

Antibodies against hypocretin receptor 2 are rare in narcolepsy

Subtitle: HCRTR2 antibodies in narcolepsy

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Abstract

Study Objectives. Recently, antibodies to the hypocretin receptor 2 (HCRTR2-Abs) were reported in a high proportion of narcolepsy patients who developed the disease following Pandemrix® vaccination. We tested a group of narcolepsy patients for the HCRTR2-Abs using a newly established cell-based assay.

Methods. Sera from 50 narcolepsy type 1 (NT1) and 11 with type 2 (NT2), 22 patients with other sleep disorders, 15 healthy controls and 93 disease controls were studied. CSFs from 3 narcoleptic patients were also subsequently included. Human embryonic kidney cells were transiently transfected with human HCRTR2, incubated with patients' sera for 1 hour at 1:20 dilution and then fixed. Binding of antibodies was detected by fluorescently-labelled secondary antibodies to human IgG and the different IgG subclasses. A non-linear visual scoring system was used from 0 to 4; samples scoring ≥ 1 were considered positive.

Results. Only 3/61 patients (5%) showed a score ≥ 1 , one with IgG1- and two with IgG3-antibodies, but titers were low (1:40 – 1:100). CSFs from these patients were negative. The three positive patients included one NT1 case with associated psychotic features, one NT2 patient and a NT1 patient with normal hypocretin CSF levels.

Conclusions. Low levels of IgG1 or IgG3 antibodies against HCRTR2 were found in 3/61 patients with narcolepsy, although only one presenting with a full-blown NT1. HCRTR2-Abs are not common in narcolepsy and their clinical and pathogenic significance is unclear.

Keywords: narcolepsy, antibodies, HCRTR2, autoimmune

Statement of significance. Very low titre antibodies against HCRTR2 are rare in patients with narcolepsy and no clinical association was identified.

Introduction

Narcolepsy is a rare central disorder of hypersomnolence mainly characterized by excessive daytime sleepiness (EDS) and cataplexy, a sudden loss of muscle tone triggered by strong emotions. Other features include hallucinations at sleep onset and awakening, and sleep paralysis¹. Two subtypes of the disease are recognized, narcolepsy type 1 (NT1) and narcolepsy type 2 (NT2). NT1 is associated with loss of hypocretin (HCRT) secreting neurons in the lateral hypothalamus²⁻⁴ without a parallel loss of neurons expressing melanin-concentrating hormone^{3,4}. The strong HLA DRB1*06:02 association^{5,6} suggests an autoimmune driven process⁷. Both NT2 and NT1 have EDS with sleep onset REM periods. However, in NT2 the hypocretin-1 CSF levels are reduced in only 10 to 24%^{8,9} and the HLA DRB1*06:02 association is weaker; present in only 40-50%^{10,11} of patients. Therefore the pathophysiology of NT2 remains elusive.

The role of autoimmunity in NT2(?) has been strengthened by the increased rates of narcolepsy onset in children following streptococcus pyogenes¹² and influenza A H1N1 infections,¹³ as well as after exposure to selected H1N1 vaccine preparations such as Pandemrix®¹⁴. These observations suggest that, in an appropriate genetic setting, exposure to some infections can lead, through unknown mechanisms, to a hypocretin-neuron specific autoimmune destruction. There is no evidence for disease specific antibodies directed against neuronal¹⁵⁻²⁰ or non-neuronal autoantigens²¹ in any form of narcolepsy, but a recent study noted homology between the H1N1 influenza virus nucleoprotein A and the first extracellular domain of the hypocretin receptor 2 (HCRTR2). Antibodies to the HCRTR2 (HCRTR2-Abs) were found in 85.0% of NT1 patients with a history of Pandemrix® vaccination, although also in up to 34.7% of the non-narcolepsy control groups²². The diagnostic and pathological significance of the antibodies in vaccine-related narcolepsy is, therefore, not clear²³. Moreover, other narcolepsy patient groups such as NT2(?) were not investigated.

We used a live cell-based assay approach, as now used in many diagnostic antibody tests²⁴, to establish an antibody test for HCRTR2-Abs and tested sera from patients with narcolepsy, other sleep disorders, healthy and disease controls.

Materials and methods

Patients and serum samples

Sera from sixty one narcolepsy patients (41 adults, 20 children), including 50 patients with NT1 and 11 with NT2, 22 patients with other sleep disorders (11 patients with Idiopathic Hypersomnia (IH) and 11 patients complaining of EDS with normal sleep studies (subjective-EDS, sEDS), 15 healthy controls (HC) and 93 disease controls (39 epileptic and 54 patients with known positivity for neuronal surface antigens - i.e. AQ4, CASPR2, LGI1, MOG) were studied (and CSF from ...). Sleep disorders and epileptic patients' sera and CSFs, when available, were retrieved from the Sleep Center of the Neurologic clinic of Bologna. Antibody positive and healthy control sera, all anonymized, were retrieved from the Neuroimmunology lab of the Nuffield Department of Clinical Neurosciences. Narcolepsy and IH were diagnosed accordingly to the International Classification of Sleep Disorders, 3rd edition²⁵. Subjective daytime sleepiness was assessed using the Epworth Sleepiness Scale (ESS). All patients underwent MSLT the day after 48 hours continuous polysomnography (PSG). CSF HCRT-1 levels and HLA status at locus DQB1*0602 were available in all narcoleptic patients. Local ethical committee approved the study and written informed consent was obtained from all study participants.

Plasmid construct and transfection of human embryonic kidney cells

Human *HCRT2* cDNA in the pcDNA3.1(-) mammalian expression vector was used for transient transfection of human embryonic kidney (HEK) 293 cells. Briefly, HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma®) supplemented with 10% fetal calf serum (FCS) (Biowest®) and 1% each of penicillin G and streptomycin in 175 cm³ tissue culture flasks at 37°C 5% CO₂. 4.6×10^5 HEK cells were seeded into 6 well plates containing 4 borosilicate glass coverslips (13 mm, VWR) previously coated with poly-L-lysine. 24 hours after seeding, at about 60% of confluence, the cells were transiently transfected with the *HCRT2* encoding plasmid. A total of 3 µg of DNA was diluted in 50 µL DMEM, 0.82 µL of 20% glucose and 1.5 µL of polyethylenimine per well. After 12-16 hours of incubation (37°C) the media was replaced and cells cultured for a further 24 hours before use.

HCRT2-IgG cell based assay

A cell-based assay (CBA) for the HCRT2-Abs was performed as for other routine antibody assays in the Oxford diagnostic laboratory. Briefly, coverslips were incubated either with commercial goat anti-*HCRT2* antibody (Abcam, ab65093, 1:300 dilution) or with patient or control serum (1:20) or

CSF (1:3), in incubation buffer (1% bovine serum albumin (BSA), 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) in DMEM) at room temperature (RT) for 1 hour. After washing the cells were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 10 minutes at room temperature, washed and incubated with the appropriate secondary antibodies: donkey anti-goat IgG (H+L) Alexa Fluor® 568 conjugated antibody (1:750) or goat anti-human IgG (H+L)-Alexa Fluor® 568-conjugated antibody (1:750) (ThermoFisher scientific).

To confirm the presence of IgG antibodies, goat anti-human IgG-Fc (1:750) or mouse anti-human IgG1, IgG2, IgG3 or IgG4 (Sigma) (1:50) were used followed by donkey anti-goat IgG-Alexa Fluor® 568 goat or anti-mouse IgG-Alexa Fluor® 568 conjugated secondary antibody (1:750). The presence of IgM antibodies was assessed by goat anti-human IgM Alexa Fluor® 568-conjugated antibody (Thermo Scientific, #31125) (1:750).

Immunofluorescence analysis

Coverslips were coded and the immunofluorescence was scored for the frequency and intensity of surface antibody binding, using a visual non-linear scoring system from 0 to 4, as previously described²⁶. All samples were tested twice, any serum showing a score ≥ 0.5 was repeated at least 3 times. Samples scoring ≥ 1 were considered positive and evaluated for specificity by testing against at least one other antigen.

Statistical analysis

The data were analyzed using GraphPad PRISM 6.

Results

Clinical data

The clinical data, the HCRT-1 levels and DQB1*0602 status, when available, are given in the Table. Among narcolepsy patients, eighteen (7 children) were considered to have had an acute onset (≤ 3 months elapsed between EDS and cataplexy onset) and in all but one cataplexy and EDS appeared in the same month. The interval between onset and serum sampling, however, was highly variable (see Table 1), and only 4 acute patients were sampled at 2 (3 patients) and 4 (1 patient) months after onset.

The narcolepsy cohort included rare cases with uncommon combinations of clinical features, HLA status and CSF HCRT-1 levels, and atypical cases, including patients with NT1 and associated psychosis (Table 1). One patient presented NT1 after receiving the Focetria® H1N1 vaccine.

HCRT2 antibody detection

HCRT2 was expressed on the surface of the live HEK cells as shown by strong binding of the commercial antibody against the extracellular domain of the receptor (Figure 1A). All control sera were negative for binding to HCRT2. Only 3/61 narcolepsy (5%) sera showed a positive score of ≥ 1 (Figure 1B, C). Serum dilutions were performed in the 3 sera scoring ≥ 1 and none of them reached a score of 1 at a dilution beyond 1:100 (Figure 1 D). CSFs from the three positive patients were negative.

The presence of IgG antibodies was confirmed using an IgG-Fc secondary antibody and also by determining the IgG subclasses in the three positive patients (Figure 2A, B). IgG3 HCRT2-Abs were present in two patients (one patient with NT1 and one patient with NT2). The third patient (NT1) had IgG1 and IgG2 HCRT2-Abs. IgM antibodies were negative in each case (data not shown).

The clinical features of these 3 patients are summarized in Table 1. Patient #1 presented with a progressive psychiatric disorder with severe psychotic features after the diagnosis of NT1. Patient #2 presented at age 16 with severe and progressively worsening EDS, daily nightmares and rare episodes of sleep paralysis. From age 17 she developed episodes of muscle tone loss induced by emotions. Her MSLT (performed twice) was typical for the diagnosis of narcolepsy but CSF hypocretin-1 levels were normal and the patient did not carry the HLA DQB*06:02. The clear-cut cataplexy and the typical MSLT and clinical history, lead to a diagnosis of NT1. Patient #3 presented with EDS since childhood; she never experienced cataplexy and hypocretin CSF levels were normal, but her MSLT supported the diagnosis of NT2.

Discussion

There has been much interest in looking for antibodies to potential neuronal targets in narcolepsy, and a recent study identified autoantibodies against the HCRT2 in patients with post-Pandemrix® narcolepsy. We established a cell-based assay to detect these antibodies but only 3 (5%) narcolepsy patients had IgG antibodies to HCRT2. Moreover, the titers were low in all 3, and no HCRT2-Abs were found in their CSFs. Only 1/3 had classical NT1, and that patient also had psychosis. Tanaka et al.¹⁸ also found a low proportion (5/181 patients; 4 NT1 and 1 NT2) of

patients with antibodies immunoprecipitating [³⁵S]-HCRTR2. Thus HCRTR2-Abs in patients with idiopathic narcolepsy appear to be rare. This, together with the low titers in serum and the absence of CSF antibodies, which are usually present in patients with typical antibody-mediated diseases,²⁴ suggest that they may not be clinically relevant.

There are, however, other possible reasons for the low titers and the absence in the CSF. None of the three patients with HCRTR2-Abs were acute onset, a time at which the initiating immune response might be more evident. The absence in CSF may be related to the relatively low titers found in the serum where total IgG levels and most systemic antibodies are typically 300-400 times higher than those in the CSF.

Our and Tanaka and colleagues¹⁸ results contrast with the much higher rate (85%) reported in patients post-Pandemrix® vaccination, although also in 34.7% controls²². Our results could reflect different methodologies since we used a live-cell assay with visual inspection of the binding whereas the Pandemrix® study used a cell-based ELISA²². Also, the different frequency may suggest a major role of these antibodies in vaccine related narcolepsy but not in idiopathic cases. The different frequency may suggest a major role of these antibodies in vaccine related narcolepsy but not in idiopathic cases or at least not in typical patients. Indeed, all our positives were atypical, as one presented NT1 and psychosis and two were DQB1*0602 negative including one patient initially diagnosed with NT2 who subsequently developed cataplexy despite normal HCRT-1 CSF levels. Rare cases of narcolepsy-like syndromes, often atypical, have been observed in the course of antibody-mediated disorders which in turn can be associated with low HCRT-1 levels, suggestive of a specific vulnerability of the hypocretin neurons independent from the HLA status^{27,28}. Considering the high frequency of the DQB1*0602 allele in narcolepsy the finding of antibodies in two HLA negative patients supports a phenomenon occurring only in a rare subgroup of patients, which may be unrelated to classical narcolepsy.

Moreover, a major question regarding HCRTR2-Abs relates to whether they could be responsible for the loss of the HCRT-producing neurons in narcolepsy, and where they might act in the brain. The pathogenic potential of autoantibodies is determined not only by their titers but also by their isotype. IgG1 and IgG3 are the most efficient in promoting C1q deposition and complement-dependent cytotoxicity. Therefore, if these antibodies are able to access the brain parenchyma, they could bind to and cause complement-mediated damage to HCRTR2 expressing cells. One would need to postulate, however, that the hypocretin neurons, which appear to be selectively destroyed in typical narcolepsy,^{3,4} express autoreceptors making the cells vulnerable to the effects of the antibodies. This is controversial; Yamanaka and coworkers, using a neurophysiological/immunoelectron microscopic approach, found that orexin neurons are directly

and indirectly activated by hypocretin via the HCRT2²⁹. By contrast, however, Vassalli et al. failed to demonstrate the expression of HCRT2 on mouse hypocretin neurons²³. Alternatively, since hypocretin neurons project widely in the brain where they modulate several crucial functions³⁰, such as sleep and wake, feeding behavior and energy homeostasis, autonomic function and reward system regulation, it is possible that the neuronal damage is not limited to the hypocretin neurons themselves but also affects other neurons that receive the hypocretin-signals via HCRT1 and HCRT2. One postmortem narcolepsy³¹ study suggested that gliosis was not restricted to the lateral hypothalamus which would be consistent with more widespread expression of an autoantigen. Until these issues have been resolved, the significance of any HCRT2-Abs, even in a subset of patients with narcolepsy, needs to be interpreted with caution.

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Figure Legend

Figure 1. (A) Representative image of a CBA using the commercial antibody or (B) a positive patient serum (Patient #1). Note that the intensity of binding is similar between A and B but the commercial antibody has been diluted 300 fold, and the patient serum only 20 fold. (C) Screening of the 191 serum samples using anti-IgG secondary antibody detected only 3 narcoleptic patients with scores of 1 or above. (D). Serial dilutions of the sera showed that the endpoint dilutions, giving a score of 1, were only 1:40 or 1:100 indicating low antibody titers. The results are given as mean \pm SEM of 3 or more determinations. (D). CBA= cell based assay; DC= disease controls; AI= autoimmune controls.

Figure 2. The three positive sera were tested for IgG, using a IgG specific anti-IgG Fc antibody and for the different IgG subclasses. Two patients had mainly IgG3 (A, Patient #1) and one mainly IgG1 and 2 (B, Patient #3). The results are given as mean \pm SEM (C).

Table 1. Demographics, CSF Hcrt-1 levels and HLA DQB1*0602 status

Diagnosis	N. of patients	Sex (female /male)	Age EDS at onset (mean \pm SD)	Time to cataplexy (mean \pm SD)	Time to sampling (mean \pm SD)	HLA-DQB1*0602	Hcrt-1 levels	Additional features
NT1	47	17/30	18 \pm 11.3	2.6 \pm 4.5	12.6 \pm 11.1	+	<110	Psychosis (n=3); post-vaccine (n=1)
*	1	M	15	12	23	+	<110	Psychosis, HCRTR positive (IgG3) (Patient 1)
	1	F	14	0	1	-	<110	Psychosis
*	1	F	16	1	7	-	Normal	HCRTR positive (IgG1>IgG3) (Patient 2)
NT2	5	3/2	27.5 \pm 14.1	-	7.7 \pm 5.7	-	Normal	
*	1	F	childhood	-	-	-	Normal	HCRTR positive (IgG3) (Patient 3)
	4	2/2	12 \pm 4.3	-	8 \pm 8.1	+	Normal	
	1	M	6	-	18	+	<110	
IH	10	3/7	21.1 \pm 16.4	-	20.2 \pm 17.3	-	Normal	
	1	F	20	-	11	+	Normal	

sEDS	9	4/5	20.5 ± 13.7	-	10.4 ± 8.1	-	Normal	
	2	1/1	27.5 ± 10.6	-	8.5 ± 9.1	+	Normal	
HC	15	-	-	-	-	NA	NA	
DS	39	-	-	-	-	NA	NA	
AI	54	-	-	-	-	NA	NA	

Hcrt-1= hypocretin 1; Normal levels > 200 pg/ml; NA= not available, DS= disease controls; AI= autoimmune controls. * indicates patients positive for HCRTR2-Abs.