Contactin-1 IgG4 antibodies cause paranode dismantling and conduction defects

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Paranodal axoglial junctions formed by the association of contactin-1, contactin-associated protein 1, and neurofascin-155, play important functions in nerve impulse propagation along myelinated axons. Autoantibodies to contactin-1 and neurofascin-155 define chronic inflammatory demyelinating polyradiculoneuropathy subsets of patients with specific clinical features. These autoantibodies are mostly of the IgG4 isotype, but their pathogenicity has not been proven. Here, we investigated the mechanisms how IgG subclasses to contactin-1 affect conduction. We show that purified anti-contactin-1 IgG1 and IgG4 bind to paranodes. To determine whether these isotypes can pass the paranodal barrier, we incubated isolated sciatic nerves with the purified antibody or performed intraneural injections. We found that IgG4 diffused into the paranodal regions in vitro or after intraneural injections. IgG4 infiltration was slow and progressive. In 24 h, IgG4 accessed the paranode borders near the nodal lumen, and completely fill the paranodal segments by 3 days. By contrast, control IgG, anti-contactin-1 IgG1, or even anti-contactin-associated-protein-2 IgG4 did not pass the paranodal barrier. To determine whether chronic exposure to these antibodies is pathogenic, we passively transferred anti-contactin-1 IgG1 and IgG4 into Lewis rats immunized with P2 peptide. IgG4 to contactin-1, but not IgG1, induced progressive clinical deteriorations combined with gait ataxia. No demyelination, axonal degeneration, or immune infiltration were observed. Instead, these animals presented a selective loss of the paranodal specialization in motor neurons characterized by the disappearance of the contactin-associated protein 1/contactin-1/neurofascin-155 complex at paranodes. Paranode destruction did not affect nodal specialization, but resulted in a moderate node lengthening. The sensory nerves and dorsal root ganglion were not affected in these animals. Electrophysiological examination further supported these results and revealed strong nerve activity loss affecting predominantly small diameter or slow conducting motor axons. These deficits partly matched with those found in patients: proximal motor involvement, gait ataxia, and a demyelinating neuropathy that showed early axonal features. The animal model thus seemed to replicate the early deteriorations in these patients and pointed out that paranodal loss in mature fibres results in conduction defects, but not conduction slowing. Our findings indicate that IgG4 directed against contactin-1 are pathogenic and are reliable biomarkers of a specific subset of chronic inflammatory demyelinating polyneuropathy patients. These antibodies appear to loosen the paranodal barrier, thereby favouring antibody progression and causing paranodal collapse.

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Keywords: autoantibody; dysimmune; caspr; CIDP; neuritis

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**Introduction**

Chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) is a heterogeneous disease affecting peripheral nerves. Insofar, the diagnosis of CIDP mostly relies on electrophysiological criteria (Dalakas, 2011). The molecular basis and physiopathogenic mechanisms responsible for conduction abnormalities in CIDP have only been partially resolved (Lim et al., 2014; Mathey et al., 2015). Several recent reports document the presence of autoantibodies directed against contactin-1 (CNTN1) and neurofascin-155 (NF155, encoded by NFASC) in a subpopulation of CIDP patients (Ng et al., 2012; Querol et al., 2012, 2014; Devaux et al., 2016; Doppler et al., 2015; Miura et al., 2015).

CNTN1 and NF155 are two important cell adhesion molecules involved in the formation of paranodal domains (Faivre-Sarrailh and Devaux, 2013). CNTN1 and its partner contactin-associated protein-1 (CASPR1, encoded by CNTNAP1) are expressed on the axonal surface and interact with NF155 located on the closely apposed glial loops (Peles et al., 1997; Rios et al., 2000; Charles et al., 2002). Deletions of either NF155, CASPR1, or CNTN1 lead to important conduction deficits and to the loss of the septate-like junctions at paranodes (Bhat et al., 2001; Boyle et al., 2001; Zonta et al., 2008). The latter are believed to form a barrier to the lateral diffusion of particles and to participate in myelin insulation (Rosenbluth, 2009).

The presence of antibodies to CNTN1 and NF155 implicates paranodal dysfunctions in the pathogenesis of CIDP and in the processes of demyelination. In keeping with this view, clinical remission follows the depletion of antibodies to paranodal proteins in CIDP patients treated with rituximab (Querol et al., 2015). Moreover, destruction of the paranodes in skin biopsies were recently linked to the presence of autoantibodies directed against contactin-1 (CNTN1) and neurofascin-155 (NF155, encoded by NFASC) in a subpopulation of CIDP patients (Lim et al., 2014; Mathey et al., 2015). The latter are believed to form a barrier to the lateral diffusion of particles and to participate in myelin insulation (Rosenbluth, 2009). The presence of antibodies to CNTN1 and NF155 implicates paranodal dysfunctions in the pathogenesis of CIDP and in the processes of demyelination. In keeping with this view, clinical remission follows the depletion of antibodies to paranodal proteins in CIDP patients treated with rituximab (Querol et al., 2015). Moreover, destruction of the paranodes in skin biopsies were recently linked to the presence of autoantibodies directed against contactin-1 (CNTN1) and neurofascin-155 (NF155, encoded by NFASC) in a subpopulation of CIDP patients (Lim et al., 2014; Mathey et al., 2015). The latter are believed to form a barrier to the lateral diffusion of particles and to participate in myelin insulation (Rosenbluth, 2009).

Of interest, the subclass of anti-CNTN1 and anti-NF155 autoantibodies found in CIDP patients is generally of the IgG4 isotype (Ng et al., 2012; Querol et al., 2012, 2014; Devaux et al., 2016; Doppler et al., 2015; Miura et al., 2015). IgG4 antibodies are considered to have anti-inflammatory activity and are associated with numerous neurological disorders (Huijbers et al., 2015). Notably, IgG4 antibodies are suspected to interfere with extracellular protein complex. For instance, anti-muscle-specific kinase (MuSK) IgG4 antibodies appear to trigger the disease in MuSK+ myasthenia by blocking the interaction between MuSK and low-density lipoprotein receptor-related protein 4 (Huijbers et al., 2013). Similarly, anti-CNTN1 IgG4 were shown to block the interaction between CNTN1/CASPR1 complex and NF155 in vitro (Labasque et al., 2014). This raised the possibility that anti-CNTN1 IgG4 antibodies may perturb paranode structure and thereby affect conduction.

Here, we investigated whether human IgG1 and IgG4 against CNTN1 from two patients with CIDP are pathogenic in animal models. We demonstrate that anti-CNTN1 IgG4 can readily penetrate through paranodal segments, and cause nerve activity loss by disrupting the CNTN1/CASPR1/NF155 complex.

**Materials and methods**

**Patients’ antibodies**

Plasmapheresis fluids were obtained from plasma exchange of two CIDP patients with high titre CNTN1 antibodies and similar clinical phenotypes. Plasmapheresis fluids were pooled together to create a single batch of antibodies sufficient for all the experiments described herein. Plasma was also obtained from a patient with autoimmune encephalitis without neuromyotonia associated with high titre contactin-associated protein-2 (CASPR2) antibodies. The patients gave informed consent to participate and the study was approved by the institutional ethics committee of Hospital de la Santa Creu i Sant Pau. Plasma from a healthy donor negative for CNTN1, NF155, or CASPR2 antibodies was used as control (Etablissement Français du Sang). IgG1 and IgG4 were successively purified using CaptureSelect™ affinity matrix according to manufacturer’s instructions (ThermoFisher scientific).

Briefly, the matrix was first equilibrated with phosphate-buffered saline (PBS), then 10 ml of matrix was added to the plasma diluted 1:1 in PBS and was agitated overnight at 4 °C. The day after, the matrix was packed into a glass column, washed with 200 ml of PBS, then antibodies were eluted with 10 ml of 0.1 M glycine pH 3.0, and the pH was neutralized with 1/30 volume 1 M Tris pH 9.0. Antibody concentration was determined using absorbance at 280 nm. Control IgG were purified by affinity chromatography with protein G sepharose (Sigma-Aldrich) as described above and eluted similarly. Fractions were dialyzed to PBS and sterilized by filtration. Fraction purity was verified by western blot, cell-based assays on CNTN1-transfected human embryonic kidney (HEK) cells, and by tests on mouse sciatic nerve as previously described (Miura et al., 2015). In addition, we verified that the binding of purified human IgG1 and IgG4 onto native CNTN1 did not mask the epitopes and the recognition by the goat anti-CNTN1 antibodies (Supplementary Fig. 1).
Western blot

Purified antibody samples were denatured in sodium dodecyl sulphate (SDS) sample buffer for 2 min at 90 °C, loaded on 7.5% SDS-polyacrylamide (PAGE) gels, transferred, and immunoblotted with mouse monoclonal antibodies against human IgG1 (1:1000; 4E3; Abcam), IgG2 (1:1000; HP6014; Abcam), IgG3 (1:1000; HP-6050; Sigma-Aldrich), or IgG4 (1:2000; HP6035; Abcam). Immunoreactivity was revealed using peroxidase-coupled donkey anti-mouse secondary antibodies (1:5000; Jackson ImmunoResearch) and BM chemiluminescence kit (Sigma-Aldrich).

Animals

All animal experiments were in line with the European Community’s guiding principles on the care and use of animals (86/609/CEE) and were approved by the local ethics committee. For in vitro nerve incubation experiments, male Lewis rats were euthanized and the sciatic nerves were quickly dissected out and transferred into oxygenated artificial CSF: 126 mM NaCl, 3 mM KCl, 2 mM CaCl2, 2 mM MgSO4, 1.25 mM NaH2PO4, 26 mM NaHCO3, and 10 mM dextrose, pH 7.4–7.5. Nerves were desheathed, cut in 1 cm segments, and incubated for 10 min, 30 min, 1 h or 3 h with 10 μg of purified control IgG, anti-CNTN1 IgG1, anti-CNTN1 IgG4, or anti-CASPR2 IgG4 diluted in artificial CSF. Then, nerve segments were washed 3 × 5 min with artificial CSF, fixed in 2% paraformaldehyde in PBS for 1 h at 4 °C, and processed for immunolabelling, as detailed below.

For intraneural injections, anaesthesia was induced and maintained with Isovet®. Animals also received a subcutaneous injection of buprenorphine for pain relief. The right sciatic nerve was exposed at the level of the sciatic notch and injected with 5 μl of antibody (2 μg/μl) using a glass micropipette. One, three, or five days after surgery, injected nerves were dissected out, fixed in 2% paraformaldehyde in PBS for 1 h at 4 °C, then processed for immunolabelling, as detailed below.

For passive transfer, male Lewis rats were sensitized with 50 μg of synthetic peptide of bovine P2 myelin protein as previously described (Devaux, 2012). At 12, 19, 26 and 33 days post-immunization (dpi), the animals received intravenous injections of purified anti-CNTN1 IgG1, anti-CNTN1 IgG4, or control IgG (500 μg). Animals were weighed and examined daily for clinical signs. Clinical signs were graded as follows: 0 = no illness; 1 = tail tip hanging; 2 = limp tail; 3 = tail paralysis; 4 = gait ataxia; 5 = mild paralysis; 6 = severe paralysis; 7 = paraplegia; 8 = tetraparesis; 9 = moribund; 10 = death. To monitor gait abnormalities, animals were allowed to walk freely along a 2 m long surface (Supplementary Video 1). The hindlimb grip strength was monitored before antibody injection and at 35 dpi using a BIO-GS3 grip test (Bioseb). For footprint analysis, the animal hind paws were dipped in ink and the animals walked down a corridor on white paper. The angles of the footprints were measured with ImageJ version 1.43u software (National Institutes of Health).

Immunolabelling and histopathology

L6 ventral and dorsal spinal nerves, as well as the attached dorsal root ganglion were dissected out and fixed in 2% paraformaldehyde in PBS for 1 h at 4 °C, then rinsed in PBS. Axons were gently teased, dried on glass slides and stored at −20 °C. Dorsal root ganglia were cryoprotected in 30% sucrose in 0.1 M PBS overnight at 4 °C, then cut into 5- to 10-μm thick cryosections. Sections and teased fibres were permeabilised by immersion in −20 °C acetone for 10 min, blocked at room temperature for 1 h with PBS containing 5% fish skin gelatin and 0.1% Triton™X-100, then incubated overnight at 4 °C with primary antibodies: rabbit antiserum against gliomedin (1/500) (Eshed et al., 2005), NF186 (1/500) (Southwood et al., 2004), ankryrin-G (1/2000) (Bouzidi et al., 2002), or CASPR1 (1/2000) (Menegoz et al., 1997); mouse monoclonal antibodies against MAG (513; 1/200; Merck-Millipore); NaV channels (K58/35; 1:500; Sigma-Aldrich); human antiserum against NF155 (1/500) (Querol et al., 2014); goat antibody against CNTN1 (1/2000; R&D Systems); or chicken antibody against neurofilament heavy (1/2000; Abcam). The slides were then washed several times and incubated with Alexa conjugated donkey antiserum against rabbit, mouse, human, goat or chicken IgG (1/500; Jackson ImmunoResearch) or FITC conjugated donkey antisera against rabbit, mouse, human, goat or chicken IgG (1/200; Abcam). Slides were mounted with Mowiol® plus 2% DABCO, and examined using an ApoTome fluorescence microscope (Carl Zeiss MicroImaging GmbH). Digital images were manipulated into figures with CorelDraw and Corel Photo-Paint. Teased fibres were analysed from 10 animals for each group. For quantification of nodal alterations, the percentage of paranodes showing loss of nodal clusters of ankryrin-G, NaV channels, gliomedin or NF186 was quantified in 10 animals (~100 axons counted in total). In addition, percentage of myelinated axons (identified using MAG and neurofilament heavy) showing loss of nodal clusters of gliomedin or NF186 was quantified in four animals (~100 axons counted in total). Dorsal root ganglion sections were analysed from five animals for each group.

For histopathological analysis, L6 spinal nerves were fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M PBS overnight at 4 °C and post-fixed in 1% OsO4 in 0.1 M PBS for 1 h. Nerves were dehydrated and embedded in epoxy resin. Transverse semi-thin sections were stained with Toluidine blue and examined by light microscopy.

Electrophysiology

Recordings were performed at 38 dpi as previously described (Lonigro and Devaux, 2009). Briefly, the L6 spinal nerves were dissected out and transferred into artificial CSF. Recordings of compound action potentials (CAPs) were made at 36 °C in a three compartment recording chamber. Conduction velocities were estimated from latencies and were calculated at maximal and at half the maximal CAP amplitude.

Statistics

Statistical significance was assessed by unpaired two-tailed Student’s t-tests, Kolmogorov-Smirnov 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

Purification of anti-CNTN1 IgG1 and IgG4

The clinical details of two patients showing anti-CNTN1 IgG have been previously reported (Querol et al., 2012). These two patients presented with high titre anti-CNTN1 IgG4, and low titre IgG1, IgG2, or IgG3 (Supplementary Fig. 1 and Labasque et al., 2014), and received plasma exchange therapy. To further confirm the pathogenic potentials of these IgG isotypes, we pooled these plasmapheresis fluids and tested these on teased sciatic nerve fibres. We found that anti-CNTN1 IgG1 and IgG4 immunolabelled the paranodal regions (Fig. 1A), but not IgG2 and IgG3, indicating that solely IgG1 or IgG4 might have pathogenic potentials. These results were in keeping with our previous observations (Miura et al., 2015). Using CaptureSelect™ affinity matrix, we purified IgG1 and IgG4 from the pooled plasmapheresis fluids. As controls, we purified total IgG from healthy donor plasma using protein G sepharose affinity column. Immunoblots demonstrated that purified IgG4 fractions were not contaminated by IgG1, IgG2, or IgG3, and reciprocally that purified IgG1 fractions were not contaminated by IgG2, IgG3, or IgG4 (Fig. 1B). As shown for anti-CNTN1 IgG4 in Fig. 1C and D, the purified antibodies did not lose their binding capacity after purification, and purified antibodies specifically bound to human CNTN1 on the surface of CNTN1-transfected HEK cells, and at paranodal regions in teased sciatic nerve fibres. These latter results further confirmed the absence of contamination in the samples.

Anti-CNTN1 IgG4 selectively accesses paranodes in vitro

The paranodal domains have been shown to form a barrier to the lateral diffusion of large molecules (Mierzwa et al., 2010). We thus conjectured that, in order to be pathogenic, autoantibodies CNTN1 should be able to access the paranodal regions. To test this hypothesis, we incubated sciatic nerve segments in vitro (n = 3 nerves for each condition) with 10 µg of anti-CNTN1 IgG1, anti-CNTN1 IgG4, or control IgG for extended periods of time (from 10 min to 3 h). Anti-CNTN1 IgG1 or control IgG did not stain the paranode at any time tested (Fig. 2A and B), albeit anti-CNTN1 IgG1 could stain paranodes after permeabilization (Fig. 2F). Because CNTN1 is a glycosylphosphatidylinositol-linked protein and patients’ autoantibodies recognized the extracellular domain of CNTN1 (Supplementary Fig. 1), it seems unlikely that paranodal staining after permeabilization is due to the binding of the antibodies to cryptic antigens. In contrast, anti-CNTN1 IgG4 faintly labelled the paranode borders after 1 h of incubation (Supplementary Fig. 1), and paranodes were importantly labelled after 3 h of incubation. At that incubation time, anti-CNTN1 IgG4 were detected within the paranodal regions, but did not completely overlap with CNTN1 labelling (Supplementary Fig. 1 and Fig. 2C). To ascertain that this was not due to the steric hindrance of IgG4, we tested purified anti-CASPR2 IgG4 from a patient with autoimmune encephalitis without neuromyotonia. We did not find diffusion or binding of anti-CASPR2 IgG4 at juxtaparanodal regions (Fig. 2D), albeit these antibodies stained juxtaparanodal regions in permeabilized fibres (Fig. 2H). These results thus indicated that only anti-CNTN1 IgG4 penetrates the paranodes.

Next, we performed intraneural injection in vivo, and monitored IgG4 penetration to confirm our previous observations. One day after injection, strong IgG4 deposition was found at paranode borders in most sciatic nerve axons (Fig. 3A and D). Only a small percentage of these presented IgG4 staining that completely overlapped with CNTN1 labelling. Three days after injection, the length of IgG4 penetration across paranodes was significantly increased (Fig. 3C; P < 0.001 by unpaired two-tailed Student’s t-tests and Kolmogorov-Smirnov test), and IgG4 co-localized completely with CNTN1 labelling in most paranodes (Fig. 3B and D). To quantitate the level of IgG4 diffusion across the paranodes, we measured the length of IgG4 and CNTN1 labelling and calculated the ratio of IgG4 length/paranode length. Of interest, the progression of IgG4 was more important in small diameter axons (node diameter < 2 µm) compared to larger axons (Fig. 3E). In keeping with our previous findings, control IgG, anti-CNTN1 IgG1, or anti-CASPR2 IgG4 did not stain paranodes or juxtaparanodes 1 or 3 days after injection (n = 3 animals for each condition). These results thus confirmed our previous observations and ascertained that anti-CNTN1 IgG4 infiltration was not due to paranode stretching during desheathing and dissection. Although our original goal was to determine whether antibodies are pathogenic, we did not detect significant node or paranode alterations, demyelination, electrophysiological changes, or even behavioural deficits after intraneural injection. Antibodies appeared to have a short lifespan in the interstitial fluid, as the intensity of IgG4 labelling was already strongly decreased 3 days after injection and became almost undetectable by 5 days.

Passive transfer of anti-CNTN1 IgG4 causes chronic progressive deteriorations in Lewis rats

To test the long-term effects of anti-CNTN1 IgG4, we thus passively transferred three groups of 10 Lewis rats with anti-CNTN1 IgG1, anti-CNTN1 IgG4, or control IgG. Animals were first immunized with low doses of P2 peptide to induce a mild neuropathy and blood–nerve barrier leakage. Then, intravenous injections of 500 µg of antibodies were performed weekly at 12, 19, 26, and 33 dpi. Passive transfer of anti-CNTN1 IgG4 slowly, but progressively,
exacerbated the severity of the clinical signs (Fig. 4A). No recovery and no relapse-remitting course were observed in any of these animals. On the day of sacrifice (38 dpi), the animals exhibited complete tail paralysis, and 9 of 10 animals showed gait abnormalities (Supplementary Video 1). The gait abnormalities were confirmed by footprint pattern analysis (Supplementary Fig. 2). By contrast, animals treated with control IgG progressively remitted, whereas animals treated with anti-CNTN1 IgG1 showed a persistence of their clinical symptoms from 12 to 38 dpi. No control animals and only 3 of 10 IgG1-treated animals showed gait abnormalities (Fig. 4B). The hindlimb grip strength was measured at 35 dpi. No significant difference in grip strength was observed between the groups (Fig. 4B). This pointed out that gait abnormalities are more likely due to coordination defects rather than to neuromuscular weaknesses.

Histopathological examinations of transverse semi-thin sections of sciatic and spinal nerves did not reveal gross alterations in the treated animals at 38 dpi (Fig. 4C). No signs of demyelination, degeneration, or immune cells infiltration were encountered. Because CNTN1 is strongly expressed in large dorsal root ganglion neurons (Miura et al., 2015), we thus inferred that treated animals could exhibit sensory ganglionopathy. No overt alterations were found in dorsal root ganglia, and the proportion of CNTN1-positive dorsal root ganglia were unchanged between control and anti-CNTN1 IgG4-treated animals (Supplementary Fig. 3;
Figure 2 Anti-CNTN1 IgG4 penetrates paranodal regions in vitro. (A–D) Desheathed rat sciatic nerves (n = 3 per conditions) were incubated in vitro with purified control IgG (A), anti-CNTN1 IgG1 (B), anti-CNTN1 IgG4 (C), or anti-CASPR2 IgG4 (D) for 3 h in artificial CSF, then nerve segments were rinsed, fixed, and immunolabelled for human IgG (green) and CNTN1 (red). Anti-CNTN1 IgG4 entered the paranodes and partially co-localized with CNTN1 (arrows). (E–H) As controls, teased sciatic nerve fibres were permeabilized and immunolabelled for CNTN1 (red) and control IgG (E), anti-CNTN1 IgG1 (F), anti-CNTN1 IgG4 (G), or anti-CASPR2 IgG4 (H). As expected, anti-CNTN1 IgG1 and IgG4 labelled the paranodes, whereas anti-CASPR2 IgG4 labelled the juxtaparanodes. Scale bar = 10 μm.
n = 5 animals for each group; \( P > 0.05 \) by unpaired two-tailed Student’s \( t \)-tests and by one-way ANOVA followed by Bonferroni’s post hoc tests).

**CNTN1 CIDP IgG4 induces the loss of CNTN1/CASPR1/NF155 complex at paranodes**

In animal models of demyelinating or axonal neuropathies, structural changes often consist of paranodal demyelination and node disruption (Novakovic et al., 1998; Susuki et al., 2007; Lonigro and Devaux, 2009; Devaux, 2012). In anti-CNTN1 IgG4 or IgG1-treated animals, ventral and dorsal root axons did not show signs of demyelination or node disorganization (Supplementary Fig. 4). All nodes showed normal clusters of gliomedin, NF186, NaV channels and ankyrin-G. However, many nodes lacked CNTN1, CASPR1, and NF155 staining at paranodes in anti-CNTN1 IgG4-treated animals (Fig. 5 and Supplementary Fig. 4). These alterations were significantly more prominent in animals treated with anti-CNTN1 IgG4 (35.7 ± 3.1%) compared to IgG1 (18.4 ± 3.2%) or control IgG (7.2 ± 1.4%), and selectively affected ventral root axons (Fig. 6A–C and E). We noticed that these alterations appeared more prominent in small diameter axons. To confirm this observation, we measured the diameter of affected and normal nodes. We did not see a difference in the overall distribution of the axonal diameter between control, IgG1, or IgG4-treated animals (Fig. 6A and B), confirming the absence of axonal loss. However, the diameter of nodes lacking paranodal specialization were significantly smaller than that of normal nodes (Fig. 6D and F; \( P < 0.005 \) by unpaired two-tailed Student’s \( t \)-tests). This indicated that anti-CNTN1 IgG4 selectively affected small motor neurons and partly explained the ataxic phenotype. The loss of the paranodal specialization was also associated with a discrete lengthening of the nodes (Supplementary Fig. 4G and H; \( P < 0.001 \) by Kolmogorov-Smirnov test). No paranodal alterations were seen in the sciatic nerves of the treated animals.

**Disturbed motor nerve conduction in anti-CNTN1 IgG4-treated animals**

Loss of paranodal specialization causes severe conduction slowing in CNTN1, CASPR1, or NF155-deficient mice (Bhat et al., 2001; Boyle et al., 2001; Pillai et al., 2009).
In contrast, CAPs from ventral spinal roots of anti-CNTN1 IgG4-treated animals had decreased amplitude associated with an increase in nerve conduction velocity measured at peak amplitude compared to control or IgG1-treated animals (Fig. 7A and B). Recruitment was unaffected in the treated nerves (Supplementary Fig. 5). In addition, nerve conduction velocity measured at CAP first inflection appeared normal (Supplementary Fig. 5). Thus, the increase
in velocity measured at maximal amplitude seems only apparent and is likely due to the fact that fast conducting axons (presumably large diameter axons) are unaffected, while slow conducting axons (presumably small diameter axons) present a loss of conduction (see superimposed traces in Fig. 7A). In contrast, dorsal root axons did not show significant conduction alterations (Fig. 7C and D), which is in accordance with the lack of morphological alterations in sensory nerves. Taken together, these data attest that anti-CNTN1 IgG4 are pathogenic and induce specific morphologic alterations in small motor neurons leading to motor defects.

**Discussion**

Over the past few years, several groups have proven that antibodies directed against CNTN1 or NF155 are specifically associated with a subset of CIDP patients (Ng et al., 2012; Querol et al., 2012, 2014; Devaux et al., 2016; Doppler et al., 2015; Miura et al., 2015). In our recent reports, we demonstrated that these antibodies are mostly of the IgG4 isotype (Querol et al., 2014; Devaux et al., 2016; Miura et al., 2015). The clinical implication of these antibodies was further substantiated as clinical recovery correlated with IgG4 depletion in CIDP patients treated with rituximab (Querol et al., 2015). Here, we demonstrate that human anti-CNTN1 IgG4 antibodies purified from CIDP patients can access paranodal regions, induce the loss of the paranodal specialization, motor conduction deficits, and ataxia. By comparison, anti-CNTN1 IgG1 did not have the potency to access paranodal domains and only maintained marginal clinical deficits. These findings clearly attest that IgG4 are pathogenic and reliable biomarkers for CIDP.

IgG4-treated animals developed predominantly motor nerve alterations associated with conduction defects and gait abnormalities. These clinical deficits partly matched with those seen in the CNTN1 CIDP patients (Querol et al., 2012). These patients showed predominant motor involvement, gait ataxia, and partial motor conduction block. As inferred by compound muscle action potential study, the patients presented with axonal involvement in the early stage of the disease, then decreased nerve conduction velocities at the onset of the disease, thus fulfilling the criteria for CIDP. The passive transfer of IgG4 thus appeared to replicate the initial stage of the disease (the first weeks), and longer exposure time may be required to induce demyelination and conduction slowing. Of course, these animal models may not fully represent the physiopathogenic mechanisms taking place in patients, as antibodies required intraneural injection or active immunization in order to pass the blood–nerve barrier and to be pathogenic.

The exact reasons why anti-CNTN1 IgG4 specifically affects motor axons, notably small diameter axons, is unclear. Because small axons have shorter paranodes, one may easily imagine that these are more vulnerable and are affected by a lower amount of IgG4 by contrast to
Figure 6  Anti-CNTN1 IgG4 preferentially affects small motor axons. (A and B) The distribution of node diameter was examined at 38 dpi in L6 ventral roots (A) and dorsal roots (B) of animals treated with control IgG (n = 859 nodes for ventral roots and 848 nodes for dorsal roots from 10 animals), anti-CNTN1 IgG1 (blue; n = 933 nodes for ventral roots and 946 nodes for dorsal roots from 10 animals), or anti-CNTN1 IgG4 (yellow; n = 870 for ventral roots and 940 nodes for dorsal roots from 10 animals). The distribution of the total axonal population is represented on top, and the respective proportion of normal nodes (filled bars) or nodes lacking paranodes (dashed bars) is represented on the bottom. Note that paranodal alterations were more prominent in ventral root axons than in dorsal roots, and predominantly affected small diameter axons.  

(C–F) The percentage of nodes showing paranodal alterations were calculated in ventral (C) and dorsal roots (E) in animals treated with control IgG, anti-CNTN1 IgG1 (blue), or anti-CNTN1 IgG4 (yellow). In addition, the mean diameter of the normal nodes (filled bars) or nodes lacking paranodes (dashed bars) were calculated in animals treated with control IgG or anti-CNTN1 IgG4. Paranodal alterations were significantly more frequent in ventral root axons than in dorsal roots. In addition, paranodes were more affected in anti-CNTN1 IgG4-treated animals than in control or IgG1 treated animals (P < 0.001 by unpaired two-tailed Student’s t-tests and by one-way ANOVA followed by Bonferroni’s post hoc tests). The diameter of nodes with disrupted paranodes was significantly smaller compared to that of normal nodes (P < 0.005 by unpaired two-tailed Student’s t-tests and by one-way ANOVA followed by Bonferroni’s post hoc tests), thus indicating that anti-CNTN1 IgG4 selectively targets small motor neurons. Bars represents mean and standard error of the mean.
large axons. In keeping, anti-CNTN1 IgG4 penetrated more efficiently small diameter axon paranodes after intra-neural injection. Longer exposure time or higher IgG4 concentration may thus be required to affect larger axons, as in CNTN1 CIDP patients (Querol et al., 2012). Another explanation could be that IgG4 selectively affects the function of a different form of CNTN1 that is expressed in motor axons. Indeed, CNTN1 is a highly glycosylated protein that coexists in two forms in the brain and plays an important function, notably in sensory axon guidance (Rios et al., 2000; Perrin et al., 2001; Labasque et al., 2014). Our experimental data argue against the hypothesis of a ‘selective targeting’ because CIDP patients’ IgG4 labelled paranodes in both small and large sensory or motor axons in teased nerve fibres. However, anti-CNTN1 IgG4 are likely polyclonal antibodies and we cannot exclude that some antibodies target common CNTN1 domains whereas others have selective function blocking activity. It is worth mentioning that anti-CNTN1 IgG4 were also detected in a homogenous subgroup of Japanese CIDP patients presenting with sensory ataxia (Miura et al., 2015). It is thus plausible that anti-CNTN1 IgG4 dismantle different protein complexes involving different CNTN1 forms, and thereby lead to different clinical phenotypes. The function of these CNTN1 forms and complexes need to be further investigated in adult sensory and motor axons, in order to understand the selective effects of the antibodies.

The electrophysiological examination pinpointed that the paranodal destruction leads to conduction loss in small and slow conducting motor neurons. These results were somehow unexpected, as in genetic models the deletion of CNTN1, CASPR1, or NF155 results in conduction slowing (Bhat et al., 2001; Boyle et al., 2001; Pillai et al., 2009). A likely explanation could be that the loss of paranode specialization has different impacts on developing and mature myelinated axons. Indeed, in constitutive and conditional knock-out animals for CNTN1, CASPR1, or NF155, electrophysiological measurements were performed at early ages because these animals die at young ages. By contrast, the disappearance of the paranodal axo-glial complex was, here, induced in adult myelinated axons that primarily exhibited normal paranodal junctions. In one study, Pillai et al. (2009) generated inducible knock-out to ablate NF155 in adult myelinating glia. In keeping with our
finding, NF155 ablation in adults induced conduction loss, but modestly affected conduction velocity (Pillai et al., 2009). Altogether, this pinpoints that paranodal junctions play a central role in conduction, and that their deletion abolish propagation in adult myelinated axons.

As previously mentioned, paranodal regions are believed to form a barrier to the diffusion of particles along the lateral axis of the axon (Rosenbluth, 2009). Small fluorescent toxin can cross the paranodal regions and bind Kv1.1/ Kv1.2 channels at juxtaparanodes (Devaux and Gow, 2008). However, paranodes are 20 times less permeant to large 70 kDa fluorescent dextrans compared to small 3 kDa dextrans (Mierzwa et al., 2010). Here, we found that anti-CNTN1 IgG1 or anti-CASPR2 IgG4 did not cross the paranodal regions, albeit they bound the extracellular domains of these proteins, and their paranodal or juxtaparanodal targets following permeabilization. This further proved that paranodal regions are impermeable to large molecules such as antibodies if their architecture is intact. By contrast, anti-CNTN1 IgG4 slowly and progressively infiltrate the paranodal regions indicating that IgG4 antibodies progressively dismantle the paranodal adherent junction and thereby enable their progression. Anti-CNTN1 IgG4 were previously shown to inhibit the interaction between the CASPR1/CNTN1 complex and NF155 in vitro (Labasque et al., 2014). This function blocking activity is likely responsible for the pathogenic effect of IgG4. Because antibodies access the entire paranodal length within a few days, paranodes may just collapse in an all-or-nothing manner, which could explain the lack of progressive alterations in our model.

A previous attempt proposed that anti-NF155 IgG are not pathogenic, at least in an animal model of multiple sclerosis (Lindner et al., 2013). It is important to mention that anti-NF155 antibodies were raised in rats and were mostly of the IgG1 isotype in that study. Here, we found that IgG4 antibodies have a stronger pathogenic potential compared to IgG1. Although it is difficult to understand conceptually how IgG4 antibodies could be pathogenic, it has been shown that a number of neurological diseases are IgG4-mediated (Huijbers et al., 2015). It appears that antibodies (but not complements or cells) disrupt the function of the target antigens (desmoglein in pemphigus, MuSK in myasthenia gravis, or PLA2R in nephropathy). Similarly, anti-CNTN1 IgG4 disrupts the interaction between CASPR1/CNTN1 and NF155 in vitro (Labasque et al., 2014) and at paranodes (this study). IgG1 only produced mild deteriorations in our passive model, which may reflect maintenance of nerve inflammation as the clinical signs plateaued. Nonetheless, we cannot exclude that once the paranodal barrier is weakened by inflammation or anti-CNTN1 IgG4, the long-term exposure to IgG1 or other isotypes may exacerbate demyelination, nerve conduction slowing, and axonal degeneration as it is seen in patients (Querol et al., 2012; Doppler et al., 2015; Muira et al., 2015). Some patients have been reported to show predominant IgG3 response in association with IgG4 (Ng et al., 2012; Doppler et al., 2015). Here, we did not test the pathogenic potential of IgG3 as our patients did not show IgG3 reaction against paranodes. But these isotypes may likely have pathogenic implication.

The discovery of novel immune targets in CIDP has been an important step forward for improving patients’ diagnosis and treatment. Rituximab was recently found effective in CIDP patients with antibodies to paranodal proteins (Querol et al., 2015). Here, our findings confirm the strong pathogenic function of IgG4 antibodies and unravel the mechanisms responsible for this disorder. These data further emphasize the impact of IgG4 autoantibodies in dysimmune neurological syndromes (Huijbers et al., 2015). Efforts must now be driven to identify additional immune targets in a wider population of patients with peripheral neuropathies, notably novel targets of IgG4 autoantibodies.

Acknowledgements

We thank Drs Laurence GoutoBroze, Elior Peles, Josep Dalmau and Gisèle Alcaraz for generous gift of antibodies, and Axel Fernandez for technical assistance.

Funding

J.D., L.Q., and I.I. acknowledge grant support from the Agence Nationale pour la Recherche and Instituto de Salud Carlos III CIBERER (ACAMIN) under the frame of E-Rare-2, the ERA-Net for Research on Rare Diseases, and from the Association Française contre les Myopathies (MNMI 2012-14580). J.D. received a research grant in immunology from CSL Behring. L.Q. received support from Fondo de Investigaciones Sanitarias, Subprograma Jual Rodes (JR13/00014). I.I. received received research support from Fondo de Investigaciones Sanitarias, ISCIII, Ministry of Health (Spain) FIS 13/00937, holds a patent for dysferlin detection in monocytes and has consulted for Grifols, Genzyme, Alexion and UCB.

Supplementary material

Supplementary material is available at Brain online.

References


