Intracellular and circulating neuronal antinuclear antibodies in human epilepsy

Philip H. Ifland a,b,f, Juliana Carvalho-Tavares a,b,g, Abhishek Trigunaite c, Shumei Man c, Peter Rasmussen a,h,c, Andreas Alexopoulos d, Chaitali Ghosh a,b,c,f, Trine N. Jørgensen e, Damir Janigro a,b,c,g

a Department of Neurological Surgery, Cleveland Clinic Foundation, Cleveland, OH, USA
b Department of Cellular and Molecular Medicine, Cleveland Clinic Foundation, Cleveland, OH, USA
c Cerebrovascular Research, Cleveland Clinic Foundation, Cleveland, OH, USA
d Department of Neurology, Cleveland Clinic Foundation, Cleveland, OH, USA
e Department of Immunology, Cleveland Clinic Foundation, Cleveland, OH, USA
f Kent State University School of Biomedical Sciences, Kent, OH, USA
g Núcleo de Neurociências, Departamento de Fisiologia e Biofísica, ICB/UFMG, Belo Horizonte, MG, Brazil

A R T I C L E   I N F O

Article history:
Received 29 May 2013
Revised 2 July 2013
Accepted 12 July 2013
Available online 21 July 2013

Keywords:
Neuroimmunology
Seizure disorders
Autoantigen
Autoantibody
Anti-histone antibody
Anti-chromatin antibody
Autoimmune

A B S T R A C T

There are overwhelming data supporting the inflammatory origin of some epilepsies (e.g., Rasmussen’s encephalitis and limbic encephalitis). Inflammatory epilepsies with an autoimmune component are characterized by autoantibodies against membrane-bound, intracellular or secreted proteins (e.g., voltage gated potassium channels). Comparably, little is known regarding autoantibodies targeting nuclear antigen. We tested the hypothesis that in addition to known epilepsy-related autoantigens, the human brain tissue and serum from patients with epilepsy contain autoantibodies recognizing nuclear targets. We also determined the specific nuclear proteins acting as autoantigens in patients with epilepsy.

Brain tissue samples were obtained from patients undergoing brain resections to treat refractory seizures, from the brain with arteriovenous malformations or from post-mortem multiple sclerosis brain. Patients with epilepsy had no known history of autoimmune disease and were not diagnosed with autoimmune epilepsy. Tissue was processed for immunohistochemical staining. We also obtained subcellular fractions to extract intracellular IgGs. After separating nuclear antibody–antigen complexes, the purified autoantigen was analyzed by mass spectrometry. Western blots using autoantigen or total histones were probed to detect the presence of antinuclear antibodies in the serum of patients with epilepsy. Additionally, Hep-2 assays and antinuclear antibody ELISA were used to detect the staining pattern and specific presence of antinuclear antibodies in the serum of patients with epilepsy.

Brain regions from patients with epilepsy characterized by blood–brain barrier disruption (visualized by extravasated albumin) contained extravasated IgGs. In the brain from patients with epilepsy, neurons displayed higher levels of nuclear IgGs compared to glia. IgG colocalized with extravasated albumin. All subcellular fractions from brain resections of patients with epilepsy contained extravasated IgGs (n = 10/10), but epileptogenic cortex, where seizures originated from, displayed the highest levels of chromatin-bound IgGs. In the nuclear IgG pool, anti-histone autoantibodies were identified by two independent immunodetection methods. Hep-2 assay and ELISA confirmed the presence of anti-histone (n = 5/8) and anti-chromatin antibodies in the serum from patients with epilepsy.

We developed a multi-step approach to unmask autoantigens in the brain and sera of patients with epilepsy. This approach revealed antigen-bound antinuclear antibodies in neurons and free antinuclear IgGs in the serum of patients with epilepsy. Conditions with blood–brain barrier disruption but not seizures, were characterized by extravasated but not chromatin-bound IgGs. Our results show that the pool of intracellular IgG in the brain of patients with epilepsy contains autoantibodies recognizing nuclear targets. Seizures may be the trigger of neuronal uptake of antinuclear antibodies.

© 2013 The Authors. Published by Elsevier Inc. Open access under CC BY license.

* Corresponding author at: Cleveland Clinic Foundation, Department of Cellular and Molecular Medicine, 9500 Euclid Ave., NB-20, Cleveland, OH 44195, USA. Fax: +1 216 444 9404.
E-mail addresses: ifland@ccf.org (P.H. Ifland), carvalho@ccf.org (J. Carvalho-Tavares), trigunaite@ccf.org (A. Trigunaite), mano@ccf.org (S. Man), rasmusp@ccf.org (P. Rasmussen), alexopoul@ccf.org (A. Alexopoulos), ghoshc@ccf.org (C. Ghosh), jorgent@ccf.org (T.N. Jørgensen), janigro@ccf.org (D. Janigro).

Available online on ScienceDirect (www.sciencedirect.com).

0969-9961 © 2013 The Authors. Published by Elsevier Inc. Open access under CC BY license.
http://dx.doi.org/10.1016/j.nbd.2013.07.006
Introduction

Studies on inflammatory mechanisms in epilepsy have been burgeoning, with a 300% increase in published articles on PubMed from 1993 to 2003 compared to the previous decade. It is thus not surprising that new models of seizures have emerged. These models take into account the knowledge gained from clinical studies, and are based on mechanisms, receptors, and pathways that were formerly reserved for the immunologist (Galanopoulou, 2013; Marchi et al., 2007b, 2009; Vezzani et al., 2012). Evidence to support a role for inflammation and autoimmunity in epilepsy has come from indirect and direct sources. For example, the anticonvulsant activity of steroids in some epilepsies (indirect (Marchi et al., 2011)), together with the presence of inflammatory signs and markers in the serum or cerebrospinal fluid (CSF) of patients (direct (Nabbout, 2012)) have been interpreted as clues suggestive of a role for the immune response. In addition, well-established models of seizures which were developed to specifically target neurons have been re-examined to reveal an underlying inflammatory etiology. For example, research has shown that a putative muscarinic convulsant, pilocarpine, acts by immune activation and not as previously suspected by a CNS exclusive action on muscarinic receptors (Faber et al., 2008; Marchi et al., 2007b, 2009; Uva et al., 2007).

The role of inflammation in seizure disorders has therefore been recognized as an etiologic reality and as an important target for therapy (Marchi et al., 2009, 2010, 2012b; Vezzani et al., 2011).

There are three groups of “inflammation-related seizures” (IRS): 1) Seizures caused by the presence of a pathogen. These are perhaps the least studied cluster of IRS and include seizures due to meningitis, neurotropic pathogens, etc. In developing countries, pathogens are considered the highest risk factor for acute seizure and increase the risk of epilepsy by eleven fold (Bharucha et al., 2008). 2) A large family of IRS encompasses autoimmune epilepsy syndromes, where one of the etiological mechanisms is believed to be the presence of anti-neuronal autoantibodies typically targeting either ion channels, intracellular epitopes or neurotransmitter receptors (Graus and Dalmau, 2012; Lancaster and Dalmau, 2012; McKnight et al., 2005). 3) A number of seizure disorders lacking either of these features (pathogen or autoantibodies) can be classified as IRS based on a therapeutic response to immunomodulators (Granata et al., 2008, 2011), vascular changes consistent with an ongoing inflammatory process (e.g., blood–brain barrier disruption; for a review see Janigro, 2012), or concomitant brain changes that mimic some, but not all, signs of inflammation (Granata et al., 2011; Nabbout, 2012; Nabbout et al., 2011).

As mentioned above, the third type of IRS may be linked to blood–brain barrier (BBB) disruption. The BBB is the gatekeeper of immune privilege in the CNS (Bechmann et al., 2007; Galea et al., 2007). The BBB maintains ionic homeostasis which, in turn, controls neuronal excitability (de Vries et al., 2012; Janigro, 2012; Marchi et al., 2012b; Seiffert et al., 2004). Thus, BBB disruption (BBBD) not only causes loss of immune privilege but may also directly result in seizures (Marchi et al., 2007a). A reporter of BBB failure, extravasated albumin levels in CSF, has also been used to detect focal BBBD by immunohistochemistry. Interestingly, after diffusion through the CNS extracellular space, albumin accumulates in neurons and glia (David et al., 2009a,b; Seiffert et al., 2004). Regions of focal BBBD can also be measured by detection of extravasated IgGs. Whether extravasated IgGs also enter into the brain cells it has not been fully elucidated (Michalak et al., 2012).

The presence of IgGs in the brain from patients with epilepsy, together with our understanding of the pathophysiology of multiple sclerosis (MS), has been used to propose autoimmunity as an etiologic factor in seizure disorders. Autoantibodies to the NMDA, GABA_ receptors, as well as the voltage-gated potassium channel and its components LGI1 and CASPR2 have been detected in the CSF or serum of patients with seizures (Lancaster and Dalmau, 2012). In addition, autoimmune diseases such as systemic lupus erythematosus (SLE) greatly influence seizure susceptibility (Adelow et al., 2012). Thus, seizure threshold can be lowered by direct action on CNS targets (e.g., glutamate receptors), by exposure to endotoxin (Galic et al., 2008) or by autoimmune targeting of a specific antigen, such as nuclear components. A recent paper has shown that even in the absence of autoimmune disease, IgG can be found in the brain of mice after lithium/pilocarpine-induced seizures (Michalak et al., 2012). This is also consistent with previous work showing that BBBD, as seen in regions of seizure generation in the human brain, is characterized by large deposits of extravasated IgG (Michalak et al., 2012). However, to date, the significance or consequences of IgG extravasation into the CNS has not been fully elucidated.

The CNS of patients with epilepsy provides a unique environment where the coupling of seizure with inflammation, loss of immune privilege and cell death may provide a mechanism for the generation and uptake of autoantibodies against intracellular proteins. Therefore, we examined whether or not autoantibodies against intracellular proteins existed in the CNS and serum of patients with epilepsy where an autoimmune or infectious etiology was ruled out. By using a number of techniques and an approach based on comparison of different pathologies all characterized by BBBD, we isolated autoantigens from subcellular fractions of the brain from patients with epilepsy. MS was used as a comparative “auto-immuno” disease, and brain sections derived from cerebrovascular malformations as a means to study BBBD independent of seizures. Our results demonstrate the presence of antinuclear antibodies in the brain and serum from patients with epilepsy, and the accumulation of autoantibodies in neuronal nuclei.

Materials and methods

The multimodal approach used for the experiments detailed in this section is depicted graphically in Fig. 1.

Patient selection

Brain tissue specimens were obtained from patients conforming to the guidelines of the Declaration of Helsinki. All patients signed an informed consent according to institutional review protocols at the Cleveland Clinic Foundation. Patient information and experimental use of patient samples are summarized in Table 1. All brain tissue samples were obtained from surgical resections with the exception of post-mortem MS brain. Post-mortem samples were a generous gift of Dr. Bruce Trapp’s laboratory at the Cleveland Clinic Foundation Lerner Research Institute. Inclusion criteria were willingness to participate to the study and lack of positive diagnosis for an autoimmune disease. One patient was identified as RA post-facto and is considered a positive control (Fig. 6).

Detection and discovery

Immunohistochemical staining of brain tissue sections

The brain tissue was mounted using Tissue-Tek OCT compound (Sakura Finetek Europe B.V., The Netherlands) and sectioned at approximately 25 μm on a Leica CM3050 cryostat (Leica Microsystems Inc., Buffalo Grove, IL). Nine patients with epilepsy, 4 multiple sclerosis patients, and 3 arteriovenous malformation (AVM) patients were included in these experiments.

Immunofluorescent detection of IgG and albumin in neurons, glia and brain parenchyma

Free-floating sections were stained for IgG and albumin. Non-specific binding was minimized by incubation in a 3% goat serum blocking solution at room temperature for 1 h. Sections of the brain tissue were incubated with monoclonal mouse anti-human albumin antibody (1:1000; Sigma-Aldrich, St. Louis, MO). Fluorescently-labeled secondary antibodies used were as follows: Alexa Fluor 594 polyclonal donkey anti-mouse IgG (1:100; Jackson ImmunoResearch, West

207

P.H. Ilffland II et al. / Neurobiology of Disease 59 (2013) 206–219
Grove, PA), and fluorescein conjugated polyclonal goat anti-human IgG (1:200, Vector Labs, Burlingame, CA).

3,3′-Diaminobenzidine staining of AVM patient brain tissue
3,3′-Diaminobenzidine (DAB) staining of brain tissue sections was achieved using the method from Marchi et al. (2010).

Immunofluorescent detection of IgGs in neurons and astrocytes
Free-floating brain sections were stained for IgG and microtubule-associated protein 2 (MAP2). Adjacent sections were stained for IgG and glial fibrillary acidic protein (GFAP). Non-specific binding was minimized by incubation in a 3% goat serum blocking solution at room temperature for 1 h. The following primary antibodies were used to stain the tissue sections: mouse monoclonal anti-human MAP2 (1:1000; Covance, Princeton, New Jersey), mouse monoclonal anti-human GFAP (1:500; Sigma-Aldrich, St. Louis, MO). The following secondary antibodies were used: goat anti-mouse polyclonal Alexa Fluor 594 (1:400, Jackson Immunoresearch, West Grove, PA), fluorescein goat polyclonal anti-human IgG (1:200; Vector Labs, Burlingame, CA). Auto-fluorescence was minimized using Sudan Black B. Finally, tissue slices were placed on glass slides and mounted using a glass coverslip and Vectorshield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei (Vector Labs, Burlingame, CA). Images were obtained using a Leica Leitz fluorescence microscope and a Leica Microsystems upright confocal microscope with attached cameras (Leica Microsystems, Allendale, New Jersey). Fluorescence intensity and co-localization were measured using Q-Capture software (Q-Capture, Surrey, BC, Canada). Three-dimensional reconstruction of confocal images was performed using Velocity (PerkinElmer, Waltham, Massachusetts).

Subcellular fractionation of brain tissue resections
Snap frozen tissue stored at −80 °C was processed according to the protocol provided with the Thermo Scientific Subcellular Protein Fractionation Kit for Tissue (Thermo Fisher Scientific, Rockford, IL). 200 mg of tissue was used for each fractionation. Ten patients with epilepsy and 4 AVM patients were used for these experiments.

Western blots for IgG from subcellular fractions
Proteins from subcellular fractions were separated via SDS-PAGE electrophoresis under non-denaturing conditions and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Billerica, MA). Membranes were incubated overnight at 4 °C with horseradish peroxidase (HRP) conjugated anti-human IgG (1:2000; Calbiochem-Novabiochem Corporation, CA, U.S.A.). Proteins were visualized using Western Lightning Plus ECL (PerkinElmer, Waltham, Massachusetts) and developed on Kodak Biomax MR Film (Eastman Kodak Company, Rochester, New York). IgG volume was quantified using Phoretix 2D software (Nonlinear USA Inc., Durham, North Carolina).

Purification and analysis
Indirect Immunoprecipitation
Nuclear fractions were divided into soluble nuclear and chromatin-bound samples. Typically, indirect immunoprecipitation requires a step to allow the antibody–antigen complex to form. However, as antibody–antigen complexes were already present in cell nuclei, no antibody–antigen complex forming step was required. Nuclear fractions were incubated with protein-A coated agrose beads (Santa Cruz Biotechnology Inc., Dallas, Texas) for 3 h at 4 °C with gentle mixing. Beads were washed and centrifuged 4 times in 20% Tween–PBS to remove as much unbound protein as possible. Samples were then placed in microcentrifuge tubes and exposed to a low pH (2.6) glycine–HCl solution for 3, 15 or 30 min. After centrifugation and removal of supernatant (containing antigen), the pH of the supernatant was neutralized with 1 M Tris at pH 8.5.

Liquid chromatography–mass spectrometry analysis
Antigen samples, obtained as described above, were analyzed by SDS-PAGE electrophoresis. Bands were cut out of the gel, washed and dehydrated in acetonitrile. Bands were reduced with dithiothreitol and alkylated with iodoacetamide. Proteins were digested in gel overnight at room temperature using 10 ng/μl trypsin in 50 mM ammonium bicarbonate. Proteins were extracted from the polyacrylamide with acetonitrile (50%) and formic acid (5%). Extracts were evaporated in a SpeedVac and resuspended in acetic acid (1%). Five microliter volumes of extract were injected on a Dionex 15 cm × 75 μm i.d Acclaim Pepmap C18, 2 μm, 100 Å, reversed-phase capillary chromatography column for liquid chromatography separation (Thermo Fisher Scientific Inc., Rockford, IL). Peptides were eluted from the column by acetonitrile/formic acid (0.1%) gradient at a flow rate of 0.25 μl/min and introduced to the mass spectrometry source on-line. For mass spectrometry analysis, a Finnigan LTQ-Orbitrap Elite hybrid mass spectrometer system (Thermo Fisher Scientific Inc., Rockford, IL) was used. The microelectrospray ion source was operated at 2.5 kV. The peptide digest was analyzed using the data-dependent multitask capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and ion spectra to determine the amino acid sequence.

Data from this experiment were analyzed using all collisionally-induced dissociation spectra collected. These were used to search the NCBI Mascot program with a human taxonomy filter. Manual interpretation, Sequest and Blast were used to verify Mascot matches.

Identification of potential antigens from mass spectrometry data
In our patient sample, time-dependent change in total antigen content was evaluated. The procedure (see above) was used to study both soluble nuclear and chromatin-bound fractions. Before collection, columns were washed 4 × to remove unbound proteins. Thus, the remaining proteins were considered putative autoantigens; weakly-bound non-specific IgGs were also present in this sample. Qualitative analysis was achieved by examining the change in putative autoantigen count observed over time. A positive increase in a given autoantigen abundance (spectral count) was interpreted as elution of antigen tightly bound to IgGs. In contrast, a negligible increase or decrease was interpreted as non-specific binding of antigen to IgG. A transient increase or decrease at 15 min followed by a return to levels less than or equal to that observed at 3 min were not further analyzed. In short, putative autoantigens were further analyzed only if they were not obvious contaminants (e.g., keratins) and if their kinetic dissociation was indicative of specific antibody–antigen binding.

An additional validation step was used to unveil autoantigen. We compared the kinetics of soluble nuclear (presumably non-specific) vs. chromatin-bound (specific) complexes. This analysis revealed that, in the soluble nuclear fraction, the dissociation of protein from IgG was not time-dependent. In fact, increase, decrease or no change in total protein content were equally common. In contrast, the chromatin-bound IgG–antigen dissociation was observed by a definite time-dependent increase of dissociated autoantigen. The comparisons of these two behaviors for each putative autoantigen (see Fig. 5) together with the observations above were merged into the following:

\[
[(30\text{ minCB}−15\text{ minCB})−(15\text{ minCB}−3\text{ minCB})]−[(30\text{ minSN}−15\text{ minSN})−(15\text{ minSN}−3\text{ minSN})] = Q
\]

where Q is a coefficient and where each value equals the normalized spectral count of a given autoantigen. CB = chromatin bound fraction and SN = soluble nuclear fraction. Q was used to discern between soluble unbound IgG, and chromatin–IgG or histone–IgG complexes.
All proteins associated with negative coefficients were considered to be likely autoantigen specifically bound to antibodies. Proteins were then ranked from the most negative to least negative and any non-nuclear proteins were eliminated. The nuclear proteins with the most negative number were considered the primary targets of the autoantibodies (Table 2).

Identification of antibody and antigen

**Western blots using serum to detect isolated nuclear antigen**

Protein from 1 patient with epilepsy, serum from 8 patients with epilepsy, 2 control patients and 1 positive control patient with rheumatoid arthritis (RA) were used in this experiment. Isolated antigens from the immunoprecipitation experiment were separated via SDS-PAGE electrophoresis under non-denaturing conditions and transferred onto a PVDF membrane. Membranes were incubated at room temperature for 1 h with the serum from patients with epilepsy (1:1000). After repeated washing, membranes were incubated at room temperature for 2 h with HRP-conjugated goat anti-human IgG (1:2000; Calbiochem-Novabiochem Corporation, CA, U.S.A.). Proteins were visualized using Western Lightning Plus ECL and developed on Kodak Biomax MR Film.

**Western blots using serum to detect purified histones**

Total histones were separated by SDS-PAGE electrophoresis under non-denaturing conditions and transferred onto a PVDF membrane. Membranes were incubated at room temperature for 1 h with the serum from patients with epilepsy (1:500). After repeated washing, membranes were incubated at room temperature for 2 h with HRP-conjugated goat anti-human IgG (1:2000). Proteins were visualized using Western Lightning Plus ECL and developed on Kodak Biomax MR Film.

**Anti-histone and anti-chromatin ELISA using serum**

Serum obtained from human patients with epilepsy was diluted 1:300 in serum diluent (sterile filtered 0.5% bovine gamma-globulin, 5% gelatin, 0.05 mM Tween in 1× PBS) and analyzed for levels of anti-chromatin and anti-histone IgG autoantibodies. Microtiter plates (Immulon 2HB) were coated with purified chromatin or total histones overnight at 4 °C, blocked in 5% gelatin/PBS for 2 h, and incubated with serum samples for 2 h. Secondary HRP-conjugated anti-human IgG antibodies were added for 1.5 h and plates were developed using 10 mg/ml 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in McIlwain’s buffer (0.09 M NaH2PO4, 0.06 M citric acid, pH 4.6). Samples were read on a spectrophotometer at 405 nm.
| Patient ID | Age | Gender | Seizures Y/N | Epilepsy Y/N | Diagnosis | Etiology | Pathology | Autoimmune/inflammatory disease Y/N | Previous path./surgery | Current AEDs | Sample type | Experimental use |
|------------|-----|--------|--------------|--------------|-----------|----------|----------|-------------------------------------|------------------------|-------------|-------------|------------------|
| 1          | 45  | F      | Y            | Y            | Frontal lobe epilepsy | Encephalomalacia | Cortical atrophy and WM degeneration | N | Meningioma | Carb, Lev | Brain | WB, BHC, IP, MS, |
| 2          | 18  | M      | Y            | Y            | Temporal lobe epilepsy | Cortical malformation | Neuronomegaly and loss of cortical architecture | N | | Lam, Lev, Phen, Clor | Brain | WB |
| 3          | 2 m | M      | Y            | Y            | Temporal lobe epilepsy | Cortical malformation | Cortical dysplasia, neuronal cytomegaly/dysmorphi, gliosis and microcalcifications | N | | Lev, Lam, Oxc, Phen | Brain | WB |
| 4          | 23  | M      | Y            | Y            | Temporal lobe epilepsy | Hippocampal sclerosis | Cortical dysplasia | N | | Lam, Lev | Brain | WB, BHC |
| 5          | 19  | M      | Y            | Y            | Occipital lobe epilepsy | Cortical malformation | Cortical dysplasia | N | Vagus nerve stimulator | Val, Zin, Lev | Brain | WB, BHC |
| 6          | 1   | M      | Y            | Y            | Temporal lobe epilepsy | Hemimegalencephaly | Cortical dysplasia, WM atrophy and gliosis | N | Vagus nerve stimulator | Topi, Phen | Brain | WB, BHC |
| 7          | 12  | F      | Y            | Y            | Frontal lobe epilepsy | Cortical malformation, encephalomalacia w/porencephaly | Cortical dysplasia and gliosis | N | | Val, Zin, Lev | Brain | WB, BHC |
| 8A         | 14  | M      | Y            | Y            | Temporal lobe epilepsy | Cortical malformation, encephalomalacia w/porencephaly | Cortical dysplasia, fibrotic leptomeninges | N | | Topi, Lev, Phen | Brain | WB |
| 8B         | 14  | M      | N            | Y            | Temporal lobe epilepsy | Cortical malformation, encephalomalacia w/porencephaly | Cortical dysplasia, fibrotic leptomeninges | N | | Topi, Lev, Phen | Brain | WB |
| 9          | 7   | M      | Y            | Y            | Parieto-occipital lobe epilepsy | Ganglioglioma | Ganglioglioma w/ cortical dysplasia | N | | Carb, Lev | Brain | WB, BHC |
| 10         | 27  | M      | Y            | Y            | Temporal lobe epilepsy | Unknown | Unknown | N | Unknown | Unknown | Brain | IHC |
| 11         | 46  | F      | Y            | Y            | Temporal lobe epilepsy | Unknown | Unknown | N | Unknown | Unknown | Brain | IHC |
| 12         | 48  | M      | Y            | Y            | Temporal lobe epilepsy | Unknown | Unknown | N | Unknown | Unknown | Brain | IHC |
| 13         | 14  | F      | Y            | Y            | Temporal lobe epilepsy | Cortical malformation | Cortical malformation | N | | Carb, Phen, Val, Gabap, Lam, Phen | Brain | IHC |
| 14         | 36  | M      | Y            | Y            | Temporal lobe epilepsy | Cavernous angioma | Cavernous angioma | N | | Serum | WB-IP, WB-H, ELISA, HEp-2 | | |
|   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 15 | 29 | F | Y | Y | Temporal lobe epilepsy | Unknown | Unknown | N | Anterior temporal lobectomy (hippocampal sclerosis) | Carb | Serum | WB-IP, WB-H, ELISA, HEp-2 |
| 16 | 32 | M | Y | Y | Temporal lobe epilepsy | Unknown | Unknown | N | Lam, Lev | Serum | WB-IP, WB-H, ELISA, HEp-2 |
| 17 | 20 | M | Y | Y | Epilepsy | Cortical malformation | Perisylvian polymicrogyria | N | Vagus nerve stimulator | Laco, Val | Serum | WB-IP, WB-H, ELISA, HEp-2 |
| 18 | 21 | F | Y | Y | Temporal lobe epilepsy | Unknown | Unknown | N | Topi | Serum | ELISA, HEp-2 |
| 19 | 33 | M | Y | Y | Temporal lobe epilepsy | Unknown | Unknown | N | Lev, Oxcarb, Clon | Serum | ELISA, HEp-2 |
| 20 | 30 | F | Y | Y | Temporal lobe epilepsy | Unknown | Unknown | N | Val | Serum | ELISA, HEp-2 |
| 21 | 38 | M | Y | Y | Temporal lobe epilepsy | Unknown | Unknown | N | Vagus nerve stimulator | Laco, Val | Serum | ELISA, HEp-2 |
| 22 | Unknown | Unknown | N | N | Multiple sclerosis | Unknown | Unknown | N | Vagus nerve stimulator | Laco, Val | Serum | ELISA, HEp-2 |
| 23 | Unknown | Unknown | N | N | Multiple sclerosis | Unknown | Unknown | N | Val | Serum | ELISA, HEp-2 |
| 24 | Unknown | Unknown | N | N | Multiple sclerosis | Unknown | Unknown | N | Val | Serum | ELISA, HEp-2 |
| 25 | Unknown | Unknown | N | N | Multiple sclerosis | Unknown | Unknown | N | Val | Serum | ELISA, HEp-2 |
| 26 | 48 | M | N | N | AVM w/ med. controlled seizure | AVM | N | Embolization | Val, Zon, Lev | Brain | WB, IHC |
| 27 | 23 | M | N | N | AVM w/ no history of seizure | AVM | N | Embolization | Val, Zon, Lev | Brain | WB, IHC |
| 28 | 37 | F | Y | N | AVM w/ epilepsy | AVM | N | Embolization of the left vertebral artery | Levo, Oxcarb, Pheny, Clon | Brain | WB, IHC |
| 29 | 28 | F | N | N | AVM | N | | | Brain | MRI |
| C1 | 26 | M | N | N | Healthy control | N | | | Serum | ELISA, WB-IP, WB-H, HEp-2 |
| C2 | 33 | M | N | N | Healthy control | N | | | Serum | ELISA, WB-IP, WB-H, HEp-2 |
| Pos. C 1 | 19 | F | Y | Y | Epilepsy | Unknown | Unknown | Y (rheumatoid arthritis) | Carb, Lam | Serum | WB-IP, ELISA, HEp-2 |

AED abbreviations: Carb = carbamazepine, Lev = levetiracetam, Lam = lamotrigine, Pheno = phenobarbital, Clor = clorazepate, Oxcarb = oxcarbazepine, Clon = clonazepam, Topi = topiramate, Val = valproate, Zon = zonisamide, Pheny = Phenytoin, Laco = lacosamide, Preg = pregabalin.

Experimental use abbreviations: WB = Western blot, IHC = immunohistochemistry, IP = immunoprecipitation, MS = mass spectrometry, WB-IP = Western blot using immunoprecipitation samples, WB-H = Western blot using purified histones, ELISA = enzyme linked immunosorbent assay, HEp-2 = human epithelial cell assay.

* Indicates post-mortem brain tissue samples. All other brain tissue specimens were obtained from surgical resections.
**Human epithelial type 2 (HEp-2) cell assay**

Slides provided by the manufacturer (Bio-Rad, Hercules, CA) were placed into a humidity chamber. Positive and negative controls provided with the manufacturers’ kit were added (1:64 dilution) to two wells of the slides in addition to 3 human serum controls and the serum from 8 patients with epilepsy (1:10 dilution). Slides were incubated for 20 min and then washed for 10 min in PBS. 25 μl of fluorescein conjugate was added to each well of the slide and incubated in a humidity chamber for 20 min. Images were obtained on a Leica Leitz fluorescent microscope with attached camera.

**Results**

For the results presented here we used a total of 32 subjects (including control). 21 brain samples and blood from 11 donors were analyzed. Of the brain samples, 13 were obtained from patients affected by multiple drug resistant seizures, 4 were from patients treated by a neurosurgeon to repair AVMs and 4 were post-mortem brain samples from patients affected by MS. Brain samples were used for Western blotting, immunohistochemistry and general morphology. Eight patients with epilepsy, 2 healthy volunteers and 1 patient with epilepsy and RA were enrolled to donate blood samples used for ELISA, HEp-2 and Western blot data. The AVM samples were obtained from 1 patient with seizures responding to treatment, 1 patient with multiple drug resistant seizures and 2 with no seizure history. None of the MS patients had a history of epilepsy or seizures. Data obtained from the medical records of patients used, including volunteers, are shown in Table 1.

Our experimental design also encompassed another layer of distribution, namely the presence or absence of recurring seizures. Thus, 1 AVM patient where seizures were present at time of surgery was grouped together with resected brain from patients with epilepsy. These criteria were only used for Fig. 4.

**BBB disruption in multiple sclerosis, epilepsy, and AVM brain**

One of the goals of this study was to detect and localize immunoglobulins in the CNS. We first wished to understand the mechanisms by which these macromolecular complexes gain entry into the brain. There are two known mechanisms for IgG CNS ingress, namely passage across the BBB or synthesis by CNS B lymphocytes (Ankeny and Popovich, 2010). Our results suggest that the former was the predominant source of CNS IgGs in the brain from patients with epilepsy. Figs. 2A–B demonstrate the topographic overlap of extravasated immunoglobulins and albumin. Extravasated IgGs and albumin were found in the extracellular as well as the intracellular compartments (Figs. 2B1–B3). The arrows in B1–B3 points to a neuron filled with IgGs and albumin.

Table 2 Candidate autoantigens.

| Protein | CB-SN | Accession #   | Mass (kDa) | Autoimmune Association | Support         |
|---------|-------|---------------|------------|------------------------|-----------------|
| Histone H4 | −2.83  | 4504301      | 11         | SLE                    | Burlingame et al. (1994) |
| Histone H2A type 1-C | −2.07  | 4504245      | 14         | SLE                    | Inoue et al. (1990) |
| Histone H2A type 1 | −1.96  | 4504239      | 14         | SLE                    | Gu et al. (2013)   |
| Histone H2B type 2-E | −1.82  | 4504277      | 13         |                       |                 |
| Histone H3.3 | −0.92  | 4504279      | 15         | SLE                    | Van Bavel et al. (2011) |
| Histone H1.3 | −0.74  | 4685377      | 22         |                       |                 |
| Histone H2AZ | −0.70  | 4504255      | 13         |                       |                 |
| Core histone macro-H2A 1 isoform 2 | −0.47  | 4758496      | 39         |                       |                 |
| Heterogeneous nuclear ribonucleoproteins C1/C2 isoform a | −0.18  | 117189975  | 33         |                       |                 |
| Heterogeneous nuclear ribonucleoprotein A3 | −0.16  | 34740329     | 39         | SLE, scleroderma       | Siapka et al. (2007) |
| Histone H1.0 | −0.07  | 4685371      | 20         |                       |                 |
| Nucleoside diphosphate kinase B isoform a | −0.03  | 4505409      | 17         |                       |                 |

Autoimmune Association abbreviations: SLE = systemic lupus erythematosus.
intracellular accumulation of IgG was absent (arrow in Fig. 3C1). The profile lines in Fig. 3C2 show two continuous neurons to demonstrate the lack of any IgG content in these cells.

In order to determine the specific location of IgGs, subcellular fractionation was performed. The Western blot in Fig. 4A shows the presence of intracellular IgGs in each subcellular fraction. The
75 kDa band in the cytoskeletal fraction represents an IgG fragment corresponding to the heavy chain regions. Figs. 4B, C and D show the difference between pathologies with BBB leakage with no seizures vs. BBB leakage in patients affected by seizures. AVM patients’ brain displayed abundant IgGs in soluble nuclear fractions but a less remarkable level in the chromatin-bound fraction. In samples from patients with epilepsy, results demonstrated comparable amounts of IgGs in both soluble nuclear and chromatin-bound fractions. In addition, the AVM patient who also experienced seizures had similar levels of chromatin-bound IgGs as patients with epilepsy (Figs. 4B, C and D patient #28). “Non-
spiking" brain from patients with epilepsy displayed chromatin-bound IgG levels similar to those found in AVM patients without seizure (Figs. 4B, C and D patient #8B).

**Purification and analysis**

A second major goal was to determine the molecular profiles of putative autoantigen in the nuclei of neurons from patients with epilepsy. The strategy used to unveil the nature of these autoantigens is described in detail in the Materials and methods section. Fig. 5 summarizes the experimental design and results. After disassociation of antigen from the antibodies by low pH the soluble nuclear fraction displayed a dramatic drop in protein content over time (Fig. 5A); this was the opposite of what was observed in the chromatin-bound fraction where a dramatic increase in protein content was observed (Fig. 5A). This was interpreted as the presence of specific binding of IgG to nuclear proteins and chromatin in comparison to free-floating or non-specifically bound protein–IgG complexes in the soluble fraction. When mass spectrometry analysis was performed, a number of non-nuclear proteins were found and discarded (see Supplemental Table 1). When narrowing down likely nuclear autoantigen candidates, the most prominent family of putative autoantigen was found in histones. The legend in Fig. 5B highlights the molecular descriptions of the histones found to be significantly associated with specific binding to IgG in the chromatin fraction. All histones tested behaved as predicted by the equation shown in the Materials and methods section and according to the time-dependent association of IgG and antigen shown in Fig. 5A. This also implies that IgG–histone binding displayed high affinity owing to the fact that the kinetic profile was consistent with tight binding antibodies. This is in contrast to what was observed in the soluble fraction where histones were rapidly dissociated from antibodies. A summary of the molecular properties and quantitative analysis of histones and other putative autoantigens is shown in Table 2.

**Identification of antigen and antibody**

While the results in Fig. 5 and Table 2 show an indirect approach to determine the molecular nature of putative autoantigens found in the brain from patients with epilepsy, these results heavily rely on statistical analysis of mass spectrometry data. We performed three additional immunodetection experiments to confirm or disprove the presence of histone-specific IgGs in patients with epilepsy. We first used Western blotting to qualitatively analyze histones by molecular weight. To this end we prepared Western blots using protein extracts from the immunoprecipitation experiments shown in Fig. 5. In other words, we used a mixture of nuclear protein that contained histones and other nuclear proteins obtained from brain tissue resections of patients with epilepsy as a target for patients’ serum IgGs. In doing so we wished to test the hypothesis that the putative autoantigens isolated from the brain were also targets of IgGs present in the serum of patients with epilepsy. The results are shown in Fig. 6A. Note that, when control serum was used, no significant immunosignal was detected, whereas with the serum from patients with epilepsy a distinct band was apparent. This band reflects the binding of IgGs isolated from the serum of patients with epilepsy to autoantigen isolated from IgG present inside cells of the human brain with epilepsy. This band also corresponds to the molecular weight of histones (kDa). To confirm that these bands indeed reflected the presence of histones, we ran a similar gel (Fig. 6A; patient 17) to demonstrate that total histones are recognized by the same serum...
used in the previous experiment. These results show that the molecular target of IgG extracted from neuronal nuclei of brain tissue resections of patients with epilepsy consists of histones.

While Western blots used to screen and confirm the presence of specific nuclear protein are useful for a few experiments, screening of a larger number of subjects is less convenient. To this end we used ELISA to quantitatively detect and quantify the presence of anti-histone IgG (Fig. 6B). Note that in 5 of 8 patients with epilepsy a signal suggestive of autoimmunity against histones was present. Also note that in control subjects (labeled as C1) the signal was not present. These results confirm the Western blot findings shown in Fig. 6A but also underscore the fact that these autoantibodies are not present in all patients with epilepsy. However, some patients with epilepsy have higher levels of antinuclear antibodies than the positive control rheumatoid arthritis patient (labeled Pos, C 1).

The presence of anti-histone antibodies can be indicative of a broader autoimmune response against multiple nuclear components. That is, many antinuclear antibodies recognize not just histones, but the histone–DNA complex (chromatin). Therefore, we tested the hypothesis that in addition to anti-histone antibodies, the serum from patients with epilepsy also contains autoantibodies targeting chromatin (Fig. 6C). Using the HEP-2 assay, a distinct pattern of staining was discovered. The serum from patients with epilepsy, but not the serum from control subjects (Fig. 6C1) displayed various types of antinuclear staining. The insets in the figures show specific binding to nuclei or other sub-nuclear fractions (specifically nucleoli (Pt. 14) and centro-meres (Pt. 16)). Note the similarity between the manufacturer’s negative control serum and the serum control provided by the volunteer. The HEP-2 assay results demonstrate that a clinical tool for the diagnosis of autoimmune disease is able to detect the antinuclear antibodies in the serum of patients with epilepsy.

Discussion

The main finding of this research is the presence of antinuclear antibodies in the brain and serum from patients with epilepsy. These findings were obtained in patients who did not present any symptoms or clinical diagnoses of a typical autoimmune disease (such as SLE). The serum from a patient with epilepsy also diagnosed with RA was used as a positive control to underscore that the levels of auto-antibody in subjects with epilepsy were comparable to bona fide patients with autoimmune disease. Nuclear antibodies were predominantly found in neurons and appeared to spare glia. Nuclear targets included histones and chromatin. Autoimmune IgGs are derived from the systemic circulation as their presence overlapped with areas of albumin leakage across the BBB. An alternative interpretation may support an indirect role of B lymphocytes in BBBD; this hypothesis needs to be tested by ad hoc experiments.

Significance

Many clinical studies suggest that aberrations of the immune system may be associated with seizures. Our results suggest the existence of a new category of autoimmune epilepsy. Additionally, while a number of studies have demonstrated the presence of antinuclear antibodies in sera of patients with epilepsy, we have for the first time shown that these antibodies are able to enter the nuclei of neurons. We focused on a population where antibodies against traditional “epilepsy related autoantigen” were not present and discovered that the misguided immune response in this population targeted the nuclear antigens histones and chromatins. A previous study by others concluded that nuclear antigens are associated with recurrent seizures in patients with refractory focal epilepsy (Peltola et al., 2000). Furthermore, these results were exclusively based on the HEP-2 assay, which is qualitative, and not with a clinical-grade ELISA test. In addition, the same group recently reported that antinuclear antibodies are associated with recurrent seizures in patients with refractory focal epilepsy (Peltola et al., 2000). Our results show a convergence of positive findings, since both qualitative (HEP-2) and quantitative tests (ELISA) demonstrated the presence of antinuclear antibodies in patients with epilepsy. We also used an extracted nuclear antigen fraction to confirm the presence of IgG-bound autoantigen which was found to be histones or chromatins.
Given the fact that areas of IgG leakage corresponded to areas of albumin extravasation, we concluded that ingress of IgGs in neurons occurred by a trans-endothelial route either across altered tight junctions or by other trans-cellular means. We found no evidence of local production of these immune molecules. Others also confirm the lack of significant B cell presence in brain tissue resections of patients with epilepsy where an infectious trigger is not suspected (Prayson and Frater, 2002).

In typical autoimmune epilepsy the presence of a pathogen is often suspected but not always demonstrated (Nabbout, 2012). Both bacterial and viral mechanisms have been proposed. The onset and development of the autoimmune response may be due to molecular mimicry or breaking of a barrier maintaining immunological privilege. The latter is typical for CNS disorders owing to the presence of a BBB. The question, however, remains of why an autoimmune disease should cause seizures which are characterized by neuronal hyperexcitability and synchronization, or alternatively do seizures trigger an autoimmune disease? There are several lines of evidence pointing to the devastating effect of antibodies targeting ion channels (Lang et al., 2003), glutamate receptors, voltage gated calcium and potassium channels, etc.(Graus and Dalmau, 2012; Hoftberger et al., 2012). In these pathologies the proposed and accepted cascade of evidence/events is as follows: 1) The immune system mounts a response against a pathogen or against a self-molecule perceived as such; 2) antibodies are produced to target these proteins or a specific sequence; 3) upon binding to its target the antibody promotes loss of function or excessive function of one of the several crucial components that regulate neuronal resting potential or synaptic function (see Vincent et al., 2006 for details). We did not directly investigate whether autoantibodies may have an influence on and/or produce a downstream event that may impact comorbidities such as cognitive decline. Antinuclear IgGs may be yet another mechanism of seizure-induced neuronal cell loss originally described in hippocampal sclerosis but nowadays recognized also for neocortical seizure disorders. However further experiments are needed to determine the pathological mechanisms involves.

Fig. 6. A multimodal analysis of the serum from patients with epilepsy reveals the presence of specific anti-histone and anti-chromatin antibodies. (A) Immunoprecipitation isolated nuclear antigen taken from the brain tissue of a patient with epilepsy was ran on a gel and probed with the serum from patients with epilepsy and healthy controls. While control serum produces no signal, the serum from patients with epilepsy produces a signal corresponding to histone complexes. Additionally, the serum from Pt. 26 was used to probe a gel containing only purified histones (A-Pt. 17*). Note the similar binding pattern between both gels using the serum from patient 17. (B) ELISA quantifying anti-histone antibodies using the serum of patients with epilepsy and control patients. The ELISA did not detect anti-histone antibodies in control serum but did detect them in 5 of 8 patients with epilepsy. Going further, a Hep-2 assay using the same serum detected the presence of anti-chromatin antibodies (C). Patient 15 shows the speckled pattern of general antinuclear staining and patients 14 and 16 show the specific binding pattern of anti-nuclear and anti-centromere antibodies, respectively. The inset at the top of the figure refers to the phase of experimental design discussed in Fig. 1.
Consequences of intranuclear brain IgGs

How IgGs pass through a breached BBB is not known, nor is it understood how once they enter the cellular compartment IgG may act as “neurotoxins” (Levite and Ganor, 2008). There are many possible mechanisms for their “toxic” actions, including cell-induced death by an immune response, altered transcription owing to binding of IgGs to DNA, altered mechanisms of cell cycle or apoptosis, etc. Our results show no evidence of widespread neuronal cell death in the regions of albumin and IgG extravasation. In fact, the cells displaying the most intranuclear IgG content (for example Fig. 3A) were characterized by healthy-appearing chromatin and nuclear content (Marchi et al., 2004). Others have suggested that IgGs are “toxic” and that they promote neuronal cell death in the lithium/pilocarpine model of status epilepticus or in the human cortex (Levite and Ganor, 2008; Michalak et al., 2012).

Alternative hypotheses to speculate on the consequences of nuclear IgGs in the human brain from patients with epilepsy may focus on comorbidities rather than seizures themselves. It is well known that complex sequelae of pathologies follow the onset and progression of epilepsy. This is particularly true for pediatric populations where developmental delays, mental illness and other noxious consequences of prolonged seizures are often encountered. It is possible that given a similar family history, seizure severity and age/gender patients deteriorate more rapidly when neuronal cells are exposed to nuclear IgGs. It is also possible, though it appears unlikely, that IgGs have a neuroprotective effect.

A role for the blood–brain barrier?

Seizures are characterized by widespread vascular changes that span from hyperemia to vascular leakage (Abe et al., 1997; Diehl et al., 1998; Ivens et al., 2010; Janigro, 2012; Marchi et al., 2011, 2012a, 2013; Weinand et al., 1997). We and others have shown that BBB dysfunction precedes seizures in patients with epilepsy and that BBBD causes seizures in humans and animals even in absence of a prior history of epilepsy (for review see Marchi et al., 2012a). There is therefore strong evidence that the BBB is impaired in patients with epilepsy, at least at time of seizure onset. BBBD leakage results in extravasation of IgGs which is the first step towards their ingress into the nuclear compartment of neurons (Rigau et al., 2007). Thus, either BBBD, seizures, or both may be necessary for this to happen. To address this hypothesis, we performed experiments using two human pathologies also characterized by BBB leakage but not seizures; we used post-mortem samples of MS brain as well as brain samples isolated during surgeries to repair AVM. We also studied samples from patients with BBBD and seizures and compared those to BBBD but no ongoing seizures or negative seizure history. To this end, in addition to resections of the brain from patients with epilepsy, we used a “non-spiking” region of resected temporal lobe (as a BBBD + but no seizure) and AVM tissue from a patient presenting with multiple drug resistant seizures (as BBBD + and seizure +) (see Table 1). The data in Fig. 4 show that chromatin-bound autoantibodies were elevated in all samples from “spiking” cortex, regardless of its origin. This group contained all tissue isolated from foci of patients with epilepsy as well as AVM brain associated with seizures. The group with the least chromatin-bound IgGs encompassed brain samples from non-epilepsy patients and the brain tissue resected from “non-spiking” brain of patients with epilepsy. These preliminary results led us to conclude that leakage of the barrier is necessary for entry of IgG into the parenchyma but is not sufficient to allow binding of antibodies to chromatin.

If BBBD dysfunction is not sufficient to cause accumulation of IgGs into the nucleus of neurons, what is the likely mechanism? We propose a scenario where seizures themselves are responsible for uptake of IgGs into the nuclear compartment of neurons. According to this scenario, prolonged excitation of neurons and other cellular elements occurring during a seizure, and the subsequent metabolic mismatch, act synergistically to decrease selective permeability of the cell membrane. According to this hypothesis, a specific receptor is not necessary, but rather, this uptake occurs due to a non-specific spreading depolarization-like episode. However, this scenario explains how IgGs enter into the cells but do not account for the presence in the nucleus. In fact, spreading depression affects all cells in a certain region but nuclear uptake was only present in neurons. How IgG may migrate from cytosol to nucleus remains unknown. A recent paper has shown that electrical stimulation characterized by low intensity (µA) and a frequency comparable to neuronal firing during an ictal event (50 Hz) causes translocation of membrane-bound protein to the nucleus (Janigro et al., 2006). It is thus possible that field potential changes alone are sufficient to cause subcellular redistribution of macromolecules.

Potential pitfalls

One of the potential confounders in this study is the autoimmune side-effects of certain anti-convulsants. For example, carbamazepine is known to induce lupus-like symptoms or full-blown disease; this has been shown to occur primarily in female patients (Schoonen et al., 2010). This does not appear to be a significant factor in our study because of all the patients enrolled, only 5 were under carbamazepine therapy. In addition to carbamazepine, anecdotal reports have shown that lamotrigine and valproate can produce lupus-like symptoms (Bonnet et al., 2003; Chang and Cole, 2012). However, this was unlikely to be a factor in antinuclear antibody generation in our patients as none of them presented with symptoms of anti-convulsant drug-induced lupus. The probability of finding antinuclear antibodies was, in other words, unrelated to drug regimen. Others also found cohorts of patients with epilepsy undergoing carbamazepine therapy with no symptoms or signs suggestive of SLE (Ramua et al., 2004). It has to be noted, however, that in this study the presence of antinuclear antibodies was more common in female patients compared to male. Of the three patients with undetectable levels of auto-IgG, all were male while 60% of the patients with detectable levels of anti-histone antibodies were female. This finding is in agreement with numerous findings linking autoimmune disease to gender differences (Danchenko et al., 2006).

Another possible limitation of this study is the fact that we did not use CSF to test for autoantibodies. CSF analysis is a routine clinical approach to diagnosis infectious or autoimmune diseases. We did not have access to CSF samples for the patient population whose data is shown. Further experiments paired CSF and serum samples are being performed to confirm the diagnostic potential of antinuclear antibodies in epilepsy.

Conclusions

Our results point to a sterile inflammation mechanism (Rock et al., 2010) by which failure of the BBB promotes neuronal dysfunction. We focused on three neurological conditions (epilepsy, multiple sclerosis, AVM) all characterized by leakage of the BBB but found nucleus specific IgGs only in the brain from patients with epilepsy and within these samples only in a population of neurons. IgGs directed towards histones and chromatin were subsequently found in sera from other patients with epilepsy suggesting that both compartments (circulatory and CNS) contain these antibodies and that antibodies found in the serum are able to bind nuclear protein extracted from brain sample of patients with epilepsy. Thus, antinuclear autoantibodies may be a new mechanism of seizures-induced neuronal death.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2013.07.006.

Disclosure of conflicts of interest

None.
