 4 minutes and 20 seconds to perform. The SART error score consists of the total number of errors, expressed as the sum of the times a key was pressed when a 3 was presented, and the times when no key was pressed when it should have been.

Statistical Analysis

Differences between groups were assessed using the Mann-Whitney test. For each subject, the mean of the 5 MSLT latencies and SART scores were computed and used in

the analysis. Receiver operator curves (ROC) were employed to compare the diagnostic yield of the MSLT and the SART. Sensitivity and specificity were computed using the commonly used 5- and 8-minute cutoffs for the MSLT. For the SART a 5-error cutoff point was used, which was derived from the 95th percentile in controls (5.4 errors). As variables were not normally distributed, Spearman ρ was used to investigate the correlation within the separate groups between the MSLT, the SART, and the ESS. Effects of testing time were first evaluated using the Friedman-Test and analyzed posthoc using the Wilcoxon Signed Rank Test.

Results

Test Results

Data are presented as median (25th-75th percentile). The median sleep latency was 2.5 (0.8-4.7) minutes in patients and 12.2 (8.6-14.2) minutes in controls (Figure 7.1a). Controls showed a broader range (12.6 minutes) in MSLT sleep latencies than patients (8.0 minutes). The median SART error score was 10.6 (6.1-18.7) errors for patients and 2.0 (1.3-4.0) errors for controls (Figure 7.1b). SART error scores of patients showed a much broader range (33.0 errors) than did error scores of controls (5.0 errors). On the ESS, patients obtained a median score of 17.4 (16.1-19.8), whereas controls had a median score of 4.3 (2.6-7.0). The differences between patients and controls were significant for all these tests ($p < .01$; Table 1).

In contrast with that of the patient group, the sleep latency significantly differed between testing times in the control group (controls: $p < .01$, patients: $p = .59$; Figure 7.2a). In controls, the sleep latency at noon was significantly shorter, as compared with the sleep latency at 9:00 AM and 10:30 AM (9:00 AM: $p < .01$, 10:30 AM: $p = .04$). In controls, the SART error score significantly differed between testing times. This effect was not significant in patients (controls: $p < .01$; patients: $p = .46$; Figure 7.2b). In controls, the SART error score at 9:00 AM was higher, as compared with the SART error scores at all other testing times (all testing times: $p < .02$).

Diagnostic Yield

Areas under the ROC were 0.97 for the MSLT and 0.95 for the SART. The sensitivity and specificity for the MSLT were 80% and 100% using a 5-minute cutoff. With an MSLT cutoff point at 8 minutes (stage 1), sensitivity and specificity for the MSLT were 93% and 80%. For the SART, these were 87% and 100%, using the 5 error cutoff.

Correlations

No significant correlation emerged between SART error score and MSLT latency in either controls or patients (controls: $\rho=0.29$, $p = .33$; patients: $\rho = 0.15$, $p = .60$; Figure 7.3). No correlations between the SART error score, the MSLT latency, or the ESS were significant (all: $\rho < .27$, $p > .33$).

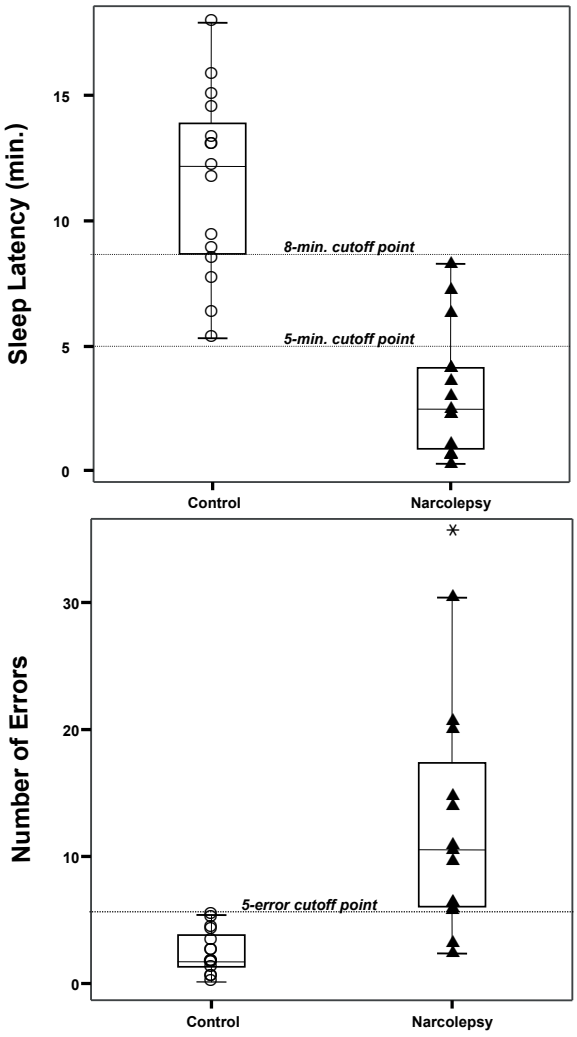

Figure 7.1
Boxplots
Multiple Sleep Latency Test (MSLT) sleep latency (a) and Sustained Attention to Response Task (SART) error score (b). This figure shows box plots indicating the median, 25th-75th percentiles, and the range. An asterisk indicates an out-of-range value. With cutoff points for the MSLT and SART, respectively, at 5 minutes (stage I) and 5 errors, sensitivity and specificity for the MLST were 80% and 100%. For the SART, these were 87% and 100%. With an MSLT cutoff point at 8 minutes (stage I), sensitivity and specificity for the MSLT were 93% and 80%.

Discussion

In this explorative study, we investigated the SART as a tool to measure vigilance as an important indicator of the severity of narcolepsy and compared it to the MSLT, known to reflect the tendency to fall asleep. In their respective roles, both tests performed equally well, as shown by excellent ROC and high sensitivity and specificity. There was clear evidence that both tests indeed measured different phenomena: there was no correlation between MSLT and SART results. Moreover, the range of MSLT latency was considerably larger in controls than in patients, while the reverse applied to an even stronger degree for the range of SART error scores. The large variability of SART results in patients may be advantageous, in that it may offer a better resolution to quantify vigilance as a severity indicator of narcolepsy, which may be of use in measuring treatment effects.

Our results are in line with Broughton and colleagues, who found that narcoleptic patients have more lapses (response omissions) and false-positive responses (errors of commission) during the Wilkinson Auditory Vigilance Task.[2] The SART error score consists of the sum of errors of omission as well as those of commission. Another benefit of the SART is that it is considerably shorter than other vigilance tasks, while having a high resolution to analyze vigilance. Subjects have to respond to a continuous sequence of stimuli but have to inhibit a response at an unexpected moment. In contrast, most vigilance tests consist of responding to unexpected stimuli over a longer time period. In the time between stimuli, no action by the subject is required and, as such, no information about the level of vigilance in these periods is acquired. How well does the SART compare with the ESS? The SART showed a low correlation with the ESS in both groups, which might be seen as evidence that both tests are sensitive to different features, ie, vigilance and sleepiness. However, there was no correlation between MSLT and ESS results either, which is surprising, as these tests are both thought to reflect sleepiness. This corroborates earlier reports of low or absent correlation between ESS and MSLT in narcolepsy; in fact, correlations are only moderate in controls.[9,12,20,23-26] Apparently, subjective assessment of sleepiness and a latency measure reflect significantly different aspects of sleepiness.

As for the diagnostic use of the SART, several remarks need to be made. Firstly, the new International Classification of Sleep Disorders-2 allows narcolepsy to be diagnosed by establishing a lack of hypocretin in the cerebrospinal fluid or by performing an MSLT.[21] This new approach will remove the need to perform an MSLT in a number of cases, particularly in those with cataplexy. In such cases, an assessment of impaired vigilance as a functional indicator of the severity of narcolepsy may be of use. Secondly, we compared narcoleptic patients with healthy controls, which explains the excellent sensitivity, specificity, and ROC of the SART and the MSLT. The contrast in results between groups is very likely to be less pronounced in a comparison with patients suffering from other disorders that also cause impaired vigilance. At present, we therefore do not advocate

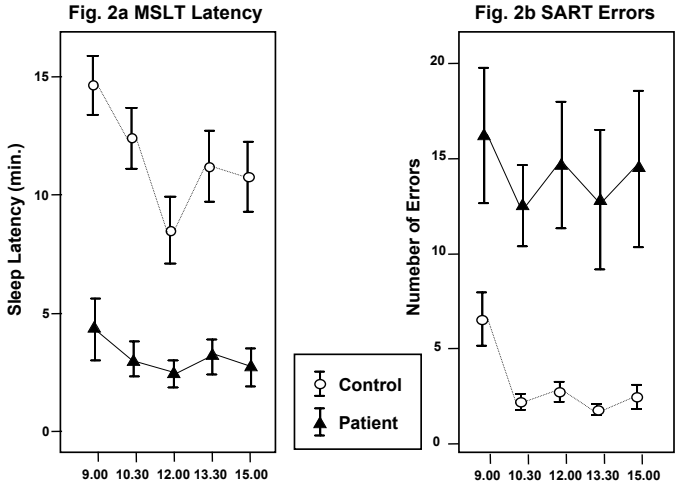

Figure 7.2
Diurnal effects
Multiple Sleep Latency Test (MSLT) latency (a) and Sustained Attention to Response Task (SART) error score (b) over the day. The MSLT latency and SART error score significantly differed between testing times in the control group. Error bars indicate SEM.

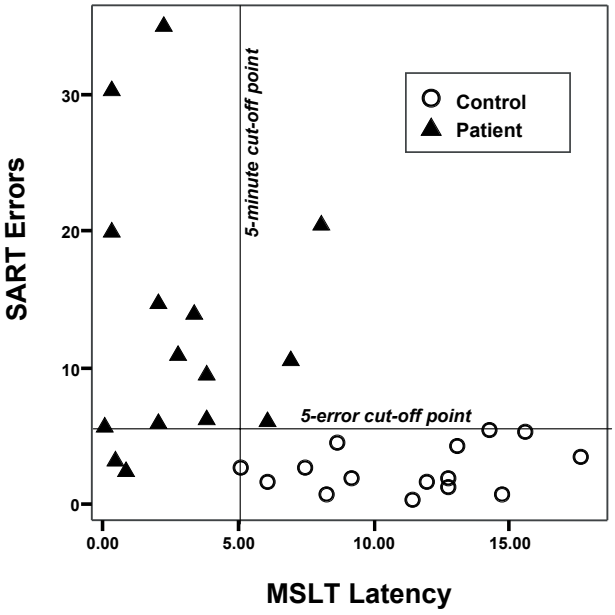

Figure 7.3
Correlation
Correlation between Sustained Attention to Response Task (SART) error score and Multiple Sleep Latency Test (MSLT) sleep latency. Circles represent controls, triangles represent patients. In the figure, the 5-error cutoff point of the SART and the 5-minute cutoff point of the MSLT are indicated by a line. There was no significant correlation between SART error score and MSLT sleep latency.

using the SART to distinguish between such disorders causing sleepiness; we contend that it is of use to measure vigilance.

There were diurnal effects on SART performance and MSLT latency. In controls, the noon MSLT latency was shorter than that of earlier MSLT periods, as has been found in earlier studies.[27-29] This effect was not significant in patients. In controls, the 9:00AM SART error score was significantly higher than that of other times. A possible explanation is that this reflects a brief learning effect, not fully covered by the 30-second introductory session. Another cause could be a diurnal effect. This effect meant that SART error scores of patients and controls were closer together at 9:00 AM than at other times, but there was still a clear difference at this time as well (Figure 7.2b).

Conclusion

The inability to remain vigilant during the day may be the most serious complaint in narcolepsy, since it impairs performance. The SART quantifies this neglected aspect and is valid, easy to administer, and takes little time to perform. Further studies are needed to probe the ability of the SART to measure treatment effects.

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Increased Heart Rate Variability but Normal Resting Metabolic Rate in Hypocretin/ Orexin-deficient Human Narcolepsy

Based On: *Fronczek R, Overeem S, Reijntjes R, Lammers GJ, Van Dijk JG, Pijl H. submitted*

CHAPTER 8

Increased Heart Rate Variability but Normal Resting Metabolic Rate in Hypocretin/Orexin-deficient Human Narcolepsy

Objective We investigated the possible role of abnormalities in autonomic balance and resting metabolic rate to explain obesity in hypocretin/orexin-deficient narcoleptic subjects.

Methods Resting metabolic rate (RMR) and variability in heart rate and blood pressure were determined in the fasted, resting state. Subjects were 15 untreated, hypocretin-deficient male narcoleptics and 15 male controls matched for age and body mass index.

Results Spectral power analysis revealed greater heart rate and blood pressure variability in hypocretin-deficient male narcoleptic patients (heart rate: $p=0.01$; blood pressure systolic: $p=0.02$; diastolic: $p<0.01$). The LF/HF ratio was normal ($p=0.48$). Resting metabolic rate did not differ between hypocretin-deficient male patients and controls (controls=1767 ± 226 kcal/24h, patients: 1766 ± 227 kcal/24h, $p=0.99$).

Conclusion Using indirect calorimetry we did not find a reduced resting metabolic rate in hypocretin-deficient narcoleptic men. However, heart rate and blood pressure variability was increased, which may point to a reduced sympathetic tone. The role of this latter finding in the pathophysiology of obesity in narcolepsy remains to be elucidated.

Introduction

Narcolepsy is a sleep disorder that affects 20-60 per 100,000 in western countries. The syndrome is classically characterized by the tetrad of excessive daytime sleepiness, cataplexy, sleep paralysis and hypnagogic hallucinations.[1] The first report of obesity as a metabolic feature of narcoleptic patients dates back as early as the 1930s[2, 3] and the observation was confirmed repeatedly since.[4-11] The identification of hypocretin/orexin deficiency as the cause of human narcolepsy with cataplexy and the potential role of hypocretin peptides in metabolic control has sparked interest in the pathophysiology of the obesity accompanying narcolepsy. Indeed, it not only is a consistent feature of human narcolepsy, but also in hypocretin-deficient animal models.[12, 13] Furthermore, patients suffering from idiopathic hypersomnia who are also suffering from excessive daytime sleepiness, but are not hypocretin-deficient, are not obese.[11]

Hypocretin peptides are involved in the control of autonomic nervous system activity, food intake and energy balance.[14-16] In particular, injection of hypocretins into the lateral cerebral ventricle stimulates food intake.[15] Accordingly, ablation of hypocretin neurons leads to hypophagia in mice[13] and narcoleptic humans eat less than age and sex matched controls.[17] Paradoxically, both in mice and men this is accompanied by increased body weight. To reconcile these apparently contradictory corollaries of hypocretin deficiency, it may be necessary to consider the effects of hypocretin peptides on wakefulness and sympathetic activity. In rats, injection of hypocretins into the lateral ventricle also stimulates arousal and activates the sympathetic nervous system to increase arterial blood pressure, heart rate, oxygen consumption, body temperature and plasma catecholamine levels.[18-21] Thus, hypocretin deficiency and daytime sleepiness may reduce physical activity, which could diminish energy expenditure. Also, hypocretin deficiency might directly reduce sympathetic tone and resting metabolic rate, and thereby induce obesity. Moreover, adipose tissue is under neuronal control and is innervated by both sympathetic (catabolic) and parasympathetic (anabolic) pathways[22] and autonomic imbalance could thus lead to fat accumulation.[23]

We studied resting metabolic rate and variation in blood pressure and heart rate in hypocretin-deficient narcoleptic subjects and healthy controls. We hypothesized that sympathetic tone might be diminished and that resting metabolic rate would be reduced in narcoleptic subjects.

Materials and Methods

Subjects

The study was approved by the local medical ethical committee. All narcoleptic patients were male and fulfilled the ICSD-2 criteria of narcolepsy with cataplexy.[24] They did not take any medication and hypocretin/orexin was undetectable in their cerebrospinal fluid, as measured by a standard radioimmuno-assay (Phoenix Pharmaceuticals, Inc., Belmont, CA). Healthy male controls were recruited using an advertisement in a local newspaper. Groups were matched for age and body mass index (BMI). As BMI is a very strong confounder of metabolic rate in itself, BMI-matching is mandatory. The pathogenesis of obesity is a multifactorial (increased caloric intake, sedentary lifestyle and predisposing genetic make-up). However, narcoleptics do not eat more or move less than healthy individuals, which led us to hypothesize that a lowered metabolic rate is the sole causative factor in narcolepsy. As said above, BMI is a strong determinant of metabolic rate by itself. In other words, our hypothesis was that narcoleptic subjects have a metabolic rate that is too low for a given BMI. Therefore, we matched the control group for BMI.

Metabolic Measurements

In fifteen patients and fifteen controls metabolic values were measured. Subjects were instructed to fast and drink only water from 22.00 hrs the night before until the metabolic measurement was performed. Subjects arrived at the hospital at 09.00 hrs and

lied down in a supine position for a 30-minute period. Special care was taken to keep subjects awake during this period by talking to them. RMR was measured by indirect calorimetry[25] using a computerized open-circuit ventilated hood system (Oxycon B; Jaeger, Breda, The Netherlands).[26] Because subjects have to get used to the recording circumstances, the first 10 minutes of the test period were discarded.

Oxygen consumption ($VO_{2,}$ L/min) and carbon dioxide production ($VCO_{2,}$ L/min) were used to calculate the respiratory quotient (RQ=VCO2/VO2). Resting metabolic rate and carbohydrate (C) and fat (F) combustion were calculated using the Weir formula and were expressed as kilocalories per 24h (per kilogram body weight) and grams per minute (per kilogram body weight) respectively.[27, 28] The following formulas were used:

$$RMR\ (kcal/24h) = (3.9 \times VO2) + (1.1 \times VCO2) \times 1.44$$

$$C\ (g/min) = (4.55 \times VCO2) - (3.21 \times VO2)$$

$$F\ (g/min) = (1.67 \times VO2) - (1.67 \times VCO2)$$

Autonomic Measurements

Since measurement of autonomic function was added in a later stage, autonomic activity was measured in nine patients and nine controls, a subpopulation of the subjects included for the metabolic measurements. This was done simultaneously with the metabolic measurements. Heart rate was determined by ECG, measured continuously using standard Ag-AgCl electrodes. Beat-to-beat arterial blood pressure was noninvasively monitored (Finometer, TNO-Biomedical Instruments, The Netherlands). The hand used for these finger blood pressure measurements was held in a constant position at heart level. The complete last 20 minutes of the actual testing period were used to calculate heart rate and blood pressure.

Blood pressure and heart rate calculations were performed using software written in MatLab (MatLab v7.0, Mathworks, Massachusetts). Heart Rate Variability (HRV) was estimated by calculating the mean and SD of consecutive R-R intervals and with spectral analysis performed by interpolating the series of RR intervals by cubic splines, resampling the signal at 3 Hz and performing a Fast Fourier Transformation (FFT) using a Hamming-window.[29] Power was calculated for the following bands: Very Low Frequency (VLF, 0-0.04 Hz), Low Frequency (LF, 0.04-0.15 Hz), and High Frequency (HF, 0.15-0.4 Hz). Total power was calculated by adding the powers of the VLF, LF and HF bands. Increases in total power can be caused by a reduction in sympathetic tone.[30, 31] Furthermore, the LF/HF ratio was calculated. The LF band is usually considered to represent the sympathetic part of the baroreceptor reflex, while the HF band, largely derived from respiratory influences, mostly concerns parasympathetic activity. The

LF/HF ratio is generally used as another measure for the autonomic parasympathetic-sympathetic balance.[32] However, some authors regard it as an indication of sympathetic activity.[33] Mean systolic (SBP) and diastolic (DBP) blood pressures were calculated and the total power in their frequency spectrum (calculated similarly to the HRV spectrum) was taken as an estimate of variability. Note that finger blood pressure measurement using the aforementioned finometer has a tendency to underestimate blood pressure. This can be prevented by calibrating the device using using the Riva-Rocci method.[34] However, blood pressure values were not corrected in this study, since groups were compared.

Statistics

Differences between groups were calculated using Student's t-test for unpaired samples. Pearson's correlation coefficient was used to evaluate potential correlations. P-values below 0.05 were considered significant.

Results

Resting Metabolic Rate

Data are shown in Table 8.1. Patients and controls did not differ for age or BMI (Figure 8.1a). There were no significant differences in resting metabolic rate (RMR), oxygen consumption (VO2), carbodioxide consumption (VCO2), respiratory quotient (RQ) and carbohydrate or fat substrate combustion between narcoleptic patients and controls. Since the groups were matched for BMI, correcting RMR and carbohydrate or fat combustion for bodyweight in kilograms did not influence the results (Table 8.1).

Autonomic data

Data are shown in Table 8.2. Patients and controls had similar age and BMI. There were no significant differences in mean heart rate (HR), systolic blood pressure (SBP) and diastolic blood pressure (DBP). However, heart rate variability (HRV) and blood pressure variability differed between the two groups: the total power in the spectrum of both the diastolic and systolic blood pressure was significantly higher in patients compared to controls (systolic: $p < 0.02$, diastolic: $p < 0.001$; Figure 8.1c and 8.1d). A respiratory high frequency (HF) peak was seen in the HRV spectra of all subjects (Figure 8.2). Total power ($p < 0.01$), very low frequency (VLF) power ($p < 0.03$) and low frequency (LF) power ($p < 0.02$) were higher in hypocretin-deficient patients compared to controls, while high frequency (HF) power tended to be higher as well ($p = 0.05$, Figure 8.2). In contrast, the ratio between low frequency and high frequency power (LF/HF ratio) did not differ between patients and controls ($p = 0.48$).

Table 8.1 Metabolic Measures

	Controls (N=15)	Narcolepsy (N=15)	P-value
male/ female	15/0	15/0	
Age (years)	36.3 ± 13.8	35.6 ± 13.8	0.89
BMI (kg/m^2)	26.0 ± 2.8	26.8 ± 2.3	0.42
VO2 (ml/min)	250.9 ± 34.2	252.4 ± 33.4	0.91
VCO2 (ml/min)	225.9 ± 24.6	220.3 ± 31.0	0.58
RQ	0.88 ± 0.06	0.86 ± 0.07	0.45
RMR (kcal/24h)	1767.1 ± 226.5	1766.5 ± 226.5	0.99
RMR / kg	19.9 ± 2.0	20.1 ± 2.2	0.77
C (Carbohydrate) (g/min)	222.5 ± 56.9	191.9 ± 95.0	0.29
C / kg	2.5 ± 0.6	2.1 ± 1.0	0.34
C in % of RMR	72.5 ± 18.5	62.6 ± 31.0	0.29
F (Fat) (g/min)	41.7 ± 29.5	53.7 ± 38.9	0.35
F / kg	0.5 ± 0.3	0.6 ± 0.5	0.35
F in % of RMR	30.6 ± 21.6	39.4 ± 28.5	0.35

Values in the table are means ± standard deviation. T-tests were used to assess group differences; no significant differences were found. BMI, body mass index; kcal, kilocalories; 24h, per 24 hours; RMR, resting metabolic rate; kg, kilograms; RQ, respiratory quotient; VO2, oxygen consumption; VCO2, carbon dioxide consumption; g, grams; min, minute.

Discussion

The pathogenesis of obesity in narcoleptic patients remains unexplained. Obviously, eating more or moving less are potential explanations. Hypocretin neuron-ablated narcoleptic mice[12] and human patients[17] were shown to eat less than normal controls (total daily food intake, narcoleptic humans: 8,756 ± 2,312 kilojoules; controls: 10,640 ± 3,129 kJ; $p<0.001$, data from Lammers et al.),[17] which is in accordance with the orexigenic qualities of hypocretin peptides. Actigraphy studies showed that although periods of activity and inactivity were more scattered in narcoleptic subjects versus controls, the total intensity of physical activity did not differ.[35, 36] Furthermore, narcoleptic subjects are more obese than equally active subjects suffering from idiopathic hypersomnia.[11] Thus, hypocretin deficiency must have other metabolic consequences to explain why narcoleptic animals and humans are obese. Since hypocretin peptides were shown to activate the sympathetic nervous system and increase oxygen consumption in rat,[18-21] we hypothesized that hypocretin deficiency would lead to reduction of sympathetic tone and resting metabolic rate in patients with narcolepsy.

In the present study, spectral power analysis of heart rate and blood pressure variability revealed an increase in the power across all frequency domains in narcoleptic patients, but no differences in the LF/HF ratio for heart rate. These results could point to a reduced sympathetic tone in narcoleptic patients. The HF peak is effected almost

Table 8.2 Autonomic Measures

	Controls (N=9)	Narcolepsy (N=9)	P-value
male/ female	9/0	9/0	
Age (years)	29.2 ± 4.1)	32.6 ± 16.2	0.55
BMI (kg/m2)	24.9 ± 2.6)	26.2 ± 2.1	0.31
Total Power in HRV spectrum x 10^4 s^2/Hz	27.8 ± 14.1	77.4 ± 52.0	0.01*
- VLF (0-0.04 Hz) x 104 s2/Hz	12.6 ± 8.2	30.7 ± 22.0	0.03*
- LF (0.04-0.15 Hz) x 104 s2/Hz	9.7 ± 5.5	30.6 ± 24.2	0.02*
- HF (0.15-0.4 Hz) x 104 s2/Hz	5.6 ± 2.9	16.2 ± 14.9	0.05
LF/HF Ratio	1.9 ± 1.1	2.3 ± 1.2	0.48
Mean Heart Rate (BPM)	59.6 ± 8.8	56.7 ± 5.4	0.42
Mean Diastolic Blood Pressure (DBP, mmHg)	55.8 ± 9.0	52.8 ± 6.9	0.44
Mean Systolic Blood Pressure (SBP, mmHg)	104.0 ± 13.7	99.5 ± 17.6	0.55
Total Power in SBP spectrum x 10^4 $mmHg^2$/Hz	27.5 ± 11.1	47.7 ± 19.7	0.02*
Total Power in DBP spectrum x 10^4 $mmHg^2$/Hz	6.8 ± 3.5	16.7 ± 7.9	<0.00*

Values in the table are means ± standard deviation. T-tests were used to assess group differences. yrs, years; BMI, body mass index; HRV, heart rate variability; s, seconds; Hz, hertz, VLF, very low frequency; LF, low frequency; HF, high frequency; BMP, beats per minute.

exclusively by the parasympathetic system, so an alteration in sympathetic tone may be expected to leave it unchanged. Somewhat unexpectedly, however, an increase in power in both the LF and the HF band can result from an exclusive reduction of sympathetic tone, as proven by selective sympathetic blockade studies.[30, 31] A possible explanation could be that diminished sympathetic control may lead to higher fluctuations in blood pressure, induced by respiratory or other influences that in turn cause parasympathetic heart rate responses. The lack of differences in the LF/HF ratio in our study may in part be due to the fact that sympathetic tone contributes to both the LF and HF peaks, affecting both elements of the ratio. Sympathetic tone is already low in the supine position, so any further decreases are not likely to affect the ratio under these circumstances.[30, 31] This hampers straightforward interpretation of this ratio.[33] The only finding that is not readily compatible with decreased sympathetic tone is that mean heart rate was not lower in the narcolepsy group.[30, 31]

Surprisingly, although sympathetic activity drives resting energy expenditure, at least in rodents, resting metabolic rate was similar in narcoleptic patients and controls. Accordingly, hypocretin knockout mice have normal RMRs (C.M. Sinton, personal communication). However, large cohorts of patients and controls are needed to detect small differences in energy expenditure by indirect calorimetry, and even subtle reductions of RMR may lead to body weight gain in the long term.[37]

What are the potential explanations for the increased variability in blood pressure and heart rate in our narcoleptic patients? Firstly, narcoleptic subjects may not have been as awake as the control subjects, although special care was taken to keep patients alert

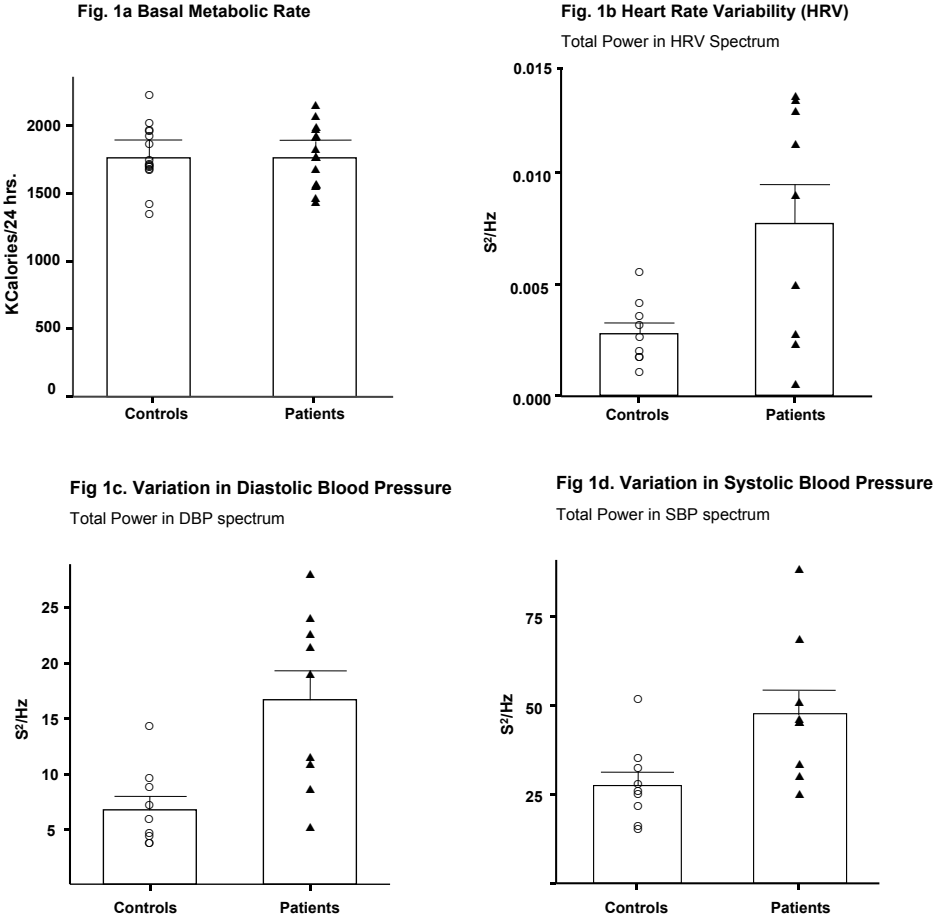

Figure 8.1 Results

Resting metabolic rate (a), heart rate variation (b), variation in systolic blood pressure (c) and variation in diastolic blood pressure (d) estimated by total power in the SBP and DBP frequency spectrum in narcoleptic patients and controls. Bars represent means, error bars indicate standard deviation, triangles represent patients and circles represent controls.

during the measurements. Narcolepsy is commonly seen as a loss of state boundary control, which means that patients are unable to remain awake steadily.[38] A tendency to drift into drowsiness could lead to a higher variability in autonomic parameters, as autonomic control differs in the various sleep stages. The transition between wakefulness and sleep affects the power in both the HF and the LF band.[39] A tendency to shift from waking to drowsiness frequently might therefore show up as increased HRV. None of the subjects was visibly asleep during any test, but we cannot exclude a contribution of drowsiness to the higher variability in blood pressure and heart rate in our narcoleptic patients. Drowsiness would not only affect autonomic parameters, but might also have lead to an underestimation of the RMR in narcoleptic subjects, since RMR is lower during sleep.[40] However, this would mean that the actual RMR in narcoleptic subjects

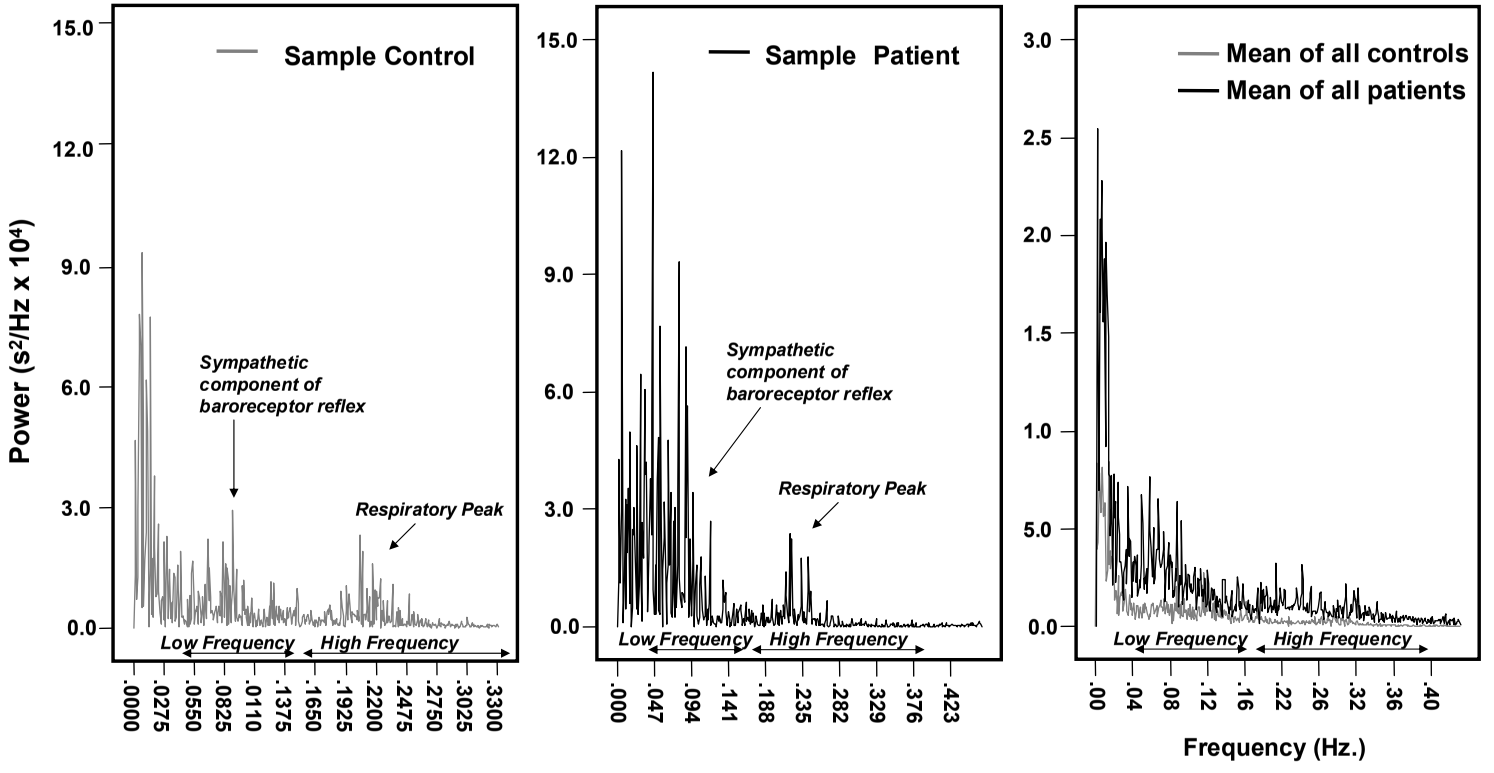

Figure 8.2 | **Power Spectra**
Example of typical power spectrum including respiratory peak, sympathetic component of the baroreceptor reflex and LF/HF regions for a control (left) and a narcoleptic patient (middle), and mean spectrum for both groups (right).

is higher, which does not explain their obesity. We suggest that further studies should take drowsiness into account. Interestingly, when looking at individual data points (Figure 8.1), there is a large variation in heart rate and blood pressure variability in narcoleptic patients. This was not correlated with BMI. The higher variation might explain or perhaps be explained by differences in phenotype, sleepiness or disease severity.

Alternatively, hypocretin deficiency may directly inhibit sympathetic activity. Various studies have shown that the hypocretin system is heavily involved in autonomic control and that hypocretins stimulate sympathetic activity.[41] Indeed, orexin neuron-ablated mice, which grow obese, have lower sympathetic vasoconstrictor outflow.[42] There is direct innervation of adipose tissue by sympathetic (catabolic) and parasympathetic (anabolic) pathways,[22] implying that a low sympathetic tone can directly promote fat accrual.[23]

Other authors who have looked at autonomic nervous function in narcoleptic patients found no abnormalities during provocations[43] and no primary disturbance between 6 and 8 PM or during sleep.[44] An increased LF/HF ratio compared with controls was found just before sleep onset, but this was thought to be related to the impairment of the sleep-wake cycle in narcolepsy and not to a primary disturbance.[44]

In conclusion, we did not find abnormalities in resting metabolic rate in narcoleptic humans when measured by indirect calorimetry. However, there are signs of reduced sympathetic activity, which may lead to fat accrual through direct effects on adipocytes. Future studies should directly measure sympathetic tone, for example using microneurography.

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Altered Skin-Temperature Regulation in Narcolepsy relates to Sleep Propensity

CHAPTER 9

Based On: *Fronczek R, Overeem S, Lammers GJ, van Dijk JG, Van Someren EJW. Sleep. 2006;29:1444-9*

Altered Skin-Temperature Regulation in Narcolepsy Relates to Sleep Propensity

Context In healthy subjects, sleep propensity increases when the distal skin temperature increases relative to the proximal skin temperature. This increase results from increased blood flow in the skin of the extremities and is, among other factors, controlled by the hypothalamic circadian clock, as is sleep. Because narcolepsy is characterized by hypothalamic alterations, we studied skin temperature in narcoleptic patients in relation to their characteristically increased sleep propensity during the day.

Methods Distal and proximal skin temperature and their gradient (DPG) were measured during a Multiple Sleep Latency Test. This allowed temperature to be studied during wakefulness, at sleep onset and during sleep.

Patients Fifteen unmedicated narcolepsy patients with cataplexy and 15 controls.

Results In subjects in the waking state, DPG was higher in narcoleptics than in controls throughout the day (time by group interaction, $p < .0001$), due to increased distal skin temperature and decreased proximal skin temperature. The increase in DPG was related to a shorter subsequent sleep-onset latency ($p = .02$). Once asleep, narcoleptics maintained their elevated distal skin temperature and DPG ($p < .0001$), whereas proximal skin temperature increased to reach normal levels.

Conclusion This is the first demonstration of a dramatic alteration of daytime skin temperature control in narcolepsy. Even awake narcoleptic patients showed a DPG higher than that which healthy controls achieve when asleep. This observation suggests that hypocretin deficiency in narcolepsy affects skin-temperature regulation and invites further examination. Skin-temperature control might ultimately even have therapeutic implications for the alleviation of narcoleptic symptoms.

Introduction

The circadian regulation of sleep and body temperature are intimately related: the core temperature is lowest during the major sleep period at night. This temperature decline at night is mainly due to an increase in skin blood flow causing skin warming and dissipation of body heat. Skin temperature in people is therefore higher during the night than the day, a rhythm that is the inverse of the core temperature curve.[1] During daytime quiet wakefulness, distal skin areas are usually cooler than proximal

ones, which is expressed as a negative distal-to-proximal gradient (DPG).[2] The distal skin warms up when hypothalamically regulated sympathetic cutaneous vasoconstrictor tone is released, opening a dense network of arteriovenous anastomoses[2,3]in the skin of the extremities. Many factors, such as light,may induce a relative cooling of the distal skin.[4,5] Fewer conditionsinduce distal skin warming; the most notable are activation of heat-loss mechanisms due to body heating, changing of body posture from upright to supine, or, at night, conditions that are under the control of the hypothalamic circadian timing system.[6] Interestingly, and recognized by Magnussen as early as the 1930s,[7] an increase in distal skin temperature may herald sleep onset. More-recent studies in healthy subjects have shown that sleep latency could in fact be predicted from skin-temperature distribution prior to the attempt to sleep. Krauchi et al[3] showed that, the more the distal temperature increased toward the proximal temperature, the less time it took to fall asleep. They hypothesized that skin temperature modulates neuronal activity in sleep-regulating brain areas and might thus promote falling asleep.[1] Indeed, animal studies have shown that afferents conveying skin-temperature information modulate the firing rate of thermosensitive neurons in the preoptic anterior hypothalamus, a region known to be crucial in promoting sleep.[8] The first direct support for a sleep-modulating role of skin temperature was recently provided in a controlled experiment in healthy young subjects, whose sleep onset could be accelerated by means of very subtle skin warming, even though this was slightly uncomfortable.[19]

Narcolepsy is clinically characterized by excessive daytime sleepiness and cataplexy.[9] The hypothalamus is crucially involved in narcolepsy, with a selective loss of hypocretin (orexin)-producing neurons in the perifornical region.[10] It should be noted that the hypocretin system not only regulates the sleep-wake cycle, but also plays a role in metabolic and autonomic functions: hypocretin administration in rodents not only increases heart rate, but also elevates body temperature.[11,12] Whereas the diurnal core body temperature rhythm is preserved in narcolepsy—possibly with minor changes as compared with healthy subjects—no prior study has systematically examined a major determinant of this rhythm, ie, skin-temperature regulation. We investigated skin-temperature regulation in narcolepsy during the day on which a standardized Multiple Sleep Latency Test (MSLT) was conducted. The protocol allowed for an investigation of skin-temperature regulation during both upright and supine wakefulness and sleep and of the relationship between skin-temperature regulation and sleep propensity in narcoleptic patients and healthy controls.

Materials and Methods

Subjects

After obtaining informed consent, we included 15 unmedicated patients with narcolepsy (6 men). All suffered from excessive daytime sleepiness and unequivocal cataplexy and thereby fulfilled the criteria of narcolepsy with cataplexy (International Classification of Sleep Disorders).[13] Twelve patients who had never received any treatment were tested shortly after diagnosis. The remaining 3 patients discontinued their medication at least

1 week prior to the study. Fifteen age- and sex-matched unmedicated control subjects, free of any neurologic or psychiatric disease, were recruited through an advertisement in a local newspaper. All subjects were instructed to follow their normal sleep routine the night before the testing day. In all subjects, subjective sleepiness was assessed using the Epworth Sleepiness Scale.[14] Results from 1 man with narcolepsy had to be excluded because of temperature-data loss.

Study Design

Continuous skin-temperature measurements were performed during a standard MSLT[15] comprising five 20-minute periods during which subjects were asked to lie down on a bed in a quiet room and instructed to try to fall asleep after lights out. Periods started around 9:30 am, 11:00 am, 12:30 pm, 2:00 pm, and 3:30 pm. Sleep was measured using the standard polysomnographic montage, with sleep stages scored in 30-second epochs according to Rechtschaffen and Kales.[16] Sleep latency was defined as the time from lights out to the first epoch of any sleep stage, including stage 1.[17]

Skin-Temperature Measurement

Skin temperature was measured using a wireless monitoring system consisting of 9 Thermochron iButtons (type DS1921H; Maxim/Dallas Semiconductor Corp, Sunnyvale, CA, USA). The iButton is a small (16 x 6 mm) coin-like device that measures and stores temperature data between +15°C and +46°C, with an accuracyof 1°C and a 0.125°C resolution.[18] Temperature was sampled once per minute; data were transferred to a computer for analysis after completion of the recordings. iButtons were fixed to the skin with Fixomull tape (Beiersdorf, Hamburg, Germany). A weighted average proximal skin temperature (Tprox) was obtained bilaterally on the middle of the frontal aspect of the thigh and the infraclavicular areas, and on the abdomen.[19] Average distal skin temperature (Tdist) was obtained from the thenar eminence of both hands and the medial plantar aspect of both feet.[19] In addition to the distal and proximal

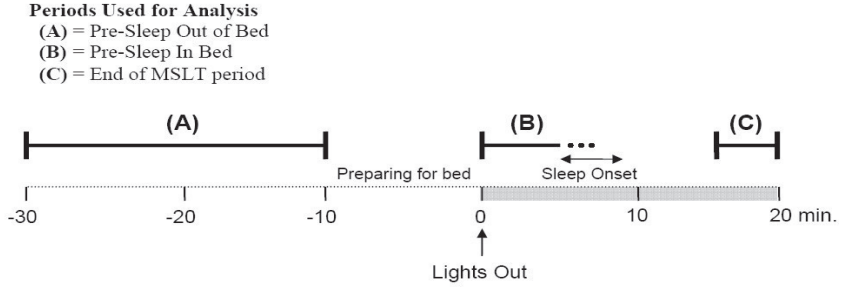

Figure 9.1 **Study Design**

This scheme shows the different conditions that were used for analysis. Temperature data (see Figure 2) were averaged for conditions A, B, and C of each of the 5 Multiple Sleep Latency Test (MSLT) tests. Condition A represents the skin temperature during the unrestrained active wakefulness state prior to the sleep-latency tests. Condition B represents skin temperature during quiet wakefulness at rest in bed, as measured from lights out until sleep onset. Condition C represents the skin temperature during the last 5 minutes of each MSLT test, where healthy subjects were also asleep during almost all their nap opportunities (88%).

skin temperatures, their gradient (distal minus proximal skin temperature, DPG) was calculated, providing an optimal estimate of distal skin blood flow.[2]

Data Analysis and Statistics

Given the skewed distribution of sleep latency, all analyses were performed on logarithmically transformed values, which effectively normalized the distribution (Kolmogorov-Smirnov test: $p = .001$ before, $p = .40$ after log-transformation). The mean Tprox, Tdist, and DPG were calculated for each of 3 different conditions (Figure 9.1): before sleep out of bed (A), before sleep in bed (B), and sleep (C). The 20 minutes running from 30 to 10 minutes prior to the start of each of the 5 MSLT periods were labeled condition A and concern the skin-temperature state during wakefulness prior to the sleep-latency tests, which may contain both upright and sitting postures. The 10 minutes directly preceding the lightsoff moment were not analyzed to preclude systemic changes associated with the preparations for bedtime. The mean Tprox, Tdist, and DPG were also obtained for the period beginning with the last measurement before lights off and ending with the first temperature sample after sleep onset (Figure 9.1). This interval, labeled condition B, represents the thermoregulatory state when lying awake in bed. Inclusion of 1 temperature sample before lights off and 1 temperature sample following sleep onset was necessary to be able to estimate the skin temperature for occasional test periods with a sleep latency of 0. When subjects did not fall asleep during a test period, sleep latency was noted as 20 minutes. Data from the last 5 minutes of the 20-minute MSLT periods, when subjects slept, were also averaged and resulted in "condition C," which therefore concerned sleep. Temperature data of conditions B and C include the onset of long-lasting changes induced by the postural change to a supine position. Differences in sleep-latency and skin-temperature variables between narcoleptic patients and control subjects were tested for the conditions A, B, and C using linear regression, including group (narcolepsy or control), time of day (1 to 5 for the 5 MSLT periods), and a group-by-time interaction, followed by posthoc analyses on individual time points in case of significant group-by-time interactions. Regression analyses were performed using the MLwiN software (Center for Multilevel Modeling, Institute of Education, London, UK), which allows the inclusion of incomplete cases. Due to technical problems, the temperature measurements prior to the first test period were incomplete in 3 cases. The within-subject influence of skin-temperature variables on sleep-onset latency was tested using linear regression analyses for the healthy control and narcolepsy groups separately. In order to prevent the detection of spurious relations due to covariance of the diurnal modulation of both sleep-latency and temperature profiles, models included time up to the second order as needed. Optimal regression models were selected using the likelihood ratio $\chi 2$ test.[20] Data and effect estimates are given as mean ± SEM. All reported p values are 2-sided, with .05 as the significance threshold.

Results

Subjects and Basic Sleep Parameters

The mean age of the included subjects was 35.9 ± 2.4 years for patients and 35.9 ± 2.5

years for controls. Epworth Sleepiness Scale results were 17.9 ± 0.7 for patients and 4.7 ± 0.8 for controls. All patients scored in the pathologic range of the Epworth Sleepiness Scale14 (> 12). As expected, the mean sleep latency was significantly shorter ($p < .0001$ for log-transformed values) in narcoleptic subjects (2.9 ± 0.6 minutes) than in controls (10.6 ± 0.8 minutes, Figure 9.2D).

Group Differences in Skin Temperature

Figure 9.2 shows the distal (a), proximal (b), and DPG (c) temperatures preceding and during the 5 MSLT test periods and the sleep latency (d). Group differences between narcoleptic and control subjects on skin-temperature values took the form of interaction effects with time of day; no differences were observed around the first MSLT, but differences grew over the day. Regression analyses confirmed strong group-by-time interactions for Tdist and DPG during wakefulness both prior to and following lights off (conditions A and B, Figure 9.1, all $p < .0001$) and during sleep (condition C, Figure 9.1, both $p < .0001$). Significant groupby- time interactions for Tprox were limited to the wakefulness condition prior to lights off (condition A, $p < .02$). Posthoc tests of individual time points indicated significantly elevated DPG in narcolepsy during wakefulness both prior to and following lights off (conditions A and B) at the third ($p = .04$ and $p = .05$, respectively), the fourth ($p = .03$ and $p = .02$, respectively), and the fifth ($p = .001$ and $p = .002$, respectively) test period. During condition C (when healthy subjects were also asleep in 88% of their nap opportunities), DPG differed at all but the first MSLT (all $p < .03$). Posthoc tests indicated that the elevated Tdist during condition B (wakefulness in bed) in narcolepsy reached significance only for the fifth MSLT period ($p = .01$). During condition C, the Tdist differed at all but the first MSLT (all $p < .03$). Posthoc tests indicated that the lowered Tprox during condition A in narcolepsy reached significance only for the fourth test period ($p = .04$) and completely normalized during condition C (all $p > .73$).

Within-Subject Relation of Skin Temperature With Sleep Latency

In the patient group, DPG prior to lights off (condition A, Figure 9.1) showed no relation to subsequent sleep-onset latency. However, under the more-controlled conditions (a supine posture and no physical activity) between lights off and sleep onset (condition B, Figure 9.1), significance was reached, indicating a 12% ± 6% shorter (log-transformed) sleep-onset latency per degree increase in DPG. The association between skin temperature and sleep latency was even stronger for proximal and distal skin temperature per se than for their difference. Every degree higher proximal skin temperature prior to lights off was associated with a 22% ± 10% ($p = .01$) shorter (log-transformed) sleep-onset latency. Similarly, every degree higher distal skin temperature prior to lights off was associated with an 11% ± 5% ($p = .007$) shorter (log-transformed) sleep-onset latency. The association between distal skin temperature and sleep-onset latency remained when measured between lights off and sleep onset: a 13% ± 5% ($p = .003$) shorter logtransformed sleep-onset latency per degree higher distal skin temperature. Within control subjects, an association between sleep-onset latency and skin temperature could only be derived from the proximal skin temperature before lights off: sleep-onset latency (logtransformed) was 11% ± 4% shorter per degree higher proximal skin temperature ($p = .0008$).

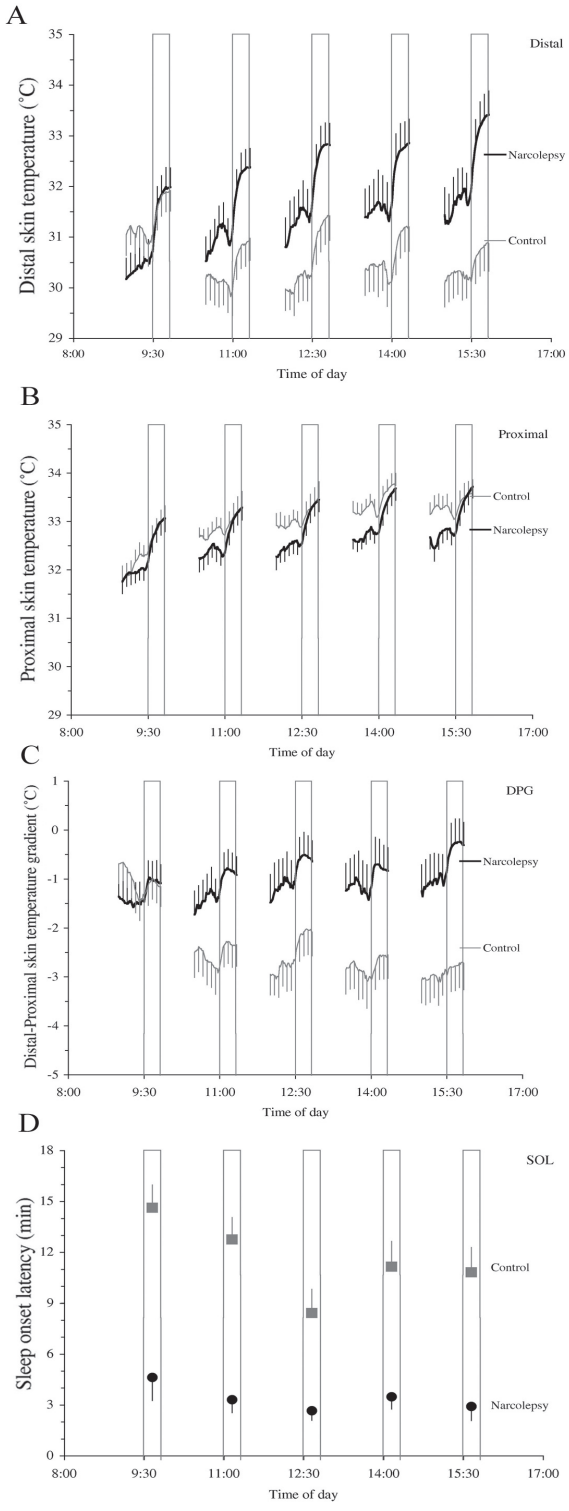

Figure 9.2

Results

Mean ± SEM profiles of (a) distal and (b) proximal skin temperature, (c) distal-to-proximal skin temperature gradient (DPG), and (d) sleep latency over the day in narcoleptic patients (black) and controls (grey). The 5 vertical bars indicate the successive 20-minute, lights-out, in-bed periods. Due to technical problems, the temperature measurements prior to the first test period were incomplete in 3 cases. The data for these cases were extrapolated in the figure but not in the data analysis. Each point represents 1 minute of temperature data. For readability, error bars are shown only at 5-minute intervals.

DISCUSSION

The most important and novel finding of the present study is that, throughout the day, distal skin temperature was elevated in narcoleptics. In healthy subjects, the occurrence of such prolonged high distal temperatures is normally restricted to 3 circumstances: the first concerns the night, in association with the circadian phase of lowered core body temperature. The second concerns prolonged heat stress during the day, and the third concerns a continuous supine posture.[6] Distal skin temperature increased over the day in narcoleptic patients, resulting in a higher DPG during both wakefulness and sleep. Even during wakefulness, narcoleptic patients maintaineda DPG that control subjects did not even reach asleep in a supine posture. Once asleep, the distal skin temperature and DPG of narcoleptic patients remained higher than that of controls, whereas their lowered proximal skin temperature found before bedtimenormalized. The second major finding of the present study was that the higher DPG during wakefulness was associated with increased sleep propensity in narcolepsy, the same as was demonstrated in healthy controls under strictly controlled laboratory conditions.[3] This relationship is therefore preserved in narcolepsy and may even be enhanced, since the relationship was stronger in the narcolepsy than in the control group. The elevated gradient between distal and proximal skin temperature that narcoleptic patients maintain might be interpreted as a "sleep-promoting" pattern of skin temperature.[3] In our control group, DPG did not predict sleep latency, in contrast to published studies.[3] We explain this through less-strictly controlled experimental conditions. However, this makes it even more remarkable that such an effect was found in the narcolepsy group even under conditions of considerable variance. A third important observation is that, even though DPG was associated with sleep propensity in narcoleptic subjects, distal and especially proximal skin temperature per se were even more strongly related to sleep latency. In controls, proximal skin temperature was even the only significant predictor of sleep-onset latency. This robust influence of proximal skin temperature on sleep propensity is in line with the recent demonstration that sleep latency is dramatically reduced when proximal skin temperature is subtly increased using a "thermosuit."[19]

Skin Versus Core Temperature

In contrast with our skin-temperature measurements, previous studies in narcoleptics focused on core body temperature. Some researchers have found no significant differences in the mean or circadian pattern of core body temperature in narcolepsy.[21-23] Pollak and Wagner reported that naps in narcoleptic patients were heralded by a small decrease in core temperature,[23] which is compatible with the present demonstration of increased distal skin blood flow, which can accommodate heat loss. Other researchers found core body temperature in narcoleptics to be elevated at nighttime.[24,25] Also, narcoleptics were reported to show an attenuated circadian amplitude and slower morning rise of the core body temperature, as well as an earlier temperature minimum,[24,25] which both fit the present finding of a continuation into sleep of the increased distal skin blood flow and consequently heat loss. Core body temperature was not measured within this study so as not to affect MSLT procedures and interpretation. However, this study focused on the difference between distal and proximal skin temperatures that are largely irrespective of the actual core body temperature.[26]

Temperature and the Nature of Sleepiness

The excessive daytime sleepiness in narcolepsy may take different forms: patients may complain of a continuous feeling of sleepiness, or sleepiness may manifest itself as a sudden falling asleep.[27] This clinical heterogeneity is reflected in 2 theoretical frameworks that have been devised to describe the pathophysiologic mechanisms for excessive daytime sleepiness in narcolepsy. The frequent sleep attacks are nicely described by a so-called loss of "state-boundary-control": the shifts between the waking and sleeping states occur too easily.[28] This theory is supported by the fact that, over the 24 hours of a day, narcoleptics do not have an increased total amount of sleep.[29] Furthermore, newly developed models of sleep-wake control propose the existence of a "sleep switch" that needs to be stabilized by the hypocretin system.[30] Hypocretin deficiency therefore destabilizes this switch, resulting in frequent state shifts in narcolepsy. In addition to this switching problem, however, narcoleptics may also have a continuously depressed level of wakefulness. Earlier authors have emphasized this continuously increased sleep propensity, or sleep "pressure."[27] If skin temperature may be taken as an indirect indicator of sleep pressure, then the fact that narcoleptics have an elevated DPG throughout the day suggests that their sleep drive is continuously increased or their waking drive decreased. This would explain why hypocretin deficiency leads not only to a loss of state-boundary-control but also to a constant state of sleepiness as well.

Within our study, no polysomnography was performed during the night prior to the MSLT. We have thus not been able to establish possible group differences in homeostatic sleep pressure, which may be presumed but are unlikely to fully account for our results. However, a number of previous findings do not favor the simple explanation of the altered skin-temperature distribution merely reflecting an increased homeostatic sleep pressure. Such an interpretation would not account for our observation that the DPG of narcoleptic subjects also stayed significantly higher than the DPG of healthy subjects when both groups were asleep. Furthermore, in healthy subjects at rest in a thermoneutral condition, sleep deprivation does not affect distal skin blood flow.[31] Recent controlled experiments have demonstrated that skin temperature shows a circadian modulation over the day but is unaffected by the homeostatic buildup of sleep pressure associated with sleep deprivation.[32] Thus, whereas sleep propensity is known to be regulated by both a circadian and a homeostatic component, the thermal state of the skin is normally linked to the circadian component, and a link to the homeostatic component appears less likely. Distal temperature does not change if prolonged wakefulness generates a homeostatic buildup of sleep pressure but is regulated to be low during the day and high at night. This circadian modulation most likely underlies the high distal skin temperature seen around the first MSLT in healthy controls, possibly combined with a need to reduce any heat load induced by traveling to the hospital, since the early-morning threshold for cutaneous vasodilation is already reached with a mild heat load.[4] The relatively high distal skin temperature decreased after the first MSLT session in healthy controls, whereas it continued to increase throughout the day in narcoleptic patients.

Relation to Hypocretin Deficiency

The altered DPG in narcolepsy is indicative of chronically decreased sympathetic distal vasoconstrictor tone in narcolepsy, which may ultimately be attributed to a hypocretin deficiency. In rats, hypocretin-labeled fibers have been demonstrated in the primary areas involved in the regulation of tail vasoconstriction, including the preoptic and lateral hypothalamic areas and the periaqueductal gray,[33-35] which show moderate to high levels of hypocretin-immunoreactive axons.[36] Hypocretin deficiency may result in lower sympathetic tone and reduced vasoconstriction, as has been shown in hypocretin-knockout mice.[37] However, the possible mechanisms involved need further study, especially since intracerebroventricular injection of hypocretin in rats did not significantly affect tail skin temperature in one study[38] and even increased distal skin (tail) temperature in another.[39] Regardless of the cause of the altered skin-temperature distribution in narcolepsy, the possible consequences of the altered distribution warrant further research. Firstly, thermoregulatory processes may be investigated in narcolepsy, such as the vasoconstrictor responses to cold stress or to standing. Secondly, further studies, now underway, may address whether manipulation of skin temperature affects sleep propensity in narcoleptic patients, as in healthy subjects.[19] If so, skin-temperature manipulation may ultimately be applied therapeutically for alleviation of narcoleptic symptoms.

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Evaluation of Wireless Determination of Skin Temperature using iButtons

CHAPTER 9A

Based On: *van Marken Lichtenbelt WD, Daanen HA, Wouters L, Fronczek R, Raymann RJ, Severens NM, Van Someren EJ. Physiol Behav. 2006;88:489-97*

Evaluation of Wireless Determination of Skin Temperature using iButtons

Measurements of skin temperatures are often complicated because of the use of wired sensors. This is so in field studies, but also holds for many laboratory conditions. This article describes a wireless temperature system for human skin temperature measurements, i.e. the Thermochron iButton DS1291H. The study deals with validation of the iButton and its application on the human skin, and describes clinical and field measurements.

The validation study shows that iButtons have a mean accuracy of – 0.09 °C (– 0.4 °C at most) with a precision of 0.05 °C (0.09 °C at most). These properties can be improved by using calibration.

Due to the size of the device the response time is longer than that of conventional sensors, with a τ in water of 19 s. On the human skin under transient conditions the response time is significantly longer, revealing momentary deviations with a magnitude of 1 °C.

The use of iButtons has been described in studies on circadian rhythms, sleep and cardiac surgery. With respect to circadian rhythm and sleep research, skin temperature assessment by iButtons is of significant value in laboratory, clinical and home situations. We demonstrate that differences in laboratory and field measurements add to our understanding of thermophysiology under natural living conditions. The advantage of iButtons in surgery research is that they are easy to sterilize and wireless so that they do not hinder the surgical procedure.

In conclusion, the application of iButtons is advantageous for measuring skin temperatures in those situations in which wired instruments are unpractical and fast responses are not required.

Introduction

Since the human skin forms the interface between the human body and the thermal environment, skin temperature is essential to quantify heat transfer. The temperature distribution over the body's surface provides useful information for many research and clinical applications. For skin temperature measurements the following thermally sensitive methods of measurement are generally applied: thermocouples, thermistors, and infrared sensors.

Thermistors and thermocouples are relatively inexpensive, but these systems have the disadvantage of wires that connect the skin sensor to a measuring device or a data logger that is worn on the body. These wires take time to apply, are prone to breaking, and may hinder the subjects. Some systems use temperature transmitters, in which case the receiver still needs to be worn on the body.

Here, a wireless temperature system for human skin temperature measurements is described, i.e. the iButton (type DS1921H; Maxim/Dallas Semiconductor Corp., USA; Figure 9a.1). The iButton is a small (16×6 mm^2), rugged self-sufficient system that measures temperature and records the results in a protected memory section. Afterwards time and temperature data can be transferred to a computer for data analysis. Although thermochron iButtons have been used in animal research,[1] there are no reports on the extended validation of the device and its application in humans.

The small size and absence of wiring indicate that the iButton may be particularly valuable with regard to the long-term and ambulatory monitoring required for human research on metabolism, sleep and circadian rhythms, nutrition, and sports physiology. Our understanding of human thermal physiology may benefit from the observations during ambulatory monitoring in everyday life. Moreover, wireless skin temperature monitoring facilitates the long-term monitoring of an important part of autonomic regulation in subjects that do not comply well with extensive wiring, e.g. children or subjects with neurodegenerative disorders, such as Alzheimer's dementia or Parkinson's disease.

The value of skin temperature assessment in sleep research is that the endogenous skin warming resulting from increased skin blood flow is functionally linked to sleep propensity. In fact, the gradient between the temperature of the distal and proximal skin may be one of the best physiological correlates of sleep propensity.[2] In metabolic studies core–skin temperature gradients measured with wired thermistors have shown significant correlates with (changes in) metabolism.[3,4] Using the iButton combined with core temperature telemetry, these studies can be extended to more natural living conditions.

Thus, the device appears to be a convenient alternative for the conventional thermistors and thermocouples. However, it is unknown whether the measurement accuracy and the response time are appropriate and how they function on the human skin compared to the generally accepted methods.

iButtons are currently being used in animal studies, as we know from symposium presentations.[5] Studies published in journals are rare. To our knowledge, only a few studies mention the use of iButtons.[1,6-8]

The aim of this study is to validate the iButton, determine its time response under both physical and physiological conditions, and describe some field and clinical applications.

Figure 9a.1
An iButton

The article has three sections. The first deals with validation studies, encompassing accuracy measurements, determination of response time, and determination of the spatial sensitivity, i.e. which side of the button is the sensitive part. The second section describes the application on the human skin, comparing the iButton with conventional thermocouples. Finally, the use of iButtons in clinical and field studies is discussed.

Description of the iButton and its key features

The Thermochron iButton® DS1291H (Dallas Maxim) has a semiconductor temperature sensor, a computer chip with a real time clock and memory, and a 3 V Lithium battery enclosed in a 16 × 6 mm^2 stainless steel can. The sensor is located towards the top of the iButton and the battery towards the bottom. Manufacturing specifications are: a temperature range between + 15 and + 46 °C, an accuracy of 1 °C with a precision of 0.125 °C, provided an eight-bit AD converter is used. The output value given by the iButton is the instantaneous value at that particular moment. During the measurements the information is stored (NV RAM). It can afterwards be transferred to a personal computer, at a maximum rate of 142 kbps. For this purpose, the iButton should be clipped into an adapter connected to the computer.

The iButton has been designed for the human temperature range. The recording is done at a user-defined rate. Up to 2048 temperature values taken at equidistant intervals ranging from 1 to 255 min can be stored. With 1-min intervals, this results in a maximum measurement period of 34 h.

Validation studies I - iButton properties: Accuracy of the iButton

The accuracy and precision of the iButton were determined using a water bath and a reference thermometer. Secondly, it was investigated whether a correction formula based on an independent calibration would result in a higher accuracy.

Materials and methods

Two experiments were conducted with 30 iButtons. The sampling rate was set at 1-min intervals. The iButtons were loosely placed in a thermostat water bath (MGW Lauda K6, Westbury, NY), in a net with small meshes, together with the tip of a calibrated certified thermometer (Testo 950 with sensor 06280016, accuracy 0.1 ± 0.05 °C, Testo, Almere, The Netherlands). There was ample space for the water to flow along

the iButtons and the calibration thermometer. The water bath was kept at a constant temperature during plateau measurements, which lasted at least 15 min. Only stable (plateau) temperatures were included in the analysis. Twelve plateau values were used ranging from 18 to 41 °C. The final five 1-min interval measurements of each plateau were averaged and used for statistical analysis. The water bath temperature variation during these measurements was always less than 0.05 °C.

Statistical analysis

In order to determine the temperature deviation of the individual iButtons, the linear regressions between the values of the standard thermometer and each iButton were calculated. For each iButton the bias (or accuracy) was calculated as the mean of the differences between the temperature of the iButton and that of the standard thermometer, and their standard deviation revealed the error (or precision).[9] In order to compare the uncorrected and corrected results, the bias of the absolute differences was calculated.

With the same iButtons, linear regression was applied to an independent data set in order to calculate individual correction formulas. The improvement due to the correction formulas was tested with a two-tailed paired T-test using the biases of the absolute values.

Results and discussion

The linear regressions between the iButtons and the calibrated thermometer were highly significant with Pearson correlation coefficients larger than 0.99. The mean bias amounted to – 0.09 ± 0.22 °C (range = – 0.40–0.40 °C). The bias of absolute values averaged 0.21 ± 0.12 °C (range = 0.05–0.40 °C). The error (or precision) averaged 0.05 ± 0.02 °C (range = 0.03–0.09 °C). These results show that if calibration is required, this should be carried out for each iButton individually.

An independent calibration was performed to determine a correction formula, based on linear regression, for each iButton. The slope calibration coefficients averaged 1.004 ± 0.004 °C (range = 0.992–1.01 °C), and the intercept calibration coefficients 0.02 ± 0.28 °C (range = – 0.65–0.59 °C). With a correction based on the individual calibration formulas the Pearson correlation coefficients all exceeded 0.999. The corrected bias averaged 0.05 ± 0.06 °C (range = 0.01–0.26 °C), and the corrected bias of the absolute values 0.06 ± 0.06 °C (range = 0.02–0.26 °C). The mean error was 0.03 ± 0.01 °C (range = 0.01–0.05 °C). The bias was significantly smaller after correction ($p < 0.001$, paired T-test on absolute values). The upper values of the bias were observed with two iButtons only. Excluding these iButtons, the remaining 28 revealed a maximum absolute bias of 0.07 °C.

It should be emphasized that after calibration, the bias and error of the iButtons become similar to the accuracy of the reference thermometer itself, i.e. satisfactory for precise measurement. Secondly, since the readout of the iButton is digitized with a resolution of 0.125 °C, improvement after calibration is relevant only when many observations are taken.

Validation studies I - iButton properties: Response time

The response time of the iButton is determined by calculating the time constant (τ) by heating in water. Because the heat capacity and conductivity of water are somewhat higher than those of the human skin and most of the iButton surface is in contact with tape and air, experiments on the human skin have been performed as well (see below). Even though the sampling rate is low (one per minute), model fitting enables determination of the time constant because the output value given by the iButton is the instantaneous value at that moment.

Materials and methods

The experimental setup consisted of two water vessels of polystyrene foam filled with water at different temperatures. The water temperature in the vessels was measured with a calibrated certified thermometer (Testo 901). Eight iButtons were loosely placed in an open test tube holder. In this way there was sufficient space between the iButtons so that they were completely surrounded by water. The iButtons were first put in the vessel with T_{water} = 18.7 °C and subsequently, immediately after the instantaneous measurement, put in another water vessel with T_{water} = 41.3 °C. The sample rate was set to one sample per minute.

Statistical analysis

The response time of the iButtons in the experiment was estimated by the following equation:

$$T(t) = T_\infty + (T_0 - T_\infty)e^{-\frac{t}{\tau}} \qquad (1)$$

with t the time in s, T the temperature, and the subscripts ∞ and 0 denoting the values at infinity and at the start. At $t = \tau$ the process was completed for 63.2%. Eq. (1) was fitted for each individual iButton temperature curve by non-linear least square fitting (MATLAB 6.5).

Results and discussion

In Figure 9a.2 the temperatures as measured with eight iButtons are shown during the switch from cold to warm water at t = 2 min. The plotted marks in the figure are the instantaneous temperatures at that time. Fitting Eq. (1) for each individual temperature curve led to T_∞ = 41.1 ± 0.3 °C, T_0 = 18.7 ± 0.3 °C, and τ as fitting parameter to a value of τ = 0.31 ± 0.06 min, i.e. or τ 19 s.

Validation studies I - iButton properties: Spatial sensitivity

The iButton has a top side with smooth edges and a bottom side with a protruding edge. Since the manufacturer does not provide information about which side can best be placed on the surface of interest, an experiment was performed in which the time responses of both sides were compared.

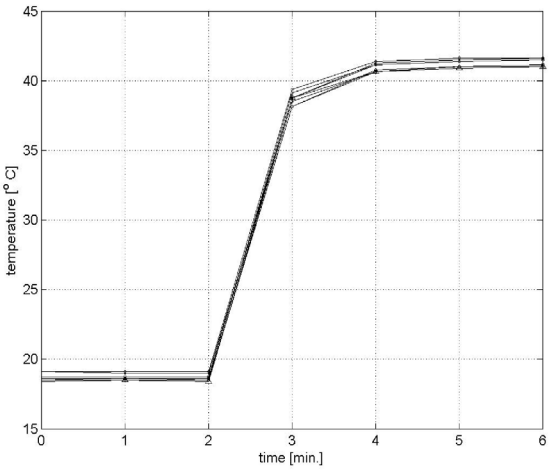

Figure 9a.2
Response Time
Temperature of eight iButtons in equilibrium with water at a temperature of 18.7 °C and after a sudden shift to 41.3 °C at t=1 min.

Materials and methods
Twelve iButtons were placed on a table to obtain the same starting temperature. Sampling rate was set at 1-min intervals. After 10 min six iButtons were placed top up and six iButtons top down on a hot plate (56.6 °C). 5 min later an ice cube big enough to cover all the iButtons was placed on top of them, thereby cooling all iButtons on one side.

Statistical analysis
The statistical difference between the temperature of the two sets of iButtons during warming and cooling was tested by means of analysis of variance.[10]

Results and discussion
The ambient temperature, measured during the first 10 min of the experiment, was 23.2 ± 0.3 °C. There was a significantly different response of the two sets of iButtons to the gradual temperature increase of the hot plate and the subsequent instant cold exposure. Upon contact with the hot plate, the iButtons with the top side facing the object were significantly warmer for the first 4 min than those with the bottom side against the object (Figure 9a.3). During exposure to the cold object the 'top-side' iButtons were significantly cooler during a 5-min period. We conclude that the top side has a faster response to thermal changes when placed on hard and flat surfaces. The difference between the bottom and top side thermal response may in part have been caused by the locations of the battery (towards the bottom) and the sensor (towards the top).

Validation studies II - Application on human skin: Response time

The response time in water has been described above. Since the heat capacity and conductivity of water are higher than those of the human skin and only part of the

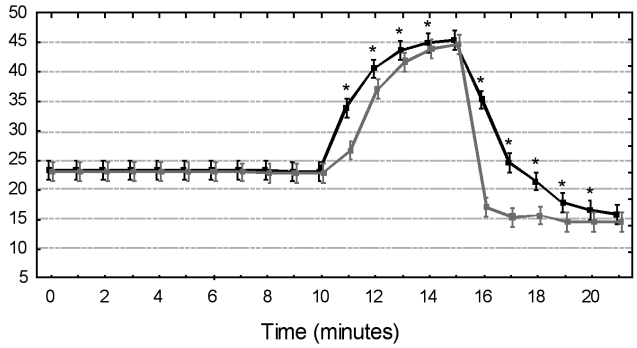

Figure 9a.3 | **Spatial Sensitivity**

Mean temperature of 12 iButtons in two experimental conditions. The upper line represents the iButtons that were in contact with the bottom of the bowl with their top side and in contact with the ice with their bottom side. The lower line represents the iButtons of the opposite condition. At 10 and 15 min the heat and a cold source were added respectively. *p<0.05. The data points were slightly shifted in time to prevent overlap.

iButton is in direct contact with the skin, it was necessary to validate the iButton for human applications. Therefore, measurements on the human skin were performed. Because local differences can be substantial, the data of mean skin temperature are presented averaged over subjects and 14 body positions and not those of the individual iButton measurements.

Materials and methods

Six subjects (three males and three females, mean age: 22.7±1.5 years, mean weight: 67.0±4.3 kg) received information about the nature of the study and signed an informed consent before participating.The temperature obtained by the iButtons (sampling rate one per minute) was compared to that of calibrated thermocouples (ULTRAKUST Electronic GmbH, Germany). According to ISO-standard 9886, skin temperature was measured at four and 14 predefined places by thermocouples and iButtons.[11] ISO 9886 specifies 14 equally weighed body locations to estimate mean skin temperature, but also allows to use only 4 of these sites (neck, scapula, hand and shin) with weighing factors of respectively 0.28, 0.28, 0.16 and 0.28. The devices were fixed onto the skin with adhesive tape (Leukoplast, BSN medical GmbH and Co. KG, Hamburg, Germany). The thermocouples were sampled four times per minute.

Measurements took place in two environmental chambers, one at an ambient temperature of 34.9 ± 0.1 °C with a relative humidity of 8.6 ± 0.5% and one at a temperature of 15.5 ± 0.3 °C and 64.0 ± 2.3% relative humidity. 10 min of exercise were performed before entering the cold chamber at 1.5 W/kg body weight on a Lode Excalibur cycle ergometer (Lode, Groningen, The Netherlands).

Statistical analysis

The statistical difference between the temperature of the iButtons and that of the thermocouples was tested by means of ANOVA, Tukey's post hoc test.[10]

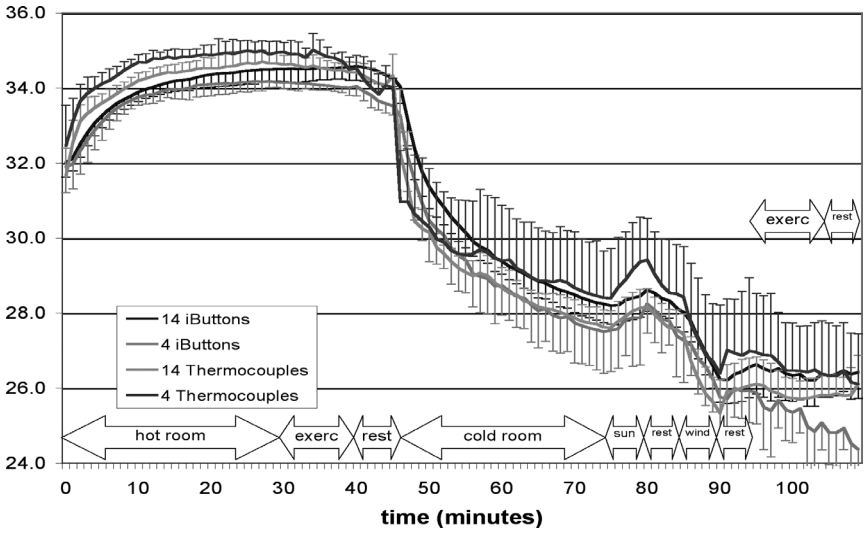

Figure 9a.4 | **Human Skin: Response Time**

Temperature plot of the thermocouples (dotted) and iButtons (solid) during the experiment. Vertical bars show the standard deviation. Temperatures between buttons and thermocouples were significantly different (pb0.05), with the exception of temperatures from 15 to 30 min.

Results and discussion

During heating in the hot chamber the iButtons showed significantly lower temperatures from t = 1 min until t = 14 min ($p < 0.05$, Figure 9a.4). After 15 min the differences were not significant anymore. In the cold chamber the temperatures of the iButtons were significantly lower during the whole 30-min interval. The mean temperature difference between the iButtons and thermocouples during both conditions (mean value of all subjects) in the hot chamber was – 0.24 ± 0.15 °C (last 10 min: 0.17 ± 0.03 °C), and in the cold chamber 0.88 ± 0.61 °C (last 10 min: 0.61 ± 0.03 °C). Thus, the iButtons appear to react slower than thermocouples. The differences were clearly smaller in the hot environment compared to the cold situation. Overall, the mean skin temperature determined using the 14 iButtons differed 0.3 ± 0.65 °C from the temperature determined using the 14 thermocouples. The mean skin temperature estimated using four versus 14 thermocouples according to ISO 9886 revealed a difference of 0.4 °C. This indicates that the difference between iButtons and thermocouples is in the same range as the difference between using four or 14 thermocouples to assess skin temperature.

The temperature yielded by the thermocouples was closer to ambient temperature than the iButton temperature (Figure 9a.4). The difference between iButtons and thermocouples was most pronounced in the cold chamber (0.6 °C), where the gradient between ambient temperature and skin temperature was much higher (about 15 °C) as compared to the hot chamber (about 2 °C). The effects of the thermal inertia of the iButtons on the temperature response are expected to be visible most clearly when the skin–environment temperature gradient is relatively high. At the end of the experiment the temperatures of the iButtons and thermocouples tended to converge.

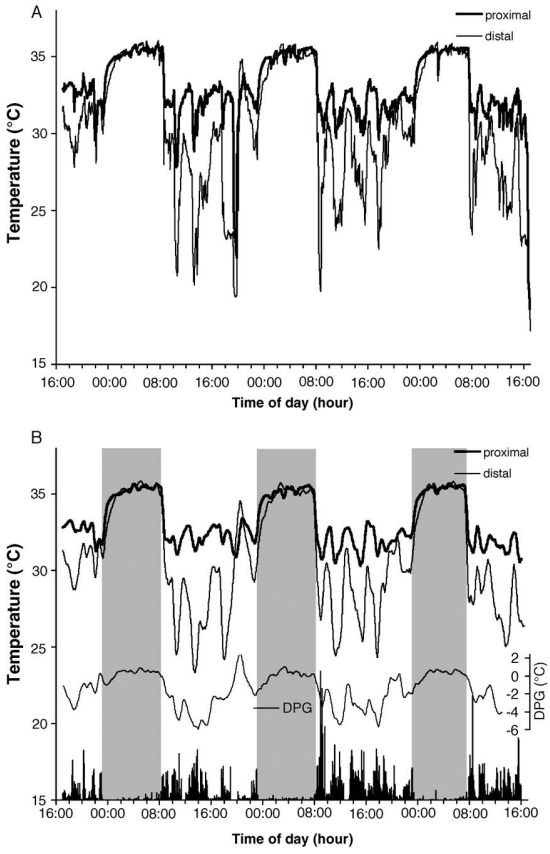

Figure 9a.5
Sleep and Circadian Rhythm Research
(A) Mean proximal (thick line) and distal temperature (thin line) during 3 days under natural living conditions in a single case. (B) The same data after outlier removal as discussed in the text. This panel shows the time spent in bed (grey area) and also the activity level (black columns, arbitrary units from simultaneous actigraphic recording). Moreover, the distal to proximal skin temperature gradient (DPG) is shown on a separate axis. Note that the marked and simultaneous nocturnal elevation of both proximal and distal temperature hardly ever occurs during wakefulness, indicative of a different thermoregulatory state during the sleep period. Note furthermore the negative correlation between activity level and skin temperatures, which is quite the reverse of the documented positive association of activity level and core body temperature.

Thus, the temperature differences between the thermocouples and iButtons are larger soon after ambient condition changes. The thermal inertia delays the response, and errors up to 1 °C may occur under realistic circumstances.

Application in field and clinical studies : Sleep and circadian rhythm research

The diurnal rhythm in core body temperature is one of the most commonly used markers for the phase and amplitude of the endogenous circadian timing system. The 24-h rhythm in core body temperature is the result of differential 24-h rhythms in heat production and heat loss. It has been demonstrated that under strictly controlled so-called 'constant routine' conditions, in which subjects remain awake in a fixed semi-supine condition without physical activity and with food and drinks taken in small portions throughout the day and night, the core temperature rhythm remains. Furthermore, the core temperature rhythm has been shown to result mostly from the circadian rhythm in heat loss, and to a lesser extent from changes in heat production.[12] Dry heat loss is caused by increased skin blood flow, allowing the dissipation of heat

from the warm blood to the cooler environment. Although the iButton is not suitable for core body temperature assessment in humans, the assessment of temperature at multiple sites of the skin provides a reliable estimate of heat loss,[12,13] suggesting a role for the iButton in long-term studies on the circadian variation in skin temperature and heat-loss which is responsible for an important part of the circadian variation in core body temperature.

The value of skin temperature assessment extends to sleep research, because endogenous skin warming, which results from increased skin blood flow, is functionally linked to sleep propensity. In addition, exogenous skin warming in the thermoneutral, comfortable range has a strong impact on sleep propensity.[7,14,15]

Therefore, the feasibility of using multiple iButtons in skin temperature studies on circadian rhythms and sleep performed outside the laboratory was investigated. The aim of the first study was to obtain diurnal rhythms in proximal and distal skin temperature under natural living conditions. The aim of the second study was to obtain proximal and distal skin temperature during standard multiple sleep latency tests (MSLTs) in a clinical setting.

Materials and methods

For both studies, the bottom side of nine iButtons was fixed onto the skin in both proximal and distal areas using Fixomul tape (Beiersdorf, Hamburg, Germany). These locations were used because for long-term recordings the slightly larger area of the flat side gives a better fixation when taped onto the skin. At the time of recording, it had not yet been evaluated that the top side had a faster response time. Apart from the iButton, the subjects wore an Actiwatch (Cambridge Neurotechnology Ltd., Cambridge, UK) on the non-dominant wrist for a continuous recording of physical activity.

Proximal skin temperature was measured at five places, i.e. the left and right mid-thigh on the musculus rectus femoris, the right and left infraclavicular area, and the abdomen (1 cm above the navel). Distal skin temperature was measured at four points: the thenar area at the palmar sites of the left and right hand, and the medial metatarsal area at the plantar sites of the left and right foot. Average distal skin temperature (T_{dist}) was calculated as the average of the average temperature of both feet and the average temperature of both hands. A weighted average was calculated for proximal skin temperature (Tprox = 0.383 average (mid-thighs) + 0.293 average (infraclavicular sites) + 0.324 abdomen) according to a modification of the method used by Kräuchi et al.[16] The first study was aimed at obtaining three full 24-h cycles. Consequently, a sample rate of once every 3 min was selected. This allowed for the assessment of four full 24-h cycles, whereas a sample rate of once every 2 min just fell short of sampling three full cycles. The second study was aimed at obtaining a single day and was performed with a sample rate of once every minute.

In contrast to most laboratory assessments, it seldom happens that field assessments are free of artefacts. Therefore an automated artefact rejection procedure was applied to exclude extreme drops and rises in temperature. Because of the bimodal rather than

normal distribution of the temperature data, no artefact rejection was applied to exclude data more than two or three standard deviations from the mean, but rather a three-step nonparametric method. Firstly, the rate of change (ROC) of all subsequent single channel data points was calculated, their quartiles Q_{25} and Q_{75}, and their interquartile distance (IQR_{ROC}). Any data point with a rate of change exceeding 1 time the interquartile distance from Q_{25} or Q_{75} was removed. This step resulted in the rejection of very fast drops or increases in temperature. Secondly, in the resulting restricted raw data, the quartiles Q_{25} and Q_{75} and their interquartile distance (IQR_{LEVEL}) were calculated. Any data point with a level exceeding 1 time the interquartile distance from Q_{25} or Q_{75} was removed. This step resulted in the rejection of very low temperatures. Thirdly, the resulting gaps in the single channels were interpolated linearly followed by an 11-point rectangular smoothing.

Results and discussion

Two single cases are discussed here to illustrate the feasibility and usefulness of multiple-site iButton skin temperature measurements in field studies on circadian rhythms and sleep. Figure 9a.5A shows three 24-h cycles of average proximal and distal skin temperature in an unmedicated male subject, aged 59, with subjective sleep complaints under natural living conditions. Although the raw data already showed a clear 24-h rhythm, some of the daytime variation may have been due to artefacts caused by poor skin contact as a result of loose tape or, e.g., dishwashing. As shown in Figure 9a.5B, the three-step artefact rejection procedure resulted in far fewer noisy distal and proximal curves, whereas the variability was maintained.

During daytime, the distal skin temperature (thin line) hardly ever exceeded proximal skin temperature. The diverging decrease in proximal skin temperature and the increase in distal skin temperature reported under laboratory conditions did not occur systematically under habitual living circumstances,[17] underscoring the value of unrestrained iButton recordings for our insight into the physiology of diurnal temperature variation. Under constant routine laboratory conditions, activity levels

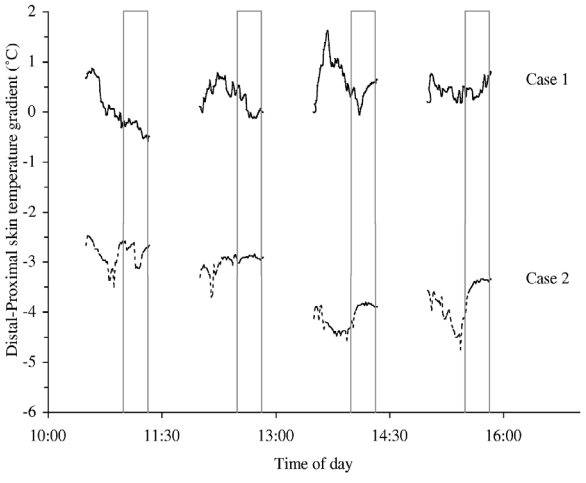

Figure 9a.6

Distal-to-Proximal Gradient
Distal minus proximal skin temperatures in two cases evaluated in a multiple sleep latency protocol. See text for details.

were minimal. Under the present unrestricted conditions, clear correlations between skin temperature and previous activity levels were observed. The correlation between activity level and distal skin temperature was strongest at a lag of 9 min (r = – 0.52 for all data, – 0.32 for out-of-bed data only). The correlation between activity level and proximal skin temperature was strongest at a lag of 12 min (r = – 0.56 for all data, – 0.50 for out-of-bed data only). The correlation between activity level and core temperature was less pronounced, but strongest at a lag of 30 min (r = + 0.11 for all data, + 0.08 for out-of-bed data only). Thus, in addition to the well-known positive correlation between activity and core body temperature, we were able to demonstrate that there is a negative correlation between activity level and skin temperature in everyday life.

During nighttime, both distal and proximal temperature reached a level not seen during wakefulness. They also showed very little variability due to the fixed supine position, lack of activity and a bed microclimate of about 34 °C.18 Thus, the diurnal rhythms of proximal and distal skin temperature under natural conditions were in phase, with the optimal levels of both occurring during the night. Note that this finding is in contrast with constant routine studies in which subjects were not allowed to sleep and were exposed to an environmental temperature much lower than that of the habitual nocturnal microclimate. Under such laboratory conditions, the distal skin temperature increased likewise, but the proximal skin temperature decreased instead of increasing during the night.12 In lab studies in which sleep was allowed, the nocturnal increases in distal and proximal temperature were still not as pronounced throughout the night as those of the present recordings under natural living conditions.17 It may be that subjects sleeping under a light cover at 22 °C laboratory temperature may not attain the microclimate of about 34 °C that is usually found with normal bedding. This example once more underscores the importance of ambulatory recordings in addition to laboratory studies to provide complete insight into everyday physiological processes. The second case illustrates the application of iButtons in clinical sleep investigation. Because under controlled conditions the gradient of distal minus proximal skin temperature (DPG) has been shown to be one of the best physiological predictors of sleep propensity, [2] it might be relevant to include skin temperature measurements in, e.g., the multiple sleep latency test (MSLT). The MSLT is widely used in sleep clinics to evaluate whether daytime sleepiness is associated with pathological conditions such as sleep apnea and narcolepsy. An MSLT consists of four or five 20-min periods at fixed time intervals during which the subject lies in bed in a darkened room, while trying to fall asleep. Polysomnography is used to determine sleep onset latency. Figure 9a.6 shows the DPG (proximal and distal assessed from multiple sites as described above) in two healthy male subjects aged 31 (case 1) and 34 (case 2) as obtained during an MSLT. The DPG is shown from 30 min prior to lights out until the end of the 20-min lights out period. Note that the DPG of Case 1 is near zero, indicative of distal temperatures that are nearly similar to the proximal temperatures in this case. The DPG of Case 2 is negative, indicative of distal temperatures 3 to 4 °C lower than the proximal temperatures. The average sleep onset latency of Case 1 was 4.9 ± 1.3 min (mean ± S.E.M.) versus 8.4 ± 1.2 min in Case 2. This example matches the earlier laboratory findings of faster sleep onsets associated with higher DPG, [2] and suggests

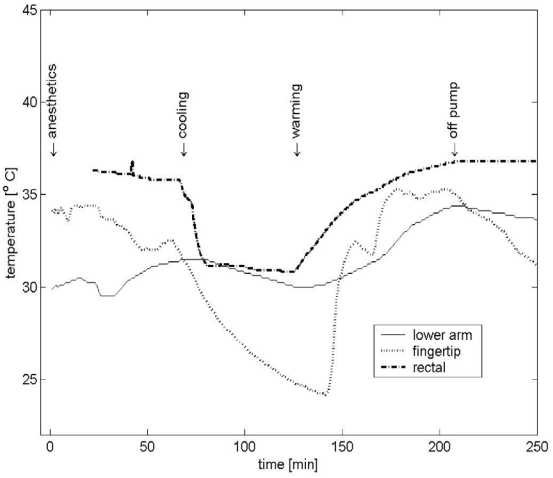

Figure 9a.7

Cardiac Surgery

The course of skin temperature (lower arm and finger tip) and core temperature (nasopharynx) during cardiac surgery and rewarming. For explanation see text.

that skin temperature assessment is of value not only in the laboratory but also within the setting of clinical sleep evaluations.

Whereas the first study example indicates considerable differences between laboratory and field studies in skin temperature physiology, the second example suggests generalizability of laboratory findings to clinical settings, underscoring the value of extending laboratory studies to field studies which have become much more feasible using the present method of wireless monitoring.

Application in field and clinical studies: Hypothermia during cardiac surgery

For over four decades, the whole body hypothermia has been used widely to reduce metabolic demand and protect vital organs during open heart surgery. During cardiopulmonary bypass surgery, cooling can be achieved using the heart lung machine (HLM). Afterwards the body is rewarmed followed by decoupling of the HLM. However, due to unnatural distribution of body heat (relatively cold periphery) often an undesirable drop of core temperature occurs.[19] This afterdrop adversely affects recovery. Simultaneous measurements of temperature distribution and blood flow provide more insight in the occurrence and prevention of afterdrop. Here we present the use of iButtons in a study on hypothermia and rewarming during cardiac surgery.

Materials

This case study is of an 78-year-old female patient. The results of an interval of 250 min are shown. At time point T = 0 anesthetics were supplied. 95 min later cooling was initiated using the HLM. At T = 125, rewarming started, again using the HLM, and at T = 198 the HLM was disconnected.

Skin temperatures were measured by means of iButtons. Only the skin temperature of the lower arm and fingertip are presented here. Core temperatures were recorded from the nasopharynx (Philips, The Netherlands).

Results and discussion

Figure 9a.7 shows the main temperature characteristics. Upon cooling with the HLM the core temperature dropped, followed by a drop in temperature of the fingertip. When rewarming with the HLM the core temperature gradually increased. After a delay, the fingertip temperature also increased. During the off-pump situation peripheral temperatures dropped again. Moreover, an afterdrop of the core temperature was evident. The varying changes between fingertip and underarm reflect changes in the skin blood flow,[13] which is low during cooling, then suddenly rises during the rewarming period. There was also a drop in the patient's skin temperature during recovery.

The advantage of using iButtons in this situation is that they are easy to sterilize and wireless, and thus do not hinder the surgical procedure. More iButtons may be used in order to map the temperature distribution of the skin. A disadvantage could be the time delay when absolute temperatures are required at a certain time point. The information obtained from iButtons is especially useful for research purposes during surgery. If online measurements are required, the iButtons should not be used.

Conclusions

We have described the accuracy, time response and usefulness of iButtons for measuring human skin temperature. The main advantages are that the iButtons are wireless, suitable for long measurement periods, sturdy, and easy to sterilize. The software is user-friendly. This means their field of application is large.

The main disadvantages are, depending on the purpose of the study, that the maximum sampling rate is one per minute, the relatively large size compared to most sensor tips (disregarding their wires), the large time constant, and the inability of the validated type to measure below 15 °C.

The validation study demonstrated that all of the 30 iButtons tested performed better than the specifications (accuracy 1 °C) provided by the firm. We observed an average accuracy of – 0.09 °C, with one sensor showing a maximum deviation of – 0.4 °C. We showed that this accuracy can be improved by using individual calibration regressions. However, it should be noted that this correction method may not always be available. It is complex because of the high demands on the specifications of the calibration bath and reference thermometer.

The response time is relatively long, i.e. a τ value of 19 s. Thermocouples perform much better in this respect, with τ values usually less than 1 s. Whether fast responses are required depends on the type of experiment. In our test on the human skin we showed that because of the thermal inertia the errors reach 1 °C. In case of faster fluctuations in skin temperature even larger errors are to be expected. On the other hand, in the sleep

and circadian studies the response time appears of negligible importance. The case studies that were described demonstrate the usefulness in field situations and in laboratory and clinical settings. Many other applications are possible. As with all measuring devices, the specifications of the measuring device should be checked beforehand against the accuracy required for the experiments. The major advantage of iButton use is that it allows multiple skin site measurements in those situations in which wired instruments are unpractical, as in long-term field studies and in poorly complying subjects. In some cases, for example in poorly complying demented subjects, the evaluation of skin thermophysiology may in fact only become possible using iButtons. Finally, we showed that field measurements may either confirm or diverge from laboratory findings, in both cases contributing considerably to our understanding of thermophysiology and its relevance during everyday life.

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Manipulation of Core Body and Skin Temperature improves Sleepiness and Maintenance of Wakefulness in Narcolepsy

Based On: *Fronczek R, Raymann RJ, Romeijn N, Overeem S, Fischer M, Van Dijk JG, Lammers GJ, Van Someren EJW. Sleep, In press*

CHAPTER 10

Manipulation of Core Body and Skin Temperature improves Vigilance and Maintenance of Wakefulness in Narcolepsy

Objective **Impaired vigilance and sleepiness are two major daily complaints of patients with narcolepsy. We previously showed their sleepiness to be correlated to an abnormally regulated skin temperature, i.e. an increased distal skin relative to proximal skin temperature. Our goal was to investigate a possible causal contribution of skin temperature disturbances to impairments in the ability to maintain vigilance and wakefulness in narcolepsy.**

Methods **In a modified constant routine protocol, the Psychomotor Vigilance Task (PVT) and the Maintenance of Wakefulness Test (MWT) were repeatedly assessed. Meanwhile, skin and core body temperatures were mildly manipulated within the thermoneutral range of the normal diurnal rhythm using a thermosuit and hot or cold food and drinks.**

Patients **Eight patients (5 males) diagnosed with narcolepsy with cataplexy according to the ICSD-2 criteria (mean age ± SD: 28.6 ± 6.4, range 18-35 years).**

Results **Compared to core cooling, core warming attenuated the typical decline in PVT response speed with increasing time-on-task by 25% (P = 0.02). Compared to distal skin warming, distal skin cooling increased the time that the patients were able to maintain wakefulness by 24% (distal warming: 1.88 min. vs. distal warming: 2.34 min.; P < 0.01).**

Conclusion **Core body and skin temperatures causally affect vigilance and sleepiness in narcolepsy. This could lead to future practical applications.**

Introduction

Narcolepsy is a syndrome characterized by excessive daytime sleepiness (EDS) and cataplexy.[1] Although sleepiness in narcolepsy is generally described as inadvertently falling asleep, a perhaps equally important aspect of it is impaired performance in the waking state due to disturbed vigilance.[2,3]

In healthy controls, both sleepiness and vigilance show a relationship with core body temperature and skin temperature. When core body temperature is high during daytime, skin temperature is relatively low, a combination that is correlated to optimal vigilance.[4-9] In contrast, core body temperature is low at night time, when skin temperature is

relatively high, and this combination is correlated to optimal sleep.[4-9] Skin temperature thus shows a circadian rhythm that is the inverse to the core body temperature rhythm.[10] Furthermore, a relatively high temperature of the distal skin (hands and feet) compared to the temperature of the proximal skin has been shown to be related to the process of falling asleep: a higher distal-to-proximal gradient (DPG) promotes sleep onset.[11] A causal contribution of core body temperature and skin temperature to vigilance and sleepiness has been shown to exist in healthy subjects, in whom mild warming of the proximal skin leads to an accelerated decline in vigilance and to an earlier onset of sleep.[12,13] It has been proposed that changes in both core body temperature and skin temperature modulate neuronal activity of thermosensitive neurons in brain areas that regulate vigilance and sleepiness.[14]

In a previous study, we reported an altered pattern of skin-temperature regulation in narcolepsy.[15] Narcoleptic subjects showed a combination of higher distal skin temperatures and lower proximal skin temperatures, which in healthy subjects is associated with the process of falling asleep.[11] We suggested that this pattern may in fact contribute to sleepiness.[15]

In this paper, we investigate whether direct manipulations of core body and skin temperature induce corresponding changes in the degree of sleepiness and vigilance in narcolepsy. We measured vigilance and the ability to maintain wakefulness in narcoleptic subjects while subtly manipulating skin and core body temperature within the thermoneutral range of the normal diurnal rhythm in a modified constant routine protocol.

Materials and Methods

Subjects

Eight narcoleptic subjects (5 males, 18-35 years of age; mean ± SD: 28.6 ± 6.4 years) participated with informed consent. All suffered from excessive daytime sleepiness (EDS) and typical cataplexy according to the ICSD-2 criteria for narcolepsy with cataplexy.[16] The protocol was approved by the local Medical Ethics Committee. All subjects were free of medication, except for one female subject using oral contraceptives. Females participated between day 4 and day 12 of the menstrual cycle (mid-follicular phase). Females participated between day 4 and day 12 of the menstrual cycle (mid-follicular phase). Subjects were excluded when they suffered from conditions that could influence their peripheral vascular bed, such as the metabolic syndrome, diabetes mellitus, thyroid function disorder, and cardiovascular pathological conditions.

Design

A previously described design was used,[12] that consisted of a modified constant routine protocol[17,18] over 2 experimental days during which vigilance was measured using the Psychomotor Vigilance Task (PVT) and sleepiness was measured using the maintenance of wakefulness test (MWT). Meanwhile, proximal and distal skin temperature were

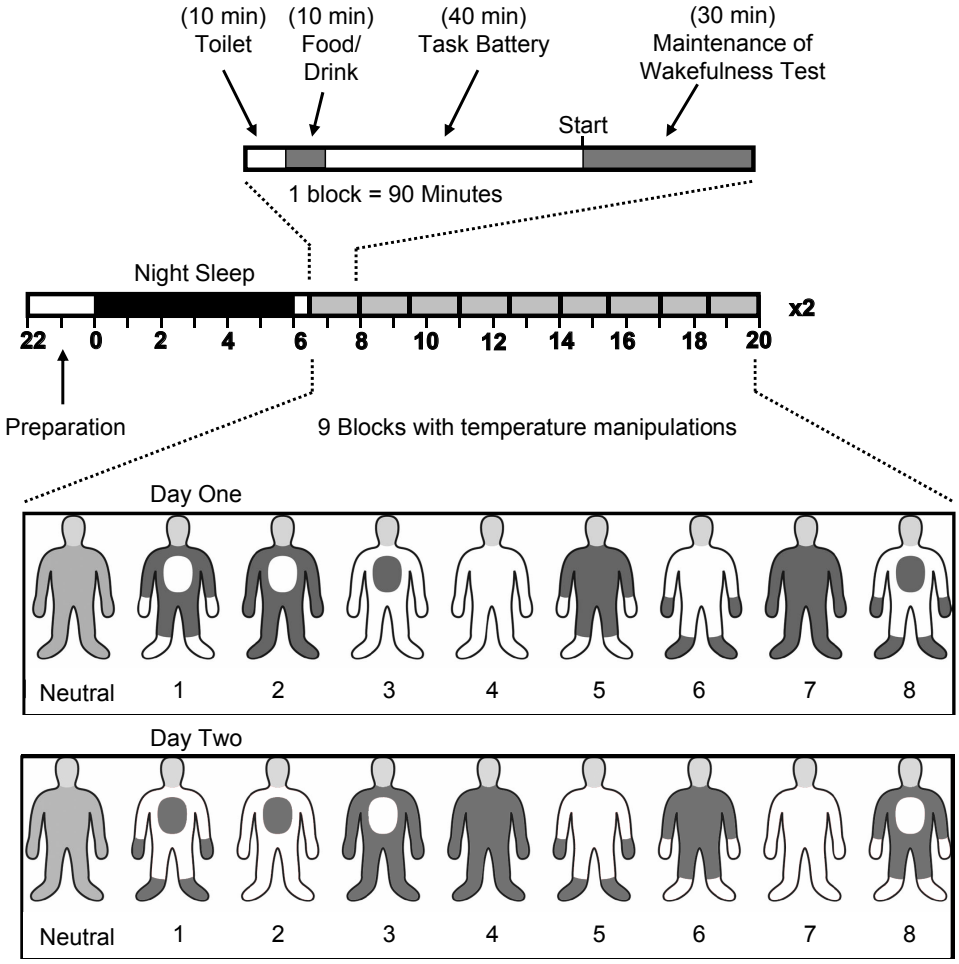

Figure 10.1 **Study Design**

This figure shows a schematic overview of the two experimental days. Each day, subjects entered the lab at 22.00 hrs and were prepared for temperature manipulation and sleep registration. After six hours of night sleep, a modified constant routine protocol was started with nine identical 90 minute blocks. During each block, subjects walked to the toilet (10 min), consumed hot or cold food and drinks (10 min), performed tests on a computerized task battery (including PVT, 40 min) and underwent a MWT (40 min). Core body, proximal and distal skin manipulation occurred during every block. Manipulation patterns are shown in the human outlines with white representing cooling and dark grey representing warming. On the second day, the protocol was identical, but temperature manipulations were exactly the opposite of day one.

subtly manipulated using a thermosuit, while core body temperature was manipulated using hot or cold food and drinks (see Figure 10.1).

Constant routine protocol

Subjects first visited the sleep laboratory to get familiar with the test environment and to practise the PVT. One week later, the actual experiment was performed. Subjects

refrained from caffeine, alcohol and tobacco for 8 hours before reporting at the sleep laboratory at 22:00 hr, where they were prepared for polysomnography and fitted with the thermosuit. At midnight, lights were turned off and subjects were allowed to sleep until 06:00 hr. The experiment started at 06:30 hr under dim-light conditions (10 lux) with a fixed body position (semi-supine) and consisted of 9 consecutive blocks with durations of 1.5 hours each (described below). At the end of the first day subjects went home and returned to the laboratory the next evening for a repeated assessment according to the same procedure, but with a different temperature manipulation scheme (see Figure 10.1).

Block Design.

Each block was similar: It started by having the subjects get out of bed and walk 5 meters, using the bathroom if needed. Ten minutes after the start of each block skin temperature manipulation was started and subjects were served a snack and a drink to consume in approximately 10 minutes. Subsequently a self-paced computerized neuropsychological task battery was completed, including the PVT (see below) and assessment of thermal comfort and temperature sensation, with the use of 100-mm visual analogue scales ranging from uncomfortable to comfortable and from cool to warm. During these tests a researcher was present to keep subjects awake if necessary. After 60 minutes the researcher left the room and subjects were asked to remain awake while lying quietly.[19] If sleep was attained (see the sleep scoring subsection)[20] subjects were awakened and kept awake for the remaining part of the MWT time of 30 minutes.

Table 1. Estimates of the effects of temperature manipulation (in gray) and time of day on core and skin temperatures during the PVT and 5 minutes before start of sleep latency test.

	Core Body Temperature (T_{re})			**Proximal Skin Temperature (T_{prox})**			**Distal Skin Temperature (T_{dist})**		
PVT - Temperature during test									
Intercept	36.47	± 0.14		34.74	± 0.15		35.11	± 0.11	
$Hour^2$	n.s.			n.s.			-0.004	± 0.001	
√Hour	0.08	± 0.01	***	n.s.			n.s.		
CBT:+/-	0.14	± 0.02	***	0.21	± 0.05	***	0.56	± 0.06	
PST:+/-	n.s.			0.50	± 0.05	***	0.22	± 0.06	
DST:+/-	n.s.			0.14	± 0.05	**	0.45	± 0.06	
MWT - Temperature 5 minutes before start of sleep latency test									
Intercept	36.46	± 0.14		34.78	± 0.15		35.12	± 0.10	
Hour	0.05	± 0.01	***	n.s.			n.s.		
$Hour^2$	-0.003	± 0.001	***	n.s.			-0.005	± 0.001	
CBT:+/-	0.10	± 0.02	***	0.15	± 0.04	***	0.36	± 0.06	
PST:+/-	n.s.			0.62	± 0.04	***	0.32	± 0.06	
DST:+/-	n.s.			0.15	± 0.04	***	0.57	± 0.06	

Intercepts represent means. Values are ± standard error. Significance levels are indicated as *P < 0.05, **P < 0.01, ***P < 0.001.

Temperature Manipulation Sequence

Skin and core body temperature were manipulated differentially in every block according to a method described before.[12] In short, the 2x2x2 design consisted of three body sites of manipulation: core body (CB), proximal skin (PS), distal skin (DS). At each, temperature could be increased or decreased (T+ and T-), resulting in eight 8 combinations (CBT+,CBT-,PST+,PST-,DST+,DST-). All eight were tested in one day (Figure 10.1). The sequence differed between subjects in order to balance the protocol such that over all subjects, every manipulation combination was given once in each of the 8 blocks and every transition from one to any other combination occurred no more than once for each time of day. To balance for circadian effects, the second experimental day temperature manipulation combinations were the inverse of those of the first day (for example: day 1, block 1, CBT+, PST-, DST-; day 2, block 1, CBT-, PST+, DST+, Figure 10.1).

Temperature Manipulation method

Core body temperature was manipulated by means of 200 ml hot (heated to 80°C, served 2 minutes later) or cold (0°C, crushed ice) diet decaffeinated tea 4.25 Kcal, Diet Decaffeinated Iced Tea Mix, Lipton, Englewood Cliffs, USA) together with a hot or cold snack at subjects' choice (200 Kcal). Skin temperature was manipulated using a full-body thermosuit (Coretech Cool tube suit, Med-Eng Systems Inc., Ottawa, Canada) connected to two computer-controlled circulation thermostat baths (K6KP, Lauda, Lauda-Königshofen, Germany). During the first 20 min of each block, the water in the thermostat baths changed to the desired temperature, while the bath temperature was kept constant for the remaining 70 min of the block. The water in the tubes was ~31°C and ~34°C just before entering the thermosuit. This range of skin temperature was chosen to avoid major thermoregulatory responses.

Body Temperature Recordings

Core body temperature was measured using a rectal thermistor. Proximal skin temperature was measured at three places: right on the middle of the frontal aspect of the tigh, abdomen (1 cm above the navel), and the right infraclavicular area. Distal skin temperature was measured at four points: thenar eminence of the left and right hand and medial plantar aspect of the left and right foot. Temperature was measured using thermistors (P-8432, ICBT, Tokyo, Japan) and digitally recorded at 1 Hz (Embla A10 and Somnologica software, Flaga, Reykjavik, Iceland). An automated procedure was applied to remove occasional artefacts and to calculate average distal and proximal skin temperature by a weighted average as described before.[12] Temperature data were averaged over 20 minute intervals surrounding the PVT assessments and over the 5 min before the start of the sleep latency test (Figure 10.1).

Sleep scoring

Polysomnographic sleep recordings were performed according to standard procedures.[20] Sleep onset was determined online during the experiment according to standard criteria, defined as three consecutive 30-s epochs of stage 1 sleep or one 30-s epoch of stage 2 (or deeper) sleep.[19] Sleep-onset latency was defined as the time between the start of

Table 2 Estimates of the effects of temperature manipulation on temperature sensation and thermal comfort.

Subjective Measures

	Temperature Sensation		Thermal Comfort	
Intercept	64.27 ± 4.34		47.24 ± 2.62	
CBT:+/-	11.31 ± 2.81	***	-15.00 ± 3.62	***
PST:+/-	14.95 ± 2.80	***	-13.50 ± 3.62	***
DST:+/-	n.s.		n.s.	

Values are means ± standard error. Temperature sensation was measured on a visual analog scale ranging from 0 (cool) to 100 (warm), with 50 reflecting thermoneutral. Thermal comfort was measured on a visual analog scale ranging from 0 (uncomfortable) to 100 (comfortable). Significance levels are indicated as *P < 0.05, **P < 0.01, ***P < 0.001.

the MWT and sleep onset. If the subject did not sleep during the 30 min, sleep-onset latency was scored as 30 min. One data point could not be included in the analysis due to loss of EEG data.

Vigilance

Vigilance was assessed using a seven minute version of the psychomotor vigilance test (PVT).[21,22] Subjects focused on a blank rectangle in the middle of a computer screen. At random intervals (2-10 sec.), a reaction time counter started, shown as the number of milliseconds since start, in the rectangle. Subjects had to press a key to stop it as quickly as possible. The obtained reaction time (RT) count was shown for 1 second, providing performance feedback. Because the distribution of reaction times deviates from normality, PVT results are as a standard reported as response speed, i.e. reciprocal RT (RRT=1000/RT), In order to quantify the typical performance decline with increasing time-on-task response speed averages were calculated per minute. The vigilance measure of interest was the decline of response speed with increasing time-on-task.

Statistical analyses

To determine the effects of skin and core temperature manipulations on actual measured temperatures (core body, proximal skin, and distal skin) and on PVT performance and subjective comfort, hierarchical regression analysis was applied using MLwiN software (Centre for Multilevel Modelling, Institute of Education, London, UK). Because the frequency distribution of sleep-onset latencies was skewed, longitudinal Poisson regression analysis was used to determine the effects of skin and core temperature manipulations and induced temperatures on MWT sleep onset latency. The hierarchical regression analyses take into account the interdependency of the data points inherent to the hierarchical structure of the design, in our case the sequential sleep-onset observations, i, that were nested within days, j, once more nested within subjects, k.[23] The first block of both days (the habituation block) was omitted from analyses. Analyses were run with induced body temperatures (core body, proximal skin, and distal skin), subjective comfort, thermal comfort, time-on-task decline of PVT response speed, and sleep-onset latency as dependent variables; and body temperature manipulations as

a. Effects of manipulation

	Response Speed (1/RT)			
Intercept	2.46	±	0.20	
√Minute	-0.28	±	0.02	***
CBT:+/- * √Minute	0.07	±	0.03	***
PST:+/- * √Minute	n.s.			
DST:+/- * √Minute	n.s.			

b. Effects of measured temperatures

	Response Speed (1/RT)			
Intercept	2.47	±	0.20	
√Minute	-0.25	±	0.02	***
T_{re}/°C * √Minute	0.30	±	0.10	**
T_{prox}/°C * √Minute	n.s.			
T_{dist}/°C * √Minute	n.s.			
DPG/°C * √Minute	-0.031	±	0.015	*

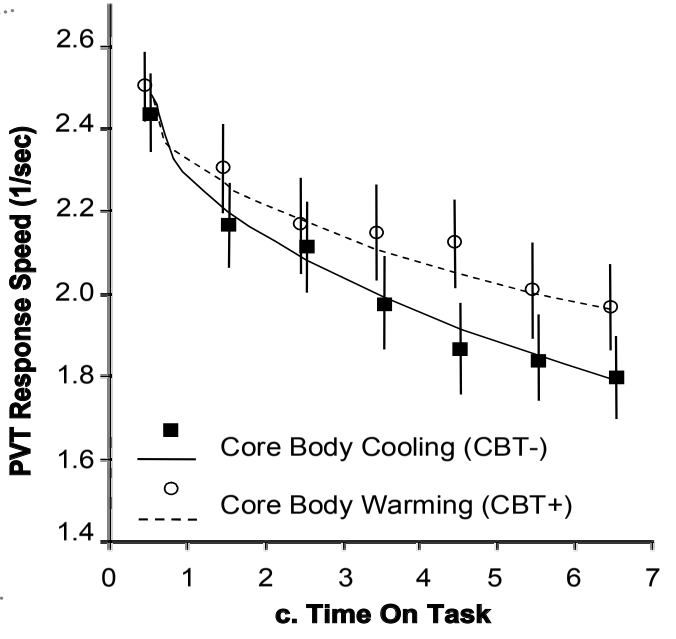

Figure 10.2 | **Effects on Vigilance**
Estimates of the effects of temperature manipulation and time on task on PVT response speed (a), estimates of the effects of actual measured temperatures and time on task on PVT response speed (b) and regression model of response speed over the seven subsequent minutes of PVT performance in the core warming (CBT+, open circles) and core cooling (CBT-, solid squares) conditions (c). Intercepts represent overall means. Values are means ± standard error. Significance levels are indicated as *:p<0.05, **:p<0.01, ***:p<0.001. Narcoleptic subjects show a low response speed and a time on task effect with a fast decline already during the first minutes. The response speed improves during the core body warming condition.

centred dichotomous predictor variables (with -0.5 reflecting the cool manipulation level and 0.5 reflecting the warm manipulation level, for ease of interpretation of the intercept, now showing the overall average). For the longitudinal Poisson regression analysis, all independent variables were centred at the within-subject single-day level.
A second series of analyses was performed, now not evaluating the manipulation conditions but rather how the actually measured core body, proximal and distal skin temperatures, and the distal-to-proximal gradient (DPG) predicted the time-on-task decline of PVT response speed and MWT sleep-onset latency.

Time (hour, hour2 and √hour; defined as the number of hours since the start of the first included PVT or MWT within each day, starting with 0 at 09:00) was allowed in the models for induced temperatures to account for possible diurnal variations in core and skin temperature.[24] For all regression analyses, we calculated the full model, with all temperature manipulation variables and covariates in the model and subsequently stepwise removed nonsignificant terms to obtain optimal models, containing only the significant contributions. Maximum likelihood was used to estimate the regression coefficients, which were tested for significance with the Wald test.[23] In order to obtain the optimal linear models, additional terms were allowed in the regression equation only if their coefficients were significant and only if their inclusion improved the regression model according to the likelihood ratio test. In order to obtain the optimal Poisson regression models, additional terms were allowed in the regression equation only if their coefficients were significant and if the residual error of the model was reduced.

The following regression models were used: [1] effects of manipulation on measured temperature: $T_{ijk} = \beta_{0ijk} + \beta_1 \times Hour_{ijk} + \beta_2 \times Hour^2_{ijk} + \beta_3 \times \sqrt{Hour}_{ijk} + \beta_4 \times CBT_{ijk} + \beta_5 \times PST_{ijk} + \beta_6 \times DST_{ijk}$ (Subscripts indicate ith observation on day j for subject k); [2] effects of manipulation on PVT response speed: $PVT_{ijkl} = \beta_{0ijkl} + \beta_1 \times CBT_{ijkl} + \beta_2 \times PST_{ijkl} + \beta_3 \times DST_{ijkl} + \beta_4 \times \sqrt{Minute}_{ijkl}$ (Subscripts indicate ith minute during the jth PVT on day k for subject l); [3] relation of measured temperatures on PVT response speed: $PVT_{ijk} = \beta_{0ijkl} + \beta_1 \times Tre_{ijkl} + \beta_2 \times Tprox_{ijkl} + \beta_3 \times Tdist_{ijkl} + \beta_4 \times \sqrt{Minute}_{ijkl}$ (Subscripts indicate ith minute during the jth PVT on day k for subject l); [4] effects of manipulation on sleep latency: $ln(latency) = \beta_{0ijk} + \beta_1 \times CBT_{ijk} + \beta_2 \times PST_{ijk} + \beta_3 \times DST_{ijk} + \beta_4 \times Hour^2_{ijk}$ (Subscripts indicate ith observation on day j for subject k); [5] effects of measured temperatures on sleep latency: $ln(latency)_{ijk} = \beta_{0ijk} + \beta_1 \times Tre_{ijk} + \beta_2 \times Tprox_{ijk} + \beta 3 \times Tdist_{ijk} + \beta_4 \times Hour^2_{ijk} (+ \beta_5 \times DPG_{ijk})$ (Subscripts indicate ith observation on day j for subject k); [6] effects of manipulation on temperature sensation and comfort: $Outcome\text{-}variable_{ijk} = \beta_{0ijk} + \beta_1 \times CBT_{ijk} + \beta_2 \times PST_{ijk} + \beta_3 \times DST_{ijk} + \beta_4 \times Hour^2_{ijk}$. In the tables the optimal models are shown, with only the significant contributing factors. Note that the values in the tables represent the effect coefficients as estimated from the Poisson regression analysis: in order to transform these coefficients to the number of 30-sec epochs, one should calculate e to the power of the regression equation. Transformation to minutes subsequently takes a division by two. In the results section, sleep latencies have thus been transformed and are reported in minutes. Two-tailed significance levels were set at 0.05.

a. Effects of manipulation

	Sleep Latency (min)		
	mean	confidence interval	
Intercept	2.10	1.52 – 2.90	
Hour^2	0.50	0.50 – 0.50	***
CBT:+/-	n.s.		
PST:+/-	n.s.		
DST:+/-	-0.40	0.34 – 0.47	**

b. Effects of measured temperatures

	Sleep Latency (min) (factorial model)			Sleep Latency (min) (DPG model)		
	mean	confidence interval		mean	confidence interval	
Intercept	2.10	1.52 – 2-91		2.10	1.52 – 2-91	
Hour^2	0.50	0.50 – 0.50	***	0.50	0.50 – 0.50	***
T_{re}/°C	n.s.					
T_{prox}/°C	n.s.					
T_{dist}/°C	-0.40	0.34 – 0.48	**			
DPG/°C				-0.40	0.33 – 0.49	*

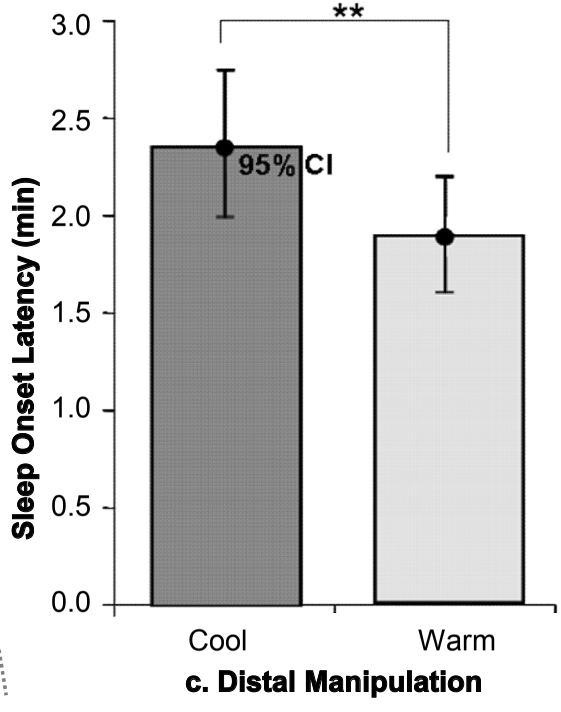

Figure 10.3 | **Effects on Maintenance of Wakefulness**
Estimates of the effects of temperature manipulation and time of day on sleep latency (a), estimates of the effects of measured temperatures and time of day on sleep latency (b) and the means (± 95% Confidence Interval) of sleep latency in the distal cooling (left bar) and distal warming (right bar) conditions (c). Intercepts represent overall means. Values in tables are means ± standard error. Significance levels are indicated as *:p<0.05, **:p<0.01, ***:p<0.001. Narcoleptic subjects were characterized by very short sleep latencies. Sleep latency was longer in the distal skin cooling condition as compared to the distal skin warming condition (**p < 0.02).

Results

Effects of manipulation on temperature and comfort

The effects of the manipulations on core body and skin temperatures during the PVT and before the start of the MWT are shown in Table 1. Core body and distal skin temperature were significantly modified by time of day ($p < 0.001$), accounting for 10-25% of variance during the PVT and the MWT.

The core body temperature manipulation was the sole factor influencing core body temperature during the PVT and the MWT (effect size: 0.10 - 0.12 °C, $p < 0.001$) and accounted for 29% of the variance during the PVT and 17% of the variance during the MWT.

Proximal and distal skin temperatures during the PVT and the MWT were mainly affected by their respective skin temperature manipulation (effect size: 0.45 – 0.62 °C, $p < 0.001$), but also to a lesser extend by core body temperature and by the other skin temperature manipulation (effect size: 0.14 – 0.56 °C, $p < 0.001$). The temperature manipulations accounted for 60% of the variance during the PVT and the MWT.

The effects of the manipulations on thermal comfort and temperature sensation, measured before the MWT are shown in Table 2. In summary, the warm conditions were experienced as less comfortable and warmer than the cool conditions. Comfort was significantly lower when the core body and proximal skin were warmed ($p < 0.001$), with a trend for warming of the distal skin ($p = 0.06$). The highest comfort was achieved when cooling was induced at all three sites. Temperature was perceived as higher in the core body and proximal skin warming condition ($p < 0.001$). Subjects did not perceive the distal skin warming condition as a significantly warmer condition than the distal skin cooling condition ($p = 0.26$).

Effect of temperature manipulation on psychomotor vigilance

The overall average RRT of narcoleptic subjects was 2.46 ± 0.20 sec^{-1}. There was a typical worsening, i.e. a decline in response speed, with increasing time-on-task (see Figure 10.2). This decline was best approximated by a square root function of time-on-task (√Minutes, $p < 0.001$). As evident from Figure 10.2, these profiles indicate that vigilance declined quickly after starting the task. As compared to core body cooling, core body warming attenuated this decline by 25% (CBT x √Minute, $p = 0.02$), while effects induced by proximal or distal skin temperature manipulations were not significant ($p > 0.20$).

Regressing PVT on the actually induced temperatures showed essentially the same effects: a higher core body temperature was associated with an *attenuated* decline in response speed over the time-on-task (T_{re} x √Minute, $p = 0.004$). Moreover, a higher DPG was associated with an *accelerated* decline in response speed (DPG x Minute, $p = 0.04$).

Effect of temperature manipulation on maintenance of wakefulness

Figure 10.3 shows the effects of the temperature manipulations on maintenance of wakefulness as derived from the regression analysis. Overall average sleep latency was 2.10 min (95% Confidence Interval CI: 1.52 - 2.90). Sleep onset latency was significantly modulated by time (hour^2) and the distal skin manipulation, with an estimated shorter latency (1.88 min; CI: 1.60 – 2.21) in the DST+ condition compared to a longer latency (2.34 min; CI: 1.99 – 2.75) in the DST- condition ($p < 0.01$, Figure 10.3). Cooling the distal skin thus meant that subject remained awake for 24% longer as when the distal skin was warmed. Sleep latency was not significantly affected by core and proximal manipulations (all $p > 0.20$).

Regressing MWT on the actually measured temperatures resulted in significance for the same variables: sleep onset latency was significantly modulated by time (hour2) and by distal skin temperature. The regression coefficients, transformed to minutes (Figure 10.3ab), can be interpreted as follows: patients could stay awake for 1.89 (CI: 1.60 – 2.24) minutes when distal skin temperature was 0.5 ˚C higher than the average distal skin temperature, as compared to 2.34 (CI: 1.97 – 2.77) minutes when distal skin temperature was 0.5 ˚C lower than the average distal skin temperature ($p < 0.01$, Figure 10.3c). One degree of decrease in distal skin temperature thus increased the time patients succeeded to maintain wakefulness by 24%. Sleep latency was not significantly related to core and proximal temperatures (all $p > 0.31$).

Discussion

We investigated whether subtle manipulations of core body and skin temperatures within the natural range of the diurnal cycle, affected vigilance and sleepiness in narcolepsy.

Firstly, patients were better able to maintain vigilance when core body temperature was increased than when it was lowered. In short, vigilance in narcolepsy can be altered simply by altering the temperature of food and drinks. Secondly, the ability to maintain wakefulness was better when distal skin temperature was lowered than when it was increased. We were thus able to influence the process of falling asleep in narcoleptic subjects by gently cooling or warming their hands and feet. The acceleration of sleep onset by distal skin warming occurred in spite of the fact that warming was perceived as slightly less comfortable.

Our data furthermore showed a number of narcolepsy-related aspects on PVT-performance and maintenance of wakefulness under strictly controlled and balanced conditions. In agreement with previous investigations,[2] narcoleptic subjects showed a very poor average PVT response speed, not only compared to matched controls, but also compared to elderly and elderly insomniacs previously submitted to the same protocol.[13] Although untreated narcolepsy is already characterized by a very short MWT sleep latency (around 6 minutes),[19] in our constant routine protocol all patients had great difficulties remaining awake for longer than 3 minutes. These shorter MWT values relative to previous reports may be due to the restricted time allowed for sleep during

the night prior to the investigation (6 hours) and continuous low-light, stimulus-free, semi-supine circumstances, known to promote falling asleep.[25] The more remarkable it is that even under these high sleep pressure inducing conditions, distal skin cooling significantly increased sleep latency with 24%.

Note that in this study, sleep-onset was defined as 3 consecutive 30-s epochs of stage 1 sleep or one 30-s epoch of stage 2 (or deeper) sleep. This differs from the definition of 16 sec of stage 1 (or deeper) sleep that is commonly used in the clinical setting. In our study design, sleep scoring was performed online and subjects had to be woken up immediately after the onset of sleep. For this purpose, we had to be absolutely sure of sleep onset, and therefore used the three 30-s epoch criterion. To compare results with other studies, the exact definition of sleep onset needs to be taken into account. However, using the clinical definition of sleep onset would not have changed the outcome of this study, since there was no occasion when a subject showed an epoch of wake after the occurrence of stage 1 sleep.

It is known that the clinical efficacy of commonly used stimulants, such as modafinil, is not adequately revealed by its small effects on sleep latency as measured in the MWT.[26] Although the definition of sleep onset may have differed between studies, the changes in sleep latency in our study are comparable to those seen with modafinil. Temperature manipulations may thus have a more significant clinical effect.

We initiated this study based upon our previous findings that narcoleptic subjects have an increased distal relative to proximal skin temperature (distal-to-proximal gradient, DPG) that was related to an increased sleepiness.[15] In that previous study, we did not measure core body temperature. Other studies have reported conflicting results regarding core body temperature. Therefore, we compared the T_{core} measured in this study in narcoleptic subjects, with the earlier published T_{core} measured in healthy controls that underwent the same protocol and matched on age and gender.[12] core body temperature was lower in narcolepsy than in controls (T_{re}: narcolepsy 36.47 ± 0.14°C; controls, 36.88 ± 0.06°C; p = 0.01 (Z-test)). A partial normalization of the low core body temperature in the core warming condition may have been involved in its positive effects on PVT performance. This interpretation is supported by the positive relation between core body temperature and vigilance we previously found in elderly subjects in an identical protocol [13] and by previous work showing a correlation between the circadian modulation of vigilance and of core body temperature.[5-9] The effects of distal skin warming and cooling on maintenance of wakefulness in narcoleptic subjects are in line with our earlier findings of an abnormally increased distal skin temperature that correlated with the ease of falling asleep in narcolepsy.[15]

In previous studies of our group, both young and elderly subjects without sleep problems and insomniac elderly showed worsening of PVT performance and shorter sleep latencies with proximal skin warming.[12,13] Given the repeatability of the proximal warming results over the 3 groups in those previous studies, the more remarkable it is that narcoleptic subjects do not show sensitivity of vigilance performance and sleepiness to proximal warming. It is not unlikely that this difference with healthy controls—and

with elderly subjects reported previously—is related to the markedly lower core and proximal temperature of narcoleptic subjects in combination with a higher distal skin temperature, even under the strictly controlled conditions of the present experiment and found previously under less controlled circumstances.[15]

Of note, the present study differs from previous work e.g.[24,27,28] in that *mild* manipulations within the thermoneutral zone were applied. Since such manipulations induced only changes within the temperature range normally covered during everyday life, the circadian modulation of these temperatures could contribute to the circadian modulation in vigilance and sleepiness.

In conclusion, our results demonstrate a modulatory role for body temperature in the regulation of vigilance and maintenance of wakefulness in narcolepsy. Experimentally induced subtle changes in core body and skin temperature *caused* changes in vigilance and the ability to maintain wakefulness. A practical implication of our findings is that temperature manipulations may be of value in the management of vigilance and sleepiness problems in narcolepsy. An ultimate practical application could for example be clothing with integrated measurement and regulation of skin temperature. For the time being, the advice may be to utilise a warm drink or meal in combination with cooling of the extremities to aid their fight against vigilance impairment and daytime sleepiness.

Acknowledgements

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Manipulation of Skin Temperature improves Nocturnal Sleep in Narcolepsy

Based On: *Fronczek R, Raymann RJ, Overeem S, Romeijn N, Van Dijk JG, Lammers GJ, Van Someren EJW. Submitted*

CHAPTER 11

Manipulation of Skin Temperature improves Nocturnal Sleep in Narcolepsy

Objective **Besides excessive daytime sleepiness, disturbed nocturnal sleep is a major complaint of patients with narcolepsy. Previously, we showed alterations in skin temperature regulation in narcoleptic patients that were related to increased sleepiness. Furthermore, temperature manipulations improved daytime vigilance and maintenance of wakefulness. In this study, we investigated the effect of skin temperature manipulations on nocturnal sleep in narcolepsy.**

Methods **Polysomnography was obtained during two nights in eight patients (5 males) diagnosed with narcolepsy with cataplexy according to the ICSD-2 criteria (mean age 28.6 ± 6.4, range 18-35 years). Proximal and distal skin temperature was manipulated using a comfortable thermosuit and slowly cycled within a range normally observed during sleep (34.9 - 35.4 °C). The distribution of different sleep stages and nocturnal wakefulness was compared between different skin temperature conditions.**

Results **Proximal skin warming significantly suppressed wakefulness and enhanced slow wave sleep (SWS). In contrast, distal skin warming enhanced wakefulness and stage 1 sleep at the cost of SWS and REM sleep. The optimal combination of proximal skin warming and distal skin cooling led to a 160% increase in SWS, a 50% increase in REM-sleep and a 68% decrease in wakefulness, compared to the least beneficial combination of proximal skin cooling and distal skin warming.**

Conclusion **Subtle skin temperature manipulations under controlled conditions significantly improved the typical nocturnal sleep problems in narcolepsy. These results indicate that skin temperature control could have therapeutic relevance.**

Introduction

The four classical symptoms of narcolepsy are excessive daytime sleepiness, cataplexy, hypnagogic hallucinations and sleep paralysis.[1] During the last years, disturbed nocturnal sleep has gained increasing attention as a fifth core symptom that severely affects quality of life.[2] Nocturnal polysomnography in patients with narcolepsy shows a fragmentation of the normal sleep pattern with frequent arousals and a decrease in slow wave sleep.[3-5] Several hypnotics, including sodium oxybate (gammahydroxybutyrate), are currently used to improve sleep in narcolepsy.[6]

Narcolepsy is caused by a loss of the neuropeptide hypocretin (orexin), a neurotransmitter that is produced by neurons in the lateral hypothalamus.[7] Hypocretin neurons are normally active during wakefulness and hypocretin is thought to stabilize sleep/wake patterns by activating wake-promoting brain areas.[8] However, the exact role of hypocretin in stabilizing nocturnal sleep is unknown.

There is a relation between sleep and both core body and skin temperature.[9,10] In a comfortable environmental temperature, core body temperature is lower and skin temperature is higher during the night than during the day.[10,11] Conversely, sleep initiation is facilitated when the temperature of the distal skin (hands and feet) is relatively high.[12] There seems to be a causal relation as mild warming of the skin promotes daytime sleep onset.[13] Moreover, active manipulation of skin temperature does affect night time sleep in healthy controls.[14] It is thought that sensory afferents conveying information about skin temperature modulate the firing rate of thermosensitive neurons in the sleep regulating systems including the preoptic area/anterior hypothalamus, which is the major thermoregulatory center of the mammalian brain and a key structure in arousal state control.[15]

In a previous study, we reported disturbances in skin-temperature regulation in narcolepsy.[16] Narcoleptic subjects showed a combination of a higher distal skin temperature and a lower proximal skin temperature, which in healthy subjects is associated with the process of falling asleep.[17] In a follow-up study, we were able to affect both daytime vigilance and maintenance of wakefulness by manipulating skin and core body temperature.[18] To explore whether manipulation of skin temperature can also be beneficial as a tool to improve nocturnal sleep in narcolepsy, we performed subtle manipulations of proximal and distal skin temperature during two nocturnal sleep episodes in eight narcoleptic patients.

Materials and Methods

Subjects

Eight narcoleptic patients (5 males, 18-35 years of age; mean ± SD: 28.6 ± 6.4 years) participated with informed consent. All suffered from excessive daytime sleepiness and typical cataplexy according to the ICSD-2 criteria for narcolepsy with cataplexy.[19] All subjects were free of medication, except for one female subject using oral contraceptives. All females participated between day 4 and day 12 of the menstrual cycle (mid-follicular phase or pseudo-follicular phase). The protocol was approved by the local Medical Ethics Committee.

Design

A previously described design was used to differentially manipulate proximal and distal skin temperature and to determine the effects of these manipulations on sleep depth.[14] Subjects refrained from caffeine, alcohol and tobacco for 8 hours before reporting at the sleep laboratory at 22:00 hr. There they were prepared for polysomnography and fitted with a thermosuit. At midnight, lights were turned off and subjects were allowed

Table 1

Stage	OR	$T_{suit-prox}$ 95% CI	P	OR	$T_{suit-dist}$ 95% CI	P
Wake	0.81	(0.77-0.84)	***	1.11	(1.06-1.16)	***
S1		ns		1.22	(1.16-1.28)	***
S2		ns			ns	
SWS	1.23	(1.17-1.29)	***	0.85	(0.81-0.89)	***
REM		ns		0.87	(0.83-0.92)	***

The odds ratio (OR), confidence interval (CI) and significance (P) for the occurrence of each sleep state as modulated by the temperature of the thermosuit warming the distal and proximal skin (per 1˚C). S1, stage 1 sleep; S2, stage 2 sleep; SWS, slow wave sleep; REM, rapid-eye-movement sleep; Tsuit-prox, proximal suit temperature; Tsuit-dist, distal suit temperature; ns, not significant.

to sleep until 06:00 hr. From 00:30 hr till 06:00 hr, their proximal and distal skin temperatures were manipulated. After this, subjects slept one night at home after which they returned for a second hospital night, during which the temperature manipulation sequence was inverted to that of the first night.

Temperature manipulations and measurement

Starting at 0:30 hr, the temperature of the proximal skin ($T_{skin-prox}$) and the temperature of the distal skin ($T_{skin-dist}$) were differentially manipulated by slowly cycling the temperature of thermosuit water perfusion (figure 1). The thermosuit (Coretech Cool tube suit, Med-Eng Systems Inc., Ottawa, Canada) was connected to two sequence programmed computer-controlled bath/circulation thermostats (K6KP, Lauda, Lauda-Königshofen, Germany). The suit temperature (T_{suit}) stayed at constant plateaus of either 15 or 30 minutes with slow (15 min) transitions in-between. The order of skin temperature manipulations was different for each subject using a balanced design. Tsuit cycled between 31.9 ± 0.1 ºC (mean ± SE) in the 'cool' and 34.8 ± 0.1 ºC in the 'warm' condition, as measured once per minute on the isolated inflow tubes at their proximal and distal connections with thermosuit (PT100 thermistors, RTD-3-3105, Omega, Stanford, USA). This range was specifically chosen to match the previously reported range of temperatures normally present in the bed microclimate.[20] The temperature of the environmental air was kept at 21˚C.

Core body temperature was measured rectally. Proximal skin temperature was measured at three places: right on the middle of the frontal aspect of the thigh, abdomen (1 cm above the navel), and the right infraclavicular area. Distal skin temperature was measured at four points: thenar eminence of the left and right hand and medial plantar aspect of the left and right foot. Temperature was measured using thermistors (PT100 thermistors, RTD-3-3105, Omega, Stanford, USA) and digitally recorded at 1 Hz using a Embla A10 recorder (Flaga, Reykjavik, Iceland). Data was analysed using Somnologica software (Flaga). An automated procedure was applied to remove occasional artefacts and to calculate average distal and proximal skin temperature by a weighted average as described before.[21] Temperature data were averaged over 30 second intervals synchronized to the sleep stage epochs.

Sleep recordings

Polysomnographic sleep recordings were performed according to standard procedures and consisted of electroencephalography (EEG), electromyography, and electrooculography.[22] Polysomnography signals were also recorded with the A10 recorder. Sleep was scored by one person blinded to the temperature conditions in 30 sec. epochs according to the Rechtschaffen and Kales criteria using Somnologica software.[22] Sleep stages 3 and 4 were combined into the single class 'slow wave sleep'.

Statistical Analysis

The main outcome measures of this study were the effects of proximal and distal skin warming or cooling (per 1° Celsius) on the odds ratios for the occurrence of each sleep stage (stage 1, stage 2, slow wave sleep, REM-sleep and wakefulness). Multilevel regression modelling was applied to account for the interdependency of the data points inherent to the hierarchical structure of the dataset: sleep epochs within nights within subjects (MLwiN software, Centre for Multilevel Modelling, Institute of Education, London, UK).[23] The regression models included parameters to account for nonlinear changes over time that could lead to correlated residual error. The analyses included all epochs during the skin temperature cycles (from 00:30 hr. until 6:00 hr.). To determine the effects of skin temperature manipulation on the probability of occurrence of sleep stages, longitudinal multilevel logistic regressions were applied for each sleep stage classification, with the current presence or absence of that stage as dummy coded dichotomous dependent variable and $T_{suit\text{-}prox}$ and $T_{suit\text{-}dist}$ as predictor variables. Optimal regression models were selected using the likelihood ratio chi-square test.[23] Odds ratios were translated into sleep stage probabilities at every time point during the night for the maximal and minimal thermosuit temperature levels using the transformation ex/(1+ex), where x represents the regressor part of the best fitting model. A separate plot was generated to visualize the regression prediction for the cumulative sleep stage probability during the mean upper (34.8 ± 0.1 °C) and lower (31.9 ± 0.1 °C) T_{suit} levels. Two-tailed significance levels were set at 0.05.

Results

Induced temperatures

The thermosuit was able to differentially manipulate proximal and distal skin temperature in narcoleptic patients (see example of one night in one patient in Figure 11.1). The temperature manipulations of the proximal part of the thermosuit accounted for 53.8% of the variance in mean $T_{skin\text{-}prox}$. For the warm and cool periods, $T_{skin\text{-}prox}$ averaged 35.1 ± 0.1 °C versus 34.7 ± 0.1 °C respectively. Likewise, the independently manipulated temperature of the distal part of the thermosuit accounted for 44.0% of the variance in mean $T_{skin\text{-}dist}$. $T_{skin\text{-}dist}$ averaged 35.5 ± 0.05 °C versus 35.1 ± 0.05 °C for the warm and cool levels respectively. Thus, the manipulations forced the skin temperature to slowly cycle within a very subtle 0.4 °C range (see temperature graph in Figure 11.1). The manipulations left core body temperature virtually unchanged (skin temperature manipulations accounted for only 2.5% of the variance in core body temperature).

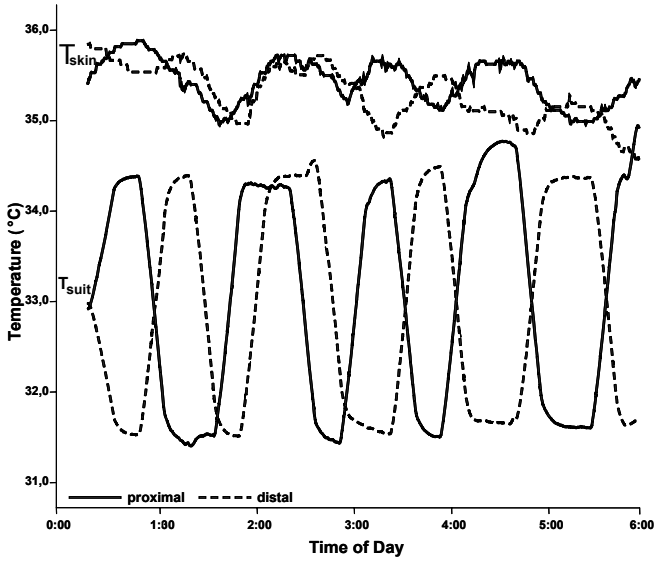

Figure 11.1
Sample night of one patient
Example of a temperature profile induced in one patient during a single night. The lower traces show the temperature of the proximal (straight line) and distal (dotted line) parts of the thermosuit. The upper traces show the actually induced proximal and distal skin temperatures.

Effect of temperature manipulation on sleep stage distribution
Thermosuit manipulation of the temperature of the proximal and distal skin significantly affected sleep depth and the occurrence of wakefulness. Table 1 shows that proximal warming (per 1°C increase in $T_{suit\text{-}prox}$) suppressed wakefulness (OR 0.81 [0.77-0.84], p<0.001) and enhanced slow wave sleep (OR 1.23 [1.17-1.29], p<0.001). In contrast, distal warming (per 1°C increase $T_{suit\text{-}dist}$) enhanced wakefulness (OR 1.11 [1.06-1.16], p<0.001) and stage 1 sleep (OR 1.22 [1.16-1.28], p<0.001) sleep at the cost of slow wave sleep (OR 0.85 [0.81-0.89], p<0.001) and REM sleep (OR 0.87 [0.83-0.92], p<0.001). There were no significant effects on the occurrence of stage 2 sleep.

A graphical representation of the sleep stage distribution is given in Figure 11.2, showing the optimal thermal condition of proximal skin warming and distal skin cooling (right bar) and least beneficial combination of proximal skin cooling and distal skin warming (left bar). The optimal skin temperature combination led to a 160% increase in slow wave sleep, a 50% increase in REM-sleep and a 68% decrease in wakefulness.

Discussion

This study shows that subtle manipulation of proximal and distal skin temperatures has beneficial effects on nocturnal sleep in narcolepsy. When the proximal skin was warmed, slow wave sleep increased and wakefulness was suppressed. In contrast, warming of the distal skin suppressed slow wave and REM-sleep, while enhancing wakefulness and stage-1 sleep.

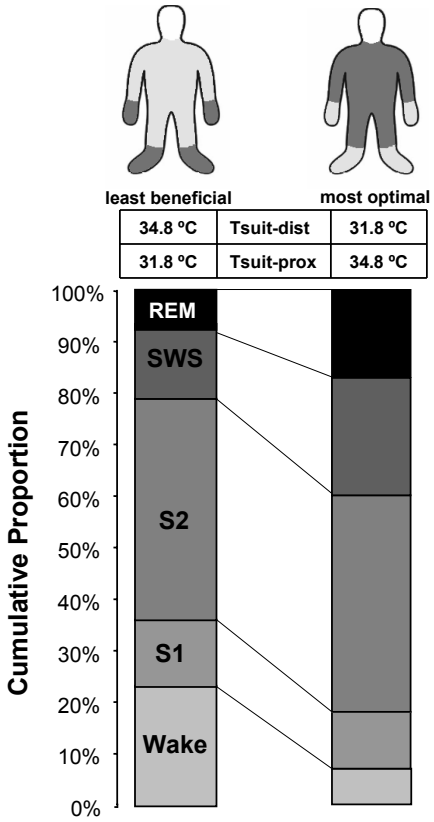

Figure 11.2

Sleep stage distribution

A graphical representation of the proportion of the sleep stages during the optimal (distal cooling and proximal warming) and least beneficial (distal warming and proximal cooling) manipulation scheme. The proportions were derived in separate logistic regressions for each sleep stage. For graphical purposes only, the figure was rescaled to 100%.

Fragmented nocturnal sleep is a major and difficult to treat problem for many patients with narcolepsy. Currently, treatment of this invalidating symptom is based on hypnotics, most notably sodium oxybate,[24] which increases SWS and REM-sleep, while suppressing wakefulness.[25,26] Of note, effects of these hypnotics are at best similar in quality as to the effects found in this study using manipulation of skin temperature. Moreover, this method is non-invasive and did not produce any adverse effects.

This study was designed in such a way that different manipulation schemes were equally and randomly distributed over the test subjects in a balanced way. As such, the effects cannot have been caused by time of night or circadian effects, but can be solely attributed to the manipulation of skin temperature. Skin temperature manipulations were applied while keeping a constant temperature of the ambient air, which was breathed and to which the face was exposed. We do not expect that elevating ambient temperature would lead to any comparable sleep profit, because it is essential to differentially manipulate proximal and distal temperatures. Worse sleep has indeed been reported with an air temperature of 30˚C, as compared to 18˚C and 23˚C.[27]

The fact that subtle changes in skin temperature affect sleep in both narcoleptic patients and healthy controls,[14] shows that the basic hypothalamic circuitry involved in temperature and sleep regulation is intact in narcolepsy and is uninfluenced by

the hypocretin deficiency. Temperature manipulations can have beneficial effects in narcolepsy, both during the night as well as during the day. Previously, we showed that distal cooling improve maintenance of wakefulness during the day while distal warming increased sleep propensity. Here we show that, although warm hands and feet promote the onset of sleep, having a higher distal skin temperature or actively warming the distal skin during the whole night does not improve sleep quality.

In this study, no subject experienced the optimal or least beneficial combination of proximal and distal manipulations continuously during a full night. It would be of interest to confirm the positive effects found in this study using a controlled trial in which the optimal and least beneficial temperature conditions are compared to one another and with the baseline situation.

In conclusion, selective manipulation of skin temperature can be applied to ameliorate one of the core symptoms of narcolepsy, disturbed nocturnal sleep. Effects of temperature manipulation were of such a magnitude, that this new approach could potentially supplement other established methods to improve nocturnal sleep in narcolepsy.

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Summary & General Discussion

Summary & General Discussion

In the first part of this thesis the question was examined whether or not hypocretin neurons are lost in neurological disorders in which sleep disturbances similar to those in narcolepsy occur. Furthermore, a screening for auto-antibodies was described, aimed at finding evidence for a putative autoimmune aetiology of human narcolepsy, followed by a report on a placebo-controlled double-blind N=1 trial with intravenous immunoglobulins (IVIg) in one narcoleptic patient.

In the second part of this thesis the consequences of a loss of hypocretin neurons were examined, with a focus on non-sleep-related symptoms of narcolepsy, i.e. obesity (metabolism and autonomic control), vigilance impairment and skin temperature regulation.

Part I: The Hypothalamus and its Hypocretin Neurons

We investigated hypocretin function in other neurodegenerative disorders that are often accompanied by narcolepsy-like sleep disturbances, such as Alzheimer's Disease, Parkinson's Disease and Huntington's Disease. Furthermore, hypocretin functioning was assessed in patients with Prader-Willi syndrome, because of case reports describing cataplexy and sleep disturbances in this syndrome.[1]

Prader-Willi Syndrome.

Prader-Willi Syndrome is characterized by mental retardation, hypogonadism, growth deficiency and most notably by an insatiable hunger. Prader-Willi Syndrome is the most common genetic cause of obesity.[2] Furthermore, patients suffer from excessive daytime sleepiness,[3] and some case reports suggest that a minority of patients experience cataplexy-like attacks.[1] CSF hypocretin values are normal in Prader-Willi Syndrome,[4] although slightly lower than normal values have been reported.[5] To determine whether the hypocretin system is involved, we studied post-mortem hypothalami of eight adult and three infant Prader-Willi Syndrome patients and 11 controls. No difference in the total number of hypocretin-containing neurons was found between Prader-Willi Syndrome patients and controls.

> The number of hypothalamic hypocretin neurons is not abnormally low in Prader-Willi Syndrome. A decline in the number of hypocretin neurons is not the most likely cause of the excessive daytime sleepiness and possible cataplexy reported in this syndrome.
>
> ***Chapter 1***

Future perspectives

Unfortunately no clinical information about sleep disturbances or cataplexy was available for the hypothalami that we studied. The possibility remains that a reduction in the total number of hypocretin neurons can still be found in a few Prader-Willi Syndrome patients with clear-cut cataplexy. Evidence for changes in hypocretin gene expression in Prader-Willi Syndrome has been reported.[6] To further study this possibility, post-mortem material would have to be collected from specific patients with sleep disturbances and clear-cut cataplexy. However, this is unlikely to occur in the near future. Another possibility would be to collect cerebrospinal fluid from these patients, since it can be expected that a loss of hypocretin large enough to result in cataplexy will also be reflected in low CSF levels.

Another explanation for sleep disturbances and cataplexy in Prader-Willi Syndrome might be found in malfunction of the hypocretin receptors. Therefore, we have tried to visualize the hypocretin receptor in post-mortem material from Prader-Willi Syndrome patients, but this proved to be a difficult task due to the low expression of the receptor, specificity problems with the available commercial antibodies and the fixation method of the available Prader-Willi Syndrome and control material. Future experiments should focus on visualizing the hypocretin receptor in frozen tissue using in situ hybridization or more specific antibodies.

Normal Aging

When the control subjects from the Prader-Willi Syndrome study were analyzed together, the total number of hypocretin neurons tended to decline with age.

> The number of hypothalamic hypocretin neurons tends to decline with age in healthy control subjects.
>
> ***Chapter 1***

Future perspectives

The finding that the total number of hypocretin neurons tends to decline with age, raises the interesting question whether this would mean that the hypocretin system would be affected to a greater extent in a brain showing advanced ageing, i.e. Alzheimer's Disease. In addition, it would be of interest to relate the number of hypocretin neurons in controls to the presence of the major histocompatibility complex (MHC) subtype of the immune system that is seen in more then 90% of all narcolepsy with cataplexy patients (HLA, Human Leukocyte Antigen, DQB1*0602). The majority of these patients are hypocretin deficient, which raises the intriguing question whether healthy controls with the same HLA type but without the narcoleptic phenotype, would have a partial loss of hypocretin.

Parkinson's Disease

Although Parkinson's Disease (PD) is primarily characterized by motor symptoms such as tremor and rigidity, sleep disturbances occur often, and include excessive daytime sleepiness, fragmented nocturnal sleep and rapid eye movement (REM)-sleep behavior disorder.[7,8] The combination of these symptoms suggests an overlapping etiology with narcolepsy.[9] Hypocretin levels in CSF were reported to be normal in Parkinson's Disease when samples were obtained using a spinal tap, [10-12] but another study reported low or even absent levels in ventricular CSF.[13] To assess hypocretin function in Parkinson's Disease we determined the total number of hypocretin containing neurons in nine PD patients and nine controls. Hypocretin levels were also determined in post-mortem ventricular CSF of these subjects. Furthermore, cortical brain tissue hypocretin levels were determined in nine PD patients and 16 controls. We found that the hypocretin system was affected in PD. The hypocretin concentration in the cortex was almost 40% lower in PD patients than in controls. Ventricular CSF levels were lower by almost 25%. The total number of hypocretin neurons was about one half of that of controls. In rodents, a reduction in the number of hypocretin neurons of 60-70% results in REM sleep disturbances, which suggests that the cell loss in PD can explain at least part of the sleep disturbances commonly seen in this disorder.

> The hypocretin system is affected in Parkinson's Disease. The total number of hypocretin neurons is 50% lower, the hypocretin concentration in the cortex is 40% lower and the concentration in ventricular CSF is 25% lower in Parkinson's Disease compared to controls. This could at least partly explain the sleep disturbances commonly seen in this disease.
>
> ***Chapter 2***

Future perspectives

The functional relevance of a loss of hypocretin neurons in Parkinson's Disease still needs to be studied. This could involve studying post-mortem hypothalami of Parkinson's Disease patients, combined with a thorough documentation of their sleep disturbances in the last few years of their lives. This would be a difficult task to accomplish. Regrettably, sleep problems, such as excessive daytime sleepiness and REM sleep behavior disorder, are not objectively documented in the clinical histories of the currently available post-mortem material in a way that permits systematic research. Another possibility for future research in patients with Parkinson's Disease could be to perform a treatment trial with narcolepsy medication, for instance gamma hydroxybutyrate or hypocretin-agonists when available for human use. Another option would be administration of the now available hypocretin antagonists to healthy controls to assess the effects of a partial loss of hypocretin neurotransmission.

Huntington's Disease

Huntington's Disease is a neurodegenerative genetic trinucleotide repeat disorder with a dominant mode of inheritance characterized by abnormal dance-like body movements (chorea) and personality changes. Furthermore, patients suffer from severe weight loss, sleep disturbances and autonomic dysfunction, which could partly be due to alterations in hypocretin signalling.[14] Although spinal CSF hypocretin levels were normal in human patients,[15-18] the density of hypocretin neurons was reported to be decreased in two mouse models of the disease.[19] In order to validate and extend these data in Huntington's Disease patients, we counted the total number of hypocretin neurons in 8 HD patients and 8 controls. Hypocretin levels were also measured in post-mortem ventricular CSF of these subjects. Furthermore, cortical brain tissue hypocretin levels were determined in 19 HD patients and 16 controls. Both the total number of hypocretin neurons and the hypocretin concentration in the cortex were 30% lower in HD patients. However, ventricular CSF hypocretin levels were similar to controls. This reduction in hypocretin signalling is in contrast with the strong reduction seen in the R6/2 mouse model of the disease and the contribution to the clinical symptoms of HD patients remains to be investigated.

> The number of hypocretin containing neurons is 30% reduced in Huntington's Disease. This is in contrast with the strong reduction seen in the R6/2 mouse model of the disease. The contribution to the symptoms of HD patients remains to be investigated.
>
> ***Chapter 3***

Future perspectives

As in Parkinson's Disease, the contribution of a loss of hypocretin neurons to the sleep disturbances seen in Huntington's Disease needs to be studied. Again, sleep problems in Huntington's Disease patients are neither well described nor objectively documented. As such, the currently available post-mortem material does not permit systematic research. As in Parkinson's Disease, narcolepsy medication may improve sleep symptoms in Huntington's Disease.

Narcolepsy: Screening for Autoantibodies

In narcolepsy there is a severe decrease (>95%) of hypocretin containing neurons in the lateral hypothalamus,[20] leading to a general absence of hypocretin in the cortex[21] and in CSF.[22] It is not known how these neurons disappear. The most popular hypothesis concerns an autoimmune process that selectively targets hypocretin neurons. However, no direct evidence for this putative autoimmune process has so far been found. We screened the CSF of 54 patients and the serum of 76 patients and 63 controls for the presence of autoantibodies directed against neurons in the lateral hypothalamus. Detectable autoantibodies were present in only two patients, but also in two controls. Therefore, as shown by immunostaining, humoral immune mechanisms appear not to

play a major role in the pathogenesis of narcolepsy, at least not in the clinically overt stage of the disease.

> As shown by immunostaining, no direct evidence has been found to support the prevailing theory that humoral immune mechanisms (autoantibodies) play a role in the selective loss of hypocretin containing neurons in narcolepsy.
>
> ***Chapter 4***

Future Perspectives

Since the screening was performed on formalin fixed paraffin embedded tissue, a future screening on frozen hypothalamic tissue, although technically challenging, might be advisable. At this stage, it is still a mystery why hypocretin disappears. Lacking evidence for an autoimmune hypothesis, it is important to keep an open mind regarding the cause of narcolepsy. T cells are a fundamental part of many auto-immune diseases, and cellular immune mechanisms should also be examined. In type 1 diabetes mellitus, islet-cell antibodies are abundant around disease onset, but 5 to 10 years later, titers are much lower. Because the symptoms of narcolepsy usually stabilize within the first few years, it is likely that any auto-immune process settles down over time. It is thus advisable to study serum and CSF very soon after disease onset, presumably when the inflammation is ongoing.

Narcolepsy: Trial with Intravenous Immunoglobulins

In line with the prevailing autoimmune theory to explain the pathogenesis of narcolepsy, treatment with high-dose prednisone after acute manifestation of hypocretin deficiency has been tried in an 8-year old boy.[23] This was not effective. However, two open-label studies suggested that treatment with intravenous immunoglobulins (IVIg) shortly after disease onset may dramatically reduce the frequency and severity of cataplexy.[24,25] We performed a double-blind N=1 study in a 55 year old female narcolepsy patient who was suffering from typical narcolepsy with severe cataplexy for 7 years. Open label treatment with IVIg resulted in what appeared to be a dramatic success. However, this striking effect disappeared during the subsequent double-blind placebo-controlled n=1 trial, in which there was no difference between placebo and IVIg treatment. Nevertheless, the placebo effect was impressive. The patient reported fewer cataplectic attacks after the first drug administration of the study, which concerned the placebo. Our findings stress the need for strict adherence to common methodological standards involving blinding and the use of a placebo for future trials.

> During a double-blind placebo-controlled n=1 trial there was no difference between placebo and IVIg treatment. The placebo effect was impressive.
>
> ***Chapter 5***

Future Perspectives

Regarding the effect of IVIg on cataplexy, a double-blind placebo controlled study is needed, in which well-documented baseline measurements are mandatory to control for placebo effects.

Part II: When Hypocretin Neurons are Absent: Narcolepsy

Vigilance

Excessive daytime sleepiness (EDS) is considered to be the main complaint in narcolepsy.[26] However, this focus on inadvertently falling asleep may have led to undervaluation of the perhaps most serious complaint: impaired performance in the waking state.[27] This realisation suggested that tests aiming to measure vigilance might be useful in narcolepsy. The Sustained Attention to Response Task (SART) appeared to be a good candidate.[28] This test takes only a short time to perform and is easy to administer, which make it useful in a clinical setting. To explore the role of the SART in quantifying vigilance as an essential aspect of the severity of narcolepsy, we compared the SART with 2 instruments commonly used to measure sleepiness: the MSLT [29] and the Epworth Sleepiness Scale (ESS).[30] We found that the SART, measuring attention, was abnormal as often as the MSLT, measuring sleepiness. Still, the two tests measure different aspects of the disease, as SART and MSLT results showed no correlation with each other or with the Epworth Sleepiness Scale. The range of the MSLT latency was considerably larger in controls than in patients, while the reverse applied to an even stronger degree for the range of the SART error scores.

> Difficulty in remaining vigilant during the day may be the most serious concern in narcolepsy, since it impairs performance. The Sustained Attention to Response Task quantifies this neglected symptom and is valid, easy to administer and takes little time to perform.
>
> ***Chapter 7***

Future Perspectives

Vigilance impairment is an important aspect of narcolepsy that deserves more attention. The fact that the range of SART results in narcoleptic patients is large, may be advantageous, in that it may offer a better resolution to quantify vigilance as a severity indicator of narcolepsy, which may be of use in measuring treatment effects. However, before the SART can be used in treatment trials and other studies, its sensitivity to treatment effects has to be studied in more detail. This could involve administration of the SART on multiple timepoints during several testing days as a long-term measure for vigilance before and after treatment in narcolepsy and possibly other sleep disorders.

Obesity

Obesity is a consistent feature of narcolepsy.[31] The identification of hypocretin deficiency as the cause of human narcolepsy with cataplexy and the potential role of hypocretin peptides in metabolic control has sparked interest in the pathophysiology of obesity in narcolepsy. Obviously, eating too much or moving too little are straightforward explanations. In contrast to such expectations narcoleptic subjects in fact consumed less food than healthy controls,[32] while there were no signs pointing to a reduced amount of physical activity.[33] Therefore, the link between hypocretin deficiency and obesity must be less straightforward than assumed. We studied basal metabolic rate and variation in blood pressure and heart rate in hypocretin-deficient narcoleptic subjects and healthy controls, hypothesizing that sympathetic tone might be diminished and/or that basal metabolic rate would be reduced in narcoleptic subjects. We did not find a reduced basal metabolic rate in narcoleptic subjects. However, we did find a higher variability in heart rate and blood pressure, which could point to a changed sympathetic tone. The role of this latter finding in the pathophysiology of obesity in narcolepsy remains to be elucidated.

> Using indirect calorimetry no reduced basal metabolic rate in hypocretin deficient narcoleptic humans can be detected. However, a higher variability in heart rate and blood pressure could point to a reduced sympathetic tone. The role of this latter finding in the pathophysiology of obesity in narcolepsy remains to be elucidated.
>
> ***Chapter 8***

Future Perspectives

We did not find a change in basal metabolic rate in human patients. A normal basal metabolic rate has also been observed in hypocretin knock-out rodents (C. Sinton, personal communication). However, it was found that energy expenditure is reduced in the ataxin-3 mouse model of narcolepsy, with profound sleep/wake fragmentation as the leading cause.[34] Indirect calorimetry in the fasted, rested state could be too insensitive to detect subtle changes in basal metabolic rate. It is thus of interest to study metabolism using more sensitive methods. A study using a 24-hour metabolic chamber in combination with doubly labelled water should provide the most reliable information about metabolism in narcoleptic patients. Furthermore, research might address autonomic regulation in narcolepsy. However, this is not an easy task, as the sympathetic and parasympathetic efferent command streams to relevant target organs cannot be measured directly. Concepts such as sympathetic tone and sympathovagal balance are frequently used, but difficult to validate. The finding of an increased variation in heart rate and blood pressure should be replicated.

Skin Temperature

In healthy subjects there is a relation between skin temperature and sleep. When the temperature of the distal skin (hands and feet) increases relative to the temperature

of the proximal skin, the process of falling asleep is facilitated.[35] This increase in the temperature of the hands and feet results from increased blood flow in the skin of the extremities and is, among other factors, controlled by the hypothalamic circadian clock, as is sleep.[36] Because narcolepsy is characterized by hypothalamic alterations, we studied skin temperature in narcoleptic patients. We found that the distal skin temperature was higher in narcoleptic subjects compared to healthy controls throughout the day in the waking state, while the proximal skin temperature was lower. The increase in the gradient between the distal and the proximal skin temperature (the distal-to-proximal gradient, DPG) was related to a shorter subsequent sleep latency. Once asleep, narcoleptics maintained their elevated distal skin temperature and DPG, whereas proximal skin temperature increased to reach normal levels. This dramatic alteration of daytime skin temperature control in narcolepsy suggests that hypocretin deficiency in narcolepsy affects skin temperature regulation, which in turn may affect sleep and vigilance.

The temperature of the distal skin (hands and feet) is higher in narcoleptic subjects in the waking state, while the temperature of the proximal skin is lower. These alterations are related to a shorter subsequent sleep-onset latency. Hypocretin deficiency affects skin temperature regulation.

Chapter 9

Our next goal was to investigate a contribution of skin temperature regulation disturbances to impairments in the ability to maintain vigilance and wakefulness, two major complaints of patients with narcolepsy. The Psychomotor Vigilance Task [37] and the Maintenance of Wakefulness Test [38] were repeatedly assessed, while skin and core body temperature were manipulated using a thermosuit and hot or cold food and drinks.[39] Compared to core cooling, core warming improved the time-on-task decline in Psychomotor Vigilance Task response speed by 25%. Slightly increasing core body temperature, -which was relatively low in the narcolepsy patients-, towards a more normal level, thus improves vigilance. As compared to distal skin warming, distal skin cooling increased the time that the patients were able to maintain wakefulness by 24%. Cooling the hands and feet and warming the proximal skin thus decreases daytime sleepiness in narcolepsy. Core body and skin temperatures causally affect vigilance and sleepiness in narcolepsy. This may have future therapeutic consequences.

In narcoleptic subjects vigilance can be improved by slightly increasing core body temperature towards normal levels, while sleepiness can be decreased by cooling the hands and feet and warming the proximal skin. Although these effects were moderate, this may have future therapeutic consequences.

Chapter 10

Apart from sleepiness and decreased vigilance, disturbed night time sleep is another core symptom of narcolepsy that can severely affect quality of life.[40] Nocturnal polysomnography shows a fragmentation of the normal sleep pattern and frequent arousals.[40,41] To investigate a causal contribution of temperature alterations to the

disturbed sleep in narcolepsy, we manipulated proximal and distal skin temperature during nocturnal polysomnography. Throughout the night, skin temperature was manipulated to slowly cycle within a range normally observed during sleep. The sleep-inducing combination of proximal skin warming and distal skin cooling led to a 160% increase in the duration of slow wave sleep, a 50% increase in REM-sleep and a 68% decrease in wakefulness, compared to the wakefulness-inducing combination of proximal skin cooling and distal skin warming (note, that due to the protocol used, temperature manipulations can only be compared to one another, but not to a 'thermoneutral' situation). These effects are similar in magnitude to the effects of the currently used hypnotic sodium oxybate (gamma hydroxybutyrate).[42] Skin temperature manipulations under controlled conditions thus ameliorated the typical nocturnal sleep problems of narcoleptic patients - i.e. they led to increased slow wave sleep and decreased wakefulness–, making their sleep more comparable to that of healthy persons. These results indicate that skin temperature control could have clinical relevance in the management of disturbed nocturnal sleep in narcolepsy.

In narcoleptic subjects night time sleep can be improved by subtle skin manipulations (distal skin cooling and proximal skin warming) that counteract the temperature alterations found earlier, making their sleep more comparable to that of healthy control subjects. Skin temperature control could have clinical relevance in the management of disturbed nocturnal sleep in narcolepsy.

Chapter 11

Future Perspectives

The studies into regulation and manipulation of temperature in narcolepsy were performed in a laboratory setting under strictly controlled circumstances. The extent to which such fairly subtle effects also occur in daily life is as yet unknown, suggesting the need for monitoring under normal circumstances in patients' homes. An intriguing consequence of monitoring thermoregulatory profiles could be the prediction of sleep

Table 1. Overview Part 1

	Hypocretin System			
	In Vivo	Post Mortem		
Disorder	Spinal CSF	Ventricular CSF	Tissue Level	Hcrt-1 IR Cell Number
Narcolepsy	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓ (-95%)
Prader-Willi Syndrome	=	=	=	=
Huntington's Disease	=	=	↓ (-30%)	↓ (-30%)
Parkinson's Disease	=	↓ (-25%)	↓ (-40%)	↓↓ (-50%)
Alzheimer's Disease	?	?	?	?
Normal Aging	=	?	?	=/↓?

CSF, Cerebrospinal Fluid; IR, immuno-reactie; hcrt-1, hypocretin 1.

attacks. Furthermore, the effects of manipulation of skin temperature on night time sleep are promising. These effects were obtained using slowly cycling manipulation patterns. The most optimal pattern found (proximal skin warming and distal skin cooling) might then be applied during multiple nights and compared to other patterns to confirm its beneficial effect in the long run.

General conclusions

Neuronal Loss and Narcoleptic Symptoms

As stated in the first part of this thesis, a loss of hypocretin containing neurons is not limited to narcolepsy, but also occurs in other disorders. The speed of the process that targets hypocretin containing neurons in narcolepsy is unknown, but the process is highly selective and complete, in contrast to the situation in Parkinson's and Huntington's Disease, where the hypocretin cell loss is not complete and where various neuronal populations are at risk. We know that other cell types are reduced to a lesser extent (for example the melanin-concentrating hormone neurons in Huntington's Disease in chapter 3), but maybe other cell types to an even greater extent (for example dopamine neurons in Parkinson's Disease) than the hypocretin containing neurons.

A complicating issue is the fact that it is difficult to distinguish a loss of neurons from a loss of a cell marker, such as hypocretin-1. Strictly speaking, a decreased number of neurons that express hypocretin-1 does not mean that there is actually a loss of these neurons. Neurons that used to express hypocretin-1 could still be present and functionally active. In fact, some researchers hypothesize that hypocretin neurons are not actually lost in narcolepsy, but just stop making hypocretin.[43] The fact that the expression of dynorphin and neuronal activity-regulated pentraxin (NARP), which are normally co-expressed by the majority of hypocretin neurons,[44,45] is also lost in narcoleptic hypothalami, does still not prove that the neurons are really gone. These cells may produce fewer peptides on a global level. However, despite this unanswered question, the functional consequences of a 'real' loss of neurons on the one hand or a loss of 'only' the marker on the other hand, are similar. But if the neurons that formerly produced hypocretin are still alive and can be turned intro active hypocretin neurons again, this could potentially be a way to cure narcolepsy.

The link between the complete narcoleptic phenotype and a complete loss of hypocretin has been well established. Hypocretin is undetectable in the spinal CSF of narcoleptic patients with cataplexy[22] and hypocretin knockout rodent models show the complete narcoleptic phenotype.[46] However, the exact relationship between loss of hypocretin containing neurons and the occurrence of clinical symptoms remains unknown. At this moment, it is impossible to quantify hypocretin neurons in vivo and as such the only clues originate from post-mortem research.

Some clues to solve this issue originate from research into disorders other than narcolepsy. In brains from patients who had been suffering from Parkinson's Disease for some years, the degree of cell loss in the substantia nigra pars compacta turned out to be at least 75%.[47,48] However, the question still remains how many dopamine neurons have to

be lost before clinical symptoms will start to appear. The only disorders in which in vivo evidence for the relationship between cell loss and function is available, are disorders of the motor neuron. In Amyotrophic Lateral Sclerosis (ALS) the number of remaining motor units can be estimated using an electrophysiological calculation method (Motor Unit Number Estimation, MUNE). It has been shown that subjects can lose up to 25% of their motor units without a reduction of muscle strength, meaning that function can be maintained through compensatory processes. [49]

Whether the moderate loss of hypocretin containing neurons in Parkinson's Disease and Huntington's Disease will lead to clinical symptoms remains an intriguing question. In rodents a loss of 60-70% of hypocretin neurons results in REM-sleep disturbances. This would imply that the more than 50% loss we found in Parkinson's Disease (reflected in lower ventricular CSF levels) could at least partially explain the sleep disturbances (excessive daytime sleepiness, REM-sleep Behavior Disorder) commonly seen in this disorder. The loss of hypocretin containing neurons in Huntington's Disease is less severe (with normal ventricular CSF levels) and is thus less likely to result in clinical symptoms.

The exact contribution of a loss of hypocretin neurons to sleep disturbances still needs to be studied. This should involve studying post-mortem hypothalami of patients, whose sleep disturbances were well documented during the last few years of their lives. Furthermore, we found an indication for a decrease in hypocretin cell number with age in the controls of our study looking into hypocretin functioning in Prader-Willi Syndrome. This raises the interesting question whether this would mean that the hypocretin system would be affected to an even greater extent in a brain showing advanced ageing, i.e. Alzheimer's Disease. Interestingly, preliminary observations pointing to changes in the hypocretin system in Alzheimer's Disease were indeed mentioned in an abstract by Harper et al.[50] Sleep disturbances and autonomic disorders are frequently present in Alzheimer's Disease.[51] A loss of hypocretin neurons could partly explain these phenomena. We are, therefore, currently examining the hypocretin system in normal ageing and in Alzheimer's Disease. In addition, as mentioned before, it would be of interest to relate the number of hypocretin neurons in controls to the presence of the HLA DQB1*0602 subtype of the immune system that is seen in more then 90% of all narcolepsy with cataplexy patients.

Body Temperature and Narcolepsy

In narcolepsy a relatively high skin temperature of the hands and feet compared to the temperature of the proximal skin is related to a shorter sleep latency. By manipulating skin and core body temperature we were able to influence sleepiness, vigilance and night time sleep. Note that the beneficial manipulations were all in a direction that counteracted the narcoleptic alterations in temperature that we found earlier. This indicates that the hypothalamic circuitry involved in the coupling between temperature and sleep are basically intact in narcolepsy and that manipulation of core body and skin temperature can causally affect sleep and vigilance in narcolepsy.

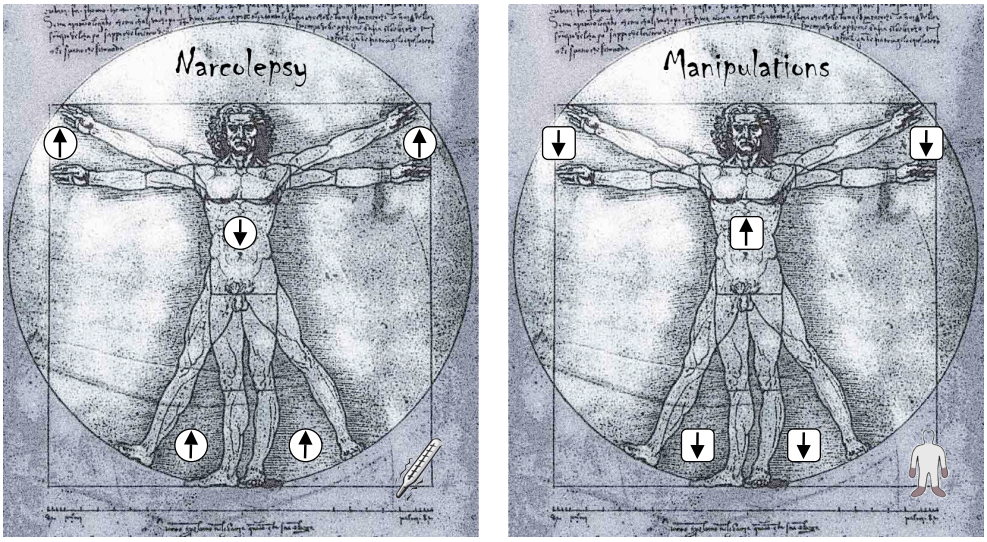

Figure 12.1 | **Overview Temperature Studies**
Scheme indicating the alterations in skin temperature control we found in narcolepsy (left, circles) and the manipulations of skin and core body temperature that had beneficial effects on vigilance, sleepiness and night time sleep (right, squares). Note that the beneficial manipulations (right) are all in the opposite direction compared to the alterations found (left).

Of course, these findings were obtained in a laboratory setting under strictly controlled circumstances. The narcoleptic alterations in skin temperature control and the beneficial effects of manipulation thus need to be replicated and confirmed in a different setting: outside the hospital and at home. As described in chapters 10 and 11, one could then even think of practical applications such as chairs or bedding that measure and differentially manipulate proximal and distal skin temperature. At this point, the findings can be summarised as follows: To stay alert, drink your hot coffee, but don't hold it, and hold your ice cream, but don't eat it.

Narcolepsy: Search for the Cause and Therapy

The last few years have seen an enormous increase in knowledge concerning the role of hypocretin in the regulation of sleep. However, more studies are needed to assess the effects of hypocretin deficiency on other important aspects: metabolism, vigilance, autonomic control and temperature regulation in humans.

Regarding narcolepsy, the two most exciting future fields of research are the search for the cause and the search for a new therapy.

Cause

As of yet no evidence for the autoimmune hypothesis has been found. However, immune mechanisms could still be involved. An environmental factor, such as a pathogen, may lead to a B- or T-cell mediated immune reaction and a selective cell

loss in genetically susceptible individuals. Nonetheless, it is important to keep an open mind regarding the cause of narcolepsy. It has been shown that hypocretin neurons are specifically vulnerable to N-methyl-D-aspartate (NMDA) mediated excitotoxicity.[52] One could also hypothesize that a loss of hypocretin is not directly responsible for the narcoleptic symptoms, but just an epiphenomenon of another, unknown disease process in narcolepsy. However, knock-out animal models of the disease show the complete narcoleptic phenotype including cataplexy,[46] which renders it less likely that additional factors are involved. Clues may be found in familial cases of narcolepsy with cataplexy without a loss of hypocretin neurons. However, it is most likely that mutations in those families will affect either the hypocretin signalling pathway or susceptibility to the narcolepsy disease proces. But families with multiple cases are scarce and rarely have more than two affected individuals.[53] Genome-wide studies have reported potential linkage to chromosome 4p13-q21 in eight small multiplexes, Japanese families,[54] and evidence for linkage to chromosome 21q in a large French family.[55]

The existence of narcolepsy without cataplexy, where hypocretin is most often present, shows that the narcoleptic phenotype of excessive daytime sleepiness with sleep-onset REM can occur without hypocretin deficiency. This raises the intruiging question whether *narcolepsy with cataplexy* and *narcolepsy without cataplexy* might be two completely distinct disease entities with different pathophysiological mechanisms. It even may be the case that what we now classify as *narcolepsy without cataplexy* is in fact no single disease entity. Some of these subjects will be narcolepsy patients that are going to develop cataplexy in the future, being most often hypocretin deficient. A few subjects in this group will have various other pathology causing excessive daytime sleepiness and sleep-onset REM. For many subjects, however, their complaints could be due to lifestyle (sleep deprivation). One could even hypothesize these complaints may be due to HLA DQB1*0602 positivity.[56]

Therapy

The most obvious new treatment option for narcolepsy with cataplexy would be administration of hypocretin. However, systemic hypocretin-1 hardly crosses the blood-brain barrier to produce therapeutic effects. So treatment with the peptide itself does not seem to be a good option. The development of hypocretin analogues that do reach the brain will be needed to further explore this therapeutic pathway in humans. Reportedly, pharmaceutical companies are working on a hypocretin agonist. However, it has been almost 8 years since the discovery that hypocretin deficiency is the cause of human narcolepsy with cataplexy. So far, it has been disappointingly silent on this topic.

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Nederlandse Samenvatting

Narcolepsie is een slaap/waak stoornis die gekarakteriseerd wordt door instabiliteit van waak en de verschillende slaapstadia. De klassieke symptomen van narcolepsie zijn overmatige slaperigheid overdag, kataplexie (een plotseling, symmetrisch verlies van spierspanning uitgelokt door sterke emotionele stimuli - zoals bij lachen - met behouden bewustzijn), hypnagoge hallucinaties (zeer levendige en vaak beangstigende droomervaringen die voorkomen bij de overgang van waak naar slaap of andersom) en slaapparalyse (waarbij een patiënt zich na het ontwaken of vlak voor het in slaap vallen niet kan bewegen maar wel wakker is). Andere belangrijke symptomen zijn een gefragmenteerde nachtslaap, verminderde alertheid en overgewicht. De oorzaak van narcolepsie is een gebrek aan hypocretine (ook wel orexine genaamd), waarschijnlijk veroorzaakt door een selectief verlies van de cellen in de hypothalamus die deze neurotransmitter maken. Bij patiënten met narcolepsie is hypocretine niet of nauwelijks aantoonbaar in het hersenvocht.

Deel 1 - De hypothalamus en hypocretine neuronen

In het eerste deel van dit proefschrift wordt het hypocretinesysteem bestudeerd in neurodegeneratieve aandoeningen die gepaard gaan met slaapstoornissen lijkend op die van narcolepsie, zoals de ziekten van Parkinson en Huntington. Ook wordt het hypocretinesysteem onderzocht bij het Prader-Willi syndroom.

Ziekte van Parkinson

Hoewel de ziekte van Parkinson primair gekenmerkt wordt door motorische symptomen, zoals tremor, traagheid en rigiditeit, komen ook slaapstoornissen frequent voor. Deze lijken op de symptomen van narcolepsie. De hypocretineconcentratie in het lumbale hersenvocht blijkt normaal te zijn. Echter, een verlaagde concentratie of zelfs afwezigheid van hypocretine in de hersenventrikels werd gerapporteerd. Wij bestudeerden post-mortem het hypocretinesysteem van negen patiënten met deze ziekte en negen controlepersonen. Het hypocretinesysteem bleek aangedaan bij de ziekte van Parkinson. Het aantal hypocretineneuronen is gemiddeld met 50% afgenomen. De hypocretineconcentratie in de cortex was 40% lager en de concentratie in het hersenvocht 25% lager dan bij gezonde controlepersonen. Deze bevindingen zouden de slaapstoornissen die vaak optreden bij de ziekte van Parkinson ten dele kunnen verklaren.

Ziekte van Huntington

De ziekte van Hungtinton is een neurodegeneratieve, genetisch bepaalde 'repeat'-stoornis met een dominante overerving, gekenmerkt door abnormale dans-achtige lichaamsbewegingen (chorea) en dementie. Patiënten hebben tevens last van ernstig gewichtsverlies, slaapstoornissen en autonome dysfunctie. Deze symptomen zouden

veroorzaakt kunnen worden door veranderingen in de neurotransmissie van hypocretine. Hoewel de hypocretineconcentratie normaal is in het spinale hersenvocht van patiënten, bleek de dichtheid van hypocretineneuronen verminderd bij twee muismodellen van deze ziekte. Om na te gaan of dit ook bij mensen het geval is, onderzochten wij post-mortem het hypocretinesysteem bij patiënten met de ziekte van Huntington en controlepersonen. Het aantal hypocretineneuronen en de hypocretineconcentratie in de cortex bleek 30% afgenomen. De concentratie in het hersenvocht uit de ventrikels was normaal. Deze bevinding staat in schril contrast met de sterke afname die aangetoond is bij het R6/2 muismodel van de ziekte. In hoeverre deze afname bijdraagt tot de symptomen van patiënten met de ziekte van Huntington moet verder worden onderzocht.

Prader-Willi Syndroom

Het Prader-Willi syndroom is een genetisch bepaald syndroom met mentale retardatie, hypogonadisme en groeideficiëntie. Het meest kernmerkende symptoom is onverzadigbare honger. Het is de meest voorkomende genetische oorzaak van overgewicht. Patiënten met dit syndroom hebben tevens last van slaapstoornissen en er zijn zelfs beschrijvingen van kataplexie. De hypocretineconcentratie in het hersenvocht is echter niet abnormaal laag. Wij bestudeerden de hypocretineneuronen in de hypothalamus van acht volwassenen en drie kinderen met het Prader-Willi syndroom en elf gezonde controlepersonen. Het aantal hypocretineneuronen bleek niet verlaagd bij patiënten. Het is onwaarschijnlijk dat een afname van het aantal hypocretineneuronen de oorzaak is van de overmatige slaperigheid en kataplexie die gerapporteerd worden bij dit syndroom.

Oorzaak van hypocretinecelverlies

Bij narcolepsie is er een verlies van meer dan 95% van hypocretine-bevattende neuronen in de laterale hypothalamus, met als gevolg een afwezigheid van hypocretine in het hersenvocht en de cortex. Het is onbekend hoe deze neuronen verdwijnen. Volgens de meest gehuldigde hypothese is een auto-immuun proces verantwoordelijk voor deze selectieve celdood. Wij onderzochten het hersenvocht van 54 narcolepsiepatiënten en het serum van 76 patiënten en 63 controlepersonen op de aanwezigheid van auto-antilichamen gericht tegen hypocretineneuronen in de laterale hypothalamus. Detecteerbare auto-antilichamen waren aanwezig bij twee patiënten, maar ook bij twee controlepersonen. Immuunkleuring leverde dan ook geen direct bewijs voor de theorie dat autoantilichamen een rol spelen bij het selectief verlies van hypocretineneuronen bij narcolepsie.

Behandeling met intraveneuze immunoglobulinen

In lijn met de heersende auto-immuun theorie om de pathogenese van narcolepsie te verklaren, heeft men geprobeerd een jongen van 8 jaar oud bij acute manifestatie van de symptomen van narcolepsie te behandelen met een hoge dosis prednison. Dit was niet effectief. Twee open onderzoeken met intraveneuze immunoglobulinen (IVIg) kort na aanvang van de ziekte lieten echter een dramatische reductie in de frequentie en ernst van kataplexie zien. Wij voerden een dubbelblinde, placebogecontroleerde n=1 studie uit in een patiënte die al zeven jaar last had van ernstige kataplexie die niet op medicatie reageerde. Een voorafgaande open behandeling met intraveneuze immunoglobulinen resulteerde in een dramatische verbetering. Dit effect verdween echter in de daarop

volgende dubbelblinde, placebogecontroleerde N=1 studie. Er was geen verschil tussen de placebo en de intraveneuze immunoglobuline behandeling. Het placebo-effect was indrukwekkend groot.

Deel 2 - Afwezigheid van hypocretine neuronen: Narcolepsie

In het tweede deel van dit proefschrift worden de gevolgen van het verlies van hypocretine onderzocht, met name wat betreft alertheid, metabolisme, het autonoom zenuwstelsel en temperatuurregulatie.

Alertheid

Overmatige slaperigheid overdag wordt beschouwd als het hoofdsymptoom van narcolepsie. Dit focus op ongewild in slaap vallen heeft ervoor gezorgd dat wellicht de meest serieuze klacht onderbelicht is gebleven, namelijk verminderde prestatie tijdens het wakker zijn. Dit gebrek aan vigilantie (alertheid) overdag mag worden beschouwd als een van de meest invaliderende klachten bij narcolepsie. Deze realisatie leidde tot het besef dat een test die dit aspect meet nuttig zou kunnen zijn. De Sustained Attention to Response Task (SART) leek een goede kandidaat, omdat deze test slechts vier minuten duurt en gemakkelijk uitgevoerd kan worden. We vergeleken de SART met twee instrumenten die worden gebruikt om slaperigheid te meten: de Multipele Slaap Latentie Test (MSLT) en de Epworth Sleepiness Scale (ESS). De SART bleek even vaak abnormaal als de MSLT en de ESS en bleek een ander aspect dan in slaap vallen te meten. Er bestond geen significante correlatie tussen de instrumenten. Geconcludeerd wordt dat de SART een valide, eenvoudig uit te voeren test is die weinig tijd kost en een vaak verwaarloosd aspect van narcolepsie kan kwantificeren.

Metabolisme en autonoom zenuwstelsel

Overwicht is een kenmerkend symptoom van narcolepsie met een vooralsnog onbekende oorzaak. Natuurlijk zijn te veel eten of te weinig bewegen verklaringen die al snel gesuggereerd worden in relatie tot narcolepsie. Tegen de verwachting in lijken narcolepsiepatiënten echter minder te eten dan gezonde controlepersonen, terwijl er geen overtuigende aanwijzingen waren voor een veranderd activiteitsniveau. De link tussen een gebrek aan hypocretine en overgewicht moet dus complexer zijn dan aanvankelijk werd gedacht. Wij bestudeerden het basaal metabolisme en autonome regulatie van hartslag en bloeddruk in hypocretine-deficiënte narcolepsiepatiënten en gezonde controlepersonen. We verwachtten een verlaagd basaal metabolisme en een verlaagde sympathische tonus bij de narcolepsie patiënten. Gebruik makend van indirecte calorimetrie kon echter geen verschil in basaal metabolisme gevonden worden. Narcolepsiepatiënten hadden wel een grotere variatie in hartslag en bloeddruk, hetgeen op een verlaagde sympathische tonus zou kunnen wijzen. De rol van deze bevinding in de pathofysiologie van het overgewicht bij narcolepsie moet nog opgehelderd worden.

Huidtemperatuur regulatie

Bij gezonde controlepersonen is er een relatie tussen huidtemperatuur en slaap. Wanneer de distale huidtemperatuur (handen en voeten) stijgt ten opzichte van de

proximale huidtemperatuur (romp), wordt in slaap vallen gefaciliteerd. Deze stijging van de huidtemperatuur wordt veroorzaakt door toegenomen doorbloeding van de huid van de extremiteiten en wordt onder andere gecontroleerd door de hypothalame circadiane klok, net zoals slaap. Omdat hypothalame veranderingen kenmerkend zijn voor narcolepsie, bestudeerden wij huidtemperatuur bij narcolepsie en gezonde controlepersonen in relatie tot slaap. We vonden dat de temperatuur van de distale huid hoger is bij patiënten met narcolepsie in wakkere toestand, terwijl de temperatuur van de proximale huid lager is. Deze veranderingen zijn geassocieerd met de verhoogde slaapneiging. Tijdens de slaap bleef de distale huidtemperatuur relatief hoog, terwijl de proximale huidtemperatuur normaliseerde. Geconcludeerd wordt dat een gebrek aan hypocretine de regulatie van huidtemperatuur beïnvloedt.

Ons volgende doel was te onderzoeken of veranderingen in huidtemperatuur bijdragen aan de klachten van narcolepsie. Hiertoe werden de Psychomotor Vigilance Task (PVT), die aandacht meet, en de Maintenance of Wakefulness Test (MWT), die meet hoe lang men wakker kan blijven overdag, herhaaldelijk uitgevoerd terwijl de lichaams- en huidtemperatuur gemanipuleerd werden door middel van een speciaal temperatuurpak en warme of koude hapjes en drankjes. Bij patiënten met narcolepsie was het mogelijk het aandachtsniveau overdag te verbeteren door de kerntemperatuur licht te verhogen tot een normaal niveau. Slaperigheid overdag kon verminderd worden door de handen en voeten te koelen. Alhoewel deze effecten beperkt van omvang waren, zou dit in de toekomst therapeutische consequenties kunnen hebben.

Naast slaperigheid en verminderde aandacht overdag, is een verstoorde nachtslaap een kernsymptoom van narcolepsie met een grote invloed op de kwaliteit van leven. Nachtelijke meting van de slaap laat een fragmentatie van het normale slaappatroon en frequent ontwaken zien. Om een causale relatie tussen temperatuurveranderingen en de verstoorde nachtslaap bij narcolepsie aan te tonen, manipuleerden we de proximale en distale huidtemperatuur door middel van het eerder genoemde temperatuurpak tijdens een nachtelijke meting van de slaap. Gedurende de nacht werd de huidtemperatuur zodanig gemanipuleerd dat deze langzaam schommelde binnen de grenzen die normaal tijdens de nacht geobserveerd worden. We waren op deze manier in staat de nachtslaap te verbeteren door het subtiel koelen van de distale huid en het verwarmen van de proximale huid, zodat de eerder bij narcolepsie gevonden veranderingen in huidtemperatuur werden tegengegaan. Op deze manier werd de nachtslaap van narcolepsiepatiënten meer vergelijkbaar met de nachtslaap van gezonde controlepersonen. Het manipuleren van de huidtemperatuur zou klinische relevantie kunnen hebben in het tegengaan van de verstoorde nachtslaap bij narcolepsie.

Curriculum Vitae

Rolf Fronczek was born February 10, 1981 in Sittard. In 1987, his education began at "Basisschool Hulsberg". He went to the "Sintermeerten College" in Heerlen in 1993, where he obtained his "gymnasium" diploma cum laude. In 1999 he started his study of medicine at Leiden University, where he passed his "propedeuse" level examination cum laude in 2000. While also working towards a minor in classical solo singing at the Royal Conservatory in The Hague through the Leiden Faculty of Arts, he obtained his Master's Degree in medicine in 2005. During those years, he worked as a Student Assistant as well, teaching microscopy, brain anatomy and body dissection. He started his PhD training as part of the Leiden University Medical Centre (LUMC) excellent student program at the Department of Neurology of the LUMC and the Netherlands Institute for Neurosciences in Amsterdam. During his PhD training, he worked as an Autopsy Assistant for the Netherlands Brain Bank. In 2006, the European Sleep Research Society (ESRS) awarded his temperature and sleep research with the Helgi Kristbjarnarson Award for innovative research. Part of his research was performed in the Beth Israel Deaconess Medical Centre of Harvard Medical School in Boston.

List of Publications

1. **Fronczek R**, Overeem S, Lee SY, Hegeman IM, van Pelt J, van Duinen SG, Lammers GJ, Swaab DF. Hypocretin (orexin) loss and sleep disturbances in Parkinson's Disease. Brain 2007, in press.

2. **Fronczek R**, Verschuuren J, Lammers GJ. Response to intravenous immunoglobulins and placebo in a patient with narcolepsy with cataplexy. J Neurol. 2007 Sep 4;

3. **Fronczek R**, van der Zande WL, van Dijk JG, Overeem S, Lammers GJ. [Narcolepsy: a new perspective on diagnosis and treatment]. Ned Tijdschr Geneeskd. 2007 Apr 14;151(15):856-61. Review. Dutch.

4. **Fronczek R**, Overeem S, Lee SY, Hegeman IM, van Pelt J, van Duinen SG, Lammers GJ, Swaab DF. Hypocretin (orexin) loss in Parkinson's disease. Brain. 2007 Jun;130(Pt 6):1577-85.

5. **Fronczek R**, Overeem S, Lammers GJ, van Dijk JG, Van Someren EJ. Altered skin-temperature regulation in narcolepsy relates to sleep propensity. Sleep. 2006 Nov 1;29(11):1444-9.

6. van Marken Lichtenbelt WD, Daanen HA, Wouters L, **Fronczek R**, Raymann RJ, Severens NM, Van Someren EJ. Evaluation of wireless determination of skin temperature using iButtons. Physiol Behav. 2006 Jul 30;88(4-5):489-97.

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8. **Fronczek R**, Middelkoop HA, van Dijk JG, Lammers GJ. Focusing on vigilance instead of sleepiness in the assessment of narcolepsy: high sensitivity of the Sustained Attention to Response Task (SART). Sleep. 2006 Feb 1;29(2):187-91.

9. **Fronczek R**, Lammers GJ, Balesar R, Unmehopa UA, Swaab DF. The number of hypothalamic hypocretin (orexin) neurons is not affected in Prader-Willi syndrome. J Clin Endocrinol Metab. 2005 Sep;90(9):5466-70.

10. **Fronczek R**, Raymann RJ, Romeijn N, Overeem S, Fischer M, van Dijk JG, Lammers GJ, Someren EJW. Manipulation of core body and skin temperature improves vigilance and maintenance of wakefulness in narcolepsy. Sleep, in press.

11. Aziz NA, **Fronczek R**, Maat-Schieman MLC, Unmehopa UA, Roelandse FWC, Overeem S, Van Duinen SG, Lammers GJ, Swaab DF, Roos RAC. Hypocretin and Melanin-Concentrating Hormone in Patients with Huntington Disease. Brain Pathology, in press.