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REPORT

Autoantibodies to nodal isoforms of neurofascin in chronic inflammatory demyelinating polyneuropathy

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Chronic inflammatory demyelination polyneuropathy is a heterogeneous and treatable immune-mediated disorder that lacks biomarkers to support diagnosis. Recent evidence indicates that paranodal proteins (contactin 1, contactin-associated protein 1, and neurofascin-155) are the targets of autoantibodies in subsets of patients showing distinct clinical presentations. Here, we identified neurofascin-186 and neurofascin-140 as the main targets of autoantibodies in five patients presenting IgG reactivity against the nodes of Ranvier. Four patients displayed predominantly IgG4 antibodies, and one patient presented IgG3 antibodies that activated the complement pathway *in vitro*. These patients present distinct clinical features compared to those with anti-neurofascin-155 IgG4. Most patients had a severe phenotype associated with conduction block or decreased distal motor amplitude. Four patients had a subacute-onset and sensory ataxia. Two patients presented with nephrotic syndromes and one patient with an IgG4-related retroperitoneal fibrosis. Intravenous immunoglobulin and corticosteroids were effective in three patients, and one patient remitted following rituximab treatment. Clinical remission was associated with autoantibody depletion and with recovery of conduction block and distal motor amplitude suggesting a nodo-paranodopathy. Our data demonstrate that the pathogenic mechanisms responsible for chronic inflammatory demyelination polyneuropathy are broad and may include dysfunctions at the nodes of Ranvier in a subgroup of patients.

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Abbreviations: Caspr1 = contactin-associated protein 1; CIDP = chronic inflammatory demyelinating polyneuropathy; ELISA = enzyme-linked immunosorbent assay; GBS = Guillain-Barré syndrome; IVIg = intravenous immunoglobulin; Nfasc = neurofascin

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Introduction

Guillain-Barré syndrome (GBS) and chronic inflammatory demyelination polyneuropathy (CIDP) are rare and heterogeneous autoimmune diseases that affect peripheral nerves. Recent evidence indicates that the nodes of Ranvier can be the targets of the immune attack in GBS and in CIDP (Devaux *et al.*, 2012). Particularly, cell adhesion molecules at paranodes, contactin 1 (CNTN1), neurofascin-155 (Nfasc155), and contactin-associated protein 1 (Caspr1, encoded by *CNTNAP1*), have been shown to be selective targets of the IgG in subsets of CIDP patients (Ng *et al.*, 2012; Querol *et al.*, 2012, 2014; Doppler *et al.*, 2015*b*, 2016; Miura *et al.*, 2015; Ogata *et al.*, 2015*b*; Devaux *et al.*, 2016). Patients with anti-Nfasc155 and CNTN1 IgG4 antibodies present with distinct clinical phenotypes including rapid severe onset, ataxia, tremor, and a poor response to intravenous immunoglobulin (IVIg) (Ng *et al.*, 2012; Querol *et al.*, 2012, 2014; Miura *et al.*, 2015; Devaux *et al.*, 2016). By contrast, patients with anti-Caspr1 IgG showed neuropathic pain (Doppler *et al.*, 2016). Evidence indicates that these antibodies are pathogenic as these affect the paranodal axo-glial junctions and induce conduction defects (Doppler *et al.*, 2015*b*, 2016; Manso *et al.*, 2016). The proportion of positive patients is still small (<10%), but such antibodies can be helpful for patient prognosis and to guide treatments (Querol *et al.*, 2015).

Nodal antigens also seem to be targeted by autoantibodies in CIDP patients (Devaux *et al.,* 2012*)*; however, the nature of these antigens is unclear so far. Here, we identified neurofascin as the target of the autoantibodies using proteomic approaches. We detail the clinical features of five patients with CIDP.

Materials and methods

Patients and sera

Sera from patients fulfilling the diagnostic criteria for CIDP (Joint Task Force of the EFNS and the PNS, 2010) were received from hospitals throughout France ($n = 129$), Spain ($n = 72$), Italy ($n = 42$) and Singapore ($n = 3$), to screen for antibodies against nodal and paranodal proteins. As disease controls, we used sera from 26 patients with GBS, 32 with Charcot-Marie-Tooth disease, and 52 with multiple sclerosis. In addition, serum from 50 healthy control subjects was used. Written informed consent was obtained from each participant. The study was approved by the Ethics Committee of Aix-Marseille Université. Sera were tested by enzyme-linked immunosorbent assay (ELISA), western blot, cell-binding assay and immunohistochemistry, as described in the Supplementary material.

Constructs

Human Nfasc140 (XM_011509328.1), Nfasc186 (NM_001005388.2), gliomedin (NM_181789.2), and Caspr1 (NM_003632.2) were amplified by PCR from a human brain cDNA library and subcloned into pcDNA3.1 (ThermoFisher scientific). Human *CNTN1* and Nfasc155 constructs have been previously described (Miura *et al.*, 2015; Devaux *et al.*, 2016). Myc epitope was inserted at the intracellular C-terminal extremity of neurofascin isoforms using a site-directed mutagenesis kit (Agilent technologies). All truncations were constructed from Myc-tagged human Nfasc140 using the site-directed mutagenesis kit.

Complement binding assay

Microtitre plates were coated with 50 ng of Nfasc140 or Nfasc155, or with 100 ng of GM1 (Sigma-Aldrich). Wells were blocked and incubated overnight at 4°C with the sera diluted 1:20 in blocking buffer. Serum from a patient with acute motor axonal neuropathy, with antibodies to GM1, was used as a positive control. Serum from a healthy control was used as a negative control. Serum dilution was adjusted to the antibody titre. The next day, the wells were incubated for 2 h at room temperature with normal human sera as a source of complement diluted 1:10 in blocking buffer. Rabbit anti-human C1q antibodies (1:200; Abcam) were added for 1 h at 37°C. Peroxidase-conjugated anti-rabbit IgG (1:2000; Jackson Immunoresearch) was finally added for 1 h at 37°C, and ELISA was developed as described in the Supplementary material.

Immunoprecipitation and mass spectrometry

See Supplementary material.

Statistics

Statistical significance was assessed by unpaired two-tailed Student's *t*-tests or by one-way ANOVA followed by Bonferroni's *post hoc* tests using GraphPad Prism (GraphPad Software). *P*-values < 0.05 were considered significant.

Results

Identification of neurofascin as a target for autoantibodies at the nodes of Ranvier

A patient with CIDP presented with conduction block, prompt worsening and improvement of motor amplitudes after therapy, which were suggestive of a nodo-paranodopathy (Supplementary Fig. 1 and Supplementary Tables 1 and 2). The patient serum presented a strong IgG reactivity toward the nodes of Ranvier on teased nerve fibres and bound to surface antigens on the axon initial segments of neocortical neurons (Fig. 1). Two protein bands around 100–140 kDa and 150–190 kDa were immunoprecipitated from neocortical neuron cultures with the CIDP patient serum (Fig. 1C). Both bands were identified as neurofascin by mass spectrometry, and the peptide identified by mass

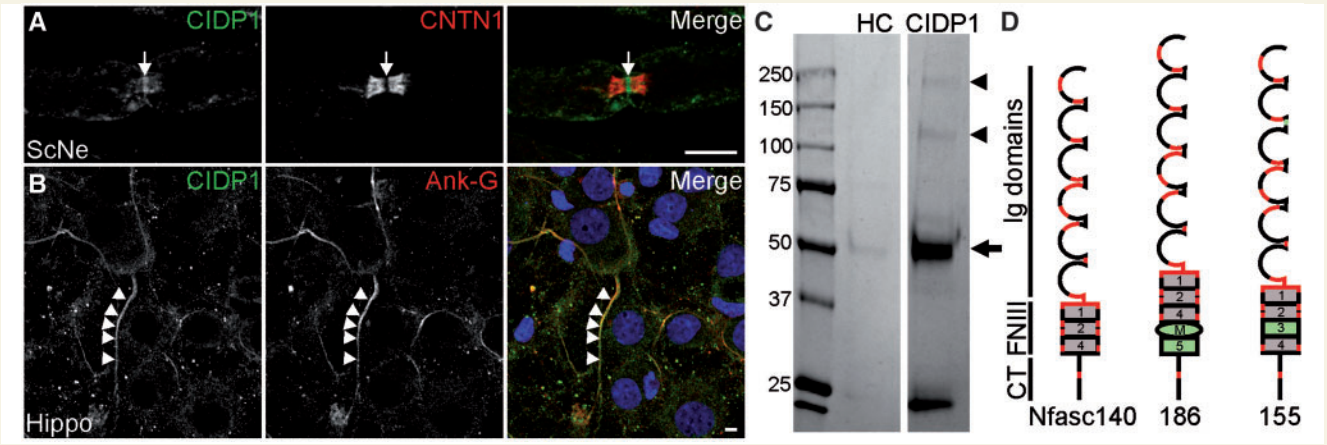

Figure 1 Identification of neurofascin as a nodal target for autoantibodies in CIDP. (**A**) Mouse sciatic nerve fibres stained with a CIDP patient's serum (Patient CIDP1; green) and for CNTN1 (red) to label paranodes. Human IgG bound specifically to the node of Ranvier (arrows). (**B**) The patient's IgG (green) bound to surface antigens expressed at the axon initial segment of cultured neocortical neurons (arrowheads), here stained with ankyrin-G (Ank-G; red). Scale bars = 10 μm. (**C**) Neocortical neurons were incubated with sera from a healthy control (HC) subject or Patient CIDP1 for 1 h, and the target antigens were immunoprecipitated, separated on SDS-PAGE gels, and stained with Imperial blue. Protein bands (arrowheads) were excised and identified by mass spectrometry as neurofascin. The arrow indicates the immunoprecipitated IgG bands. Molecular weight markers (kDa) are shown on the *left*. (**D**) Scheme of Nfasc140, 155, and 186 structures showing the position of the immunoglobulin (Ig) and fibronectin type III (FnIII) domains, and of the cytosolic tail (CT). The position of the peptides identified by mass spectrometry is shown in red. The protein sequence difference between neurofascin isoforms is indicated in green.

spectrometry matched with all isoforms of neurofascin (Fig. 1D and Supplementary Fig. 2). Three main isoforms of neurofascin have been described in the peripheral nervous system: Nfasc140, Nfasc155 and Nfasc186 (Zhang *et al.*, 2015). Nfasc155 is expressed by glial cells and located at paranodes (Tait *et al.*, 2000). By contrast, Nfasc140 and Nfasc186 are neuronal and found at nodes and axon initial segments (Sherman *et al.*, 2005; Zhang *et al.*, 2015). The fact that patient's IgG reacted against nodes and axon initial segments suggested that it recognized Nfasc140 and Nfasc186. In keeping, the IgG strongly reacted against Nfasc140 and Nfasc186, and much less to Nfasc155 (Supplementary Fig. 3). For clarity, we propose to call the latter anti-Nfasc140/186 IgG.

Epitope and isotype identification of anti-Nfasc140/186 autoantibodies

To characterize the prevalence and specificity of anti-Nfasc140/186 antibodies, cohorts of patients with CIDP ($n = 246$), GBS ($n = 26$), Charcot-Marie-Tooth ($n = 32$), or multiple sclerosis ($n = 52$) and a cohort of healthy donors ($n = 50$) were screened against Nfasc140, Nfasc155, Nfasc186, CNTN1, or Caspr1. Anti-Nfasc140/186 IgG were identified in five CIDP patients (2%), but not in patients with GBS, Charcot-Marie-Tooth and multiple sclerosis or from healthy control subjects. The serum IgG from four patients reacted toward Nfasc140, Nfasc155 and Nfasc186 by ELISA. Only one patient (Patient CIDP2) reacted against Nfasc186 (Supplementary Table 1). The patients' IgG also reacted against nodes and axon initial segments (Supplementary Fig. 4). The IgG subclass was predominantly IgG4 in four patients, and IgG3 in one patient (Supplementary Table 3). The reactivity to neurofascin isoforms and subclass determination were tested independently in two centres (CRN2M and Neuromuscular Diseases Unit), and gave consistent results.

The cohorts were also screened for antibodies to CNTN1, gliomedin, Caspr1, and Nfasc155. We identified nine patients reactive solely against Nfasc155 (4%), two against CNTN1 (1%), two against Caspr1 (1%), but none reactive against gliomedin. Patients with anti-Nfasc140/186 IgG did not present anti-CNTN1 or Caspr1 antibodies. Anti-Nfasc155 IgG4 autoantibodies bound paranodes, but did not bind axon initial segments or nodes (Supplementary Fig. 4) or react against Nfasc140 or Nfasc186 by ELISA (Supplementary Table 3) or cell-based assays (Supplementary Fig. 5).

Neurofascin isoforms are composed of six immunoglobulin (Ig) domains, and three to four fibronectin type III (FnIII) domains. Nfasc155 only differs from Nfasc140 by the presence of a fourth FnIII domain. To determine whether anti-Nfasc140/186 IgG recognizes a common region in Nfasc140, Nfasc155, or Nfasc186, the positive sera were preincubated with the neurofascin isoforms *in vitro*, then the depleted sera were tested against Nfac155 or Nfasc186 by ELISA (Fig. 2A). Preincubation with any neurofascin isoforms significantly decreased the ELISA signal against Nfasc155 or Nfasc186 for Patients CIDP1–5. This indicated that anti-Nfasc140/186 IgG recognizes a common epitope that is comprised within the peptide sequence of Nfasc140. By contrast, preincubation

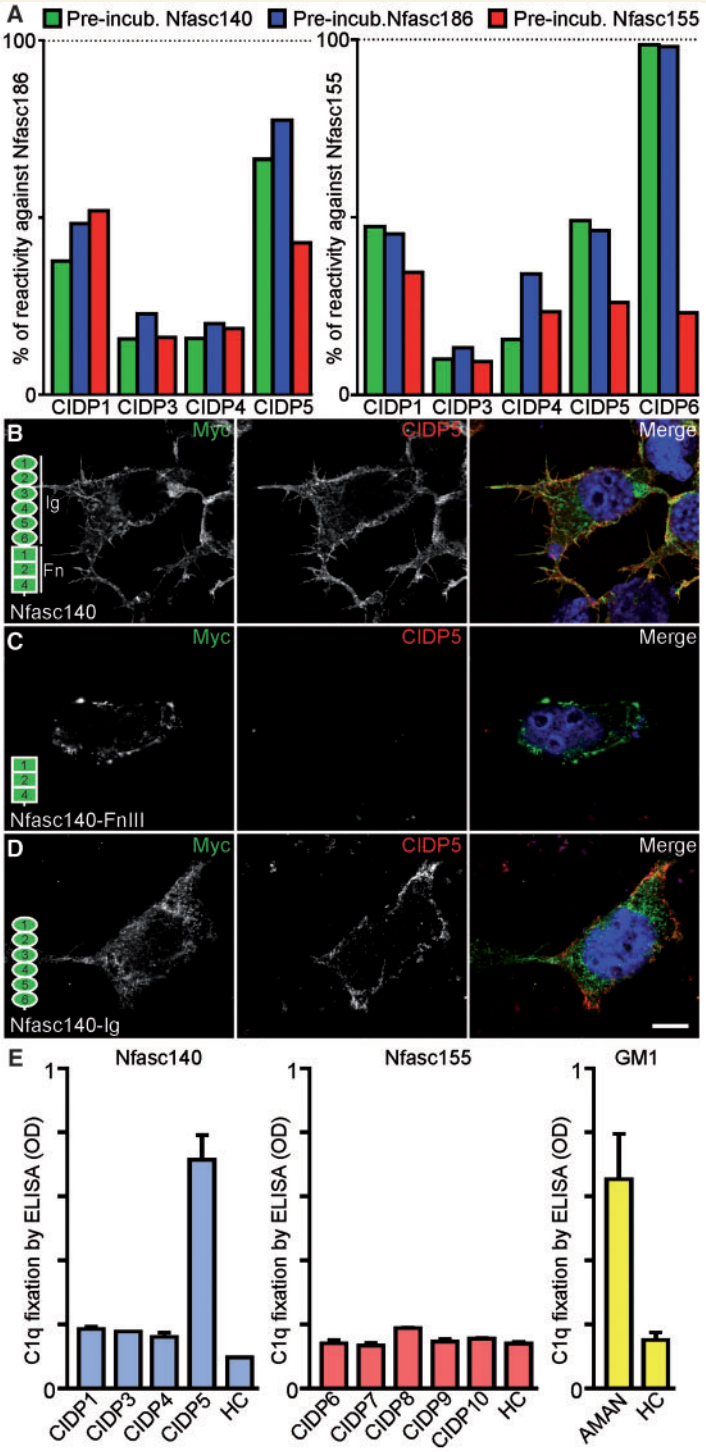

Figure 2 CIDP IgG predominantly recognizes the Nfasc140 isoform. (**A**) Sera from patients with CIDP were preincubated with Nfasc140 (green), Nfasc186 (blue), or Nfasc155 (red), then tested by ELISA against Nfasc186 (*left*) or Nfasc155 (*right*). The reactivity after depletion was normalized to that of the CIDP sera prior to depletion (control, dashed line). The preincubation with Nfasc140, Nfasc155 or Nfasc186 abolished the reactivity against Nfasc186 and Nfasc155 in most patients with anti-Nfasc140/186 IgG (CIDP1-5). By contrast, sole preincubation with Nfasc155 abolished the reactivity of patients with anti-Nfasc155 IgG4 (CIDP6). (**B–D**) CIDP sera were tested on living HEK cells transfected with full-length Myc-tagged Nfasc140 (**B**) or with constructs encoding solely the fibronectin type III (FnIII) (**C**) or the immunoglobulin (Ig) domains (**D**). A scheme of Nfasc140 constructs is inserted in each panel. Nfasc140 was revealed with a monoclonal antibody to Myc (green), and nuclei were stained with DAPI (blue). Anti-Nfasc140/186 IgG reacted predominantly against the Ig domains of Nfasc140. Scale bars = 10 μm. (**E**) C1q fixation of patients' antibodies was examined by ELISA. Only one CIDP patient (Patient CIDP5) reactive against Nfasc140 activated complement *in vitro*. No anti-Nfasc155 IgG4 activated complement. As positive control, complement fixation was tested with an acute motor axonal neuropathy sample against GM1. Serum from a healthy control (HC) subject was used as a negative control in all experiments.

of serum CIDP6 with Nfasc140 or Nfasc186 did not alter ELISA signal against Nfasc155, indicating that the anti-Nfasc155 IgG4 specifically targets the FnIII domain exclusive to Nfasc155.

To determine the target epitopes within the sequence of Nfasc140, sera were tested against deletion constructs of Nfasc140. All the sera reacted against the Ig domains, but not against the FnIII domains (Fig. 2C). This further indicated that anti-Nfasc140/186 IgG targets epitopes different from those recognized by anti-Nfasc155 IgG4.

Because one patient showed a predominant IgG3 response, we examined antibody potency to fix C1q *in vitro*. As a positive control, the serum from a patient with acute motor axonal neuropathy presenting IgG against GM1 was used. None of the patients with anti-Nfasc155 IgG4 fixed C1q. Only the patient with a predominant IgG3 response to Nfasc186 fixed complement *in vitro*.

Clinical features of anti-Nfasc140/186 IgG-positive CIDP patients

Clinical data of the five patients are detailed in Tables 1 and 2, Supplementary material and Supplementary Tables 2 and 3. All patients had a symmetric sensory and motor polyradiculoneuropathy. The neuropathy was severe at disease nadir: all patients were unable to walk without aid, and two patients had cranial nerve impairments and needed a transient mechanical ventilation in an intensive care unit. On the contrary, after therapy, all the patients were able to walk without aid at the last follow-up. Three patients improved after IVIg, and immunosuppressive drugs appeared to be effective in one of these patients. Three patients also improved after steroids. It is noticeable that four patients presented with a concomitant autoimmune disorder: one patient had a retroperitoneal fibrosis, one had anti-Ro/SSA antibodies, and two presented with nephrotic syndromes. No patients presented with evidence of tumours withstanding a paraneoplastic origin.

Nerve conduction studies revealed a demyelinating neuropathy with conduction blocks in three patients (definite CIDP) and an axonal neuropathy in the two remaining patients. The latter were diagnosed as probable CIDP based on the good response to corticosteroids and high proteinorachia (Supplementary material; median 1.79 g/l, range 0.8–2 g/l). Patients with Nfasc140/186 IgG antibodies were compared to 74 previously reported patients with anti-Nfasc155 IgG4 antibodies (Ng *et al.*, 2012; Querol *et al.*, 2014; Ogata *et al.*, 2015*b*; Devaux *et al.*, 2016; Kadoya *et al.*, 2016) and to 76 patients with CIDP without antibodies against neurofascin isoforms (Table 2). Patients with Nfasc140/186 IgG antibodies did not demonstrate tremor and seemed more responsive to IVIg than patients with anti-Nfasc155 IgG4. They also presented more frequently with a subacute-onset and a more severe phenotype than seronegative CIDP patients.

Serum samples from two CIDP patients (Patients CIDP1 and CIDP4) were available after clinical recovery. Titres of anti-Nfasc140/186 antibodies were negative after clinical improvement in both patients (Supplementary Fig. 6). In addition, serum IgG binding to nodes of Ranvier, axon

Table 1 Clinical features of patients with anti-Nfasc140/186 IgG

	CIDP1	CIDP2	CIDP3	CIDP4	CIDP5
Gender	M	F	M	M	F
Age at onset	61	70	2	75	50
Previous infection	Sore throat and bronchitis	Infection	No	No	No
Other dysimmune disease	Anti Ro/SSA	RPF	No	FSGS	FSGS
Onset	Subacute	Subacute	Subacute	Chronic	Subacute
Sensory ataxia	Yes	Yes	No	Yes	Yes
Neuropathic pain	No	No	No	No	No
Cranial nerve involvement	Yes	No	No	No	Yes
Modified Rankin Scale	5	4	4	4	5
Tremor	No	No	No	No	No
Respiratory failure	Yes	No	No	No	Yes
Intensive care unit	Yes	No	No	No	Yes
Nerve conduction study					
Demyelinating or axonal	Demyelinating	Demyelinating	Demyelinating	Axonal	Axonal
Axonal loss	Yes	Yes	No	Yes	Yes
Conduction blocks	Yes	Yes	Yes	No	No
Nerve biopsy	Mild axonal loss	ND	ND	ND	ND
Treatments					
IVIg	Yes	Yes	Yes	ND	No
Plasma exchange	Worsening	ND	ND	ND	Yes
Steroids	Yes	No	No	Yes	Yes
Other effective treatments	CY, RTX	ND	ND	ND	ND

CY = cyclophosphamide; FSGS = focal segmental glomerulosclerosis; ND = not done; RPF = retroperitoneal fibrosis; RTX = rituximab.

Table 2 Comparison of clinical features of patients with anti-Nfasc140/186 IgG or anti-Nfasc155 and of seronegative CIDP patients

	Anti-Nfasc140/186 IgG	Anti-Nfasc155 IgG4[a]	Seronegative
Number	5	74	76
Age in years, median (range)	61 (2–70)	29 (10–76)	58 (22–82)
Sex, male, *n* (%)	3 (60)	48 (69)	30 (39)
Subacute onset, *n* (%)	4 (80)*	13/55 (24)	4 (5)
Sensory ataxia, *n* (%)	4 (80)	45/70 (64)*	29 (38)
Tremor, *n* (%)	0	31/70 (44)*	14 (18)
Cranial nerve involvement, *n* (%)	2 (40)	7/32 (22)	7 (9)
CNS demyelination, *n* (%)	0	7/70 (10%)	0
Modified Rankin scale, median (range)	4 (4–5)*	3 (1–5)	2 (0–5)
Good response, *n* (%)			
IVIg	3/4 (75)	16/70 (23)*	48/60 (80)
Steroids	3/4 (75)	34/70 (49)	19/27 (70)

[a]Taken from Ng *et al.*, 2012; Querol *et al.*, 2014; Ogata *et al.*, 2015*b*; Devaux *et al.*, 2016; Kadoya *et al.*, 2016.
*$P < 0.001$ as compared to seronegative CIDP patients.

initial segments, and transfected HEK cells was completely abolished (Supplementary Fig. 7).

Discussion

We found that anti-Nfasc140/186 IgG are associated with a subset of CIDP patients showing subacute-onset (4/5), sensory ataxia (4/5), conduction block (3/5), and cranial nerve involvement (2/5). Previous studies have also examined the prevalence of antibodies to Nfasc155 and Nfasc186 in CIDP and multifocal motor neuropathy but concluded that patients lack reactivity to Nfasc186 (Ng *et al.*, 2012; Doppler *et al.*, 2015*a*; Ogata *et al.*, 2015*a*; Devaux *et al.*, 2016). This discrepancy may be due to the low prevalence of these autoantibodies. Here, these antibodies were detected in only 2% of CIDP patients. Most patients with anti-Nfasc140/186 IgG were responsive to IVIg treatments and showed a good response to steroids. No patients showed tremor or neuropathic pain. This contrasted with patients seropositive for anti-Nfasc155, CNTN1 or Caspr1 IgG4 (Ng *et al.*, 2012; Querol *et al.*, 2012, 2014; Doppler *et al.*, 2015*b*, 2016; Miura *et al.*, 2015; Ogata *et al.*, 2015*b*; Devaux *et al.*, 2016). Of interest, four patients (4/5) presented with a concomitant autoimmune disorder. Two patients presented with a nephrotic syndrome, one with retroperitoneal fibrosis, and one presented with anti-Ro/SSA antibodies. This association suggests that either anti-Nfasc140/186 IgG are responsible for the occurrence of both disorders, or that one disorder is secondary to the other. The fact that the autoimmune diseases occurred concomitantly is in favour of the first hypothesis. Concerning the nephrotic syndrome, neurofascin was shown to be expressed in human kidney glomeruli (Sistani *et al.*, 2013). It is thus plausible that anti-Nfasc140/186 IgG are responsible for both disorders. The occurrence of CIDP and nephrotic syndrome is not common but several cases have been reported in the literature (Kohli *et al.*, 1992; Wu *et al.*, 2001; Chen *et al.*, 2006; Smyth and Menkes, 2008; Quek *et al*, 2014). To date, the link between these two disorders has been a matter of discussion. Our data indicate that anti-Nfasc140/186 IgG should be investigated in these cases. Biopsies of the retroperitoneal fibrosis further revealed the presence of IgG4-positive plasma cells. Although the presence of neurofascin in the retroperitoneal mass is unknown, this suggests that these autoimmune disorders are mediated by IgG4 autoantibodies.

The depletion of anti-Nfasc140/186 IgG correlated with clinical remission in two patients. In both patients, nerve conduction study findings were in part consistent with the concept of nodo-paranodopathy (Uncini *et al.*, 2013). One patient showed recovery of a proximal conduction block after treatment, whereas the other presented a recovery of distal amplitudes, suggesting a reversible conduction failure. Nonetheless, all patients fulfilled the criteria for CIDP, and were negative for anti-ganglioside antibodies. These results indicate that anti-Nfasc140/186 IgG may induce dysfunctions at the nodes of Ranvier and a chronic nodo-paranodopathy characterized by reversible conduction block coexisting with demyelinating features. These latter findings challenge the original concept of nodo-paranodopathy, and show that autoantibodies against nodal components do not necessarily induce conduction block and an axonal-like pathology. The mechanisms by which these antibodies mediate conduction deficits have yet to be identified. In most patients, antibodies were predominantly of the IgG4 isotype and did not fix C1q *in vitro*. In contrast to patients with anti-Nfasc155 IgG4, IVIg treatments induced clear clinical improvements, suggesting that IVIg response depends on factors other than the complement pathway. Only one patient showed a predominant IgG3 reactivity and activated complement *in vitro*, but this patient did not respond to IVIg.

Several studies have shown that IgG4 against CNTN1 or Nfasc155 disrupts paranode axo-glial contact (Doppler *et al.*, 2015*b*; Manso *et al.*, 2016; Vallat *et al.*, 2017). Similarly, anti-Nfasc140/186 IgG may affect the axo-glial interaction at nodes and lead to conduction loss or slowing. Anti-Nfasc186 IgG were also found to exacerbate the clinical signs of experimental allergic encephalitis and neuritis and to induce axonal injury in the CNS (Mathey *et al.*, 2007; Lindner *et al.*, 2013; Yan *et al.*, 2014). This indicates that, in animal models, these antibodies can be pathogenic. In a similar manner as anti-GM1 antibodies (Susuki *et al.*, 2007), we suspect that anti-Nfasc140/186 IgG induces functional alterations at nodes that are more easily alleviated by IVIg than disruptions of the paranodal septate-like junctions. The importance of the reactivity toward Nfasc140 is unclear as this isoform is predominantly expressed at early developmental stages (Zhang *et al.*, 2015). The pathogenic effects of the autoantibodies more likely implicate the recognition of Nfasc186 at nodes. Nonetheless, Nfasc140 expression is strongly increased in demyelinated white matter regions of multiple sclerosis patients (Zhang *et al.*, 2015). In a similar manner, Nfasc140 expression may be increased in CIDP and thus favour autoantibodies attack and disease progression.

In conclusion, our data indicate that nodal neurofascin isoforms are additional autoantibody targets in CIDP patients showing different clinical features than those previously described. Our data show that CIDP is a heterogeneous autoimmune disorder with multiple immune targets and pathogenic mechanisms.

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Conflicts of interest

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Supplementary material

Supplementary material is available at *Brain* online.

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