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CACNA1H antibodies associated with headache with neurological deficits and cerebrospinal fluid lymphocytosis (HaNDL)

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Abstract

Background: Patients with the syndrome of headache with neurological deficits and lymphocytosis (HaNDL) typically present with recurrent and temporary attacks of neurological symptoms and cerebrospinal fluid lymphocytosis.
Aim and methods: To identify potential HaNDL-associated antibodies directed against neuronal surface and/or synapse antigens, sera of four HaNDL patients and controls were screened with indirect immunohistochemistry, immunofluorescence, cell-based assay, radioimmunoassay, protein macroarray and enzyme-linked immunosorbent assay (ELISA).
Results: Although HaNDL sera did not yield antibodies to any of the well-characterized neuronal surface or synapse antigens, protein macroarray and ELISA studies showed high-titer antibodies to a subunit of the T-type voltage-gated calcium channel (VGCC), CACNA1H, in sera of two HaNDL patients.
Conclusion: Our results support the notion that ion channel autoimmunity might at least partially contribute to HaNDL pathogenesis and occurrence of neurological symptoms.

Keywords

HaNDL, headache, CACNA1H, voltage-gated calcium channel, autoantibody

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Introduction

Syndrome of transient headache and neurological deficits with cerebrospinal fluid (CSF) lymphocytosis (HaNDL) is a self-limiting neurological disease characterized by several episodes of temporary neurological symptoms accompanied or followed by migraine-like headaches and associated with CSF lymphocytic pleocytosis. Typical presenting symptoms are hemi-sensory/motor deficits and aphasia. These episodes usually remit within weeks to months and are separated by asymptomatic periods or headache with normal neurological examination (1).

The etiology of HaNDL still remains a mystery, although theories focused on parainfectious and autoimmune pathophysiology have often been debated (2,3). While HaNDL cases with serological evidence for a recent viral infection have been rarely reported, an

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extensive screening for infectious agents has been found negative in most HaNDL patients (3). Recurrent and self-limiting episodes of transient neurological deficits and CSF lymphocytosis are features that are shared by autoimmune encephalitis associated with ion channel antibodies (4). To investigate whether HaNDL could be an autoimmune channelopathy, we screened the sera of four HaNDL patients for ion channel antibodies with a broad panel of immunological methods.

Methods

Patients and samples

In addition to four HaNDL patients, 20 relapsing–remitting multiple sclerosis (RRMS), 20 cryptogenic partial epilepsy, 30 migraine (eight with and 22 without aura), 10 viral encephalitis and three Lambert-Eaton myasthenic syndrome (LEMS) patients and 30 healthy controls were included. All RRMS patients fulfilled the McDonald's criteria for definite MS (5), and all HaNDL and migraine patients fulfilled the relevant criteria of the International Headache Society (6). Sera from all patients were obtained during a neurological episode and prior to initiation of any immunosuppressive treatments and kept at −80°C until assayed. An informed consent was obtained from all participants. The study was approved by the Ethics Committee of Istanbul Faculty of Medicine of Istanbul University.

Immunohistochemistry on rat brain sections

Immunohistochemistry studies were conducted with frozen rat brain sections fixed with 4% paraformaldehyde and the peroxidase-diaminobenzidine method, as previously described (7). The immunohistochemistry results were assessed by two independent observers (E.T. and M.K.), who were blind to patients' identities. Moderate to strong diaminobenzidine-induced brown color that could be localized to a discrete anatomical and/or subcellular location (e.g. cytoplasm, nucleus, axonal protrusions, etc.) was considered as anti-neuronal antibody positivity (Figure 1a and b).

Immunofluorescence on live neurons

Antibodies to neuronal surface antigens were detected by using cultured hippocampal neurons of P1 rat pups, as described (7). The cultured neurons were incubated with patients' sera (1:250\) for one hour at room temperature, followed by 3% formaldehyde fixation and by incubation with Alexa Fluor 488-conjugated anti-human immunoglobulin (IgG) (Invitrogen, Paisley, UK) for 45 minutes. Subsequently the cells were permeabilized with 0.3% Triton X-100 in phosphate buffered saline (PBS) for 15 min at room temperature and incubated with mouse monoclonal microtubule-associated protein 2 (MAP2) antibody (Sigma-Aldrich, Dorset, UK) (a marker of axonal and dendritic processes) (1:1000) or mouse monoclonal antibody to CACNA1H (Abcam, Cambridge, MA, USA) (1:500) for one hour at room temperature, followed by incubation with Alexa Fluor 568-conjugated anti-mouse IgG (Invitrogen) (1:1000) for 45 minutes. Images were photographed under a Zeiss fluorescence microscope with a digital camera using the Zeiss Axiovision software. The immunofluorescence results were assessed by two independent observers (E.T. and E.E.), who were blind to patients' identities. Moderate to strong Alexa Fluor 488-conjugated anti-human IgG-induced green color that co-localized with Alexa Fluor 568-conjugated anti-mouse IgG-induced red color (Figure 1c–h) was considered as positive.

Autoantibodies to ion channels and glutamic acid decarboxylase (GAD)

N-methyl-D-aspartate receptor (NMDAR), leucine-rich, glioma-inactivated 1 (LGI1) and contactin-associated protein-like 2 (CASPR2) antibodies were detected by binding to HEK293 cells transfected with plasmids containing the NR1/NR2 subunits of the NMDAR, LGI1 or CASPR2, respectively. Transfected cells were then incubated with patients' sera (1:20) and the appropriate Alexa Fluor secondary antibody, as described earlier (7,8). For P/Q-type voltage-gated calcium channel (VGCC) and voltage-gated potassium channel (VGKC)-complex antibodies, radioimmunoassays (RIA) using brain extracts labeled with ^{125}I-ω-conotoxin and ^{125}I-dendrotoxin were used, respectively (8,9). GAD antibodies were also measured by RIA (RSR Ltd, Cardiff, UK).

Protein macroarray, sequencing of cDNA inserts and protein expression

Sera of four HaNDL patients were screened using a high-density protein macroarray derived from human fetal brain cDNA expression library (hEX1), which contains approximately 24,000 clones (ImaGenes, Berlin, Germany), as described previously (10). Images were captured and analyzed for signal intensity (VisualGrid, GPC Biotech, Martinsried, Germany). The arrays were scored between 0 and 3. Plasmid DNAs from selected clones were isolated and sequenced (Iontek, Istanbul, Turkey). Nucleotide and translated amino acid sequences were compared with known sequences using Basic Local Alignment Search (BLAST) algorithms (National Center for Biotechnology Information, Bethesda, MD, USA).

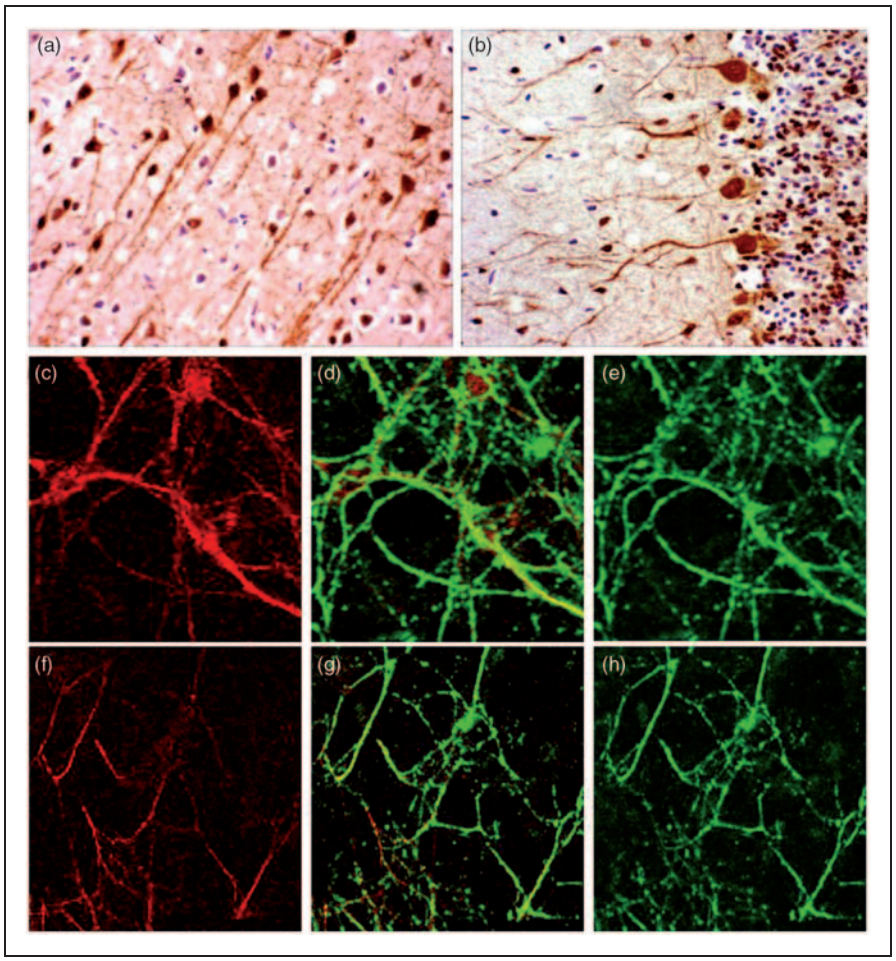

Figure 1. Immunolabeling of frozen rat brain sections (a and b) and cultured live hippocampal cells with sera of headache with neurological deficits and lymphocytosis (HaNDL) patients and commercial antibodies (c–h). Serum immunoglobulin (IgG) of Case 1 shows reactivity with the cell body and axonal/dendritic projections (neuropil) of discrete cortical neurons (a), and serum IgG of Case 2 immunolabels the dendritic projections and cell body of cerebellar Purkinje cells (b). Double immunolabeling of cultured rat hippocampal neurons (c–e) using Case 1's serum sample (e, green) and an antibody to the neuronal marker microtubule-associated protein 2 (c, red) shows the co-localization of reactivities (d). Likewise, the binding sites of Case 2's serum IgGs on cultured rat hippocampal neurons (h, green) significantly overlap (g) with those of a commercial CACNA1H antibody (f, red). Original magnification for panels a and b is × 100 and for panels c–h is × 400. Staining for panels a and b was performed with the avidin-biotin-peroxidase technique (brown color) with hematoxylin counterstaining (blue color). 220 × 238 mm (300 × 300 dots per inch (DPI)).

Following the confirmation of the selected clones, his-tagged proteins were recombinantly expressed in *Escherichia coli*, purified by affinity chromatography, and the purity of the proteins was documented by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (10). For immunoblotting experiments, the purified CACNA1H protein was denatured, electrophoresed (10% acrylamide gel) and transferred to polyvinylidene fluoride membranes (100 V, 80 min). Membranes were incubated with mouse anti-human CACNA1H (1:100) (Abcam) followed by HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) at 1:1000 dilution.

Enzyme-linked immunosorbent assay (ELISA)

The purified recombinant human proteins (10 µg/ml) were added to the wells of a 96-well plate and incubated overnight at 4°C. Non-coated wells were used as controls. Each serum sample (1:100) in Tris-buffered saline-Tween-20 (TBS-T) was added and incubated for two hours at room temperature. The plates were then incubated with alkaline phosphatase (AP)-conjugated goat anti-human IgG (1:2000) (Southern Biotech, Birmingham, AL, USA) at room temperature for one hour. After washing, 2-(2-benzothiazoyl)-6-hyroxybenzothiazole phosphate (BBTP) was added for 45 minutes at room temperature followed by addition of the

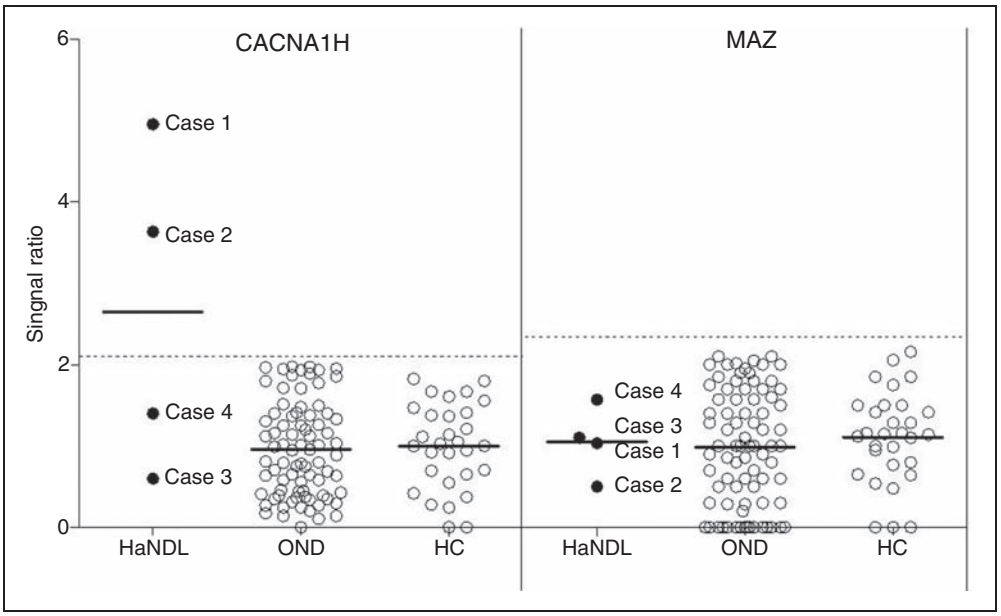

Figure 2. Enzyme-linked immunosorbent assay (ELISA) detection of immunoglobulin (IgG) antibodies directed against CACNA1H and control myc-associated zinc finger protein (MAZ) in sera of four headache with neurological deficits and lymphocytosis (HaNDL) patients (Cases 1–4), 83 patients with other neurological diseases (OND), including three Lambert-Eaton myasthenic syndrome cases, and 30 healthy controls (HC). The dashed lines represent two standard deviations above the mean of the HC samples (cut-off values for positivity). Horizontal lines indicate the mean value of each group. 153 × 92 mm (300 × 300 dots per inch (DPI)).

stopping solution (3 N NaOH). Fluorescent signals were measured at 450/50 exitation and 580/50 emission with a microplate reader. For each sample, the value obtained from the protein-coated well was subtracted from the non-coated well. The obtained results were expressed as signal ratios (sample signal/mean signal of the healthy controls). Positivity was defined as two standard deviations above the mean of healthy controls.

Results

Clinical features of HaNDL patients

The clinical features, durations of the neurological episodes and CSF findings of HaNDL patients were strictly consistent with the criteria established for HaNDL (1,6) (Table 1). Focal electroencephalogram (EEG) abnormalities and normal CSF opening pressure (Case 2) have been reported in HaNDL cases (1). None of the patients had a medical history of migraine, family history of migraine, any predisposing factors for migraine (e.g. alcohol consumption, stress, sleep deprivation, etc.), any prodromal symptoms for migraine (e.g. fatigue, mental slowness, behavioral changes, sleep problems, nausea, photophobia, etc.) or aura-like visual symptoms. The neurological examinations of all patients were normal between HaNDL episodes. Magnetic resonance (MR) angiography had been performed only in Case 2 and found normal. All cases received either one or two of the medications flunarizine, lamotrigine or acetylsalicylic acid. In all patients, routine complete blood count, biochemical analysis, thyroid function tests, sedimentation rate and a comprehensive panel for vasculitic-rheumatological diseases and infectious agents (including serology and/or CSF culture for herpes simplex virus (HSV), human immunodeficiency virus (HIV), cytomegalovirus (CMV), human herpes virus 6 (HHV-6), syphilis, *Borrelia burgdorferi*, mycoplasma, *Mycobacterium tuberculosis* and fungal infections) were normal or negative. The cytological analysis of CSF samples did not yield atypical cancer cells suggestive of leptomeningeal metastasis in any of the patients.

Anti-neuronal antibodies in HaNDL patients

In two HaNDL patients' sera (Cases 1 and 2), immunohistochemistry studies revealed IgGs immunoreacting with the whole cell body and neuropil of discrete neurons throughout the brain. Notably, the serum IgGs diffusely immunolabeled some of the neurons (Figure 1a, brown color generated by IgG binding),

whereas some others remained completely unstained (Figure 1a, neuronal nuclei stained in blue only with hematoxylin). Similarly, IgGs of Cases 1 and 2 diffusely immunolabeled the cell body and dendritic projections of Purkinje cells (Figure 1b). Serum samples of two other HaNDL patients (Cases 3 and 4) showed the widespread neuronal nuclear staining pattern that was previously described (2). Sera of control patients (including LEMS patients) and healthy controls did not show any appreciable staining.

Antibodies to neuronal surface antigens of the cultured hippocampal neurons were identified in sera of two HaNDL (Cases 1 and 2) and all LEMS patients but not in those of Cases 3, 4, and control cases. Moreover, double immunolabeling of hippocampal neuronal cultures with serum antibodies of Cases 1 and 2 and microtubule-associated protein-2 (MAP-2) antibody showed co-localization (Figure 1c–e), indicating that the identified staining was related to neurons rather than glial cells.

Since neuropil antibodies have signified the presence of antibodies directed against cell surface and synapse antigens (7,8), we examined serum antibodies against well-characterized ion channels and synapse autoantigens. Neither HaNDL nor controls other than LEMS cases had antibodies to P/Q-type VGCC, VGKC-complex (RIA), VGKC-complex antigens (cell-based assays for LGI1 and CASPR2), NMDAR and GAD. By contrast, three LEMS patients exhibited raised antibodies to P/Q-type VGCC, as expected (range 295–662 pM, normal values (nv) < 50 pM).

CACNA1H antibodies in HaNDL patients

Protein macroarray analysis identified a single ion channel-associated clone that had the highest signal intensity score, 3: T-type VGCC, alpha 1H subunit (CACNA1H) (accession no. NG_012647). ELISA studies performed with the corresponding recombinant protein revealed high-titer autoantibodies to CACNA1H in sera of two HaNDL patients (Cases 1 and 2) but not in those of Cases 3 and 4 and controls (including LEMS patients) (Figure 2). None of the examined sera gave high-titer antibody values with an irrelevant protein (myc-associated zinc finger protein (MAZ)) purified from the *E. coli* strain also used to express CACNA1H (Figure 2) and the lysate of the same *E. coli* strain with no human protein expression (data not shown). In immunoblotting experiments, the commercial CACNA1H antibody bound a single band at ~240 kDa, as predicted. In co-localization experiments, serum IgG binding sites of Cases 1 and 2 on cultured rat hippocampal neurons significantly overlapped with those of a commercial CACNA1H antibody (Figure 1f–h), whereas cultured neurons

Table 1. Clinical features of headache with neurological deficits and lymphocytosis (HaNDL) cases.

Case	Gender	Age	Total duration (days)	Number of episodes	Neurological deficits	Migraine-like headache during episodes	MRI	EEG	CSF[a] lymphocytes/mm^3; protein (mg/dl)	CSF OCB; IgG index
1	Male	34	8	4	Marching R hp-hh, aphasia; attacks last 5 min–7 hr	+	N	N	507/mm^3; 145 mg/dl	None; 0.46
2	Male	48	21	3	Marching R hp-hh, aphasia, confusion; attacks last around 1–2 hr	+	N	Slow waves in L hemisphere	182/mm^3; 56 mg/dl	None; 0.51
3	Female	46	14	2	R hp-hh, aphasia; attacks last around 6 hr	+	N	N	328/mm^3; 128 mg/dl	None; 0.47
4	Male	30	5	5	Marching L hp-hh; attacks last around 30 min	+	N	N	122/mm^3; 73 mg/dl	None; 0.57

R: right; L: left; hp: hemiparesis; hh: hemihypoesthesia; MRI: magnetic resonance imaging; EEG: electroencephalography; CSF: cerebrospinal fluid; OCB: oligoclonal bands; IgG: immunoglobulin; N: normal.
[a]Cases 1, 3 and 4 had increased and Case 2 had normal CSF opening pressure.

incubated with only Alexa Fluor-conjugated secondary antibodies or sera of the healthy controls did not yield a significant staining.

Discussion

The most notable result of our study is the presence of antibodies directed against the neuronal cell membrane antigens in HaNDL patients' sera. Our protein macroarray and ELISA studies have further shown that at least one of the target antigens of these antibodies could be T-type VGCC. The absence of neuropil or CACNA1H antibodies in sera of patients with MS, encephalitis, migraine or epilepsy suggests that the determined antibodies are probably not caused by inflammation of the central nervous system, blood–brain barrier permeability disruption, neuronal damage or vascular dilation-neuronal excitability changes experienced in migraine patients. Moreover, absence of CACNA1H antibodies in P/Q-type VGCC antibody-positive LEMS patients further indicates that these antibodies are exclusive to HaNDL. Further supporting this assumption, several parallel antibody screenings performed with sera of 20 RRMS, 25 neuromyelitis optica, 100 Behcet's disease (including 20 Neuro-Behcet's disease) and 25 migraine patients using the same cDNA expression library have not identified CACNA1H or any other ion-channel associated antigens (unpublished data).

The clinical features of HaNDL bear a resemblance to sporadic and familial hemiplegic migraine, which are associated with mutations in the gene coding for the P/Q-type VGCC α-subunit, CACNA1A. This has prompted the analysis of the CACNA1A gene in HaNDL patients. However, this study has failed to identify any mutations or shared polymorphisms in this gene (11).

One drawback of our study is that protein macroarray is not an ideal method for screening antibodies directed against the cell membrane antigens. However, novel cell membrane antibodies have previously been identified by protein macroarray or similar cDNA expression library screening methods (12). Nevertheless, a valuable method for future studies could be screening of HaNDL sera by immunoprecipitation, which might lead to detection of antibodies to other VGCC subunits or other ion channels in HaNDL patients' sera.

In conclusion, our results support the notion that autoimmunity might participate in the pathogenesis of HaNDL. CACNA1H antibodies were identified only in two HaNDL patients and thus HaNDL is conceivably a heterogeneous disorder with different mechanisms involved in its pathogenesis. Nevertheless, screening of larger HaNDL cohorts for novel ion channel and neuronal cell surface antibodies is warranted for better identification of autoimmunity in HaNDL pathogenesis.

Clinical implications

- Some headache with neurological deficits and lymphocytosis (HaNDL) patients present with high-titer antibodies to a subunit of the T-type voltage-gated calcium channel (VGCC), CACNA1H.
- Our results support the notion that ion channel autoimmunity might at least partially contribute to HaNDL pathogenesis.

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Conflict of interest

None declared.

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