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Unravelling narcolepsy : from pathophysiology to measuring treatment effects

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Citation

Heide, A. van der. (2017, May 24). *Unravelling narcolepsy : from pathophysiology to measuring treatment effects*. Retrieved from <https://hdl.handle.net/1887/49010>

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Title: Unravelling narcolepsy : from pathophysiology to measuring treatment effects

Issue Date: 2017-05-24



CHAPTER 3

IMMUNOHISTOCHEMICAL SCREENING FOR ANTIBODIES IN RECENT ONSET TYPE 1 NARCOLEPSY AND AFTER H1N1 VACCINATION

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Journal of Neuroimmunology 2015;283:58–62

ABSTRACT

Narcolepsy type 1 patients typically have undetectable hypocretin-1 levels in the cerebrospinal fluid (CSF), as a result of a selective loss of the hypocretin containing neurons in the hypothalamus. An autoimmune attack targeting hypothalamic hypocretin (orexin) neurons is hypothesised. So far, no direct evidence for an autoimmune attack was found. One of the major limitations of previous studies was that none included patients close to disease onset. We screened serum of 21 narcolepsy type 1 patients close to disease onset (median 11 months), including 8 H1N1 vaccinated patients, for antibodies against hypocretin neurons using immunohistochemistry. No autoantibodies against hypocretin neurons could be detected.

INTRODUCTION

Narcolepsy type 1 is a disorder of the regulation of sleep and wakefulness. Almost all narcolepsy type 1 patients have undetectable hypocretin-1 (orexin A) levels in the CSF.^{1,2}

Hypocretin (orexin) is a neuropeptide, produced by neurons located in the lateral and posterior hypothalamus, most abundant in the perifornical region.³⁻⁸ Post-mortem studies in narcolepsy demonstrated a selective loss of the hypocretin containing neurons in the hypothalamus,⁹⁻¹¹ explaining the undetectable CSF hypocretin-1 levels. The selectivity of this loss was supported by the fact that intermingling melanin-concentrating hormone neurons appeared to be unaffected.^{9,10} Although the mechanism behind this specific loss of hypocretin producing neurons has not been elucidated yet, an autoimmune attack targeting hypothalamic neurons that produce hypocretin is hypothesised.

This autoimmune hypothesis is mainly based on the tight association of narcolepsy with *HLA-DQB1*06.02*,^{12,13} and is supported by the identification of additional genetic factors that suggest T-cell involvement in narcolepsy: polymorphisms in the T-cell receptor alpha and beta locus (TCR α and TCR β), Cathepsin H (CTSH), Tumor Necrosis Factor (ligand) Superfamily member 4 (TNFSF4, also called OX40L), the purinergic receptor P2RY11, and the ZNF365 and IL10RB-IFNAR1 loci.¹⁴⁻¹⁷ Moreover, an increased incidence of narcolepsy was reported after infections with *Streptococcus pyogenes*, influenza type A virus, H1N1, and H1N1 vaccination.¹⁸⁻²²

In search for evidence for an autoimmune attack, several research groups (including our own) attempted to find autoantibodies against hypocretin neurons. Unfortunately, up to now none of them was convincingly successful.²³⁻²⁶ However, in 2010, three independent groups reported elevated levels of antibodies against a protein that is produced in hypocretin neurons, Tribbles homolog 2 (TRB2).²⁷⁻²⁹ Since TRB2 is not specific for hypocretin neurons, it is unlikely that these antibodies directly injure the hypocretin neurons. However, these antibodies may be a consequence of hypocretin neuronal damage or may be indirectly involved in the attack of hypocretin neurons.³⁰ In a recent study, sera and CSF from subjects suffering from narcolepsy and other sleep related disorders yielded three immunohistochemical staining patterns in the rat brains. However, none of them concerned hypocretin neurons and the patterns were not specific for narcolepsy.³¹

All these previous immunohistochemical studies were performed with serum or CSF derived from narcolepsy patients with a relatively long disease duration, or from patients with

unknown disease duration. Due to the delay after clinical onset in those studies, it might be possible that antibody levels already had decreased and no autoimmune response could be found anymore. To further investigate this aspect, we screened serum of narcolepsy type 1 patients close to disease onset, including H1N1 vaccinated patients, for antibodies against hypocretin neurons using immunohistochemistry.

MATERIALS AND METHODS

Subjects

We included 21 narcolepsy type 1 patients. Patients were recruited from the sleep clinic of the department of Neurology, Leiden University Medical Centre, Medisch Centrum Haaglanden, University Medical Centre Groningen and Erasmus MC University Medical Centre, The Netherlands. All patients suffered from narcolepsy type 1 according to the International Classification of Sleep Disorders (ICSD-3).³²

In addition, sera of 2 subjects without any medical condition were used as controls. Both were used in a previous study in which one of them demonstrated a consistent staining of hypothalamic neurons, and one no staining.²³ Serum of a narcolepsy patient who showed consistent staining of hypothalamic neurons in this same previous study was used as third control.

Brain tissue

Immunohistochemistry was performed on sections of encoded human hypothalamus and corpora mammillaria, obtained from the department of pathology of the Leiden University Medical Centre (3 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 formalin for 60–70 days, paraffin embedded and serially sectioned at 6 μ m. 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 deparaffinisation and rehydration, endogenous peroxidase activity was blocked in methanol-0.3% H₂O₂ for 20 minutes. After washes in TBS, sections were incubated for 1h at roomtemperature (room temperature) with the serum at a 1:400 dilution in supermix (0.05M Tris, 0.15M NaCl, 0.25% gelatin, 0.5% Triton X-100, pH7.6), and overnight at 4°C. The next day, after washes in TBS, sections were labelled with Goat anti-human/biotin in Supermix, dilution 1:4000, 1h at RT, followed by Avidin-Biotin Complex (Vectastain ABC-Elite Kit, Vector Lab, USA) in supermix for 30 minutes at RT. 3,3'-Diaminobenzidine (DAB) - Cobalt Chloride was used to visualise the staining.

Per subject sections of 7 different hypothalamic areas were stained. To identify the area of interest, of all these 7 areas, one section was stained with anti-orexin A/hypocretin-1 antibody (cat. no H-003-30, lot. no 01169-4, Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA). Slides incubated only with supermix served as negative control.

All sections were scored separately for staining by two blinded researchers (AvdH and RF).

RESULTS

Subjects (Table 3.1)

21 patients (13 males, 8 females) with an average age of 12 years (range 4–48 years) were included. All, but one patient, were close to disease onset, i.e. within three years after the onset of narcolepsy symptoms, with 33% of patients within 6 months of disease onset. The median duration of symptoms was 11 months (range 2–48 months). All patients suffered clear-cut cataplexy. 20 patients were HLA-typed and were positive for *HLA-DQB1*06:02*. In 17 patients hypocretin-1 was measured, all were hypocretin-1 deficient. The other four patients were expected to be hypocretin deficient as they suffered clear-cut cataplexy, had a non-familial type of narcolepsy, and were positive for *HLA-DQB1*06:02*.³³ 8 patients received a H1N1 vaccination prior to the onset of narcolepsy symptoms.

Table 3.1 Patient characteristics

	Age (years)	Sex ¹	Disease duration (months)	H1N1 vaccination ²	HLA- DQB1*060 ³	CSF Hypocretin-1 (pg/mL) ²	Staining
Patient							
1	7	M	2	-	+	≤110	-
2	4	F	2	Unknown	+	≤110	-
3	6	M	4	-	N.D.	≤110	-
4	6	M	5	+	+	≤110	-
5	7	M	5	-	+	≤110	-
6	12	F	6	+	+	≤110	-
7	4	F	6	+	+	≤110	-
8	5	F	9	+	+	≤110	-
9	9	F	10	-	+	≤110	-
10	19	F	10	+	+	≤110	-
11	17	M	11	+	+	N.D.	-
12	7	M	11	+	+	≤110	-
13	9	M	13	N.A.	+	N.D.	-
14	17	M	16	N.A.	+	N.D.	-
15	17	M	18	N.A.	+	≤110	-
16	6	M	18	+	+	≤110	-
17	10	F	27	N.A.	+	≤110	-
18	48	M	27	N.A.	+	≤110	-
19	16	M	27	N.A.	+	N.D.	-
20	7	M	36	N.A.	+	≤110	-
21	20	F	48	N.A.	+	≤110	-
Positive patient							
1	38	M	180	N.A.	+	≤110	+

Patient characteristics of the 21 included patients and 1 positive patient from the previous study.²³

¹ M: male, F: female.

² N.A.: Not applicable, i.e. patients with disease onset prior to the H1N1 vaccination campaign.

³ N.D.: Not determined.

Hypocretin staining

Hypocretin-1 positive neurons were found in all hypothalamic sections used (see Figure 3.1A for a representative section). Hypocretin-1 positive cell bodies were mainly located in the perifornical area of the lateral hypothalamus, as expected.²³

Screening immunohistochemistry

None of the 21 tested patient sera demonstrated consistent staining of neurons in the lateral hypothalamus or corpora mammillaria, i.e. no antibodies were detected, in particular not

in the patients closest to disease onset and in patients who received a vaccination against H1N1. The previous finding of consistent staining of hypothalamic neurons in one control subject and one narcolepsy subject,²³ was confirmed in the present study. The absence of staining in the previously described negative control was also confirmed. See Figure 3.1 for representative hypothalamic sections stained with the sera.

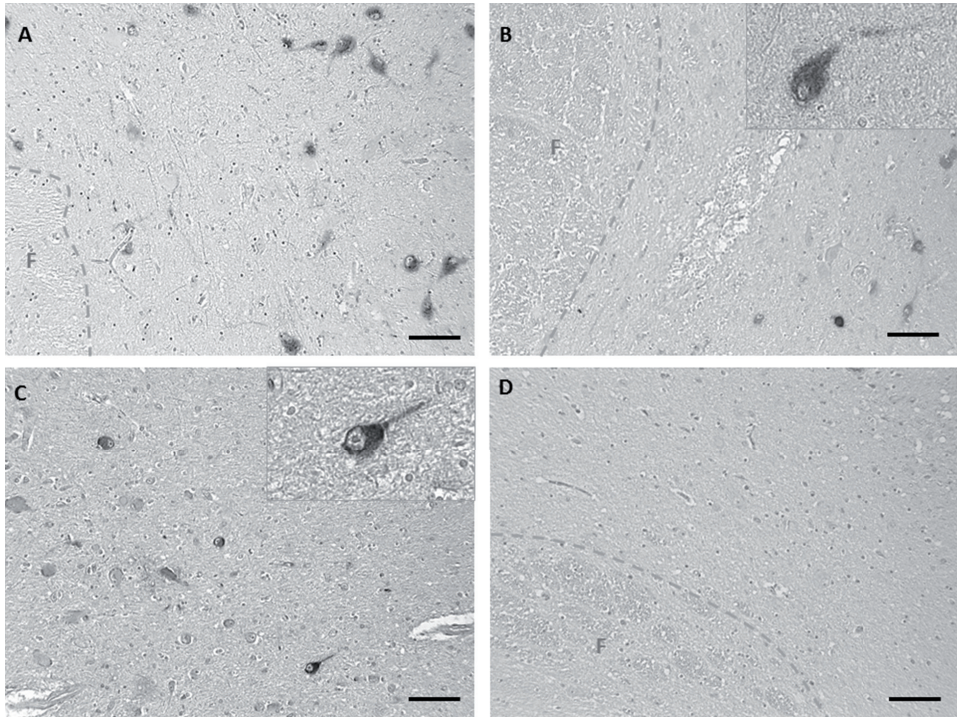


Figure 3.1 Representative examples of staining. Sections from the lateral hypothalamus stained with anti-hypocretin-1 (A), positive serum of the previously described narcolepsy with cataplexy patient (B), positive serum of the previously described control subject (C), and negative serum of 1 narcolepsy with cataplexy patient (D). Scale bars: 100 μ m. Abbreviations: F = fornix.

DISCUSSION

The aim of the present study was to find evidence for the autoimmune hypothesis in the aetiology of narcolepsy. To this end, serum of narcolepsy type 1 patients obtained close to disease onset was screened for antibodies against hypocretin neurons using immunohistochemistry. To our knowledge, this is the first study that included patients this close to disease onset.

To overcome the major limitation of these studies, i.e. the relatively long disease duration of the included patients, all patients included in the present study were close to disease onset (median 11 months). Despite this, no autoantibodies against hypocretin neurons could be detected.

The inability to detect autoantibodies in the current study does not contradict the autoimmune hypothesis; it even does not implicate an absence of autoantibodies at any time in the development of the disease. Narcolepsy with cataplexy is associated with loss of at least 90% of hypocretin containing neurons.¹⁰ It is currently presumed that narcoleptic symptoms start to occur when the vast majority of the hypocretin containing neurons have disappeared.³⁴ According to this hypothesis, the actual cell loss – i.e. the immune attack – precedes the appearance of narcoleptic symptoms. Therefore, the time window for detection of an autoimmune process might have passed by the time the patient is diagnosed. Screening patients as short after disease onset as we did, might be too late as well. Moreover, the usage of paraffin fixed hypothalamic sections could have masked the antibody epitopes.

Furthermore, narcolepsy is a disorder that seems to be confined to the central nervous system, autoantibodies produced in the brain probably hardly pass the blood-brain barrier. Subsequently, the concentration of autoantibodies might be very low in serum of narcolepsy patients. Theoretically, screening CSF could solve this problem, as the concentration of autoantibodies might be higher in CSF compared to serum. However, so far no hypocretin specific autoantibodies or signs of inflammation have been detected in CSF of narcolepsy patients at all.^{24-26,31,35,36} Moreover, when both serum and CSF were used for immunohistochemistry, CSF was used in a much higher concentration, while the staining pattern was similar, much weaker or even absent with CSF.^{23-26,31} Meanwhile, it is questionable whether narcolepsy is an antibody-mediated disease. Based on the genetic findings, narcolepsy probably is a T-cell mediated autoimmune disease,³⁷ and T-cell immunity is not necessarily associated with antibodies.

In conclusion, the present study demonstrates no evidence for autoantibodies in serum of narcolepsy patients close to disease onset and patients after H1N1 vaccination. This finding does not contradict the autoimmune hypothesis, nor implicates an absence of autoantibodies at any time in the development of the disease.

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