Received: 11 July 2017 | Accepted: 20 December 2017

DOI: 10.1111/nmo.13292

ORIGINAL ARTICLE

WILEY Neurogastroenterology & Motility NGM

High prevalence and functional effects of serum antineuronal antibodies in patients with gastrointestinal disorders

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Abstract

Background: Antineuronal antibodies can be associated with both gastrointestinal (GI) and brain disorders. For example, antibodies against the potassium channel subunit dipeptidyl-peptidase-like protein-6 (DPPX) bind to neurons in the central nervous system (CNS) and myenteric plexus and cause encephalitis, commonly preceded by severe unspecific GI symptoms. We therefore investigated the prevalence of antineuronal antibodies indicative of treatable autoimmune CNS etiologies in GI patients.

Methods: Serum samples of 107 patients (Crohn's disease n = 42, ulcerative colitis n = 16, irritable bowel syndrome n = 13, others n = 36) and 44 healthy controls were screened for anti-DPPX and further antineuronal antibodies using immunofluorescence on rat brain and intestine and cell-based assays. Functional effects of high-titer reactive sera were assessed in organ bath and Ussing chamber experiments and compared to non-reactive patient sera.

Key Results: Twenty-one of 107 patients (19.6%) had antibodies against the enteric nervous system, and 22 (20.6%) had anti-CNS antibodies, thus significantly exceeding frequencies in healthy controls (4.5% each). Screening on cell-based assays excluded established antienteric antibodies. Antibody-positive sera were not associated with motility effects in organ bath experiments. However, they induced significant, tetrodotoxin (TTX)-insensitive secretion in Ussing chambers compared to antibody-negative sera.

Conclusions & Inferences: Antineuronal antibodies were significantly more frequent in GI patients and associated with functional effects on bowel secretion. Future studies will determine whether such antibodies indicate patients who might benefit from additional antibody-directed therapies. However, well-characterized encephalitis-related autoantibodies such as against DPPX were not detected, underlining their rarity in routine cohorts.

KEYWORDS

antineuronal autoantibody, autoimmune encephalitis, gastrointestinal disorders, immunohistochemistry, inflammatory bowel disease, Ussing chamber

1 | INTRODUCTION

In recent years, autoantibodies targeting neuronal epitopes have received increasing attention in patients with neurological syndromes, as the antibodies can be directly pathogenic leading to neuronal dysfunction and various neuropsychiatric abnormalities.[1] Similar etiologies were observed in gastrointestinal (GI) disorders, for example the new clinical entity called "autoimmune gastrointestinal dysmotility", a type of autoimmune dysautonomia associated with various antineuronal antibodies targeting among others voltage-gated calcium channels, voltage-gated potassium channels, acetylcholine receptors, and intracellular Hu (ANNA-1) proteins.[2] Traditionally, autoantibodies such as anti-Hu antibodies were described in paraneoplastic disorders, characteristically in patients with small cell lung carcinoma and paraneoplastic GI motor dysfunction.[3,4] However, tumor-negative syndromes are increasingly considered, for example, with antibodies against ganglionic nicotinic acetylcholine receptors which cause a well-defined syndrome called autoimmune autonomic ganglionopathy (AAG).[5] Symptoms include orthostatic hypotension, reduced pupil constriction, and GI dysmotility, reflecting failure of the sympathetic, parasympathetic, and enteric nervous system (ENS).[6]

Autoantibodies are suspected to play a role also in more common GI syndromes such as irritable bowel syndrome (IBS). They might cause neuropathic degeneration of enteric neurons in a subgroup of patients,[7,8] although other studies have not found increased frequencies of neuronal antibodies in functional GI disorders compared to healthy controls.[9] Additionally, a rat model of postinfectious IBS has demonstrated that antibodies against cytolethal distending toxin B cross-react with the cytoskeletal protein vinculin in the ENS.[10] Clearly, further research is needed to better understand the link between functional GI disorders and autoantibodies. The same holds true for other common GI diseases, such as inflammatory bowel disease (IBD), in which antineuronal autoantibodies[11] could either represent an epiphenomenon of neuronal degeneration or a contributing factor.

Only recently, a new fascinating overlap has been discovered between autoantibodies and the fields of Neurology and Gastroenterology. Antibodies directed against dipeptidyl-peptidase-like protein-6 (DPPX), a regulatory subunit of Kv4.2 potassium channels, bind to hippocampus, cerebellum, striatum, and the myenteric plexus.[12,13] We could show that anti-DPPX antibodies are directly pathogenic *ex vivo* in both, brain and bowels, by regulating the activity of enteric neurons and membrane expression of DPPX and Kv4.2 in hippocampal neurons.[14]

The majority of DPPX antibody-positive patients complained of GI symptoms, including constipation, abdominal pain, nausea, vomiting, early satiety, and seemingly unexplained diarrhea with weight loss that often antedated immunotherapy-responsive neurological symptoms.[12,14-19] It is tempting to speculate whether earlier detection of DPPX antibodies on a gastroenterology ward could have led to immunotherapy and prevented the severe neuropsychiatric abnormalities in these patients. Toward this goal, we analyzed the associations of antineuronal antibodies with common GI diseases in a routine clinical setting. For this, we prospectively collected serum samples of patients with diverse GI symptoms. Sera were examined for the frequencies of anti-DPPX, anti-Hu, and a large panel of further antineuronal antibodies using cell-based assays, immunofluorescence staining on rat brain and intestine sections, and functional assays on guinea pig colon.

Key Points

- Antineuronal autoantibodies can cause both, neurological and gastrointestinal symptoms. We here determined the prevalence and functional effects of antibody-positive human sera.
- Autoantibodies against central and enteric nervous system antigens were frequent in patients with diverse gastrointestinal diseases. Antibody-positive sera led to increased intestinal secretion in functional assays.
- Antineuronal antibodies potentially contribute to the clinical phenotype and might help to identify gastrointestinal patients who could benefit from immunotherapy. Future work should relate functional effects to antibody target epitopes and disease course.

2 | MATERIALS AND METHODS

2.1 | Study population

We tested serum samples of 107 adult patients from the Charité—Universitätsmedizin Berlin, Department of Gastroenterology. This study was approved by the Charité University Hospital Institutional Review Board (EA1/080/15), and written informed consent was given by all subjects. Patients were selected for any kind of GI complaints (e.g., diarrhea, constipation, often more than one) being severe enough to require in-patient treatment, comprising unexplained causes or being the major symptoms of Crohn's disease (CD), ulcerative colitis (UC), IBS, or other diagnoses. Patients with an infectious cause of symptoms were excluded. Clinical documentation for each patient included major diagnoses of GI diseases, medication, coexisting neoplasia, autoimmune diseases, and accompanying neurologic or psychiatric symptoms. Serum samples of 44 healthy blood donors, not complaining of any GI symptoms, were used as a control group. Serum samples were coded and stored at −80°C.

2.2 | Immunohistochemistry

The serum of each patient or control was tested for the presence of IgG and IgA antibodies using both, rat intestinal and rat brain tissue. For brain sections, adult Wistar rats were sacrificed, the brain removed, frozen in 2-methylbutane, and stored at −20°C until 20-µm sections of the cerebellum and hippocampus were made with a cryostat. For intestinal sections, rats were perfused with 4%

paraformaldehyde (PFA), the intestine removed, washed, placed in 4% PFA overnight, followed by 30% sucrose. After freezing in 2-methylbutane, 15-µm cryostat sections of different parts of the small intestine and colon were used for immunohistochemistry. Brain sections were fixed with 4% PFA for 10 minutes. Brain and intestinal sections were washed in phosphate-buffered saline (PBS) and preincubated for 1 hour at room temperature with blocking solution which either contained 5% normal goat serum, 2% bovine serum albumin, and 0.1% Triton X-100 (for IgA detection protocol) or 4% horse serum and 0.5% Triton X-100 (for IgG). Sections were incubated with serum overnight at 4°C at screening dilutions of 1:200 on brain and 1:500 on intestinal tissue. Sections were washed with PBS and incubated with the secondary antibody for 2 hours at room temperature: fluorescein (FITC)-labeled goat anti-human IgA (1:200) or indocarbocyanine (Cy3)-labeled donkey anti-human IgG (1:500; Dianova, Hamburg, HH, Germany). Sections were washed and coverslipped with Immu-Mount (ThermoScientific, Waltham, MA, USA).

Confirmation of antibody-positive samples was performed on further sections, including addition of unfixed rat brain slices, and secondary antibodies were changed to Alexa Fluor 488-labeled goat anti-human IgG (1:1000) and Cy3-labeled goat anti-human IgA (1:500; Dianova). Additionally, double-labeling with a commercial rabbit antimicrotubule-associated protein 2 (MAP2) antibody (1:500; Millipore, Darmstadt, HE, Germany) was performed to verify intestinal plexus and dendritic labeling. Further costainings with commercial antibodies included rabbit antitau antibody (1:100; Abcam, Cambridge, Cambridgeshire, UK) and mouse antiglial fibrillary acidic protein (GFAP) antibody (1:1000; Neuromab, Davis, CA, USA). Reactive samples were titrated in increasing steps to 1:80 000 dilution. The last dilution which still showed reactivity in comparison with a negative control was defined as the titer.

2.3 | Evaluation

Fluorescence stainings were viewed using a CKX41 cell culture microscope from Olympus (Hamburg, HH, Germany); confocal and widefield photographs were taken with a LEICA DMI8 (Wetzlar, HE, Germany). Evaluation of fluorescence intensity and anatomical distribution was performed by two experienced histologists using a semiquantitative fluorescence score. This scale defined "0" as fluorescence not exceeding the background of a representative negative control, "1" = weak fluorescence (occasionally seen background intensity in serum stainings), "2" = moderate fluorescence (clearly positive with reproducible anatomical distribution), and "3" = very high fluorescence (seen, e.g., in a positive control serum containing anti-Hu antibodies). Intensities of ≥2 were defined as antibody-positive.

2.4 | Cell-based assays

Serum samples that were found to be immunoreactive on ENS or central nervous system (CNS) neurons were further tested (starting dilution 1:10 in PBS) for 18 hours at room temperature on biochip mosaics containing 4-µm cryosections of rat hippocampus, rat cerebellum, monkey cerebellum, monkey nerve, monkey intestine, and monkey pancreas (Euroimmun, Lübeck, SH, Germany). Also, the samples were screened on cell-based assays using transfected human embryonic kidney 293 cells for the presence of antibodies against DPPX, glutamic acid decarboxylase (GAD65), *N*-methyl-D-aspartate (NMDA) receptors, gamma-aminobutyric acid b (GABA-b) receptors, aquaporin-4 (AQP4), leucine-rich glioma inactivated 1 (LGI1), and contactin-associated protein-like 2 (CASPR2), following the manufacturer's standard procedure with 30-minute incubation (Euroimmun). In comparison with control stainings, the slides were evaluated by fluorescence microscopy, using 1:10 for brain tissue and 1:100 for intestinal plexus as cutoff for positivity.

2.5 | Organ bath

The motility of guinea pig muscle strip preparations of the distal colon was measured as described previously and were performed according to the German guidelines for animal protection and animal welfare and approved by the animal ethical committee of the Technical University of Munich.[20] Briefly, intestinal segments were cut open, cleaned, and the mucosa was removed. Muscle strips of approximately 1 cm^2 were cut in the longitudinal or circular direction. Strips were mounted in vertical organ baths (10 or 20 mL) and maintained at 37°C in carbogen-bubbled Krebs solution (in mmol/L: 117 NaCl, 4.7 KCl, 1.2 $MgCl_2$ 6 H_2O, 1.2 NaH_2PO_4, 20 $NaHCO_3$, 2.5 $CaCl_2$ 2 H_2O, and 11 glucose (all from Sigma-Aldrich, St. Louis, MO, USA). Initially, the strips were adjusted to a basal tension of 15 mN. After an equilibration period of 60 minutes, the preparations were stimulated by electrical field stimulation (EFS) via platinum electrodes (100 mA, 10 Hz, 10 seconds, 0.6 ms pulse width). These parameters were chosen to specifically stimulate nerve structures in the preparations. After a washing step with fresh Krebs solution the serum samples were added to the organ bath and 10-15 minutes later a second EFS was performed. We analyzed changes relative to the baseline tension in response to addition of the sera or EFS. Statistical test procedures for paired or unpaired data are indicated in the results section (SigmaPlot 12.5; Systat Software Inc., Erkrath, Germany).

2.6 | Ussing chamber

The effects of serum samples on intestinal secretion were tested with the Ussing chamber voltage clamp technique as explained previously in detail.[21] Briefly, colonic mucosa/submucosa tissue specimens were mounted in Ussing chambers (Easy Mount chambers; Physiologic Instruments, San Diego, CA, USA) with an exposed tissue area of 0.5 cm^2. Both sides of the tissue were bathed separately in 5 ml carbogenated Krebs solution at 37°C. We measured the electrogenic, active short circuit current I_{sc} in µA/cm^2 across the epithelium with positive currents indicating a net ion current from the basolateral to the apical side. After an initial equilibration period of 30 minutes, the viability of the tissue was tested with EFS (Grass SD-9; Astro-Med Inc., West Warwick, RI, USA) via platinum

electrodes that were placed on either side of the tissue (100 pulses with 20 V, 10 Hz, 1 ms pulse duration). Serum samples were added to the basolateral solution. As in the organ bath experiments, we analyzed changes ΔI_{sc} relative to the baseline I_{sc}. Statistical test procedures for paired or unpaired data are indicated in the results section (SigmaPlot 12.5; Systat Software Inc.). A $P < .05$ was considered as significant.

3 | RESULTS

3.1 | Patient cohorts and clinical data

Of the 107 patients, 60 (56.1%) were female and 50.1 ± 18 years (mean ± SD) old, and 47 (43.9%) were male and 42.9 ± 15.3 years old. The control group consisted of 20 female (45.5%; 35.8 ± 12.3 years) and 24 male (54.5%; 49.3 ± 14.0 years) healthy subjects.

Clinical diagnoses as the likely cause of GI symptoms was CD in n = 42 (39.3%), UC in n = 16 (15%), IBS in n = 13 (12.1%) and various other diagnoses in n = 36 (33.6%), including unexplained GI symptoms (n = 15, some of them with a suspected somatoform background, e.g., somatoform autonomic dysfunction of the lower GI tract or abdominal pain), GI malignancy (n = 3), autoimmune enteropathy (n = 2), celiac disease (n = 1), diabetic enteropathy (n = 1), alcoholic liver cirrhosis (n = 1), cholecystolithiasis (n = 2), proctitis (n = 1), familiar adenomatous polyposis coli (n = 1), intraabdominal abscess (n = 1), non-celiac gluten sensitivity (n = 1), edematous pancreatitis (n = 1), pancreatic insufficiency (n = 1), pylorus stenosis (n = 1), papillary fibrosis (n = 1), achalasia (n = 1), microscopic colitis (n = 1), indeterminate colitis (n = 1).

Neurological or psychiatric comorbidities were found in 33 patients (30.8%), including depression (n = 7), epilepsy/seizures (n = 5), personality disorders (n = 3), polyneuropathy (n = 4), schizophrenia (n = 2). Thirty-three patients (30.8%) had coexisting autoimmune diseases, such as myasthenia gravis (n = 2), Bechterew's disease (n = 4), or extraintestinal manifestations of IBD (n = 11, including enteropathic arthritis, erythema nodosum, primary sclerosing cholangitis, pyoderma gangrenosum, psoriasiform exanthema, acute generalized exanthematous pustulosis).

Information on immunosuppressive medication within the last 6 months was available for 71 patients (66.4%) and included corticosteroids (n = 44, e.g., prednisolone, budesonide, hydrocortisone), TNF inhibitors (n = 30), azathioprine (n = 18), methotrexate (n = 8), 5-aminosalicylic acid compounds (n = 15), vedolizumab (n = 6), cyclophosphamide (n = 2), tacrolimus (n = 2), cyclosporine (n = 2), mycophenolate mofetil (n = 1), intravenous immunoglobulins (n = 1), rituximab (n = 1).

A neoplasia was diagnosed in 15 cases (14.0%): urothelial carcinoma (n = 1), basal cell carcinoma (n = 2), lung squamous cell carcinoma (n = 1), myelodysplastic syndrome (n = 1), small bowel adenocarcinoma (n = 2), carcinoma of pancreatic head (n = 1), metastasized neuroendocrine tumor (n = 1), tubulovillous high-grade rectal/colon adenoma (n = 2), mass of pancreatic tail (n = 1), pituitary adenoma (n = 1), low-grade colon adenomas (n = 1), meningioma (n = 1).

3.2 | Frequency of autoantibodies in patient subgroups

Autoantibodies against ENS neurons, detected as fluorescence labeling of an intensity score ≥2, were found in 21 of 107 patients and in

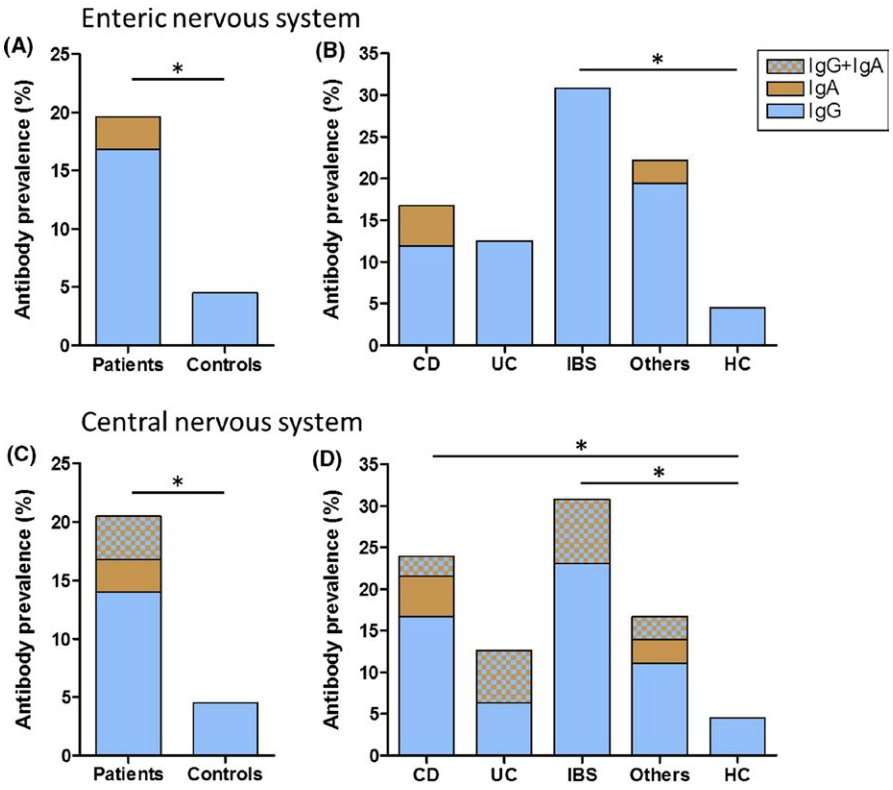

FIGURE 1 Antineuronal autoantibody frequency in patients and controls. (A) Antineuronal autoantibodies against ENS were significantly more frequent in patients (19.6%) than in controls (4.5%). (B) Antineuronal antibodies varied between patient subgroups and were most frequent in IBS patients. (C) Anti-CNS antibodies occurred significantly more frequent in patients (20.6%) than in controls (4.5%). (D) Similar to anti-ENS antibodies, anti-CNS antibodies showed a differential distribution among patient subgroups with frequent presence of both IgA and IgG isotypes in immunostainings of brain but not intestine. CD, Crohn's disease (n = 42); HC, healthy controls (n = 44); IBS, irritable bowel syndrome (n = 13); UC, ulcerative colitis (n = 16); others (n = 36)

2 of 44 controls (19.6% vs 4.5%, P = .02; chi-squared test; Microsoft Excel 2016, Redmond, WA, USA). Similarly, autoantibodies against CNS neurons were present in 22 of 107 patients and 2 of 44 controls (20.6% vs 4.5%, P = .01; chi-squared test). Ten patients (9.3%) had antibodies against both CNS and ENS neurons, while this was not detected in any of the controls (P = .035, Fisher's exact test).

Antibody-positive cases were not equally distributed among the patient subgroups (Figure 1), although differences only reached statistical significance comparing IBS patients vs controls (P = .02 for both ENS and CNS antibodies) and CD patients vs controls (P = .013 for CNS antibodies; Fisher's exact test (pairwise comparison of independent groups), Prism 5.0; GraphPad Inc., La Jolla, CA, USA). The highest prevalence was found in IBS with 4 of 13 patients (30.8%) harboring antibodies against ENS and 4 against CNS neurons, 2 patients overlapped. The lowest frequency among patients was seen in the UC group, where 2 of 16 patients (12.5%) had ENS antibodies and 2 further (12.5%) CNS-targeting antibodies. Whereas IgA and IgG autoantibodies commonly coexisted when tested on brain sections, plexus immunoreactivity was always restricted to one isotype, in almost all cases IgG.

Patients suffering from constipation had antineuronal antibodies in 5 of 14 cases (35.7%) and patients with weight loss/cachexia in 6 of 19 cases (31.6%). The group of patients with abdominal pain had antibodies in 29.2% (14/48). Antibodies were found in 20 of 69 (29.0%) patients complaining of diarrhea and in 2 of 11 (18.2%) patients with vomiting/nausea. Antibody frequencies were not significantly different between any of the symptom groups (Fisher's exact test, pairwise comparison).

Female patients (24 of 60; 40%) had ENS/CNS antibodies significantly more often than male (9 of 47, 19.1%) patients (P = .02; chi-squared test). The mean age of antibody-positive patients (48.8 ± 17.1 years) was not significantly different from antibody-negative (46.1 ± 17.3 years) patients.

Patients with autoantibodies against ENS/CNS did not receive immunosuppressive medication more often (n = 23, 69.7%) than antibody-negative patients (n = 48, 64.9%) (P = .63; chi-squared test). Similarly, no difference was seen for the presence of tumors (12.1% vs 14.9%, P = .71; chi-squared test) and neuropsychiatric comorbidities (30.3% vs 31.1%, P = .94; chi-squared test). Patients with antineuronal autoantibodies did not have a significantly higher frequency of autoimmune comorbidities (36.4% vs 28.4%, P = .41; chi-squared test). Furthermore, antibody prevalence was not related to disease duration in patients (R^2 = .039; Microsoft Excel 2016).

3.3 | Patterns of intestinal plexus immunoreactivity

Immunohistochemistry of patient sera on intestinal sections resulted in a wide range of staining patterns. In four cases, single ganglion cells of the myenteric or submucosal plexus could easily be discriminated and showed cytoplasmic reactivity (Figure 2A). Sera titers were usually high ranging from 1:1000 to 1:20 000. Several further sera with titers between 1:500 and 1:80 000 bound rather diffusely to plexus neurons, staining somatodendritic and nuclear structures (Figure 2B). One high-titer (1:20 000) serum of a patient with indeterminate colitis showed strong staining of the ENS and muscularis propria with partly granular (Figure 2C) or streaky distribution (Figure 2D), respectively.

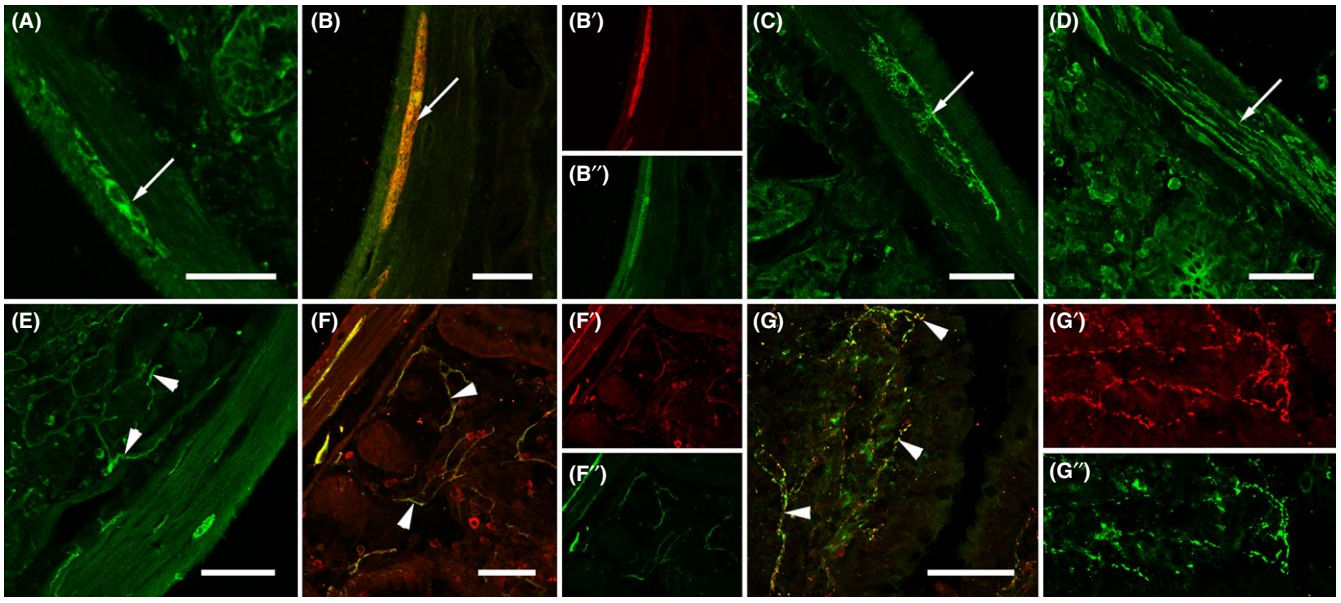

FIGURE 2 Immunofluorescence patterns of intestinal plexus reactivity. (A) Staining with a representative patient serum (1:500) showed cytoplasmic reactivity of single cells in the rat myenteric plexus (small intestine, arrow). (B) Diffuse fluorescence signal of rat myenteric plexus (colon, arrow) in a costaining of a patient serum (green, 1:500, B″) and a MAP2 antibody (red, 1:500, B′). (C) One patient serum with high IgG titers showed strong granular pattern of myenteric plexus reactivity (arrow) and a streaky muscularis signal (D, arrow) in the rat small intestine (1:1000). (E) Several sera showed strong dendritic labeling (1:500, green, arrowheads). (F) Double-labeling with MAP2 (red, F′) confirmed the dendritic structures (arrowheads). (G) One serum (red, 1:500, G′) contained IgG binding to axons in the rat small intestine (arrowheads), showing overlapping signals with tau staining (green, 1:100, G″). Scale bars = 50 μm

Similar patterns, but with lower intensities and slightly different shapes, were found with further four patient sera. Only one patient showed an intense (1:20 000 titer) nuclear staining (Figure S1A) that was not restricted to ENS cells but was detectable in almost every cell in the intestine and brain. The finding likely corresponds to the antinuclear antibodies (ANA) and extractable nuclear antigen antibodies present in this patient who had the diagnosis of mixed connective tissue disease.

Another pattern of ENS reactivity was the dendritic or axonal signal found in three sera with titers between 1:5000 and 1:20 000 (Figure 2E-G). Double-labeling with MAP2 (Figure 2F) or Tau (Figure 2G) confirmed the dendritic or axonal staining, respectively.

Apart from antineuronal immunoreactivity in the ENS, several sera showed binding to further structures in the intestine (Table S1). 25 of 107 (23.4%) patient sera and 6 of 44 (13.6%) control sera showed reactivity against epithelium, predominantly against goblet cells (Figure S1B). However, there was no significant overlap between antineuronal and antiepithelial immunoreactivity in patients and controls ($P = .79$; Fisher's exact test). The highest frequency of epithelial cell reactivity was observed in UC patients (5/16, 31.3%) and in CD patients (12/42, 28.6%). One UC patients' serum not only showed a strong goblet cell signal but also reactivity against goblet cell secretion, most probably mucins (Figure S1C). The significance of this is unclear as the use of human tissue instead of rat colon is essential for the determination of goblet cell antibodies.[22] The lamina propria was another structure commonly stained with variable patterns by patient sera in different intensities and patterns (8.4% of patients vs 0% of controls) (Figure S1D).

3.4 | Patterns of brain immunoreactivity

The predominant immunostaining pattern, seen in all 22 anti-CNS positive cases, was a cellular signal in different parts of the brain, with titers ranging from 1:200 to 1:80 000. The hippocampus frequently showed a strong signal (19 of 22 positive sera; Table S2), with variable predominance of a more intracellular (Figure 3A, antibody-negative serum shown for control in Figure 3B) vs neuronal surface distribution (Figure 3C). Similarly in the cerebellum, neurons of the cerebellar cortex showed strong immunoreactivity in 15 sera, for example, Purkinje neurons (Figure 3D) or granule cells (Figure 3E). Apart from the staining of neuronal cell bodies, immunoreactivity was also seen against somatodendritic or astrocytic structures in several brain areas (Figure 3F-H). Two sera, one of a patient with unexplained GI symptoms, one of a CD patient, showed a GFAP-like pattern with a strong staining of the radial processes of Bergmann glia and the glia limitans (Figure 3G and H). Interestingly, the reactive isotype in both sera was IgA with a titer of 1:1000.

Several sera contained antibodies that reacted with structures beyond neurons and glia cells. For example, further staining patterns included nuclear or nucleolar signals, large blood vessels, or capillaries.

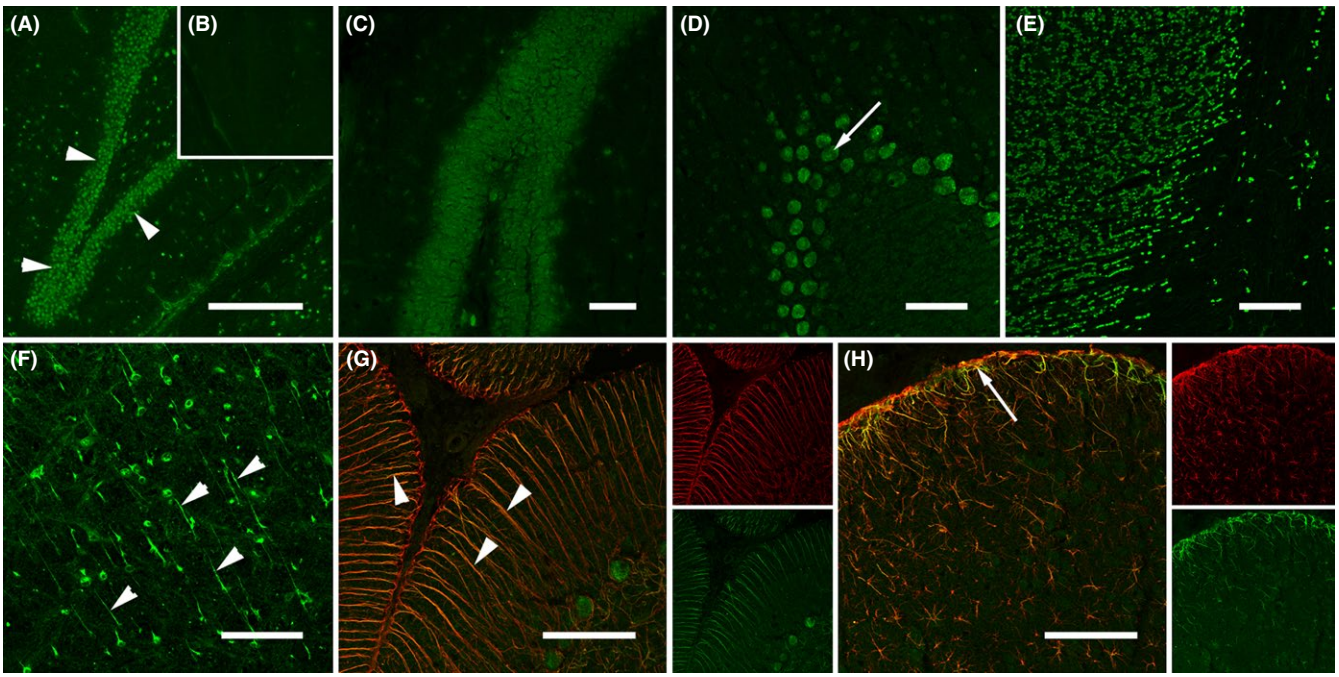

FIGURE 3 Immunofluorescence patterns of brain reactivity. (A) Patient sera (1:1000) commonly showed strong immunosignals in the rat dentate gyrus (arrowheads) with a more intracellular pattern. (B) In contrast, sections of the same anatomical area stained with representative antibody-negative serum (1:1000) were devoid of immunoreactivity. (C) Neuronal surface staining with patient sera was seen in other cases (1:1000). (D) Purkinje neurons (arrow) in the cerebellum were commonly stained with strong immunosignal (1:500). (E) Equally, cerebellar granule cells were commonly immunoreactive, here in a serum staining (1:200) on mouse brain. (F) Some sera showed somatodendritic reactivity (arrowheads), such as with this representative serum (1:200) in the mouse brain stem. (G, H) Two patient sera with IgA autoantibodies (green, 1:200) showed an astrocytic GFAP-like pattern, overlapping with a commercial anti-GFAP antibody (red, 1:1000), labeling the processes of Bergmann glia (G, arrowheads) and glia limitans (H, arrow). Scale bar = 250 μm in A and 100 μm in C-H

Immunoreactivity to blood vessels was also frequently found in control sera (18.7% patients, 20.5% controls) (Figure S1E-G).

3.5 | Targeted antibody characterization using cell-based assays

All 33 ENS- or CNS-immunoreactive patient and control sera were further analyzed for their reactivity pattern on antigen-specific cell-based assays. None of the immunoreactive serum samples contained IgA or IgG antibodies against Hu, Ri, antineuronal nuclear antibody 3 (ANNA 3), Yo, Tr, Ma, GAD65, amphiphysin, NMDAR, AQP4, GABAbR, CASPR2, or DPPX. One serum contained IgA and one serum IgA plus IgG antibodies against myelin (titers of 1:320 and 1:100, respectively), which are not considered disease-relevant according to current experience. Another serum contained IgG antibodies against LGI1 at a titer of 1:100.

Parallel investigation on biochip mosaics containing rat hippocampus and cerebellum, and monkey cerebellum, nerve, intestine, and pancreas resulted in some additional findings of antibodies with binding titers up to 1:320. Two sera contained IgA antibodies binding to intestinal plexus, unmyelinated nerve, and with a GFAP-like pattern to cerebellum (Figure 3G and H). One serum showed IgA immunoreactivity in the intestinal plexus and reacted with myelin. Two sera contained both IgG and IgA antibodies and stained myelinated nerves or intestinal plexus, respectively. The serum found positive for LGI1 antibodies on cell-based assays showed IgG immunoreactivity on intestinal plexus and monkey cerebellum. Six of the 33 serum samples had ANA of the IgA (n = 2) or IgG (n = 4) isotype.

3.6 | Functional studies in organ bath and Ussing chamber

Patient sera with the highest anti-ENS antibody titers in the staining experiments were selected and compared to sera of age- and sex-matched patients without antibodies in immunohistochemistry. Both groups did not differ regarding GI disorders and immunotherapies, but were too small for disease-specific subclassification. Antibody-positive patients had CD (n = 1), IBS (n = 2), indeterminate colitis (n = 1), pancreatic insufficiency (n = 1), and unexplained diarrhea and pain (n = 1). One serum was polyreactive in the intestine (i.e., binding to epithelium, lamina propria, and muscularis including ENS), whereas all others reacted only with ENS. Diagnoses in the antibody-negative group were CD (n = 2), IBS (n = 1), non-celiac gluten sensitivity (n = 1), microscopic colitis (n = 1), and unexplained diarrhea (n = 1). Sera were used at a final dilution of 1:200 in the organ bath. Several experiments demonstrated an initial small contraction and a longer lasting relaxation after addition of the serum samples; however, these responses were not significantly different between antibody-positive and antibody-negative patients (Figure 4A and B). Responses to EFS consisted of an "on response" during the train of stimulation pulses and an "off response" starting immediately after cessation of the stimulation. We could not find significant differences in the EFS responses before and after addition of the sera (Figure 4C-E).

In the Ussing Chamber experiments, we used the same sera and dilution (1:200) as in the organ bath experiments. Responses to all antibody-positive patient sera specifically targeting the ENS and to the one polyreactive serum showed a similar response, consisting of a slow and sustained increase in short circuit current (I_{sc}) starting approximately 10 minutes after addition of the serum to the serosal chamber. This increase in I_{sc} was significantly larger than the responses to sera from antibody-negative patients (Figure 4F and G). In fact, we observed in no case an increase in I_{sc} after addition of the sera from antibody-negative patients (ΔI_{sc} = −0.65 ± 1.3 μA/cm^2 for n = 6 antibody-negative patient sera vs 7.2 ± 2.7 μA/cm^2 for n = 5 antibody-positive patient sera, P = .00013, t test).

Tetrodotoxin, an inhibitor of fast sodium channels, did not change the response to antibody-positive sera (ΔI_{sc} = 7.1 ± 4.6 μA/cm^2 without TTX vs ΔI_{sc} = 5.4 ± 1.9 μA/cm^2 in 0.5 μM TTX, n = 5 antibody-positive sera, P = .52, paired t test).

4 | DISCUSSION

In this comprehensive analysis, we could show that the prevalence of antineuronal antibodies against the ENS and CNS is significantly higher in patients across a wider range of GI disorders compared to healthy controls. Immunofluorescence analyses revealed that autoantibodies reacted with diverse surface and intracellular structures in the intestine and brain, including not only neuronal or astrocytic elements but also epithelium and endothelium. Autoantibodies were more common in female than male patients and showed the highest prevalence in patients with IBS. However, differences were not statistically significant for most patient subgroups in our exploratory study. Higher numbers of patients are needed for sufficient statistical power in future cohorts. Additionally, no pathognomonic patterns were observed that could clearly distinguish patient groups. The findings rather suggest a high variability of antibody titers, anatomical regions and epitopes.

4.1 | Pathogenic antibodies or immunological bystanders?

The most intriguing question is whether the autoantibodies found in the patient sera contribute to the variable clinical spectrum of GI symptoms or—in particular in the many patients with neuropsychiatric comorbidities—to CNS symptoms. Although the cross-sectional design of this pilot study does not provide a definite answer to this question, data suggest a clear tendency.

The assumption of antibody pathogenicity is supported by the common binding of patient sera to soma and processes of ENS neurons, while ANA were seen in few cases only. As ANA commonly associate with autoimmunity, their absence argues against autoimmune diseases as confounders in patients. Similarly, the autoantibody prevalence did not differ significantly in patients with and without coexisting autoimmune diseases in the present cohort. However, we cannot exclude that the autoantibodies are a rather general sign of

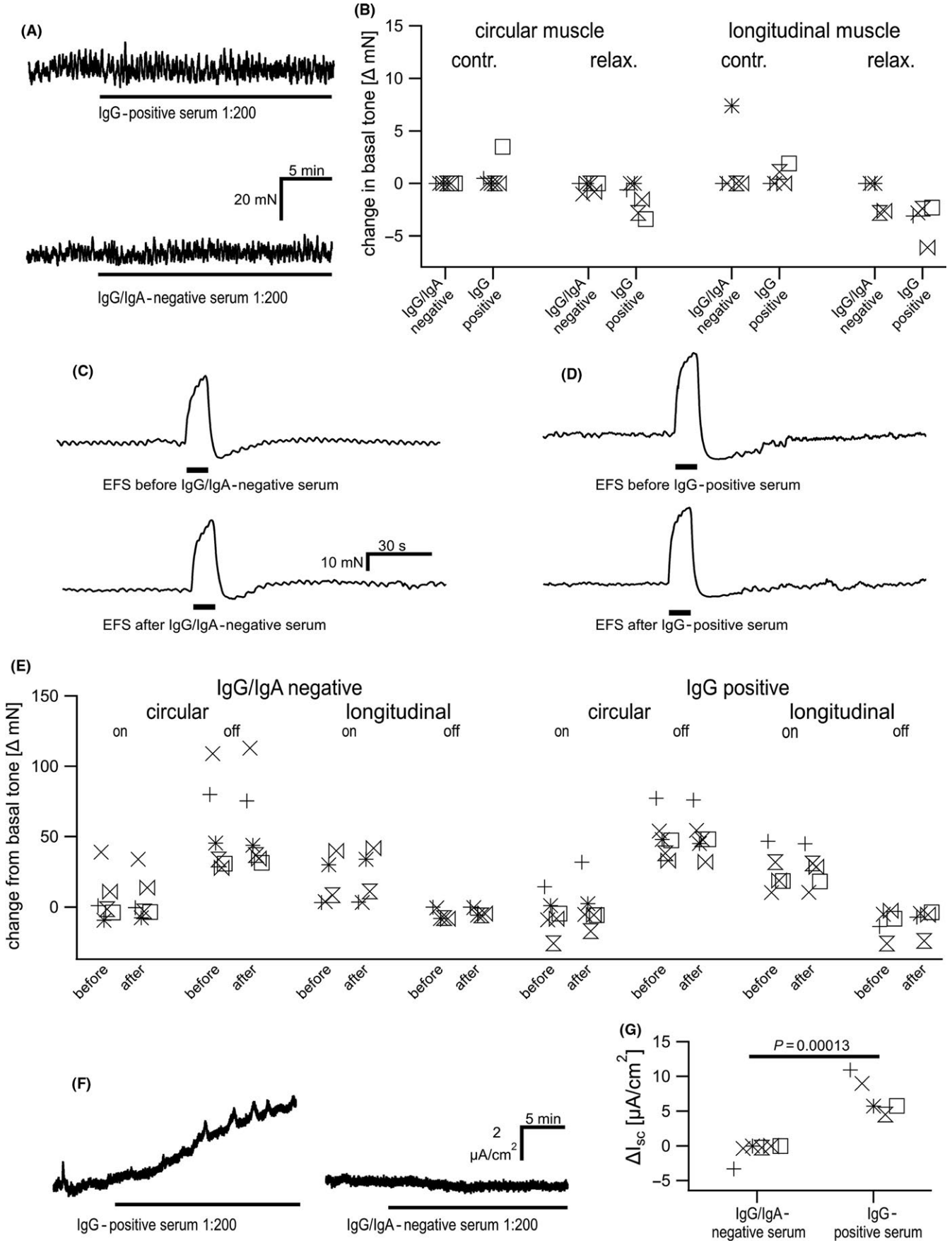
(A)
IgG-positive serum 1:200
5 min
20 mN
IgG/IgA-negative serum 1:200
(B)
circular muscle
longitudinal muscle
contr.
relax.
change in basal tone [Δ mN]
IgG/IgA negative
IgG positive
(C)
EFS before IgG/IgA-negative serum
30 s
10 mN
EFS after IgG/IgA-negative serum
(D)
EFS before IgG-positive serum
EFS after IgG-positive serum
(E)
IgG/IgA negative
IgG positive
circular
longitudinal
on
off
change from basal tone [Δ mN]
before
after
(F)
5 min
2 μA/cm²
IgG-positive serum 1:200
IgG/IgA-negative serum 1:200
(G)
P = 0.00013
ΔIsc [μA/cm²]
IgG/IgA-negative serum
IgG-positive serum

FIGURE 4 Sera with high titers of anti-ENS IgG had no effects on smooth muscle activity but enhanced mucosal secretion in guinea pig colon preparations. (A) Addition of serum (1:200) containing high-titer ENS-reactive IgG or non-ENS-reactive IgG/IgA caused no changes in the spontaneous motility pattern of guinea pig colon. (B) Although some preparations showed small contractions or relaxations after addition of the sera, these changes were not statistically different between IgG/IgA-negative and IgG-positive sera ($P > .1$ for all comparisons, Wilcoxon signed rank test). (C, D) Responses of longitudinal muscle preparations to EFS before and after addition of non-ENS-reactive IgG/IgA serum (C) or ENS-reactive IgG serum (D). (E) Comparison of responses to EFS before and after addition of patient sera showed no significant differences ($P > .1$ for all comparisons, paired *t* test or Wilcoxon signed rank test). (F) Anti-ENS IgG-positive (left) but not IgG/IgA-negative serum (right) caused an increase in short circuit currents (Δ_{Isc}) in Ussing chamber experiments. (G) The change in Δ_{Isc} was significantly larger for anti-ENS IgG-positive sera (n = 5) than for IgG/IgA-negative sera (n = 6). Scale bar in C also applies to D. All ENS-reactive or non-ENS-reactive serum samples are represented by the same symbols throughout B, E and G

autoimmunity as we did not analyze a control group of patients with non-GI autoimmune diseases.

Despite the specific binding patterns, none of the here examined established antibody targets of enteric autoimmunity (such as against DPPX or Hu proteins) were identified using both indirect immunofluorescence and cell-based assays.

In a next step, functional effects were compared between antibody-positive and antibody-negative sera. Indeed, short circuit current recordings in the Ussing chamber demonstrated increased secretion selectively with antibody-positive samples. As secretory dysfunction is relevant to both, diarrhea and constipation, the findings raise the question whether autoantibodies may be involved in GI dysfunctions of these patients. The antibody-associated effect on secretion was similar in different diseases groups, potentially reflecting downstream effects across various GI disorders and clearly worthwhile further studies. The prolonged and continuous increase in secretion suggests that the effect is not directly mediated via neuronal binding even though it is known that secretion is primarily under neural control.[23] Experiments with the sodium channel blocker TTX strengthened this impression as the observed increase in secretion was not significantly altered under the influence of TTX. Other conceivable mechanisms including a direct activation of transporters and ion channels (e.g., via calcium-dependent presynaptic release at the neuroepithelial interface) or an indirect effect via mast cell activation and histamine release clearly deserve further studies.

In contrast to secretion, organ bath experiments with smooth muscle/myenteric plexus preparations of the guinea pig distal colon did not demonstrate specific effects on contraction and relaxation of circular and longitudinal muscle strips or on muscle responses to EFS. However, given the limited amount of available patient material, we cannot exclude that significant effects would have been detected with higher serum concentrations, even though IgG-mediated cytotoxic effect in cell cultures has been shown at similar dilutions that we used here.[24] The lack of serum effects on smooth muscle/myenteric plexus structures suggests that the yet to be explored target of the serum antibodies must be specifically expressed in mucosa/submucosa preparations but not within the muscle layers.

Knowledge on the exact antibody target might be a prerequisite for prediction of pathogenic effects. Along these lines, previous studies on autoantibodies reported very different findings, ranging from clear evidence of antibody pathogenicity to bystander phenomena. For example, anti-Hu antibodies are linked to often paraneoplastic conditions in the brain and in the gut, ranging from limbic encephalomyelitis and subacute sensory neuronopathy[25] to GI dysmotility.[3] Some studies argue that the antibodies' intracellular targets prevent direct pathogenicity and that clinical effects are predominantly T-cell mediated. Others suggest that anti-Hu antibodies have direct functional effects, such as by impairing the ascending excitatory reflex in guinea pig ileum,[26] by inducing neuronal apoptosis in a human neuroblastoma cell line and in cultured myenteric neurons[27] and in direct activation of enteric and visceral sensory neurons.[28]

Another example of well-proven functional relevance is antibodies against the ganglionic (α3-type) neuronal acetylcholine receptor in AAG.[5] Rabbit and mouse models nicely reproduce the underlying sympathetic, parasympathetic, and enteric dysfunction in experimental autoimmune autonomic neuropathy.[29-31] Thereby, antibody titers correlated with the severity of dysautonomic symptoms in both, patients and immunized rabbits.[5,29] Also, organ bath functional assays with IgG from patients with systemic sclerosis and Sjögren's syndrome demonstrated an inhibitory antibody effect on M3-muscarinic receptor agonist-evoked colonic smooth muscle contraction.[32,33] A recent study discussed the role of anti-ENS antibodies in patients with multiple sclerosis, possibly linking GI dysfunction with antibody-mediated ENS degeneration.[34]

The pathogenic effects on GI dysfunction were less clear in studies on achalasia and gastro-esophageal reflux disease. Although antibodies bound to enteric neurons in the small intestine and esophagus, there was no specificity to subpopulations of enteric neurons or to disease groups suggesting that the antibodies were an epiphenomenon.[11] Similarly, a study on patients with achalasia demonstrated effects of sera on neurochemical coding in the myenteric plexus and on motility in fundic specimens despite the absence of antienteric antibodies.[35] Autoantibodies might potentially be a secondary phenomenon in some patients, for example as a response to neurodegeneration in enteric ganglionitis[36] or related to an independent autoimmune process. In this way, they could be a disease biomarker rather than driving the disease.

4.2 | Can the antibody search in GI patients accelerate diagnosis of neurological diseases?

An aim of this study was to experimentally approach potential overlapping autoimmunity in the brain and in the gut, as can be observed in anti-DPPX encephalitis. Given that none of the examined patient sera harbored antibodies against DPPX or one of the here tested brain/gut epitopes, the data support the notion that these well-defined

encephalitis-related antibodies are rare in a routine gastroenterology setting. Anti-DPPX encephalitis should still be a differential diagnosis and antibody testing should be performed when unexplained severe diarrhea, weight loss, and other intestinal symptoms precede or go along with neurological symptoms.[14] Future studies should include further antibodies targeting CNS but also enteric epitopes, for example, acetylcholine receptors.

Nonetheless, the results of our functional assays suggest that the here detected autoantibodies could potentially participate in bowel dysfunction, in particular in the regulation of secretion. Future studies will determine whether the presence of such antibodies can help to identify patients with GI disorders who might benefit from a more specific, antibody-directed therapy. Analyses will have to include the specificity of these antibodies, that is, the target epitopes, affinity, and causal relationship in functional assays.

Even more interesting, we found a high prevalence of brain-binding antibodies in patients with GI diseases, often with overlapping reactivity to ENS and CNS neurons. In one patient, antibodies were directed against the brain protein LGI1, and in two patients, IgA antibodies showed a GFAP-like pattern. Both LGI1 and GFAP antibodies were recently associated with different forms of encephalomyelitis[37,38] which could not be recognized in the clinical phenotype of the here described patients. Shared pathology is also suggested by the fact that many neuron-targeting autoantibodies were of the IgA isotype, which is normally thought to result from immunity in the GI tract and mucosal surfaces. IgA antibodies are indeed increasingly considered in CNS autoimmunity, such as in progressive cognitive impairment,[39,40] limbic encephalitis,[41] or antigliadin IgA antibodies in celiac disease cross-reacting with neuronal proteins.[42] It will be an interesting field of future research to determine the target epitopes of autoantibodies contained in patient sera. A promising strategy might be the generation of monoclonal recombinant human antibodies as recently shown by our group for brain-targeting autoantibodies in patients with encephalitis.[43] Monoclonal antibodies will enable functional assays in exactly determined concentrations, which would be superior to human samples. In addition, further studies should include functional data of larger, clearly defined patient groups and age-matched controls and should analyze correlations of sera-derived autoantibodies with disease initiation and progression.

Taken together, antineuronal autoantibodies are significantly more prevalent in patients with GI dysfunction than in healthy controls. Our data stimulate the question whether serum-derived antibodies can participate in the clinical phenotype, even though the size of the patient cohort and the study design do not allow correlations between antibody prevalence, functional data, and patient subgroups. Nonetheless, routine testing for well-established antineuronal antibodies seems not warranted on a routine gastroenterology ward at present, with only minimal risk of overlooking treatable autoimmune diseases such as anti-DPPX encephalitis. Further research should aim at the identification of the antibodies' target epitopes and prospective correlations with clinical diagnoses and symptoms.

ACKNOWLEDGMENTS

We thank Prof. R.W. Veh for excellent support of the immunohistochemical evaluation. We are indebted to Prof. Axel Pruß for providing control samples. We further thank Doreen Brandl, Birgit Kuch, and Marlene Redl for technical assistance.

CONFLICTS OF INTEREST

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

AL, KM, MV, BS, and HP designed the research study; AL, KM, DK, MH, BT, WS, MS, and HP conducted the research and interpreted the data; AL, KM, and HP wrote the manuscript; MV, MN, ES, LP, PT, and CB contributed to collection of samples. All authors approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Lütt A, Michel K, Krüger D, et al. High prevalence and functional effects of serum antineuronal antibodies in patients with gastrointestinal disorders. *Neurogastroenterol Motil*. 2018;30:e13292. https://doi.org/10.1111/nmo.13292