Journal of Neuroimmunology 174 (2006) 187 – 191

Journal of Neuroimmunology

www.elsevier.com/locate/jneuroim

Short communication

Immunohistochemical screening for autoantibodies against lateral hypothalamic neurons in human narcolepsy

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Received 13 January 2006; received in revised form 10 February 2006; accepted 14 February 2006

Abstract

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. Autoantibodies were present in two patients, but also in two controls. However, one of the patients had a clearly different staining pattern and nerve endings of immunolabeled cells were found to project onto hypocretin-producing neurons, suggesting a possible pathophysiological role. Humoral immune mechanisms appear not to play a role in the pathogenesis of narcolepsy, at least not in the clinically overt stage of the disease.

Keywords: Narcolepsy; HLA; Orexin; Hypocretin; Autoimmunity; Hypothalamus

1. Introduction

Narcolepsy is a sleep disorder affecting approximately 5 per 10,000 people. Excessive daytime sleepiness, cataplexy and fragmented nocturnal sleep are the main symptoms, and pose a severe burden on the life of patients (Overeem et al., 2001; Scammell, 2003). In humans, the disease occurs mostly in a sporadic form, with genetic factors influencing susceptibility (Mignot, 1998). Narcolepsy is associated with certain human leucocyte antigen (HLA-) subtypes, with over 90% of patients being positive for HLA-DQB1*0602 (Lin et al., 2001). 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) (Nishino et al., 2000). Post-mortem studies showed that hypothalamic neurons staining for hypocretin were virtually absent in narcoleptics, probably due to a degenerative process (Peyron et al., 2000; Thannickal et al., 2000, 2003; Crocker et al., 2005; Blouin et al., 2005).

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

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doi:10.1016/j.jneuroim.2006.02.010

antibodies against lateral hypothalamic neurons using immunohistochemistry.

2. Methods

2.1. 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. Clear-cut cataplexy was present in 67 patients (88%). In 54 patients, CSF was available so hypocretin-1 levels could be measured as previously described (Ripley et al., 2001): 46 had undetectable levels. From 59 patients, HLA typing was known and 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.

2.2. 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 [three male subjects who died of non-neurological disease, age 63 (post-mortem delay [PMD] 1.7 h), age 37 (PMD 5 h) and age 48 (PMD 19 h), respectively]. Hypothalami were freshly dissected, fixed in buffered formalin 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.

2.3. Screening immunohistochemistry

After deparaffinization and rehydration, endogenous peroxidase activity was blocked in methanol–0.3% H_2O_2 for 20 min. Sections were pre-incubated in Tris-buffered saline (TBS)–10% normal goat serum for half an hour and then incubated with patient or control 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 1 h at RT and labeled with ABC-Elite kit (Vector) in supermix for 30 min 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.

2.4. 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 (Goldstone et al., 2002) and a 48-h incubation in patient serum. The reaction was visualized using AEC (3-amino-9-ethylcarbazole) solution (5 mg/ml, Vector) for 20 min. 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 h and fast blue solution (Sigma Chemicals, Zwijndrecht, Holland) 2 mg/ml in Tris–HCl for 5 min.

2.5. Enzyme-linked immunoadsorbent assay (ELISA)

ELISA was setup according to standard protocols (Verschuuren et al., 1991). 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 min at 37 °C. Peroxidase-conjugated 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, two wells were primarily stained using rabbit–anti-hypocretin-1 or -2 at 1:4000 and secondly with peroxidase-conjugated 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 1000 A.

3. Results

3.1. Hypocretin staining

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

3.2. Screening immunohistochemistry

From the 76 patient sera, we found two that consistently stained neurons in the lateral and tuberal hypothalamus.

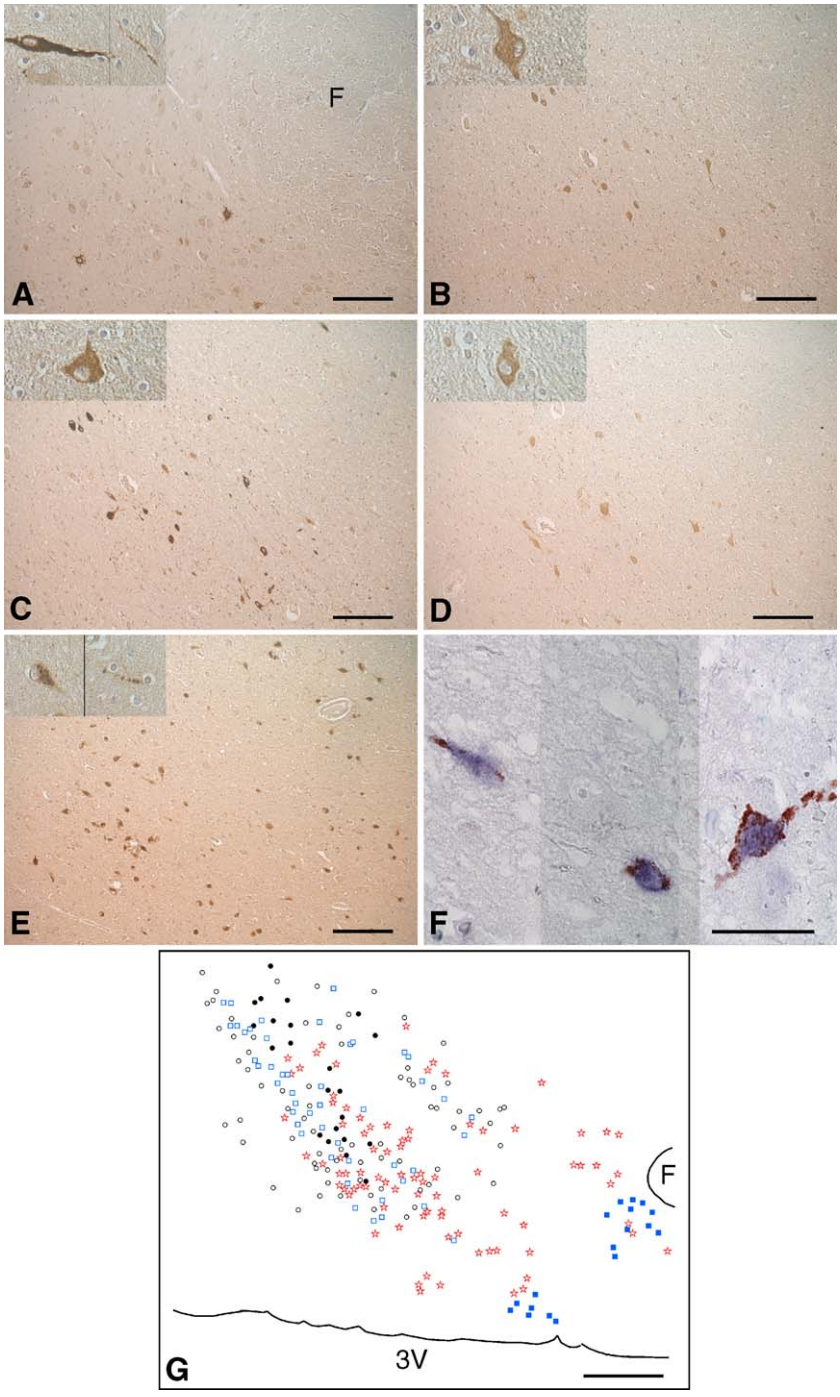

Fig. 1. Adjacent sections from the lateral hypothalamus, stained with positive sera from two patients (A, B), two controls (C, D) and anti-hypocretin-1 (E). Magnifications of representative neurons are shown in the top left corners. (G) Sketch, overdrawn from the adjacent sections, to compare the distribution of stained cells and to indicate the relative number of neurons stained in the lateral hypothalamus (hypocretin neurons [red stars], patient 1 [blue filled squares], patient 2 [open squares], control 1 [open circles], control 2 [black filled circles]). (F) Three parts from sections double-stained with anti-hypocretin-1 (blue) and serum of patient 1 (red). Note that no cell bodies are double-stained. There are multiple bouton-like structures staining red, in close proximity of hypocretin cell bodies, suggesting nerve endings (Li et al., 2002). Scale bars: (A–E) 200 μm, (F) 50 μm, (G) 1000 μm. Abbreviations: F = fornix, 3V = third ventricle.

However, we also found two control subjects with similar immunoreactivity. Both patients had clear-cut cataplexy and had no detectable hypocretin-1 levels in the CSF. See Fig. 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 (Fig. 1A and F). Furthermore, multiple neuronal fibers were visible, including ones with boutons closely resembling those of hypocretin neurons (Fig. 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 (Fig. 1B–D and F). None of these sera stained neuronal fibers resembling those of hypocretin neurons, although the distribution of positive neurons virtually overlapped with the hypocretin field (Fig. 1G).

3.3. Double-staining experiment

We further characterized the staining patterns by double-labeling 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 (Fig. 1F).

3.4. Enzyme-linked immunoadsorbent assay

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

4. 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 (Peyron et al., 2000; Thannickal et al., 2000; Crocker et al., 2005; Blouin et al., 2005). 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 (Lin et al., 2001) and several studies screening for previously described neuronal antibodies were negative (Rubin et al., 1988; Black et al., 2002; Overeem et al., 2003). Smith et al. (2004) 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.

Recently, the first study specifically looking for antibodies against the hypocretin peptides and some of their cleavage products was performed, with negative results (Black et al., 2005b,c). 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. (2005a) found that CSF from narcoleptic subjects showed immunoreactivity to rat hypothalamic protein extract on a group level. 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 two 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 (Li et al., 2002). Future experiments may shed more light on this.

Our study has some technical limitations. For example, it may be possible that the hypothalamic sections we used here (from three normal brains) lacked key antigens involved in narcolepsy. Furthermore, we used paraffin-embedded tissue, which may have altered the sensitivity of immunostaining. Finally, after the first few months of years, the symptoms of narcolepsy typically remain stable. Thus, it may be possible that patients only produce autoantibodies around disease onset.

In conclusion, we found no disease-specific increase in the presence of autoantibodies against lateral hypothalamic neurons. 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.

Acknowledgments

This study was supported by a grant from the Hersenstichting Nederland to G.J. Lammers (project number 9F01-(2)35). S. Overeem is supported by a Veni grant from the Netherlands Organisation for Scientific Research (grant no. 916.56.103). We thank P.H. de Meijer, A.J. Fogteloo and P. Kok for providing control serum samples, B.R. Roep en E. de Heer for stimulating discussions, A.M. van der Wal for technical assistance with the ELISA, and J.J. van Heerikhuize for assistance with slide photography.

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