Hypocretin deficiency : neuronal loss and functional consequences

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Immunohistochemical Screening for Autoantibodies against Lateral Hypothalamic Neurons in Human Narcolepsy

CHAPTER 4

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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

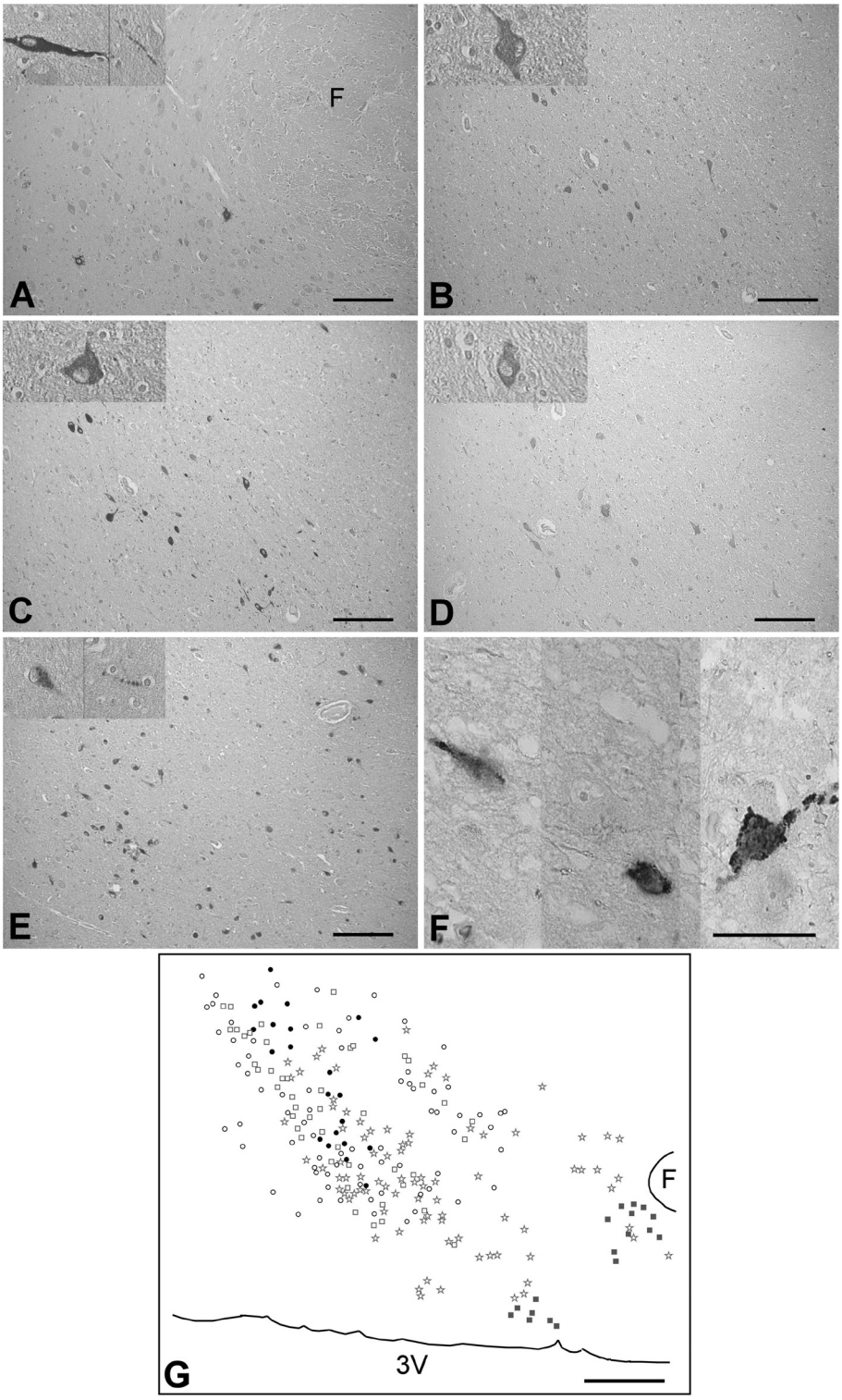
Hypocretin-1 positive neurons were found in all hypothalamic sections used (see Figure 4.1E for a representative section). Hypocretin-1 positive cell bodies were mainly located in the perifornical area of the lateral hypothalamus, as expected (Figure 4.1E and G). Fibers from hypocretin neurons, characterized 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
A
B
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D
E
F
F
3V
G

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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