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Antibodies to the Caspr1/contactin-1 complex in chronic inflammatory demyelinating polyradiculoneuropathy

Elba Pascual-Goñi,[1] Janev Fehmi,[2] Cinta Lleixà,[1] Lorena Martín-Aguilar,[1] Jérôme Devaux,[3] Romana Höftberger,[4] Emilien Delmont,[5] Kathrin Doppler,[6] Claudia Sommer,[6] Aleksandar Radunovic,[7] Alejandra Carvajal,[8] Shane Smyth,[9] Laura Williams,[9] Radim Mazanec,[10] Veronika Potočková,[10] Nigel Hinds,[11] Julien Cassereau,[12] Karine Viala,[13] Mathilde Lefilliatre,[14] Guillaume Nicolas,[15] Peter Foley,[16] Frank Leypoldt,[17,18] Stephen Keddie,[19] Michael P. Lunn,[19] Fritz Zimprich,[20] Vharoon Sharma Nunkoo,[21] Wolfgang N. Löscher,[22] Laura Martínez-Martínez,[23] Jordi Díaz-Manera,[1,24] Ricard Rojas-Garcia,[1,24] Isabel Illa,[1,24] Simon Rinaldi[2,†] and Luis Querol[1,24,†]

[†]These authors contributed equally to this work.

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Previous studies have described the clinical, serological and pathological features of patients with chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) and antibodies directed against the paranodal proteins neurofascin-155, contactin-1 (CNTN1), contactin-associated protein-1 (Caspr1), or nodal forms of neurofascin. Such antibodies are useful for diagnosis and potentially treatment selection. However, antibodies targeting Caspr1 only or the Caspr1/CNTN1 complex have been reported in few patients with CIDP. Moreover, it is unclear if these patients belong to the same pathophysiological subgroup. Using cell-based assays in routine clinical testing, we identified sera from patients with CIDP showing strong membrane reactivity when both CNTN1 and Caspr1 were co-transfected (but not when CNTN1 was transfected alone). Fifteen patients (10 male; aged between 40 and 75) with antibodies targeting Caspr1/CNTN1 co-transfected cells were enrolled for characterization. The prevalence of anti-Caspr1/CNTN1 antibodies was 1.9% (1/52) in the Sant Pau CIDP cohort, and 4.3% (1/23) in a German cohort of acute-onset CIDP. All patients fulfilled European Federation of Neurological Societies/Peripheral Nerve Society (EFNS/PNS) definite diagnostic criteria for CIDP. Seven (47%) were initially diagnosed with Guillain-Barré syndrome due to an acute-subacute onset. Six (40%) patients had cranial nerve involvement, eight (53%) reported neuropathic pain and 12 (80%) ataxia. Axonal involvement and acute denervation were frequent in electrophysiological studies. Complete response to intravenous immunoglobulin was not observed, while most (90%) responded well to rituximab. Enzyme-linked immunosorbent assay (ELISA) and teased nerve fibre immunohistochemistry confirmed reactivity against the paranodal Caspr1/CNTN1 complex. Weaker reactivity against Caspr1 transfected alone was also detected in 10/15 (67%). Sera from 13 of these patients were available for testing by ELISA. All 13 samples reacted against Caspr1 by ELISA and this reactivity was enhanced when CNTN1 was added to the Caspr1 ELISA. IgG subclasses were also investigated by ELISA. IgG4 was the predominant subclass in 10 patients, while IgG3 was predominant in other three patients. In conclusion, patients with antibodies to the Caspr1/CNTN1 complex display similar serological and clinical features and constitute a single subgroup within the CIDP syndrome. These antibodies likely target Caspr1 primarily and are detected with Caspr1-only ELISA, but reactivity is optimal when CNTN1 is added to Caspr1 in cell-based assays and ELISA.

1 Neuromuscular Diseases Unit, Hospital de la Santa Creu i Sant Pau, Universitat Autònoma de Barcelona, Spain
2 Nuffield Department of Clinical Neurosciences, University of Oxford, John Radcliffe Hospital, Oxford, UK
3 Institut de Neurosciences de Montpellier, Hospital Saint Eloi, Montpelier, France

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4 Division of Neuropathology and Neurochemistry, Department of Neurology, Medical University of Vienna, Vienna, Austria
5 Referral Centre for ALS and Neuromuscular Diseases, Hospital La Timone, Marseille, France
6 Department of Neurology, University Hospital Würzburg, Würzburg, Germany
7 Department of Neurology, Barts Health NHS Trust, London, UK
8 Complejo Hospitalario Universitario de Granada, Granada, Spain
9 Mater Misericordiae University Hospital, Dublin, Republic of Ireland
10 Department of Neurology, Medical Faculty of Charles University and University Hospital Motol, Prague, Czech Republic
11 Abertawe Bro Morgannwg University Health Board, Swansea, Wales, UK
12 Reference Centre for Neuromuscular Diseases, Department of Neurology, Angers University Hospital, Angers, France
13 Department of Clinical Neurophysiology, Hospital de la Pitié-Salpêtrière. Paris, France
14 Department of Neurology, Hospital Center University of Caen, Caen, France
15 Department of Neurology, Hôpital Raymond-Poincaré, Université Versailles-Saint-Quentin-en-Yvelines, Garches, France
16 Anne Rowling Regenerative Neurology Clinic, University of Edinburgh, Edinburgh, UK
17 Institute of Clinical Chemistry, University Hospital Schleswig-Holstein, Kiel, Germany
18 Department of Neurology, Christian-Albrechts-Universität zu Kiel, Kiel, Germany
19 Centre for Neuromuscular Diseases, National Hospital for Neurology and Neurosurgery, Queen Square, London, UK
20 Department of Neurology, Medical University of Vienna, Vienna, Austria
21 Department of Neurology, Municipal University Hospital Dr. Gavril Curteanu, Oradea, Romania
22 Department of Neurology, Medical University of Innsbruck, Austria
23 Department of Immunology, Hospital de la Santa Creu i Sant Pau, Universitat Autònoma de Barcelona, Barcelona, Spain
24 Centro para la Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Spain

Correspondence to: Dr Luis Querol
Department of Neurology, Hospital de la Santa Creu i Sant Pau Sant Pau Biomedical Research Institute
(IIB Sant Pau) Sant Quintí 77 (2nd Floor), 08041 Barcelona, Spain
E-mail: lquerol@santpau.cat

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Abbreviations: Caspr1 = contactin-associated protein 1; CBA = cell-based assays; CIDP = chronic inflammatory demyelinating polyradiculoneuropathy; DRG = dorsal root ganglion; ELISA = enzyme-linked immunosorbent assay; IVIg = intravenous immunoglobulin; mAb = monoclonal antibody

Introduction

Chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) is a disabling disorder of the peripheral nerves, diagnosed by clinical and electrophysiological criteria.[1,2] These criteria identify a heterogeneous population of patients responding to immunomodulatory therapies. Humoral factors are considered important in CIDP pathogenesis and therapies.[3,4] The recent discovery of autoantibodies against nodal and paranodal proteins such as neurofascin 155 (NF155),[5-7] nodal neurofascins (NF186 and NF140)[8,9] and contactin-1 (CNTN1)[10,11] has led to the description of subsets of CIDP patients with specific phenotypic features.[12] Antibodies to these cell-adhesion molecules are currently detected in less than 15% of all CIDP patients. Moreover, *in vitro* and *in vivo* data demonstrate their pathogenic potential[13,14] and their clinical phenotypes[7,10] and response to treatment differ from those of patients with seronegative CIDP.[15] Antibodies are usually of the IgG4 isotype, although smaller populations with predominantly IgG3 autoantibody isotypes have also been reported.[16,17] The stratification of CIDP according to their antibody reactivities has facilitated the identification of distinct pathological features[18,19] and associated HLA haplotypes[20] in some variants. Thus, autoantibodies to nodal and paranodal structures have pathophysiological relevance and have emerged as clinically useful biomarkers.

Contactin-associated protein 1 (Caspr1) is a cell-adhesion protein expressed by neurons.[21,22] Caspr1 forms a complex with CNTN1[23,24] that binds to the glial protein NF155, which together form the septate-like junctions (transverse bands) that interconnect the paranodal loops of Schwann cells to the axon at the paranode. Antibodies targeting Caspr1 have previously been reported in one patient with Guillain-Barré syndrome and in one CIDP patient with neuropathic pain.[25] Our group reported autoantibodies targeting the Caspr1/CNTN1 complex (and not their components in isolation) in one patient with an aggressive form of CIDP.[11] Three more patients with IgG4 autoantibodies against Caspr1/CNTN1 were also reported in the Italian cohort.[26] Moreover, very recently, patients with acute onset CIDP reacting against both CNTN1 and Caspr1 separately have been described.[27] It remains unclear whether patients reacting only against the Caspr1/CNTN1 complex constitute a population with distinct clinical and serological features compared to those reacting against Caspr1 or CNTN1 in isolation. The aim of our study was to analyse the clinical and immunological characteristics of CIDP patients with antibodies against the Caspr1/CNTN1 complex, elucidate the exact antigenic target (CNTN1, Caspr1 or the Caspr1/CNTN1 complex) and propose the optimal diagnostic strategy for this subpopulation of CIDP patients.

Materials and methods

Patients and sera

CIDP patients reacting against Caspr1/CNTN1 co-transfected cells, and non-reactive in CNTN1-only cell-based assays (CBA), from all participating centres were identified during routine testing for paranodal/nodal antibodies, and were selected for further characterization between 1 January and 31 December 2019. Patient 14 was identified as part of a study testing a retrospective German cohort of acute-onset CIDP. To calculate the prevalence of anti-Caspr1/CNTN1 autoantibodies in the Sant Pau CIDP cohort, we tested antibodies to Caspr1/CNTN1 in all patients fulfilling European Federation of Neurological Societies/Peripheral Nerve Society (EFNS/PNS) diagnostic criteria for CIDP[1] that were followed-up in the Neuromuscular Clinic of Hospital de la Santa Creu i Sant Pau (between 1 January and 31 December 2019). Sera collected from all patients fulfilling the inclusion criteria were further tested to specifically characterize the antigenic target of the autoantibodies. This study was conducted according to a protocol approved by the Institutional Ethics Committee of the Hospital de la Santa Creu i Sant Pau and samples were collected at each centre following local IRB approved protocols. Written informed consent was obtained from all subjects for sample handling and data collection.

Cell-based assays

Antibodies against NF155, NF140/186 and CNTN1 were investigated by immunocytochemistry as previously described.[28] Antibodies to Caspr1/CNTN1 were tested by CBA using human embryonic kidney (HEK) 293A cells and Chinese hamster ovary (CHO) Lec1 cells co-transfected with mammalian-expression vectors encoding human CNTN1 (EXA1153-MO29 Genecopoeia) and Caspr1 (EXMO417-MO2 Genecopoeia) at equimolar concentrations, or with CNTN1 or Caspr1 alone. Cells were grown in culture slides coated with poly-D-lysine for HEK293 or laminin for Lec1 cells. After 24 h, cells were transfected with Lipofectamine™ 2000 (Invitrogen), fixed with 4% paraformaldehyde (PFA) (Affymetrix Inc) and frozen at −80°C until needed. Cells were then blocked with 5% goat or rabbit sera in PBS and incubated with patient's sera (1:100) preabsorbed with HEK293 cells pellet, 1 h at room temperature, followed by anti-CNTN1 (AF904, R&D Systems) (1:1000) or anti-Caspr1 (ab133634, Abcam) (1:250) antibodies. Finally, appropriate Alexa Fluor® secondary IgG antibodies (1:1000) were used as previously described.[28] Slides were mounted with Fluoromount™ medium (Sigma-Aldrich). Images were acquired using a Leica TSC SP5 confocal microscope or a fluorescence microscope (Olympus DX51) and processed with ImageJ software. Patient 14 was recruited for another study[27] and serum of this patient was tested at the University Hospital Würzburg according to published protocols.[25,29]

Caspr1 and CNTN1 enzyme-linked immunosorbent assay

MaxiSorp™ 96-well enzyme-linked immunosorbent assay (ELISA) plates (Thermo Fisher Scientific) were coated with 5 µg/ml human recombinant Caspr1 protein (2418-CR R&D Systems), or 5 µg/ml human recombinant CNTN1 protein (Sino Biological Inc.) or 5 µg/ml of Caspr1 and 3.8 µg/ml CNTN1 protein (equimolar concentration) overnight at 4°C. Wells were blocked with 5% non-fat milk in PBS-Tween 0.1% for 1 h, incubated with sera diluted in blocking buffer for 1 h, and then incubated with peroxidase-conjugated mouse anti-human IgG secondary antibody 1:2000 (Invitrogen) or anti-IgG1, IgG2, IgG3 and IgG4 secondary antibodies for 1 h at room temperature. To calculate the anti-Caspr1 titres, the ELISA was performed with different serum concentrations (range 1:100–1:200 000). ELISAs were developed with tetramethylbenzidine solution (BioLegend), and the reaction stopped with 1 M sulphuric acid. Optical density (OD) was measured at 450 nm Multiscan ELISA reader. Samples were considered positive by ELISA when they had a ΔOD higher than average healthy control ΔOD +2 standard deviations. We used sera from healthy donors, seronegative CIDP and anti-CNTN1-positive patients as controls.

Teased nerve fibre and dorsal root ganglion immunohistochemistry

Swine sciatic nerves were obtained (discarded tissue from *Sus scrofa* used in Animal Ethics' Committee approved protocol for cardiovascular research) and pre-fixed in 2% PFA for 1 h. Nerve fibres were teased, transferred to slides, dried at room temperature, and stored at −20°C until needed. Dorsal root ganglion (DRG) sections of adult mice were prepared as previously described.[30] Slides were fixed in acetone −20°C for 10 min and incubated with the following solutions 1 h each at room temperature): (i) 5% goat serum in Triton™ 0.1%-PBS for 1 h; (ii) patient's serum (1:100); (iii) anti-neurofascin chicken antibody (AF3235 R&D Systems; 1:500) or anti-pan sodium channel monoclonal antibody (mAb) (S8809, Sigma-Aldrich; 1:500), or-anti-Caspr1 (ab133634, Abcam; 1:100), or anti-TRPV1 (ACC-030 Alomone; 1:100), or anti-IB4 (L2140 Sigma-Aldrich; 1:200); and (iv) appropriate secondary antibodies (1:1000). Slides were mounted with Fluoromount™ (Diagnostic BioSystems Inc.). Images were acquired using Leica TSC SP5 confocal microscope or Olympus DX51 fluorescence microscope, and processed with ImageJ software.

Peripheral nerve immunohistochemistry

Macaque peripheral nerve tissue slides (Inova Diagnostics, Inc.) were incubated with patients' sera and anti-Caspr1 or anti-CNTN1 antibodies. For Caspr1, peripheral nerve slides were incubated with the following solutions, 1 h each, at room temperature: (i) 5% normal goat serum in PBS; (ii) patients' sera at 1:20; (iii) anti-Caspr antibodies (ab133634, Abcam; 1:20); and (iv) monkey-adsorbed goat anti-human IgG 488 secondary antibody (2049-30 Southern Biotech; 1:400) and AF594 goat anti-rabbit secondary antibody (A11037, Thermo Fisher Scientific; 1:400). For CNTN1, slides were incubated with the following solutions, 1 h each, at room temperature: (i) UltraCruz Blocking Reagent (sc-516214, Santa Cruz Biotechnology); (ii) goat anti-CNTN1 IgG (AF904, R&D Systems, Minnesota, US; 1:50); (iii) secondary antibody donkey anti-goat IgG AF555 (A-21432 Invitrogen; 1:500); (iv) 5% normal goat serum in PBS; (v) patients' sera at 1:20; and (vi) monkey-adsorbed goat anti-human IgG 488 secondary antibodies (2049-30 Southern Biotech; 1:300). To study Caspr1 and CNTN1 staining patterns,

slides were also stained with anti-human CD56 mAb (347740, Becton Dickinson; 1:40) or anti-S100b (ab52642 Abcam; 1:50); and appropriate secondary antibodies (1:500). Slides were mounted with Fluoromount™ medium (Sigma-Aldrich). Images were acquired using Leica TSC SP5 confocal microscope and prepared using ImageJ software.

Dorsal root ganglion, hippocampal and human neuroblastoma-derived neuron immunocytochemistry

Rat DRG neurons were extracted and cultured, and immunocytochemistry was performed as previously described.[31] Rat hippocampal neurons were isolated and cultured, and immunocytochemistry was performed as previously described.[11] SH-SY5Y cells were plated in glass coverslips coated with laminin (Invitrogen) and grown in proliferation medium containing DMEM, F12, FBS (10%), L-glutamine (1%) and sodium pyruvate (1%). After 24 h, proliferation medium was replaced by differentiation medium containing Neurobasal™ (Gibco) supplemented with B27 (Gibco), GlutaMAX™ (Gibco), nerve growth factor (Invitrogen) and retinoic acid. This medium was replaced every 2 days until full differentiation was achieved. On Days 5 and 6 of differentiation, cells were fixed for 15 min with PFA 4%; and incubated with the following solutions: (i) rabbit serum 1/40 in PBS for 1 h; (ii) patients' sera diluted 1:50; (iii) anti-CNTN1 antibodies (AF904, R&D Systems; 1:100); and (iv) appropriate Alexa Fluor® secondary IgG antibodies (1:500). Slides were mounted with Vectashield with DAPI (Vector Laboratories). Sera from healthy donors and anti-CNTN1 positive CIDP patients were used as controls. Images were acquired using Leica TSC SP5 confocal microscope. Animal procedures were performed according to a protocol approved by our Institution's Animal Ethics' Committee.

Immunoabsorption experiments

Non-transfected, CNTN1-, Caspr1- or Caspr1/CNTN1-cotransfected HEK293 cells were grown in six-well plates as described above and then fixed with 4% PFA. Patients' sera diluted 1:500, 1:800 or 1:1000 in 5% goat serum in PBS were serially incubated for 1 h in each of the six wells. After incubation in the sixth and final well, the supernatant was collected, and its reactivity was tested by teased-nerve fibre immunohistochemistry as described above.

Mass spectrophotometry and immunoprecipitation

Human recombinant Caspr1 protein was analysed by mass spectrophotometry to detect CNTN1 contamination. The amount of CNTN1 was estimated based on the number of spectral counts (corrected by the molecular weight), and using the emPAI parameter as previously reported.[32] CNTN1 was immunoprecipitated from Caspr1 human recombinant protein using Pierce Classic Magnetic IP/Co-IP Kit (88804, Thermo Fisher Scientific) and goat anti-CNTN1 IgG (AF904, R&D Systems). Anti-LRP4 antibody (SC98775, Santa Cruz Biotechnology) was used as control. CNTN1 immunoprecipitation was confirmed by western blot (Supplementary Fig. 3B). ELISAs with Caspr1 protein after CNTN1 immunoprecipitation were performed to analyse sera reactivity in the absence of CNTN1.

Data collection and statistical analysis

Clinical, neurophysiological, laboratory and treatment response data of each patient were obtained retrospectively by chart review by their primary clinicians in 15 different European centres. The diagnosis of CIDP was made according to current EFNS/PNS guidelines.[1] A good response to treatment was defined as an improvement of at least two points in the modified Rankin Scale. A partial response to treatment was defined as an improvement of 1 point in the modified Rankin Scale. Statistical analysis was performed with Prism v8.0 (GraphPad Software, La Jolla, California, USA).

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its Supplementary material.

Results

Autoantibodies against Caspr1/CNTN1 co-transfected cells are detected in CIDP patients

We identified 15 CIDP patients with autoantibodies targeting Caspr1/CNTN1 co-transfected cells (and not reacting against CNTN1 alone), for clinical and immunological characterization. All samples were also negative for NF155 and NF140/186 by CBA. Table 1 summarizes the immunological features of these patients. The prevalence of antibodies to Caspr1/CNTN1 co-transfected cells in the Sant Pau CIDP cohort was 1/52 (1.9%). Patient 14 belonged to a German cohort of 23 patients with acute-onset CIDP of whom only one (4.3%) had anti-Caspr1 antibodies. All patients' sera showed reactivity against the Caspr1/CNTN1 co-transfected cells that co-localized better with CNTN1 (Fig. 1A and B) than with Caspr1 mAb (Fig. 1C and D). However, reactivity was absent when CNTN1 was transfected alone (Fig. 1G and H). Although this may be interpreted as Caspr1 being the antigenic target, five patients did not show any reactivity against HEK293 transfected only with Caspr1 (Fig. 1E), and in the 10 others only mild to moderate reactivity was seen against such cells and, again, this reactivity did not co-localize with Caspr1 (Fig. 1F). Non-transfected HEK293 endogenously express low levels of CNTN1 (Supplementary Fig. 1) and this may explain why some patients react against Caspr1-only transfected cells.

We aimed to identify whether the interaction between CNTN1 and Caspr1 during their expression in HEK cells, or the formation of Caspr1/CNTN1 complex itself, is required to generate the antigenic epitope for these autoantibodies.

Table 1 Summary of immunological features of CIDP patients with antibodies against the Caspr1/CNTN1 complex

Patient	CBA HEK Caspr1	ELISA Caspr1 Total IgG titres	ELISA Caspr1 IgG subclasses	Swine teased nerve fibres IHC	Macaque's peripheral nerve IHC	Cultured rat DRG neurons	Mouse DRG sections IHC
1[a]	Negative	1:8100	IgG3 > > IgG4 > IgG1	Paranodes	Caspr1 pattern[b]	Moderate positive	DRG mild positive Dorsal roots paranodes
2	Positive	1:24 300	IgG4	Paranodes	Caspr1 pattern[b]	NA	NA
3	Positive	1:218 700	IgG4 > > > IgG2	Paranodes	Caspr1 pattern[b]	Weak positive	DRG mild positive Dorsal roots paranodes
4	Positive	1:24 300	IgG4	Paranodes	Caspr1 pattern[b]	NA	NA
5	Positive	1:8100	IgG4	Paranodes	Caspr1 pattern[b]	NA	NA
6[a]	Negative	1:2700	IgG3 > IgG1	Paranodes	Caspr1 pattern[b]	Moderate positive	DRG mild positive Dorsal roots paranodes
7[a]	Negative	1:2700	IgG4	Paranodes	Caspr1 pattern[b]	Weak positive	DRG mild positive Dorsal roots paranodes
8	Positive	1:72 300	IgG4	Paranodes	Caspr1 pattern[b]	Strong positive	DRG mild positive Dorsal roots paranodes
9	Positive	NA	NA	NA	NA	NA	NA
10	Positive	1:24 300	IgG4 > > > IgG2	Paranodes	Caspr1 pattern[b]	Weak positive	DRG mild positive Dorsal roots paranodes
11[a]	Negative	1:900	IgG4	Paranodes	Caspr1 pattern[b]	Weak positive	DRG mild positive Dorsal roots paranodes
12	Positive	1:218 700	IgG4	Paranodes	Caspr1 pattern[b]	Strong positive	DRG mild positive Dorsal roots paranodes
13	Positive	NA	NA	NA	NA	NA	NA
14	Positive[c]	Positive[c]	IgG3 > IgG2[c]	Paranodes (mouse)[c]	NA	NA	NA
15[a]	Negative	1:2700	IgG4	Paranodes	Caspr1 pattern[b]	Weak positive	NA

IHC = immunohistochemistry; NA = assays were not performed because sera were not available.
[a]Samples were negative in the Caspr1 CBA.
[b]Paranodal staining that co-localizes with Caspr1 mAb.
[c]Tested in Würzburg.

Autoantibodies against both CNTN1 and Caspr1 bearing mannose-rich *N*-glycans were identified in one patient with an aggressive form of CIDP[11,33] using an *N*-glycosylation mutant cell line (Lec1). We used Lec1 cells to analyse if *N*-glycosylation of Caspr1 or CNTN1 was relevant for the detection of anti-Caspr1/CNTN1 antibodies, but did not find any difference in the reactivity pattern of HEK293 and Lec1 cells (Supplementary material and Supplementary Fig. 2).

IgG reactivity against Caspr1 alone was detected by ELISA

All tested sera (12/12) from CIDP patients with Caspr1/CNTN1 antibodies showed reactivity against the Caspr1/CNTN1 complex and against Caspr1 alone by ELISA (Fig. 2A and B). Anti-Caspr1 IgG titres by ELISA varied from 1:900 to 1:218 700 (Table 1). Interestingly, sera with higher Caspr1 ELISA titres were those that were positive in the Caspr1-only CBA (median 1:24 300 CBA Caspr1-positive versus 1:2700 CBA Caspr1-negative, $P = 0.0025$), suggesting that the addition of CNTN1 improves CBA sensitivity in those with lower titres. Indeed, both CBA Caspr1-positive and CBA Caspr1-negative sera showed increased ΔOD signal when CNTN1 protein was added to the Caspr1 ELISA (Fig. 2C). Autoantibodies were predominantly of the IgG4 subclass in all but two tested sera (Patients 1 and 6), in which the antibodies were predominantly of the IgG3 subclass (Fig. 2D and Table 1). Additionally, the sample from Patient 14 was tested in Würzburg by Caspr1 ELISA, and antibodies were predominantly of the IgG3 subclass. Finally, in the CNTN1 ELISA only Patient 15 showed mild positivity (Fig. 2E). Sera from Patients 9 and 13 were not available for ELISA tests.

Since Caspr1 is expressed in the cell membrane complexed with CNTN1 and considering the HEK CBA results, we wanted to investigate if the Caspr1 protein used in our ELISAs (obtained from Caspr1-transfected CHO cells) was contaminated with CNTN1, and if the presence of CNTN1

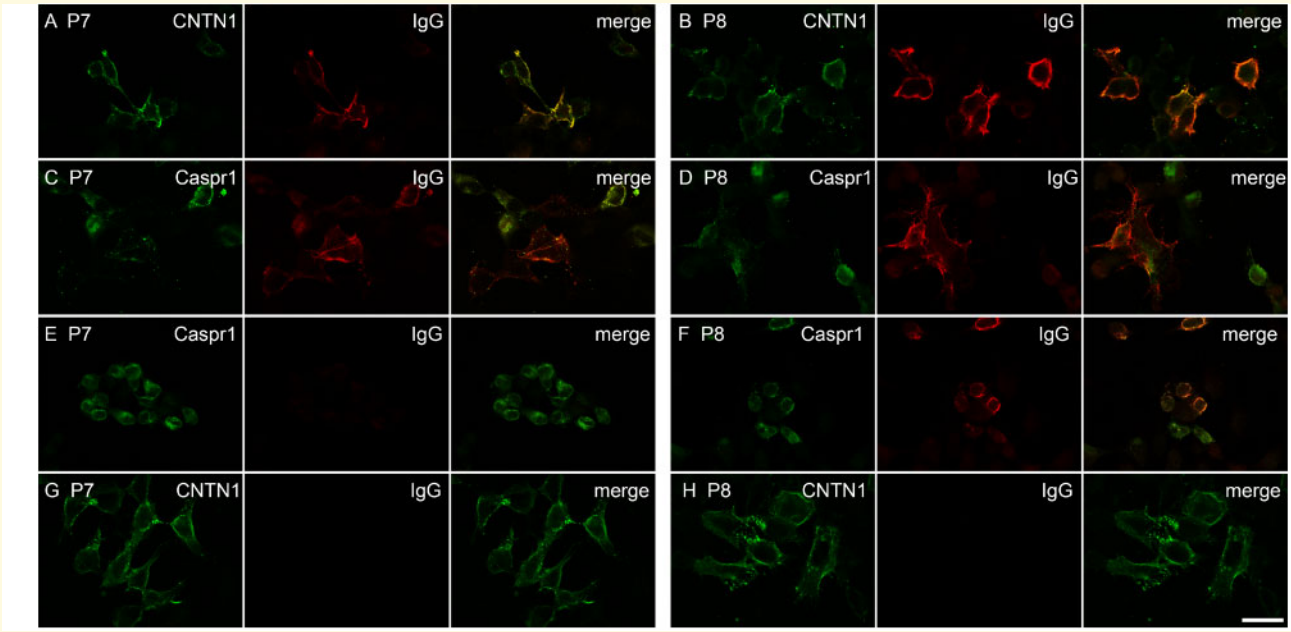

Figure 1 Immunocytochemistry of anti-Caspr1/CNTN1 antibodies. HEK293 cells co-transfected with Caspr1 and CNTN1 (**A**–**D**) or transfected with Caspr1 (**E** and **F**) or CNTN1 (**G** and **H**) alone and double labelled with CNTN1 (**A**, **B**, **G** and **H**) or Caspr1 (**C**–**F**) antibodies and patients' sera. Patients' IgG bind to cells co-transfected with CNTN1 and Caspr1 and co-localize better with CNTN1 (**A** and **B**) than with Caspr1 (**C** and **D**). While IgG from Patient 7 (**E**) does not bind to Caspr1 monotransfected cells, IgG from Patient 8 binds to cells transfected with Caspr1 only but does not co-localize with Caspr1 (**F**). Patient's IgG did not show reactivity when CNTN1 was transfected alone (**G** and **H**). Scale bars = 25 μm.

was necessary for the sera of our patients to react against Caspr1 in the ELISA. Mass spectrophotometry (Supplementary material) identified two peptides of CNTN1, with a total of two spectral counts, compared to 76 peptides and 434 spectral counts for Caspr1. The estimated ratio (molar) between the two proteins was 157 and 73, respectively, based on the number of spectral counts (corrected by molecular weight), and the emPAI parameter.[32] Thus, the amount of CNTN1 protein was estimated between 0.6% and 1.3% of the total protein amount in the purified Caspr1 protein. We confirmed the presence of CNTN1 in the Caspr1 human recombinant protein by immunoprecipitation (Supplementary Fig. 3B), and used the CNTN1-depleted Caspr1 recombinant protein to test if sera reactivity by ELISA was lost after CNTN1 depletion. We did not observe statistically significant differences in reactivity after CNTN1 depletion (Supplementary Fig. 3A). As a result, we concluded that sera from these patients primarily react against Caspr1 but that reactivity is enhanced, both in ELISA and CBA when CNTN1 is added.

Anti-Caspr1/CNTN1 antibodies bind to paranodes and co-localize with Caspr1 in peripheral nerve tissue

The presence of autoantibodies targeting paranodal structures was confirmed using swine teased-nerve fibre immunohistochemistry (Fig. 3A and B). All sera bound to paranodes and co-localized with Caspr1 mAb. The staining pattern was similar to that of other antibodies against paranodal proteins such as CNTN1. To define the IgG staining pattern in peripheral nerve tissue, we used macaque peripheral nerve sections and compared the staining pattern with that of anti-CNTN1 positive serum. IgG from both anti-Caspr1/CNTN1 and anti-CNTN1 positive patients bound to paranodes (Fig. 3C–F and Supplementary Fig. 4), but only IgG from CNTN1-positive patients and CNTN1 antibody bound strongly against NCAM (CD56)-positive non-myelinating Schwann cells (Remak bundles). Unfortunately, sera from Patients 9, 13 and 14 were not available for these experiments.

Anti-Caspr1/CNTN1 antibodies bind strongly to dorsal root paranodes and weakly to dorsal root ganglion neuron somas

Anti-Caspr1 antibodies from two previously reported patients bound to TRPV1-immunoreactive DRG neurons.[25] Hence, we investigated binding of our anti-Caspr1/CNTN1 sera to cultured DRG neurons and DRG sections. Table 1 summarizes the results of these experiments. In DRG sections, patients' IgG bound strongly to the paranodes in the nerve roots arising from the DRG (Fig. 4B), but

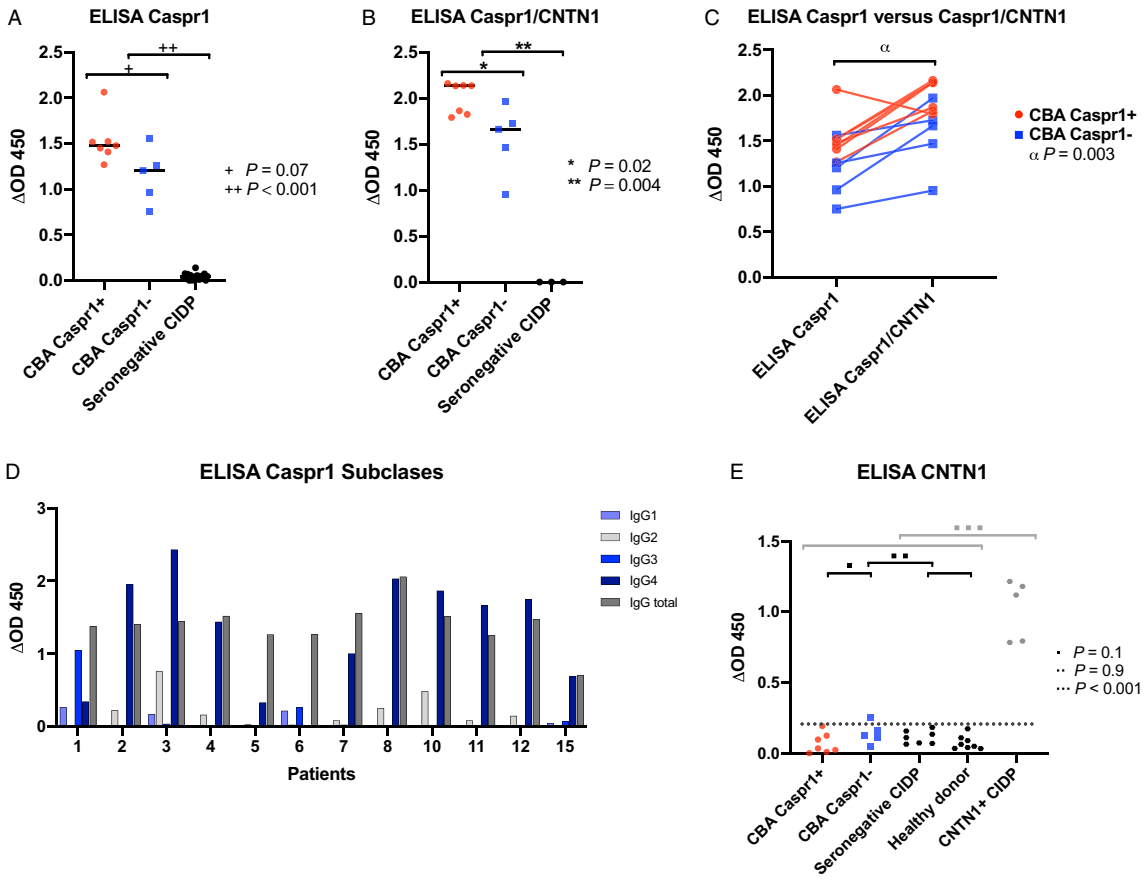

Figure 2 Sera reactivity against Caspr1 measured by ELISA. (**A** and **B**) Sera from patients with Caspr1/CNTN1 antibodies were positive in Caspr1 ELISA and Caspr1/CNTN1 ELISA. (**C**) Almost all samples showed increased reactivity (OD450) in the Caspr1/CNTN1 ELISA compared to the Caspr1 ELISA ($P = 0.003$) and there were no differences between CBA Caspr1-positive and CBA Caspr1-negative sera ($P = 0.89$). (**D**) In the Caspr1 ELISA, antibodies were predominantly of the IgG4 subclass in all patients tested except for two (Patients 1 and 6) that were predominantly of the IgG3 subclass. (**E**) In the CNTN1 ELISA only one sample (Patient 15) showed mild positivity compared with seronegative CIDP and healthy donors. (**A**, **B** and **E**) were analysed using Mann-Whitney U-test. Data in **C** were analysed using Wilcoxon matched-pairs signed rank test.

weakly to DRG neuron bodies (Fig. 4A). In contrast, the Caspr1 mAb bound strongly to DRG neuron somas (Fig. 4A) and paranodes. Interestingly, CNTN1 antibody bound weakly to DRG neuron somas (Fig. 4C) but strongly to the paranodes (Fig. 4D). We also investigated if anti-Caspr1/CNTN1-positive sera bind to small DRG neurons labelled with IB4 and TRPV1. Patients' IgG did not bind to IB4-positive DRG neurons, but bound weakly to TRPV1-positive DRG neurons. (Supplementary Fig. 5). However, this weak binding does not allow us to confirm that our patients' sera bind to small pain-conducting DRG neurons. In the cultured DRG neurons, strong binding was observed only in two samples, and moderate reactivity was observed in another two. Interestingly, patients' sera bound small but also large neurofilament heavy-positive DRG neurons (Supplementary Fig. 5). Finally, we investigated anti-Caspr1/CNTN1 IgG binding to hippocampal and motor neurons. Only serum from Patient 12 bound to hippocampal neurons. However, we observed mild to moderate IgG binding of all available patients' sera to neuronal somas and axons in neuroblastoma-derived motor neurons. Sera from Patients 9, 13 and 14 were not available for these tests.

Immunoabsorption experiments

We performed immunoabsorption experiments incubating patients' sera with HEK cells transfected with CNTN1, Caspr1 or co-transfected with Caspr1/CNTN1. Reactivity against the paranodes was abrogated after serum preabsorption with HEK cells co-expressing Caspr1/CNTN1 in all patients (Supplementary Fig. 6).

Clinical features of CIDP patients with anti-Caspr1/CNTN1 autoantibodies

Clinical features of CIDP patients with antibodies against the Caspr1/CNTN1 complex are summarized in Table 2

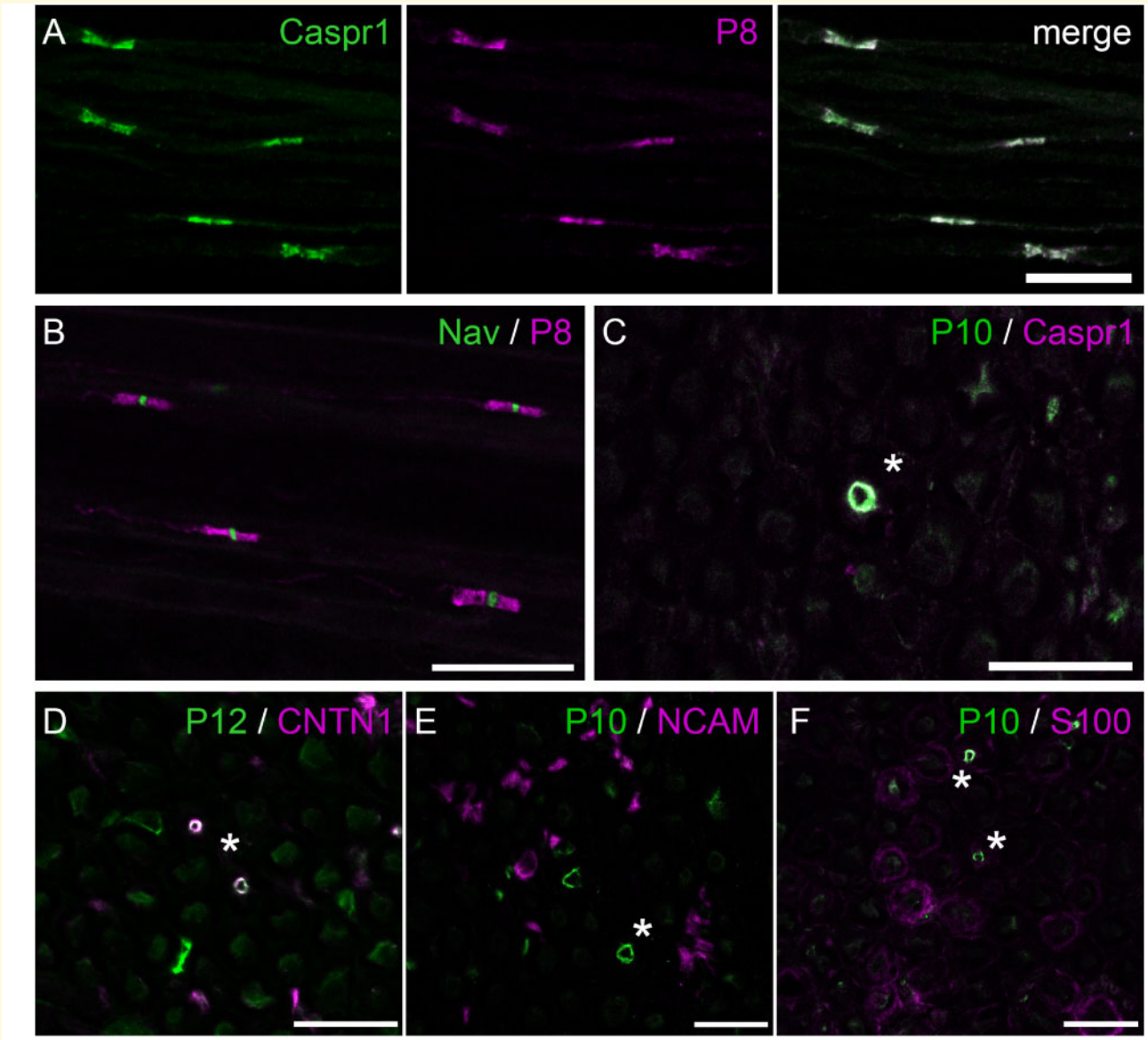

Figure 3 Anti-Caspr1/CNTN1 autoantibodies bind to paranodes in teased nerve fibre preparations and peripheral nerve sections. (**A** and **B**) Swine teased-nerve fibres preparations labelled with serum from a patient with (**A**) anti-Caspr1/CNTN1 antibodies (red) and anti-Caspr1 mAb or (**B**) anti-sodium channel antibody (green). Serum reacts against paranodes. (**C**–**F**) Macaque peripheral nerve sections stained with Caspr1/CNTN1-positive patient's serum and (**C**) Caspr1 mAb, (**D**) CNTN1 antibody, (**E**) NCAM antibody, or (**F**) S100b antibody. Sera from Caspr1/CNTN1-positive patients reacted against paranodal structures and co-localized with (**C**) Caspr1 antibody, but not with (**D**) CNTN1Ab that, besides paranodes, reacted strongly with unmyelinated fibres (Remak bundles), similar to NCAM antibody (**E**). Scale bars = 25 μm.

and Supplementary Table 2. Patients were aged between 40 and 75 at onset, 10 (67%) were male. All patients fulfilled EFNS/PNS definite diagnostic criteria for definite CIDP, 12 (80%) were typical and three had a distal acquired demyelinating symmetric (DADS) phenotype. Seven (47%) patients were initially diagnosed with Guillain-Barré syndrome due to an acute or subacute onset. All 15 patients showed areflexia or hyporeflexia and altered sensation in all four limbs. Six (40%) patients had cranial nerve dysfunction, including ophthalmoparesis, facial weakness and dysphagia, or respiratory involvement. Eight (53%) patients reported neuropathic pain. Ten (67%) patients had tremor and 12 (80%) ataxia. Neurophysiological studies (Supplementary Table 1) showed non-uniform motor nerve conduction slowing and increased (or absent) F-wave latencies. In addition, low-amplitude compound muscle action potentials and spontaneous activity suggesting acute denervation were observed in most patients. CSF protein levels were elevated in all patients and MRI demonstrated nerve root enhancement in the six patients in which imaging studies were performed. Nerve biopsies were available in four patients and showed subtotal loss of myelinated fibres and ongoing

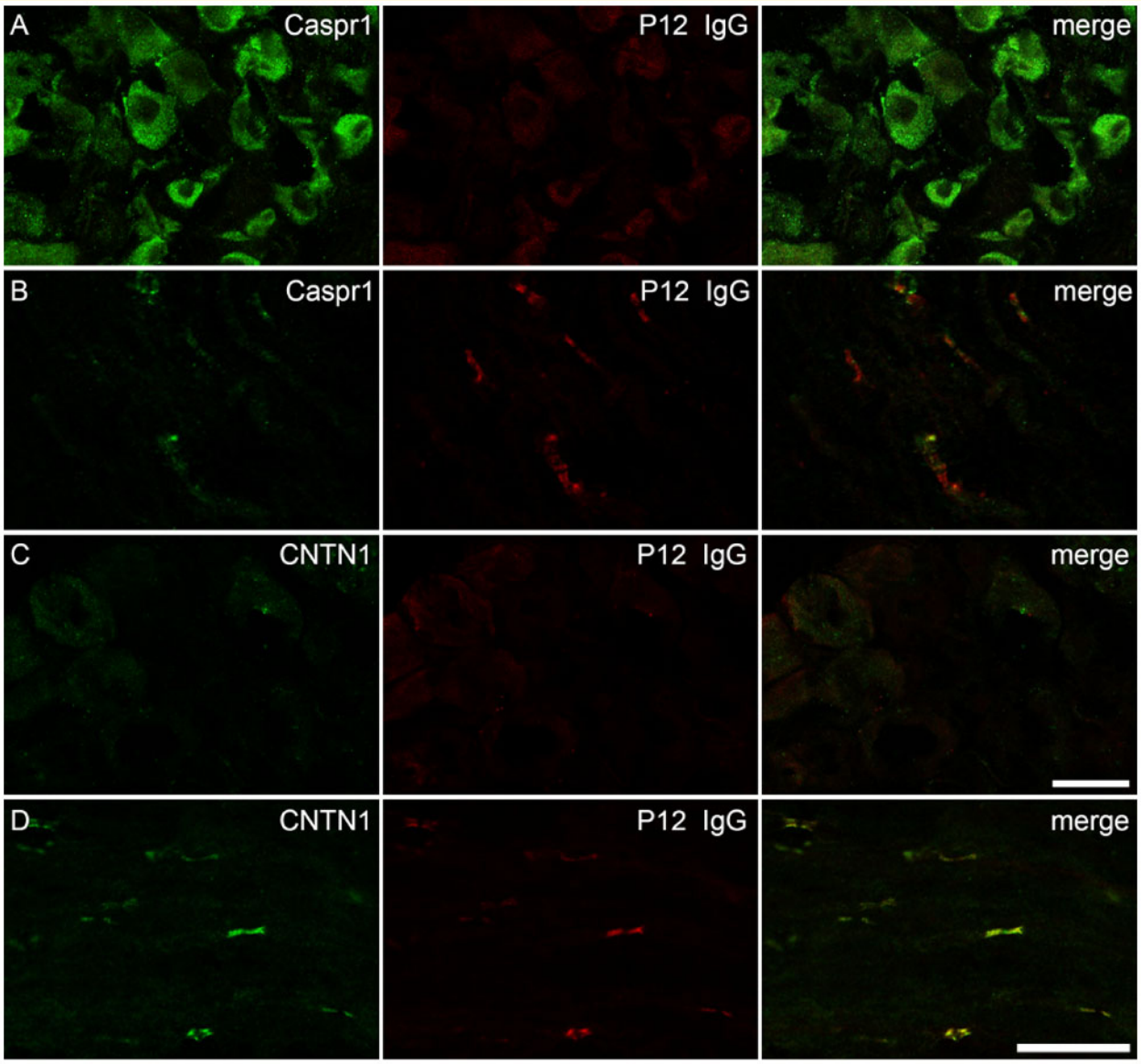

Figure 4 Anti-Caspr1/CNTN1 antibodies bind strongly to dorsal root paranodes and weakly to DRG neuron somas. (**A**–**D**) DRG neuron somas and dorsal roots fibres sections labelled with patient's serum and (**A** and **B**) Caspr1 mAb, or (**C** and **D**) CNTN1 antibody. Patient's IgG binds strongly to (**B**) paranodes of the dorsal roots, but very weakly to (**A**) DRG neuron somas. Caspr1 mAb binds strongly to (**A**) DRG neuron somas, but weakly to paranodes. Conversely, CNTN1 antibody binds slightly to (**C**) DRG neuron somas, but strongly to (**D**) the paranodes wherein it co-localizes with anti-Caspr1/CNTN1 IgG. Scale bars = 25 μm.

axonal degeneration with rare inflammatory cells and no onion bulbs. Interestingly, in three of these patients mild to moderate subperineurial oedema was observed. Figure 5 shows a representative biopsy (Patient 3) with nearly complete loss of myelinated fibres and retained small, unmyelinated axons. Complete response to intravenous immunoglobulin (IVIg) was not observed in any patient, and good response to steroids was observed in only one patient (7%). Nine of 10 (90%) patients showed a good response to rituximab defined as an improvement of at least two points in the modified Rankin Scale. Good response to rituximab was statistically significant ($P < 0.001$) compared to the response to IVIg. Data on treatments used and patients' response is summarized in Table 3. Follow-up sera following rituximab treatment were available from five patients, and all five were seronegative after a median of 20 months of follow-up.

Discussion

Patients with antibodies targeting Caspr1/CNTN1 co-transfected cells represent a rare but clinically and serologically distinct subset of CIDP patients. Such patients present with

Table 2 Clinical features and treatment data of CIDP patients with antibodies against the Caspr1/CNTN1 complex

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Sex, age	F, 75	F, 40	M, 75	M, 50	M, 51	M, 70	F, 51	M, 48	F, 58	F, 57	M, 56	M, 65	M, 53	M, 47	M, 73
Initial diagnosis	CIDP	GBS	GBS	CIDP	CIDP	GBS	CIDP	GBS	GBS	CIDP	CIDP	CIDP	GBS	GBS	CIDP
Category	Typical	Typical	Typical	Typical	Typical	DADS	Typical	Typical	Typical	DADS	Typical	Typical	Typical	Typical	DADS
Clinical features															
Onset	Subacute	Acute	Chronic	Subacute	Chronic	Subacute	Chronic	Acute	Chronic	Chronic	Chronic	Chronic	Subacute	Chronic	Subacute
Weakness	Symmetric proximal and distal	Symmetric proximal and distal	Asymmetric proximal and distal	Symmetric distal	Asymmetric proximal and distal	Symmetric predominately distal	Symmetric proximal and distal	Symmetric proximal and distal	Symmetric distal	Symmetric predominately distal	Symmetric predominately distal	Symmetric proximal	Symmetric predominately distal	Symmetric proximal and distal	Symmetric distal
Tremor	No	Yes	Yes	No	Yes	Yes	Yes	Yes	No	Yes	Yes	No	No	Yes	Yes
Ataxia	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
Cranial nerve	No	No	No	No	No	Ophthalmoparesis	No	Ophthalmoparesis and facial weakness	Facial weakness	No	Facial weakness	Dysphagia and respiratory	Facial weakness and respiratory	No	No
Pain	Yes	Yes	Yes	No	Yes	No	No	No	No	Yes	Yes	Yes	No	Yes	No
CSF															
Proteins, g/l	3.8	3.2	1.8	2.7	>5	2.5	5.1	4.6	4.4	4.0	5.0	2.2	5	4.1	0.9
WBC, c/mm^3	13	19	1	3	4	0	NA	8	5	0	0	0	NA	3	3
Treatment (given/response)															
IVIg	Yes/partial	Yes/NR	Yes/NR	Yes/partial	Yes/partial	Yes/partial	Yes/partial	Yes/partial	Yes/NR	Yes/NR	Yes/NR	Yes/NR	Yes/NR	Yes/partial	Yes/NR
Steroids	Yes/partial	Yes/partial	Yes/NR	Yes/NR	Yes/partial	Yes/NR	Yes/partial	Yes/partial	Yes/partial	Yes/partial	Yes/good	Yes/NR	Yes/NR	No	Yes/NR
PLEX	No	No	No	Yes/NR	Yes/partial	Yes/partial	Yes/partial	Yes/partial	No	No	Yes/good	Yes/NR	Yes/NR	No	Yes/good
Rituximab	Yes/good	Yes/good	Yes/good	Yes/good	Yes/good	No	No	Yes/good	No	Yes/good	Yes/good	Yes/good	Yes/ongoing	No	No
Cyc	No	No	No	Yes/NR	No	No	Yes/good	Yes/partial	No	No	No	No	No	No	No

Cyc = cyclophosphamide; DADS = distal acquired demyelinating symmetric; F = female; GBS = Guillain-Barré syndrome; M = male; NA = not available; NR = no response; OCB = oligoclonal bands; PLEX = plasma exchange; WBC = white blood cells.

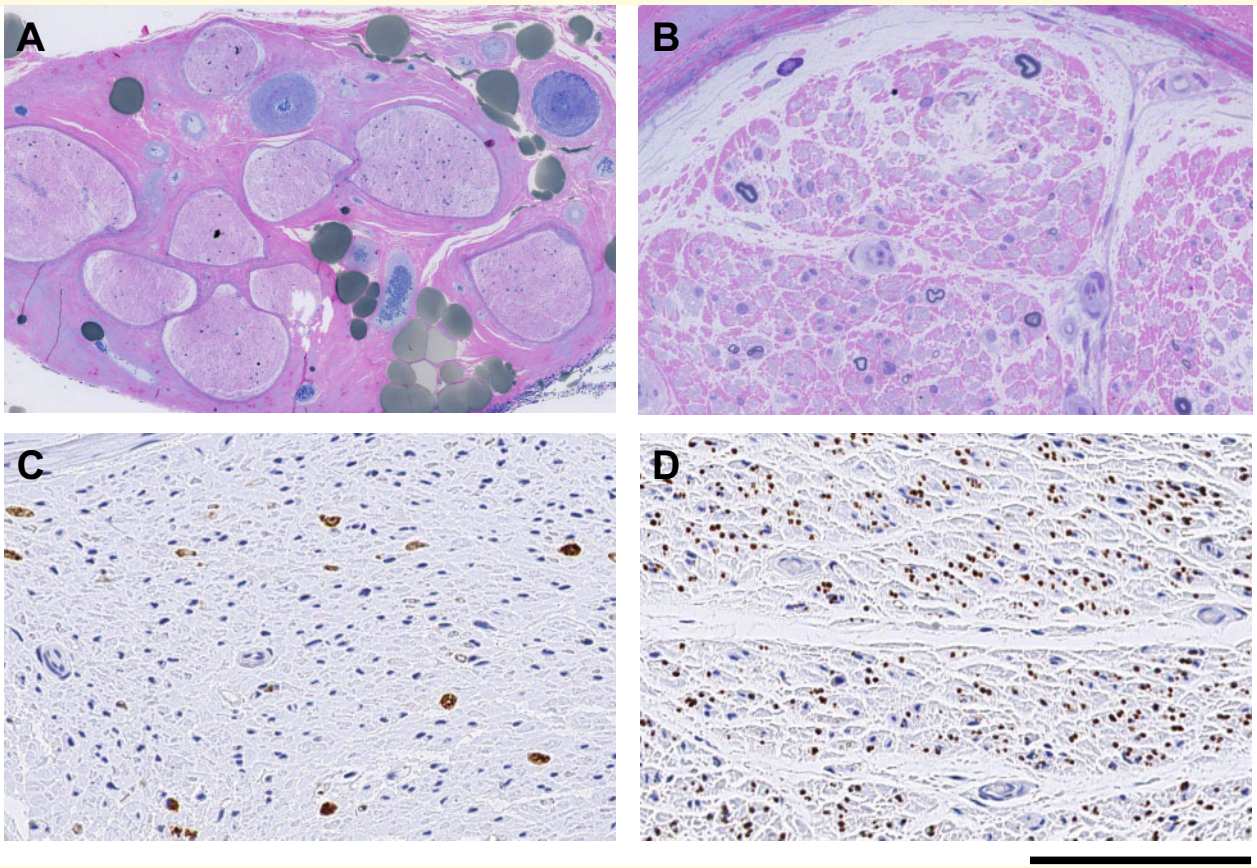

Figure 5 Nerve biopsy of Patient 3 with loss of myelinated axon and subperineurial oedema. (**A**) Overview of the transfers semithin section showing multiple fascicles depleted of myelinated fibres. (**B**) High power magnification of one of these fascicles showing subperineurial oedema and rare remaining large and small myelinated fibres. (**C**) Myelin basic protein showing residual myelinated fibres, whilst (**D**) the density of small axons remains within normal limits. Scale bar = 800 μm in **A**, and 100 μm in **B**–**D**. Images courtesy of Sebastian Brandner, UCL Queen Square Institute of Neurology.

Table 3 Treatment and response in CIDP patients with antibodies to Caspr1/CNTN1 co-transfected cells

Treatment	Response			*P* (versus IVIg)*
	Absent	Partial	Good	
IVIg (*n* = 15)	8 (53%)	7 (47%)	0 (0%)	–
Steroids (*n* = 14)	6 (43%)	7 (50%)	1 (7%)	0.48
PLEX (*n* = 9)	3 (33%)	4 (44%)	2 (22%)	0.13
Rituximab (*n* = 10)	0 (0%)	1 (10%)	9 (90%)	<0.001
Cyclophosphamide (*n* = 3)	1 (33%)	1 (33%)	1 (33%)	0.11

A good response was defined as an improvement of at least two points in the modified Rankin scale. A partial response was defined as an improvement of one point in the modified Rankin scale. PLEX = plasma exchange.
*Fisher's exact test comparing good response to each treatment relative to good response to IVIg. A *P*-value of 5% or lower was considered statistically significant.

rapidly progressive and disabling neuropathies, and generally respond poorly to IVIg, but very well to rituximab. Although two subpopulations can be defined, based on whether antibodies are detectable using the Caspr1 singly-transfected CBA or not, these sera behave identically when antibodies are tested by ELISA or by nerve immunohistochemistry, suggesting that they harbour the same autoantibody specificity at different titres. Our work aimed to elucidate if antibodies target Caspr1 only, or an epitope emerging from the Caspr1/CNTN1 complex. Despite the interpretation of our assays was complicated by the fact that Caspr1 protein used for the ELISA was contaminated by CNTN1, and HEK cells, used in the Caspr1 CBA, express low but detectable levels of CNTN1, Caspr1 ELISA detected antibodies against Caspr1 in all patients suggesting that Caspr1 is the primary target. The addition of CNTN1 to the serological assays increased both the ELISA signal intensity and the sensitivity of the CBA but reactivity in Caspr1-only ELISA was not abrogated by depletion of CNTN1 from the recombinant Caspr1 preparation. Altogether these findings strongly suggest that the primary target in these patients is Caspr1 and reactivity against it is enhanced in the presence of CNTN1. Thus, the Caspr1 ELISA could serve as a good screening method for the presence of antibodies against Caspr1 that need then to be confirmed by CBA or immunohistochemistry in the routine diagnostic process.

Caspr1 is a transmembrane glycoprotein expressed by neurons in the paranode where it forms a complex with CNTN1 that binds to the glial protein NF155 to form the septate-like junctions linking the axon and the paranodal loops of Schwann cells.[23,34,35] In 2013, our group detected autoantibodies against cells co-transfected with CNTN1 and Caspr1 but not against CNTN1 or Caspr1 alone in a single CIDP patient.[11] Subsequent studies[33] showed that these antibodies recognized contactin-1 bearing mannose-rich *N*-glycans and also reacted independently with Caspr1, which also bears oligomannose-type sugars. Later, Doppler *et al.*[25] described the presence of autoantibodies against Caspr1 in two patients, one with CIDP and one with Guillain-Barré syndrome. Two additional anti-Caspr1-positive patients were reported in a CIDP cohort,[8] but their clinical characteristics were not described in detail. Antibodies to Caspr1/CNTN1 complex were also recently reported in six (1.8%) patients of an Italian CIDP cohort.[26] Three of these patients had IgG4 anti-Caspr1/CNTN1 antibodies and presented a predominantly distal phenotype without neuropathic pain. In our series, anti-Caspr1/CNTN1-positive CIDP patients were characterized by a rapid-onset neuropathy with ataxia in more than three-quarters of the patients, comparable with that described in patients with anti-CNTN1 antibodies.[10] Only half of our patients reported pain. Interestingly, cranial involvement, including ophthalmoparesis, facial weakness or oropharyngeal weakness, uncommon in seronegative CIDP, was observed in 40% of our patients. Thus, a rapid-onset with cranial nerve involvement may be a clinical clue to the presence of anti-Caspr1/CNTN1 autoantibodies in CIDP. Neurophysiological studies demonstrated nerve conduction slowing and other features attributed to acquired demyelination, but decreased CMAP amplitudes and early denervation, suggestive of axonal involvement, were also detected, as it was described in patients with anti-CNTN1[11] and anti-Caspr1[25] antibodies. The four nerve biopsies available showed loss of myelinated fibres (but retained small, unmyelinated axons) with mild to moderate subperineurial oedema and in the absence of inflammatory cells. Similar to previous descriptions of CIDP patients with anti-CNTN1 and anti-NF155 antibodies,[18,36] no signs of segmental demyelination were observed, despite nerve conduction studies suggesting demyelination.

Antibodies against paranodal proteins such as CNTN1 and NF155 are most frequently of the IgG4 subclass, but a few patients with IgG3 antibodies have been reported. Appeltshauser *et al.*[37] demonstrated that anti-CNTN1 IgG3 antibodies are able to fix complement and may associate with a better response to IVIg. In a recent study[27] they also reported one patient with an acute-onset CIDP and antibodies against both CNTN1 and Caspr1 that evolved towards anti-Caspr1 specificity only during follow-up. Importantly, these antibodies that were initially IgG3, switched to the IgG4 subclass during follow-up. In our study IgG subclasses assays revealed that IgG4 was the predominant isotype in most of the patients, but IgG3 was predominant in three. We lacked serial samples to test if the IgG3 isotype later evolved to IgG4. None of our patients had a complete response to IVIg, as has been reported in other cohorts of CIDP patients with IgG4 paranodal antibodies.[15,38] Interestingly, all three patients with IgG3 anti-Caspr1/CNTN1 antibodies had a partial response to IVIg, while only 3 of 10 patients with IgG4 antibodies responded partially to IVIg. Moreover, 9 of 10 (90%) patients presented a good response to rituximab. However, recovery was slow in most of the cases, probably explained by the established axonal degeneration at the time the treatment was begun. Long term outcomes for these patients remain to be assessed but early diagnosis and therapy with B-cell depletion therapies could help avoid permanent axonal loss in these patients.

One of the objectives of our study was to elucidate the target of these autoantibodies. The search for the antigenic target was complicated by the fact that surface expression of Caspr1 is dependent on the presence of CNTN1,[39] and that CNTN1 glycosylation is modified by the presence of Caspr1.[40] Moreover, several cell types (including, in low amounts, the HEK293 line, routinely used for CBA) endogenously express CNTN1.[41] Our study, however, suggests that, even though the main antigenic target is Caspr1, the presence of CNTN1 enhances patients' IgG binding to Caspr1. First, patients' sera co-localized perfectly with CNTN1 and Caspr1 at the paranodes (both in DRG preparations and teased nerve immunohistochemistry) but did not co-localize well (or staining is much weaker) with Caspr1 at the DRG neuron somas, where CNTN1 is less abundant according to our experiments. Second, all patients had anti-Caspr1 antibodies detected by ELISA, but the recombinant Caspr1 protein used in the ELISA was contaminated with CNTN1 (that could also be enhancing binding to Caspr1). Indeed, the addition of CNTN1 to Caspr1 improved the detection of the anti-Caspr1/CNTN1 antibodies. Third, some patients did not have detectable antibodies by CBA when only Caspr1 is transfected, but they did when those same cells were co-transfected with CNTN1. Indeed, in the first description of anti-Caspr1 antibodies, Caspr1/CNTN1-co-transfected HEK293 cells were used to detect these autoantibodies by flow cytometry.[25] When Caspr1 and CNTN1 were co-transfected, patients' IgG co-localization was better (but not exclusive) with CNTN1, likely because only those cells that had CNTN1 expressed in their surface display Caspr1 in its natural conformation. We explored the possibility that the glycosylation patterns of CNTN1 and Caspr1, when co-expressed in HEK cells, influence the immunoreactivity of these sera, as had been previously suggested.[33] CNTN1 expressed in Lec1 cells bears mannose-rich *N*-glycans,[40] unlike CNTN1 expressed in HEK cells in the absence of Caspr1, which bears complex *N*-glycans.[33] However, in our assays, transfection of Lec1 rather than HEK293 cells did not change the staining patterns significantly, so we concluded that the epitope of the autoantibodies was not influenced by the glycosylation of CNTN1. According to these observations, we believe that anti-Caspr1/CNTN1 autoantibodies target primarily Caspr1 but binding is stronger when CNTN1 is also present. We were technically unable to

elucidate why. One possible explanation is that the presence of CNTN1 would modify Caspr1 conformation and create conformational epitopes that are best detected by anti-Caspr1/CNTN1 autoantibodies. Another explanation would be that the presence of CNTN1 triggers post-translational modifications in Caspr1 that enhance its antigenicity. The detailed study of these hypotheses is beyond the scope of this study. In summary, the conformational display of Caspr1 seems to influence the detection of these autoantibodies and, therefore, both, Caspr1 ELISA or Caspr1/CNTN1 CBA are more sensitive than Caspr1 CBA to detect these antibodies.

The main limitation of our study derives from the low numbers of patients and their diverse origin, that precludes an estimation of the real frequency of these antibodies. The prevalence of anti-Caspr1/CNTN1 antibodies in the Barcelona cohort was only 1.9% (1/52) of the CIDP cases. Similarly, in the Würzburg cohort of 85 patients who fulfilled the EFNS criteria for CIDP, two (2.3%) were anti-Caspr1/CNTN1 positive, and in the Oxford cohort of 89 CIDP patients only one (1.1%) had anti-Caspr1/CNTN1 antibodies (unpublished observations). Also, one of the patients was recruited from an acute-onset CIDP cohort in which one (4.3%) of 23 patients had anti-Caspr1/CNTN1 antibodies. Considering this low prevalence, the identification of 15 patients has required a collaborative effort that has resulted in the largest series of anti-Caspr1/CNTN1 patients reported so far and the only one that is able to report the clinical-immunological features associated with these antibodies. We believe that the description of specific phenotypic features (cranial involvement) and treatment response patterns (poor response to IVIg, good response to rituximab), despite the low frequency of these autoantibodies, provides valuable information for the identification and management of these patients.

In conclusion, anti-Caspr1/CNTN1 autoantibodies associate with a rapid-onset CIDP with cranial-nerve and early axonal involvement, but poor response to IVIg. These antibodies target primarily Caspr1 but binding is stronger when CNTN1 is also present. The antibody ELISA using human recombinant Caspr1 detects these antibodies with high sensitivity, but confirmation with the Caspr1/CNTN1 CBA or immunohistochemistry should be routine in diagnostic laboratories.

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Competing interests

E.P.G. received speaking fees from Merck, Roche and Biogen. L.M.A. received speaking fees from Roche. I.I. provided expert testimony and received speaking fees and travel grants from Pfizer. A.R. has received honoraria from Sarepta, Roche and Sanofi Aventis, is a member of MND Association Clinical Advisory Board, and was a member of NICE MND Guideline Development Group. R.H. is supported by the Jubiläumsfonds der Österreichischen Nationalbank, project 16919. S.R. runs a not-for-profit diagnostic testing service for nodal/paranodal antibodies. He has received a speaker's honorarium and travel expenses from Fresenius Medical Care, Alnylam and Excemed, and payments to provide expert medicolegal advice on inflammatory neuropathies and their treatment. He has received complimentary registration and prize money from the Peripheral Nerve Society, and a travel bursary from the European School of Neuroimmunology. He is a member of the GBS and Associated Inflammatory Neuropathies (GAIN) UK patient charity medical advisory board. L.Q. has received speaker honoraria from Merck, Sanofi-Genzyme, Roche, Biogen, Grifols, CSL Behring provided expert testimony for Grifols, Johnson and Johnson, Annexon Phaarmaceuticals, Alexion, Sanofi-Genzyme, Novartis and CSL Behring and received research funds from Roche and Grifols.

Supplementary material

Supplementary material is available at *Brain* online.

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