Distinct patterns of serum immunoreactivity as evidence for multiple brain-directed autoantibodies in juvenile neuronal ceroid lipofuscinosis

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Autoantibodies to glutamic acid decarboxylase (GAD65) have been reported in sera from the Cln3–/– mouse model of juvenile neuronal ceroid lipofuscinosis (JNCL), and in individuals with this fatal paediatric neurodegenerative disorder. To investigate the existence of other circulating autoreactive antibodies, we used sera from patients with JNCL and other forms of neuronal ceroid lipofuscinosis (NCL) as primary antisera to stain rat and human central nervous system sections. JNCL sera displayed characteristic patterns of IgG, but not IgA, IgE or IgM immunoreactivity that was distinct from the other forms of NCL. Immunoreactivity of JNCL sera was not confined to GAD65-positive (GABAergic) neurons, but also stained multiple other cell populations. Preadsorption of JNCL sera with recombinant GAD65 reduced the intensity of the immunoreactivity, but did not significantly change its staining pattern. Moreover, sera from Stiff Person Syndrome and Type I Diabetes, disorders in which GAD65 autoantibodies are present, stained with profiles that were markedly different from JNCL sera. Collectively, these studies provide evidence of the presence of autoreactive antibodies within multiple forms of NCL, and are not exclusively directed towards GAD65.

Keywords: autoantibodies, autoimmunity, Batten disease, immunoreactivity, neuronal ceroid lipofuscinoses, patient-derived sera

Introduction

In recent years, an increasing number of central nervous system (CNS) disorders have been suggested to have an autoimmune basis. This autoimmune component may involve the adaptive immune system, via autoantibody production [1], or abnormal populations of activated T cells [2]. To demonstrate humoral pathogenicity, the presence of autoantibodies in a target organ, the induction of disease by passive transfer of autoantibodies and a clinical response to specific immunomodulatory therapy are all required [3]. Despite autoantibodies being reported in a growing number of disorders, a pathogenic role for these autoantibodies has only been shown convincingly in a handful of neurological disorders [1,3].
An autoimmune response has also been described recently in murine and human juvenile neuronal ceroid lipofuscinosis (JNCL) [4,5], a fatal paediatric storage disorder. JNCL, also known as Batten disease, is one of the neuronal ceroid lipofuscinoses (NCLs), a group of at least eight genetically distinct lysosomal storage disorders (LSDs) [6]. These autosomal recessive disorders (CLN1–CLN8) commonly present during childhood with an infantile (INCL), late infantile (LINCL) or juvenile (JNCL) onset [6–8]. Each form is fatal and clinical signs include visual failure leading to blindness, an increased severity of untreated seizures and neurocognitive decline; leading up to an inevitable premature death [6,8].

JNCL is the result of mutations in the CLN3 gene that codes for a transmembrane protein whose precise function remains unknown [7,9,10]. CLN3-null mutant mice (Cln3−/−) present with a JNCL-like phenotype, including the intralyosomal accumulation of autofluorescent storage material and the loss of subpopulations of GABAergic interneurons [11,12]. Both Cln3−/− mice and individuals with JNCL have circulating autoantibodies to glutamic acid decarboxylase (GAD65) that inhibit this enzyme’s ability to convert glutamic acid to γ-aminobutyric acid and results in presynaptic elevation of glutamate [4]. Whether these autoantibodies contribute to pathogenesis remains unclear, but Western blots of whole brain extracts probed with JNCL sera reveal multiple reactive bands [4], suggesting the presence of a variety of brain-directed autoantibodies.

A simple means to begin mapping these other autoantigens in the CNS is to use patient-derived sera as primary antisera upon tissue sections. This approach has been used to demonstrate brain-directed autoantibodies in sera from patients with paraneoplastic neurological diseases [13], Stiff Person Syndrome (SPS) [14], and as an investigative tool for demonstrating autoimmunity in a wide range of other CNS and non-CNS disorders [15–17]. Here we demonstrate characteristic patterns of immunoreactivity on rat and human CNS tissue using JNCL sera. This immunoreactivity is markedly different from SPS and Type I Diabetes (TID), two other conditions where GAD65 antibodies are raised. Taken together, these data demonstrate that GAD65 antibodies contribute to only a small proportion of the JNCL serum immunoreactivity. Moreover, our data provide evidence for the presence of multiple other as yet unidentified autoantibodies in JNCL and in other forms of NCL.

Materials and methods

Patient-derived serum

Serum samples from patients with NCLs [INCL, n = 2; LINCL, n = 3; JNCL, n = 59; and vLINCL (CLN6), n = 1] were provided by (i) volunteers at annual Batten Disease Support and Research Association (BDSRA) Family Meetings; (ii) Professor P. Santavuori (University of Helsinki, Finland); and (iii) Dr A. Fensom (Guy’s Hospital NHS Trust, UK). All JNCL serum samples were confirmed as GAD65 immunoreactive via binding to recombinant human GAD65, as described previously [4]. SPS patient-derived sera (n = 7) were provided by Dr M. Dalakas (National Institute of Neurological Disorders and Stroke (NINDS) (USA) and Professor A. Vincent (University of Oxford, UK); and TID patient-derived sera (n = 8) were provided by Dr M. Atkinson (University of Florida, USA). For comparison we used sera from neurologically normal individuals (n = 12), paraneoplastic controls (n = 2) and a range of other LSDs provided by Dr D. Wenger (Jefferson Medical College, USA), and Dr A. Fensom (Guy’s Hospital NHS Trust, UK) (GM1 gangliosidoses, Tay-Sachs, Sandhoff, Hurler, I-Cell, Niemann-Pick Type A, Gaalsoliosidosis and Gaucher; n = 32). Samples were obtained with appropriate informed consent and after local ethical review. The use and storage of these samples for analysis at the Pediatric Storage Disorders Laboratory (PSDL) was approved by the Institute of Psychiatry Ethical Committee (Research) (approval number 181/02). Derivation of patient sera was performed by centrifuging the samples for 10 min at 3000 g, with sera subsequently stored at −70°C.

Tissue preparation

Animals Six-week-old male Sprague-Dawley rats (250–280 g, Charles River, Margate, UK, n = 9) were housed at 21 ± 1°C under an alternating 12-h light/12-h dark cycle and perfusion procedures carried out as required by the UK Animals (Scientific Procedures) Act 1986. Animals were deeply anaesthetized with sodium pentobarbitone (100 mg/kg) and transcardially perfused with vascular rinse (0.8% NaCl in 100 mM NaHPO4) followed by a freshly made and filtered solution of 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Brains were subsequently removed, postfixed overnight at 4°C in the

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same fixative and cryoprotected at 4°C in a solution of 30% sucrose in Tris buffered saline (TBS: 50 mM Tris, pH 7.6) containing 0.05% NaN₃. To provide sections for the detailed mapping of serum immunoreactivity, brains were bisected along the midline and 40-µm frozen coronal or sagittal sections cut through either hemisphere (Leitz 1321 freezing microtome, Leica Microsystems, Milton Keynes, UK). Sections were collected, one per well, into 96 well plates containing a cryoprotectant solution (TBS/30% ethylene glycol/15% sucrose/0.05% sodium azide) and stored at −40°C prior to histological processing.

**Human CNS tissue**  Paraffin-embedded tissue samples from neurologically normal subjects (n = 2) were obtained from the MRC London Neurodegenerative Diseases Brain Bank. At autopsy, tissues were fixed immediately by immersion in 4% neutral buffered formaldehyde and subsequently routinely processed and embedded in paraffin wax. Study protocols for the use of human material were approved by the Ethical Research Committees of the Institute of Psychiatry (approval numbers 223/00, 181/02). Eight-µm sections were cut from paraffin-embedded blocks of temporal lobe using a sledge microtome (Leica SM2400, Leica Microsystems (UK) (Ltd). Sections were then floated onto a water bath at 40°C prior to mounting onto Superfrost-plus glass microscope slides (VWR International, Poole, UK).

**Immunohistochemical staining using patient-derived sera**

**Rat CNS tissue**  Human-derived sera were used as primary antisera and incubated with both sagittal and coronal rat CNS sections with no prior knowledge of condition. For each serum sample, at least two parasagittal sections were stained, together with coronal sections at the level of the primary motor cortex, rostral hippocampus, substantia nigra and cerebellum. Following classification into different staining categories (see below), representative serum samples from each staining category were retested on every sixth coronal or sagittal sections in order to map the full extent of serum immunoreactivity.

Briefly, free floating sections were incubated in 1% H₂O₂ in TBS for 15 min, rinsed in TBS and blocked for 40 min with TBS/0.3% Triton X-100 (TBST)/15% normal goat serum before incubation overnight at 4°C in the respective patient serum diluted at 1:500 in TBST. Subsequently sections were rinsed in TBS and incubated for 2 h with biotinylated goat anti-human IgG (heavy and light chain BA 3000, Vector Laboratories, Peterborough, UK) at a dilution of 1 : 1000 in TBST/10% normal goat serum. Following rinsing in TBS, immunoreactivity was detected by incubation for 2 h in an avidin–biotin–peroxidase complex in TBS (Elite ABC kit, Vectastain, Vector Laboratories). Sections were then rinsed in TBS and staining was developed by incubation for 12 min in 0.05% DAB (Sigma, Dorset, UK) and 0.001% H₂O₂ in TBS. The reaction was stopped by immersion in excess ice-cold TBS. Sections were rinsed again, and then mounted, air-dried, cleared in xylene and cover slipped with DPX (VWR). In every staining run relevant positive and negative controls were used.

**Human tissue**  JNCL patient sera immunoreactivity were mapped on human temporal lobe sections using paraffin-embedded sections with a modified version of previously published immunoperoxidase methods [18], using patient sera diluted 1 : 500 followed by biotinylated goat anti-human IgG (heavy and light chain BA 3000, Vector Laboratories, 1 : 200).

**Subtype specificity**  To determine the subtype of immunoglobulins in patient sera that bind to tissue sections, a parallel series of sections were incubated with secondaries recognizing the various subtypes of immunoglobulins [biotinylated goat anti-human IgM (µ chain BA-3020); IgG (γ chain BA 3080); IgG (heavy and light chain BA 3000); IgA (α chain); IgE (ε chain); κ chain and λ chain (all from kit BAK 1000), Vector Laboratories] at a dilution of 1 : 1000 in TBST/10% normal goat serum.

**Other conditions that raise GAD65 autoantibodies**

Comparative studies with other conditions where GAD65 antibodies are found were also performed using serum samples as primary antisera at optimized dilutions of 1 : 1000 (TID) or 1 : 5000 (SPS), using the protocol described above. To survey the distribution of GAD65, a series of sections was stained with a monoclonal antibody to GAD (Stressgen Biotechnologies, Victoria, BC, Canada, 1 : 500) using a biotinylated goat anti-mouse secondary (BA-9200 Vector Laboratories, 1 : 500) and ABC reagent as described above.
Colocalization studies

To study the extent of colocalization of patient-derived sera with GAD65, rat CNS sections were blocked for 40 min with TBS/0.3% Triton X-100 (TBST)/15% normal goat serum before incubation overnight at 4°C in a solution containing both mouse anti-GAD (Stressgen Biotechnologies) at 1:500 dilution and the respective patient serum diluted at 1:500 (NCLs), 1:1000 (TID) or 1:5000 (SPS) in TBST/10% normal goat serum. Subsequently sections were rinsed in TBS and incubated for 4 h in a mixture of secondary antisera [Alexa Fluor 568 rat-adsorbed Goat anti-mouse IgG (A11030) 1:500 with Alexa Fluor 488 Goat anti-human IgG (A11013) 1:1000, or Alexa Fluor 488 rat-adsorbed Goat anti-mouse IgG (A11001) 1:500 with Alexa Fluor 568 goat anti-human IgG (A21090) 1:1000, Molecular Probes, Eugene, OR, USA]. After rinsing in TBS, sections were mounted and coverslipped with Vectashield hard-set mounting medium (H 1400, Vector Laboratories). Immunofluorescence was observed by conventional epifluorescence microscopy (Zeiss Axioskop 2 MOT, Carl Zeiss Ltd, Welwyn Garden City, UK) and recorded digitally (Zeiss Axiocam, Axiovision 3.0, Carl Zeiss Ltd).

To determine the extent of colocalization of JNCL sera with a range of other CNS antigens, we performed similar colocalization studies using patient-derived sera together with rabbit anti-glutamate (Sigma, 1:2000); rabbit anti-parvalbumin (Swant, Bellinzona, Switzerland 1:20000); rabbit anti-somatostatin (Peninsula Laboratories, Belmont, CA, USA 1:1000) and subsequently with the appropriate secondary antisera labelled with either Alexa Fluor 488 (1:500) or Alexa Fluor 568 (1:1000) (Molecular Probes). In all colocalization studies, the distribution of antigens was confirmed by switching the fluorochromes used to label the secondary antisera. Similar results were also obtained with sequential incubation of primary antisera, rather than as a combined solution (data not shown).

Preadsorption studies

To assess the relative contribution of anti-GAD antibodies to immunoreactivity of JNCL sera, we stained sections with sera that had been preadsorbed with recombinant GAD protein, either full length or lacking the N-terminus (Kronus Inc., Boise, ID, USA). Preadsorption was performed by incubating 10 µl of patient sera in the presence or absence of 3.3 ng recombinant GAD protein overnight at 4°C with constant gentle agitation. The degree of preadsorption was qualitatively assessed by Western blotting as described previously [4].

Analysis and review

All sections were reviewed independently by two workers who were blinded and unaware of genotype and diagnosis. For each sample the distribution of serum immunoreactivity was detailed and samples that shared similar patterns of immunoreactivity were grouped together by consensus between the two observers. These patterns were categorized as staining Types 1–4, as described below. For more comprehensive mapping of JNCL serum immunoreactivity, stained series of coronal and sagittal sections through the CNS were surveyed in detail. The distribution of immunoreactive structures was noted with reference to landmarks in a rat brain atlas [19].

Results

JNCL patient-derived sera display CNS tissue immunoreactivity

In an initial pilot study, serum from two individuals genotyped to bear the 1.02-kb major deletion in CLN3 [9], and neurologically normal controls (n = 2) were used as primary antisera to stain rat CNS sections. The JNCL sera produced a characteristic pattern of cytoplasmic immunoreactivity within a variety of restricted neuronal subpopulations (defined as Type 1 staining), that was absent in adjacent series of sections stained with control sera (Figure 1A). The immunoreactivity of JNCL sera was distributed evenly throughout the cell surface and cytoplasm with no obvious vesicular or perinuclear concentration and no distinct staining of axonal processes or white matter tracts. Significantly, this JNCL serum immunoreactivity was not confined to the cell soma, but extended along the dendrites of multiple cell types. This immunoreactivity provided particularly distinct staining of interneuron populations in the hippocampal formation, cortical...
JNCL serum immunoreactivity

Figure 1. JNCL serum immunoreactivity in rat CNS sections. (A) Overview of rat CNS sections stained immunohistochemically using serum from a neurological normal control (Control) or an individual with JNCL as a primary antiserum. JNCL serum displayed widespread immunoreactivity within multiple CNS structures in both sagittal and coronal sections. In contrast, control serum displayed little or no immunoreactivity above background. Scale bar = 2 mm. (B) Examples of JNCL serum immunoreactivity in sagittal sections through the neocortex, hippocampus and cerebellum. Intensely immunoreactive neurons and apical dendrites were present in lamina V at the border of primary motor (M1) and somatosensory barrel field cortex (S1BF). Within the hippocampus, dense JNCL serum immunoreactivity labelled neurons in the hilus (Hi) and interneurons in the stratum oriens (SO) adjacent to layer CA1. Within the cerebellum JNCL serum stained Purkinje cells (Pk) and their dendrites extending into the molecular layer. Scale bar = 50 µm.

Detailed distribution of Type 1 JNCL serum immunoreactivity

A systematic survey of a one-in-six series of coronal and sagittal sections revealed the full extent of JNCL serum immunoreactivity, staining neuronal subpopulations throughout the CNS (Figure 1). Within the cortex, lamina V pyramidal neurons in many regions exhibited dense immunoreactivity with stained apical dendrites extending into more superficial laminae where scattered neurons in laminae II and III were stained (Figure 1B). Immunoreactivity of this type was particularly evident in the primary and secondary motor cortex, cingulate cortex and all subdivisions of the somatosensory cortex. Less intense immunoreactivity was present in pyramidal neurons in the prefrontal, frontal, primary and secondary visual cortices.
the medial entorhinal cortex and all divisions of the insular cortex. In the retrosplenial, granular and agranular mitral cortices, there was a predominance of stained dendrites. Within the hippocampal formation, immunoreactive neurons with stained dendrites were particularly prominent in the hilus, but scattered immunoreactive neurons were also evident in the stratum oriens and in the dentate gyrus and layers CA1–3 with dendrites extending into the stratum radiatum (Figure 1B).

Throughout the basal ganglia, there was a varying degree of faintly immunoreactive neurons, with the exception of the lateral globus pallidus which exhibited a more intense neuronal immunoreactivity with many highly branched dendrites. Within the thalamus, the majority of immunoreactivity labelled the dense afferent innervation supplying sensory relay nuclei including lateral and ventral nuclei, especially the ventral part of the lateral geniculate nucleus. Immunoreactive neurons and terminals were also present in the reticular thalamic nucleus and in the anterior dorsal nuclei. Dense immunoreactivity of terminals and occasional neurons extended ventrally into the zona incerta, subthalamic nuclei and the medial parvicular and magnocellular and preoptic nuclei of the hypothalamus. More caudally, densely immunoreactive neurons were present in the deep layers of the inferior and superior colliculi, with branched dendrites extending to more superficial layers.

Intensely immunoreactive neurons were also present in the red nucleus, the interstitial nucleus of Cajal, nucleus of Darkschewitsch and oculomotor nucleus, with processes and many immunoreactive fibres running through the reticular formation. More ventrally, staining was evident in the mammillary nuclei, and substantia nigra pars reticularis. Within the pons and medulla there was intense immunoreactivity in the central grey, mesencephalic trigeminal nucleus, cuneiform nucleus, with dense terminal labelling in the pontine reticular nucleus. Immunoreactive neurons were revealed in pedunculopontine and dorsal tegmental nuclei, parvicellular reticular nucleus, medullary reticular nucleus, spinal trigeminal nucleus, principal sensory trigeminal nucleus, facial nucleus and all divisions of the vestibular nuclei and the nucleus of the solitary tract. More caudally, intensely immunoreactive neurons were also present in the accessory and hypoglossal nuclei and in motor neurons of the ventral horn of cervical spinal cord, which displayed many immunoreactive fibres in superficial laminae of the dorsal horn.

Within the cerebellum particularly intense staining of Purkinje cells and their dendrites was evident (Figure 1B), but not equally in all lobules. Scattered Golgi cell and basket cell immunoreactivity was also present, but with no immunoreactivity of granule neurons. Neurons of the deep cerebellar nuclei were also immunoreactive particularly within the medial fastigial cerebellar nucleus.

**JNCL patient-derived sera display distinctive patterns of immunoreactivity**

We next repeated these staining experiments using extended series of JNCL serum samples (n = 59); other forms of NCL (INCL, n = 2; LINCL, n = 3; vLINCL, n = 1); and sera from neurologically normal individuals (n = 12). These diagnoses were all confirmed by appropriate genotyping and for JNCL samples Western blot analysis to confirm the presence of GAD65 autoantibodies (data not shown). Sera from patients with other neurological conditions with neurodegeneration (paraneoplastic syndrome, n = 2 and a range of other LSDs, n = 32) were also used as neurologica controls.

JNCL serum samples produced two distinctly different categories of staining (Figure 3); either prominent staining of neuronal soma and dendrites (Type 1 staining, as depicted in Figure 1) or a more diffuse cytoplasmic immunoreactivity (Type 4). Between these two extremes, there was a range of staining appearances that exhibited markedly less pronounced staining of neuronal processes (Types 2 and 3). These latter types of staining could only be distinguished reliably by the presence (Type 3) or absence (Type 2) of immunoreactive cells with glial morphology in multiple brain regions, but prominent within the stratum radiatum of the hippocampal formation (Figure 3H).

When used as primary antisera, 25% (15 out of 59) of the JNCL samples precisely reproduced the pattern of immunoreactivity described in Figure 1 (Type 1, Figure 3A.F.K). A further 25% stained with many similar characteristics, but with markedly less prominent staining of dendrites and additional diffuse cytoplasmic staining of many cell types and increased background staining of the neuropil (Type 2, Figure 3B.G.I.). Taken together they accounted for more than half of the JNCL samples tested. A further reduction in dendritic immunoreactivity was apparent in nine of the JNCL samples.
(16% of cases), which showed an additional staining of glial cell populations that was most pronounced in the stratum radiatum of the hippocampus and the cerebellar cortex (Type 3, Figure 3C,H,M). A further 25% of cases JNCL cases exhibited a diffuse cytoplasmic stain of all cell soma, but with no consistent nuclear component (Type 4, Figure 3D,I,N). The five remaining JNCL cases could not be fitted into this classification scheme providing inconclusive staining patterns. The staining of JNCL sera was in marked contrast to control patient sera that demonstrated little or no immunoreactivity (Figure 2E,J,O). However, in an extended series (n = 12) of apparently neurologically normal subjects, a small subset of individuals produced widespread diffuse cytoplasmic staining resembling Type 4 staining. Sera from patients with other LSDs and paraneoplastic neurodegeneration, used as neurologically abnormal controls (n = 32), confirmed the specificity of the above staining patterns to JNCL sera. Sera from patients with paraneoplastic syndromes demonstrated a pattern of cytoplasmic and nuclear staining that has previously been reported [20]. Interestingly, LSD patient sera display their own characteristic staining patterns showing a variety of intracellular distributions of immunoreactivity with specificity for particular CNS regions and cell types (data not shown).

The requirement for anonymity of patient serum samples restricted the availability of clinical data to gender and age. As such, we used patient age as a surrogate marker for disease severity, but did not find a correlation between age or gender and the pattern of immunoreactivity. In a small number of JNCL patients where samples from more than one time point were available (n = 10), the pattern of immunoreactivity changed with disease progression with a tendency to move towards a Type 1 pattern.

NCL patient-derived sera also display CNS tissue immunoreactivity

Cases from individuals with other forms of NCL produced patterns of immunoreactivity that either shared the fea-

Figure 3. Multiple types of JNCL serum immunoreactivity. (A–O) An extended series of JNCL sera displayed immunoreactivity that could be classified into four subtypes. Sagittal sections through the neocortex (A–E, Ctx), hippocampus (F–J, Hipp) and cerebellum (K–O, Cb) revealed prominent staining of soma and dendrites as depicted in Figure 1 (Type 1 staining). Type 2 and Type 3 immunoreactivity exhibited successively less prominent dendritic staining, with additional glial immunoreactivity in the hippocampus of Type 3 staining (inset in H). Type 4 staining comprised diffuse cytoplasmic immunoreactivity with no dendritic staining. Serum from neurologically normal controls displayed minimal immunoreactivity above background staining of the neuropil (E, J, O). Scale bar = 50 μm.
tures of Type 2 staining (one INCL case, CLN6 case), or showed little or no distinct immunoreactivity and resembled Type 4 staining (one INCL case, two LINCL cases). These data suggest the presence of autoreactive antibodies in these other forms of NCL.

**JNCL serum immunoreactivity colocalizes to multiple antigens**

On the basis of distribution and morphology, JNCL serum immunoreactivity (Type 1) appeared to involve both inhibitory and excitatory neurons (Figure 1). This staining pattern could be due to the presence of a single autoantigen that is expressed in multiple cell types, or more likely due to the existence of multiple CNS-directed autoantibodies. To investigate this issue, we undertook colocalization studies using dual channel indirect immunofluorescence to identify neuronal populations that stain with JNCL sera. As expected, these studies revealed examples of JNCL serum staining within GAD-positive neurons (Figure 4A–C) or neuronal populations that stain with other markers of GABAergic phenotype, such as the calcium-binding proteins parvalbumin (PV) or calbindin (CB) (Figure 4D–F) or the neuropeptide somatostatin (SOM). Within the neocortex, JNCL serum immunoreactivity was clearly also present within populations of lamina V pyramidal neurons labelled with an antibody to glutamate (Figure 4G–I). However, many JNCL serum immunoreactive neurons were not glutamate-, GAD-, CB-, PV- or SOM-positive, suggesting that JNCL sera recognize only a subset of neurons immunoreactive for each of these phenotypic markers.

**JNCL patient immunoglobulins that stain CNS sections are IgG subtype specific**

To identify the subtype of immunoglobulins in JNCL sera that bind to tissue sections, we used secondary antibodies that distinguish between heavy and light chains of IgG (Figure 5) and that specifically recognize IgA, IgE and IgM immunoglobulins (Figure 6). Sera from individuals with JNCL displayed IgG subtype specificity, with secondaries recognizing the IgG heavy chain (IgG\(\gamma\)) and IgG heavy and light chain (IgG_{\text{H\&L}}) components showing maximal immunoreactivity (Figure 5). Staining with IgG\(\gamma\)-specific secondary antisera retained the intensity of Type 1 JNCL immunoreactivity, but markedly reduced the amount of background immunoreactivity in the neuropil (Figure 5).

Secondary antibodies recognizing the \(\lambda\) chain also detected JNCL serum immunoreactivity (Figure 5), but with markedly less intense staining of a subset of neurons stained by IgG\(\gamma\) and IgG_{\text{H\&L}} secondaries. There was minimal immunoreactivity detectable above background with secondary antisera recognizing \(\kappa\) chains (Figure 5), or specific for IgM, IgA and IgE (Figure 6). Similar results were obtained when using these subtype-specific antisera to detect the binding of a representative series of sera from patients with other forms of NCL (data not shown).
GAD65 autoantibodies do not contribute significantly to JNCL patient serum immunoreactivity

As JNCL patients have circulating autoantibodies to GAD65 [4,5], we compared JNCL serum immunoreactivity with two other groups of patients where GAD65 antibodies are present, SPS and TID. Both SPS (n = 7) and TID (n = 6) sera demonstrated a variety of immunoreactive profiles that were clearly different from JNCL serum immunoreactivity (Figure 7 presents data from the striatum and cerebellum only). Among SPS cases, three out of seven samples stained with a pattern of immunoreactivity previously reported for this condition (Figure 7C) [14], and comparable with the immunoreactivity of a commercially available mouse monoclonal antibody against GAD (Figure 7D). The remaining SPS and TID cases stained with novel patterns of immunoreactivity that were unlike JNCL serum staining, and which are now being characterized in an expanded series of SPS and TID cases. These data suggest that although individuals with JNCL, SPS and TID each raise autoantibodies to GAD65, these three conditions display distinct patterns of serum immunoreactivity, thus questioning how much of the JNCL immunoreactivity is related to GAD65 autoreactivity.

To evaluate this issue further, we preadsorbed JNCL sera with recombinant GAD65 protein, hypothesizing that remaining immunoreactivity would be the result of binding to antigens other than GAD. As expected, unabsorbed JNCL serum samples reproduced Type 1 staining with prominently immunoreactive dendrites in the cortex (Figure 8A), hippocampus (Figure 8B) and cerebellum (Figure 8C). The same JNCL sample that had been preadsorbed either with full-length recombinant GAD or with GAD lacking the N-terminal displayed markedly reduced staining intensity, but with no obvious change in distribution (Figure 8D–F). Although background immunoreactivity was also decreased by preadsorption, the same pattern of immunoreactivity against neuronal and dendritic targets persisted in the cortex (Figure 8D), hippocampus (Figure 8E) and cerebellum (Figure 8F). Taken together with the results of SPS and TID serum staining, these data point towards the existence of other CNS-directed autoantibodies that contribute significantly to the immunoreactivity of JNCL serum.
Discussion

This study represents the first demonstration that JNCL serum binds to tissue sections, and provides a detailed description of this IgG-mediated immunoreactivity. JNCL serum stained both inhibitory and excitatory neuronal populations and displayed immunoreactivity that was distinct from serum from other disorders that raise GAD65 autoantibodies. The pattern of JNCL serum immunoreactivity was not significantly altered by preadsorption with recombinant GAD protein. Collectively, these data suggest that GAD65 autoantibodies do not contribute significantly to JNCL serum immunoreactivity. Instead, GAD65 autoantibodies appear to be just one of multiple CNS-directed autoantibodies in JNCL serum, although the identity and pathological significance of these immunoglobulins remain unclear.

JNCL serum immunoreactivity reveals multiple CNS targets for autoantibodies

The existence of autoantibodies to antigens within the CNS has been demonstrated in a number of neurological conditions by exploring the binding of patient-derived serum to tissue sections [13–17]. Using this methodology, our data reveal the potential for widespread CNS immunoreactivity of JNCL patient-derived sera, which is IgG-mediated rather than via other subclasses of immunoglobulins (Figures 5 and 6). We have previously described the presence of GAD65 autoantibodies in both human and murine JNCL [4,5]. As such, it was to be expected that JNCL serum would bind to subpopulations of GABAergic neurons positive for GAD (Figure 3), or calcium-binding proteins that are expressed by GABAergic interneurons [21]. However, JNCL serum immunoreactivity was only present in subsets of GABAergic neurons expressing these markers. More significantly, JNCL serum also clearly stained multiple populations of...
non-GABAergic neurons, most notably within the neocortex (Figure 4). Taken together with our previous Western blotting data [4], the immunostaining results presented here provide further evidence for the presence of multiple brain-directed autoantibodies in JNCL sera.

The identity of these other autoreactive CNS antigens remains unknown. However, a direct comparison of JNCL patient sera immunoreactivity with a mouse anti-GAD65 monoclonal antibody revealed dissimilar staining patterns (Figure 7). Moreover, the inability to abolish the intensity of immunoreactivity upon adsorption of JNCL sera with GAD65 (Figure 8) further suggests that GAD65 autoantibodies are just a small component of JNCL sera immunoreactivity. This is not necessarily surprising as radioimmunoassays do not consistently detect low levels of GAD65 autoantibodies in JNCL serum samples [22]. In contrast, SPS patient sera which display significantly higher levels of circulating GAD65 autoantibodies measurable by immunoassay [23] demonstrate a pattern of immunoreactivity similar to that of monoclonal anti-GAD65 (Figure 7). The individual patterns of CNS immunoreactivity that we found in these three disorders that raise antibodies against GAD65 (JNCL, SPS and TID) could be explained by the differences in the autoantibody specificity to antigenic epitopes of GAD65 in these disorders [22]. Alternatively, the pattern of JNCL serum immunoreactivity we have described is more likely to reflect the presence of multiple other brain-directed autoantigens in this disorder.

**Production of autoantibodies in JNCL patient-derived sera**

Our immunohistochemical data reinforce evidence for multiple circulating autoantibodies in JNCL serum [4,5], but do not reveal the source of these immunoglobulins. These circulating autoantibodies in JNCL could be natural autoantibodies (NAAs), may be a direct result of tissue destruction, or reflect another unidentified consequence of CLN3 mutation upon antigen presentation and self-recognition. NAAs are antibodies principally of the IgM subtype [24], exhibiting autoreactivity in normal healthy subjects and are targeted against conserved epitopes [25]. As our data suggest the immunoreactive components in JNCL patient-derived sera are predominantly IgGs, it seems unlikely that NAAs in patient sera contribute significantly to the pattern of JNCL serum immunoreactivity.

Autoantibodies found in various neurodegenerative conditions [26–29] can be generated following tissue destruction [1]. These autoantibodies may persist due to inaccurate antigen presentation, or simply abnormal lysosomal clearance. It remains unclear whether the brain-directed autoantibodies are generated systemically or within the CNS, but lymphocyte infiltration into the JNCL CNS is very limited, even at the advanced stages of disease progression (data not shown). If produced within the CNS, the presence of autoantibodies may represent a secondary response to the liberation and vascular dissemination of antigens from dead or dying neurons. However, these autoantibodies are present in Cln3–/– mice as early as 1 week of age [4], whereas these mice do not exhibit significant neurone loss before 5 months of age [Pontikis, Pearce and Cooper 12, unpub. obs.].

Alternatively, lysosomal dysfunction could play a key role in the generation or persistence of autoantibodies. Furthermore, as CLN3 is expressed in the late endosome/lysosome [10,30,31], a loss of CLN3 function may have an impact upon antigen presentation. Such a process is required for the generation of recognition of ‘self’ antigens to protect against an autoimmune response. During dendritic cell maturation, the loading of MHC class 2 complexes is a pH-dependent process [32]. As altered vacuolar/lysosomal pH has been demonstrated in both yeast [33] and NCL patient-derived fibroblast cell lines [34], the influence of pH dysregulation may be a further contributory factor in the generation and persistence of autoantibodies, probably as a result of dysfunctional antigen presentation. Indeed, the changes in JNCL serum immunoreactivity we have described during disease progression may be indicative of an ongoing and ever worsening lysosomal defect.

**Potential pathogenicity of autoantibodies in JNCL.**

Circulating autoantibodies to GM2 gangliosides and IgG deposition within the CNS have been reported in patients and a mouse model of Sandhoff disease, another paediatric storage disorder, and may contribute to pathogenesis [35]. The presence of multiple autoantibodies in the serum and cerebrospinal fluid of Cln3–/– mice and patients (data not shown), their ability to bind to the CNS, and the elevated levels of glutamate and inhibition of GAD activity demonstrated in brain tissue of Cln3–/– mice [4], collec-
tively raise similar questions for a pathogenic role of autoantibodies in JNCL. It may be tempting to think that the presence of GAD65 autoantibodies contributes to the selective loss of GABAergic interneurons that occurs in JNCL [7,12,36]. Nevertheless, GAD65 autoantibodies are not detected in other forms of NCL [5], which also exhibit significant loss of interneuron populations [7,36]. Furthermore, if the antigenic targets of autoantibodies are intracellular, such as GAD, this may preclude their pathogenicity [37,38]. As such, it will be important to investigate the identity and potential pathogenic roles of the other, as yet unidentified autoantibodies present in JNCL sera.

Antibody-mediated CNS disorders are increasingly being recognized including conditions where the autoantigen is not identified, for example, in post-streptococcal CNS disease and neuropsychiatric presentations of systemic lupus erythematosus [1]. Rigorous evidence of pathogenicity of these autoantibodies in JNCL will still require the induction of a disease phenotype by passive transfer of autoantibodies, disease induction with the autoantigen, or a response to specific immunomodulatory therapy.

NCLs and beyond

Preliminary data using sera from individuals with other forms of NCL also suggest that the presence of autoantibodies is not restricted to JNCL and it will be important to explore this possibility further in an expanded series of cases. It would also be equally important to investigate if these antibodies cross the blood brain barrier and bind to the CNS in patients. As brain-directed antibodies are present in NCL patient-derived sera, similar autoimmune responses may happen in other disorders where lysosomal dysfunction occurs. Because the lysosome plays an active role in antigen presentation, a process which is apparently abnormal in mice that model mucopolysaccharidosis VII [39], the LSDs are an interesting group in which to investigate such responses, as has been done in GM2 [35] and Gaucher disease [40]. Defining the autoimmune components of these disorders may lead to the development of immunomodulatory therapeutic strategies. Nevertheless, it remains imperative that evidence of pathogenicity of an autoimmune response exists before clinical trials are initiated, as such treatments are not without their risks.

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